Ultrasonic vocalizations induced by appetitive or aversive clitoral stimulation: foundations for an anticipatory-based model of female sexual reward and clitorodynia

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ABSTRACT

Ultrasonic vocalizations induced by appetitive or aversive clitoral stimulation: foundations for an anticipatory-based model of female sexual reward and clitorodynia

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Concordia University, 2024

Adult rats make ultrasonic vocalizations (USVs) that exhibit acoustic characteristics indicative of an immediate emotional state and its underlying neural activation. As an integral part of the rats' behavioral repertoire, USVs can be translated to human behavior, including clinical models of human diseases and disorders. Although female rats are used as predictive models of sexual function and dysfunction in women, their USVs have not yet been incorporated into existing preclinical behavioral paradigms. Female rats reliably emit USVs in response to the application of temporally-distributed, manual clitoral stimulation (CLS). It is unclear, however, whether the emissions are reflective of the hedonic properties of CLS or those of general arousal. Thus, the experiments in the thesis aimed to characterize the acoustic properties of CLS-elicited USVs to clarify their communicative function. The experiments described in Chapter 2 determined that female rats emit hedonic USVs when distributed CLS is delivered with a soft-bristle paintbrush. The ovarian hormones estradiol and progesterone modulated the acoustic parameters of hedonic USVs whereas chronic administration of the selective serotonin reuptake inhibitor fluoxetine attenuated their emission concurrently with decreases in sexually appetitive behaviors like solicitations. The experiments described in Chapter 3 explored whether altering the tactile quality of distributed CLS alters its hedonic value and its capacity to serve as a reinforcer in partner-preference conditioning. Compared to CLS applied with a soft-bristle brush, CLS applied with a hard-bristle brush elicited distinct subtypes of low frequency USVs associated with aversion. The experiment described in Chapter 4 found that the tactile quality of distributed CLS also altered the

pattern of Fos protein expression in brain areas involved in sexual reward, aversion, and sensory integration. Exposure to a neutral odour previously associated with rough-bristle CLS increased the number of neurons expressing Fos protein in brain regions subserving aversive responses and decreased expression in regions subserving reward. An opposite pattern was expressed in response to the same odour associated with soft-bristle CLS, whereas the odour associated with sham CLS did not alter Fos expression in those areas. Taken together, the data show that CLS-induced USVs can be used as a measure of both appetitive and aversive sexual affect, as well as provide a foundational model of anticipatory-induced clitorodynia.

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Contribution of Authors

Chapter 1- General Introduction

Christine A. Gerson = Manuscript writing

James G. Pfaus = Manuscript writing

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Chapter 2.1 - Effects of ovarian hormones on the emission of 50-kHz ultrasonic vocalizations during distributed clitoral stimulation in the rat

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Chapter 2.2. - Influence of chronic administration of fluoxetine on CLS elicited 50-kHz vocalizations and expression of female sexual behavior

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Chapter 3.1 - Effect of aversive clitoral stimulation on female rat sexual behavior. I: Pattern of 50- and 22-kHz ultrasonic vocalizations

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James G. Pfaus = Study design and manuscript writing

Chapter 3.2. -Effect of aversive clitoral stimulation on female rat sexual behavior. 2: Conditioned partner preference and conditioned partner aversion

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Chapter 4 - Effect of aversive clitoral stimulation on female rat sexual behavior. 3: Fos activation in response to rewarding or aversive clitoral stimulation and paired odor cues.

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Chapter 5 - General Discussion

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All authors reviewed the final manuscript and approved of its contents

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List of Abbreviations

USV: Ultrasonic vocalization

CLS: Clitoral stimulation

FM: Frequency modulated

sUSV: short 22-kHz USVs

lUSVs: long 22-kHz USVs

OVX: Ovariectomy

EB and E2: Estradiol

P and P4: Progesterone

T: Testosterone

FLS: Flank stimulation

VCS: Vaginocervical stimulation

S-CLS: soft-bristle CLS

H-CLS: hard-bristle CLS

US: Unconditioned stimulus

CS: Conditioned stimulus

PTSD: Post-traumatic stress disorder

PMDD: Premenstrual dysphoric disorder

SSRIs: Serotonin reuptake inhibitors

FLU: Fluoxetine

CPP: Conditioned place preference

5-HT: Serotonin

DA: Dopamine

CAF: Caffeine

FSH: Follicle-stimulating hormones

LH: Luteinizing hormones

NC: Non-contact tests

CPaP: Conditioned partner preference

CPaA: Conditioned partner aversion

Sc: Scented cue

UnSc: Unscented cue

ScM: Scented male

UnScM: Unscented male

mPOA: Medial preoptic area

MEA: Medial amygdala

Fos-IR: Fos-like immunoreactivity

VMH: Ventromedial hypothalamus

LH: Lateral hypothalamus

PVN: Paraventricular nucleus of the hypothalamus

nPGi: Nucleus paragigantocellularis

Nac: Nucleus accumbens

VTA: Ventral tegmental area

MeApv: Posteriorventral aspect of the medial amygdala

BNST: Bed nucleus of the stria terminalis

BLA: Basolateral amygdala

Arc: Arcuate nucleus

PAG: Periaqueductal gray

Cg: Anterior cingulate

TBS: Tris-buffered saline

H2O2: Hydrogen peroxide

NGS: Normal Goat Serum

Fos ab5: Rabbit polyclonal anti-Fos

DAB: 3,3′-diaminobenzidine

CeA: Central amygdala

NMS: Neonatal maternal separation

VBD: Vaginal balloon distension

HPA: Hypothalamic-pituitary-adrenal

CHAPTER ONE
GENERAL INTRODUCTION

Although rodents can be used to assess appetitive and aversive states and physiological systems that underlie behavioral responses, and have been used as predictive models for memory, drug addiction, sexual behavior, feeding, thirst, sleep, temperature regulation, etc. in humans, one critique of rodent models is their predictive validity in assessing subjective experiences concurrent with physiological responses in humans. Current conditioning and preference paradigms demonstrate such predictive validity, but they cannot be used to infer immediate awareness of subjective states (Pfaus et al., 2016). Through their ethotransmitter properties, USVs serve as a putative index of immediate arousal-affective states (Pfaus et al., 2016). Recording USVs throughout behavioral paradigms allows rodent models to measure changes in subjective experience, from immediate awareness to long-term affect. Concurrent physiological changes can also be recorded in tandem with additional monitoring equipment, such as fast scan cyclic voltammetry (Koiv, Tiitsaar, Laugus and Harro, 2021; Sanchez et al., 2021) or optic fiber implants (Neugebauer et al., 2022; Tong et al., 2022). Ultrasonic vocalizations have therefore been readily incorporated into preclinical rodent models of human diseases and disorders, such as Parkinson's (Simola et al., 2021; Scattoni, Crawley & Ricceri, 2009), autism spectrum disorder (Caruso, Ricceri, and Scattori, 2020), bipolar disorder (Wohr, 2021; Wendler et al., 2016) ,anxiety disorder (Demaestri, Brenhouse & Honeycutt, 2019), schizophrenia (Potasiewicz et al., 2019).

Our understanding of female sexual dysfunction has rapidly expanded in the last few decades, as new models and criteria have been developed (Agmo & Laan, 2022; Marson & Wesselmann, 2017; Snoeren et al., 2011; Agmo, 2014; Giuliano et al., 2010; Giraldi et al., 2004;

Basson et al., 2004). As knowledge of the human condition increases, existing preclinical rodent models continue to be refined through incorporating relevant behavioral endpoints and/or techniques. Female rats exhibit USVs as part of their sexual behavior repertoire, but these have not been incorporated into current preclinical models of female sexual dysfunction. In this thesis, a rat model of clitoral stimulation and sexual behavior will be used to determine whether USVs reflect a sexual arousal state.

Can we infer emotional states in animals?

Emotions are a universal experience shared by humans and other mammals alike. A majority of animal researchers - especially those studying emotion - adopt an agnostic stance against relating assumed emotional states in animals to humans in order to avoid committing the dreaded scientific sin of anthropomorphism. The current push by animal models to infer emotional states from objective behaviors thus is still subject to fierce criticism. A centuries-old conflation of affective-cognitive processes, nevertheless, underlies today's anthropomorphic avoidance (reviewed in Panksepp, 2011; Ellsworth, 1994). Scholars past and present often consider our higher cortical capabilities part of what makes us uniquely human, of which emotions are a subset (Panksepp, 2011; reviewed in Eder, Hommel, & De Houwer, 2007). It was Willam James (1884) and Carl Lange (1887) who put forth the notion that emotions reflect cortical-cognitive ‘readouts' of peripheral-unconscious body arousals during emergency situations, e.g., fleeting from a sudden encounter with a bear. Namely, sensory feedback of bodily arousal reaches the motor-sensory areas of the cerebral cortex, and as a consequence, emotional feelings are evoked through integration of high-level mental processes (Roxo, Franceschini, Zubaran, Kleber & Sander, 2011; Dalgliesh, 2004; Lange, 1887; James, 1884). James and Lange’s neocortical ‘readout’ hypothesis has led many scholars to the question of

whether other mammals have emotional feelings as most mammals possess less 'cognitive' brain matter than humans. We have since gained a better understanding of the mammalian brain's emotional network and continue to refine our behavioral paradigms, but this concept of emotionality still constrains our belief systems within behavioral neuroscience.

One potential factor contributing to this bind is inconsistent terminology within and across both human and animal literatures. There is considerable confusion as to how to define emotions as well as a lack of consensus regarding their underlying structures (de Vere & Kuczaj, 2016). This is further complicated by the interchangeability of 'emotion' with related terms such as 'affect' and 'mood' (de Vere & Kuczaj, 2016). Throughout this thesis, I will follow Panksepp's (2006) definitions, which differentiates emotion and affect, and incorporates discrete and dimensional characteristics for each. Other authors stipulate that emotion and affect are synonymous (de Vere & Kuczja, 2016; Mendl, Burnam & Paul, 2010; Izard, Libero, Putnam & Haynes, 1993) and/or define emotion strictly based on its discrete (Izard, 2007) or dimensional characteristics (Barrett, 2006).

Panksepp (2011; 2005) defines emotion through the lens of a dual-aspect monism approach. The dual-aspect monism approach asserts that emotional feelings reflect the neurodynamics of brain systems, which generate intrinsic and/or learned emotional behaviors. Namely, emotional feelings alert non-human animals to threats and opportunities within their internal and external environments to motivate avoidance and approach behaviors. An integral part of this process is the coordination of physiological, neurological, and behavioral components with subjective components of consciousness and affect.

While Panksepp (2011;2005) uses emotion as an umbrella term, he categorizes the subjective component of consciousness into three distinct process categories - primary, secondary, and tertiary. Primary processes are a subset of basic or primordial reactions - i.e., fear, anger, joy, sadness, surprise and disgust - that are a result of an innate stimulus-response chain (Panksepp, 2011; 2005). Neuronal circuits located deep within subcortical limbic and hypothalamic regions, i.e., the primal emotional system, are responsible for these unconditioned responses (Panksepp, 2011; 2005). Secondary-processes are anticipatory reactions - i.e., avoidance, anxiety, engagement, and excitement - that arise from the interaction between the immediate environment and the individual's internal feedback loop. This largely involves the interaction of upper limbic and cortical structures with the primal emotional system via learning-conditioning (Panksepp, 2011; 2005). Tertiary processes are the awareness of primary and/or secondary reactions, which includes executive function, emotional regulation, and intention, among others. Awareness functions involve the integration of multiple neocortical structures, which exert top-down control of limbic, hypothalamic, and midbrain structures (Panksepp, 2011; 2005). While primary-processed emotions are nested within the hierarchy of higher emotional brain systems, as shown in **Fig. 1**., awareness facilities are believed to make tertiary emotional processing “uniquely human.” Nevertheless, primary and secondary processes of emotion, which rely on innate and learned responses, are universal across human and non-human animals.

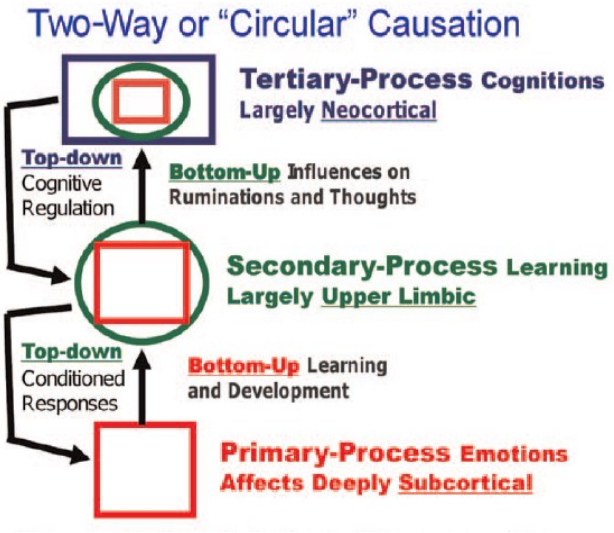

Fig.1. *Summary of the nested hierarchy of the three emotional brain systems. Adapted from Panksepp (2011). Primary-process emotions are depicted as red squares, secondary-process emotions as green circles, and tertiary-process emotions as blue rectangles. The color coding aims to highlight how lower and higher emotional brain systems interact through top-down and bottom-up integration.*

Another subjective component of emotion is affect, which Panksepp (2005) defines as the 'experiential feeling' of an emotion. As with conscious processes, emotional affect is closely linked to brain action states triggered by external and/or internal stimuli present within the body and immediate environment (Frijda, 1986; Ekman, 1992). Additionally, emotional affect can be further classified based in terms of arousal and/or of valence, as represented in **Fig.2**. Arousal is the level of autonomic and central nervous system activation, i.e. feelings of emotional intensity, which underlies emotional elicitation. Valence is the representation of opposing positive and negative states, i.e., feelings of pleasantness vs unpleasantness, within the individual's nervous system.

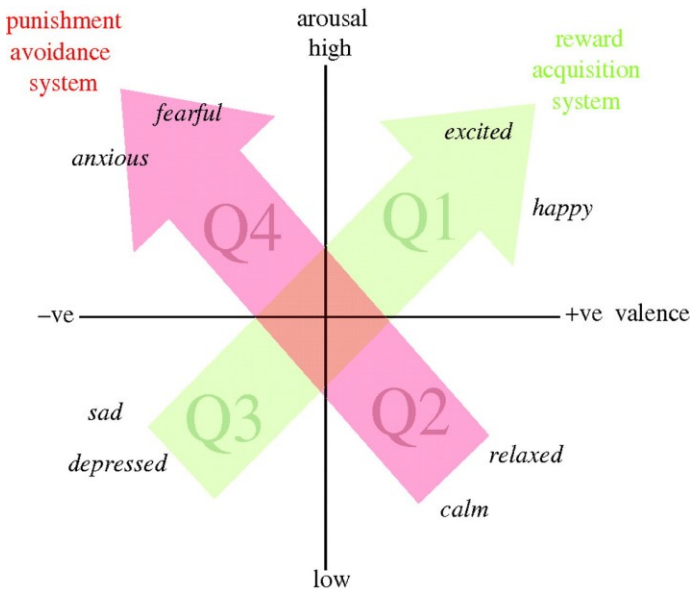

Fig.2. *Two-dimensional representation of affective arousal and valence. Adapted from Mendl, Burman & Paul (2010). Italicized words indicated possible locations for reported affective states and basic emotions. Quadrants Q1 and Q2 represent positive valenced affective states, while quadrants Q3 and Q4 represent negatively-valenced affective states. The green arrow indicates the reward acquisition system whereas the red arrow indicates punishment avoidance system.*

The combined effects of these two characteristics allow affect to enhance fitness through the formation of appropriate behaviors and physiological changes in response to events and/or stimuli (Darwin & Prodger, 1998; Ohman & Mineka, 2001; LeDoux, 2012; Nettle & Bateson, 2012; Trimmer et al., 2013; Bethell, 2015). Positive affect prompts the organism to approach stimuli that promotes fitness, i.e., rewards, whereas negative affect deter the organism to avoid stimuli that threaten fitness, i.e. punishers (Mendl et al., 2010a). Affect also serves as a form of predictive judgment, i.e. anticipation, as well as attentional bias. It enables the organism to generalize to novel situations and to track the occurrence of certain stimuli during situational events. Separation anxiety-prone dogs, for example, behave more cautiously in different

ambiguous situations, thus displaying more 'pessimistic' behavior (Mendl et al., 2010b). Those less prone to separation anxiety behave more 'optimistically' as they are quick to engage approach behaviors despite situational ambiguity (Mendl et al., 2010b). It has therefore been suggested that prior affective experiences influence the valence of future affective responses in both humans (Gripp & Johnson, 2009) and non-human mammals (Mendl et al., 2010b). Prior positive affect, however, is shown to compensate against subsequent negative experiences (Van der Harst & Spruijt, 2007) and/or situations (Reefman et al., 2012).

Studies assessing anticipatory states and/or attentional bias often conflate affect with mood. Although mood is also encompassed within the realm of emotion, it differs from affect in terms of duration and stimulus specificity. Affect is a short yet intense reaction to external and/or internal environmental stimuli; Direction towards a particular event and/or stimulus involves primary and/or secondary processes of emotional consciousness (Frijda, 1986). Moods are the accumulation of affective experiences thereby representing their valence and their level of arousal over time. They reflect one's fluctuating baseline state or general disposition, i.e., whether one is positively or negatively inclined to evaluate fitness (Mendl et al., 2010a; Nettle & Bateson, 2012; Paul et al., 2011; Nesse, 2001; Nesse 2005). Unlike affect, mood occurs without the direction of an event and/or stimulus (Russell, 2003; Trimmer et al., 2013).

The 'free floating' quality of mood falls under tertiary processes of emotion as it is directed towards executive functioning and emotional regulation as opposed to a stimulus (Rusell, 2003). It is through influencing higher cognitive functioning that future affective reactions are influenced (Rusell, 2003), creating a causal bidirectional relationship between the two emotional terms (Mendl et al., 2010a).

Human emotion can therefore be studied using animal models without being inherently anthropomorphic. Clearer operational definitions of emotional terms allows researchers and scholars alike to determine what aspects of emotionality are universally shared, i.e. affect, from those that may cross into the realm of distinctly human, i.e., moods. Primary processes are the most comparative variant of emotional consciousness to assess in animals due to shared intrinsic brain functions. While harder to study directly, secondary processes - and in some rare instances tertiary processes - of emotional consciousness can be sufficiently studied through behavioral learning procedures. By making such distinctions, we can refine our current animal models of emotion, allowing us to gain a deeper understanding of core emotional experiences.

The importance, production, and classification of vocal affect

Affect can be measured objectively in terms of behavioral outcomes, along with the underlying neurological and physiological dynamics of those outcomes. Affect can also be measured by the emotional vocalizations of animals, which have been shown to be highly translatable to the human condition (Burgdorf & Panksepp, 2006; Burgdorf, Wood, Knoes, Moskal & Panksepp, 2007; Brudynski, 2009; Panksepp, 1981; Knutson, Burgdorf & Panksepp, 2002; Panksepp, Knutson, Burgdorf, 2002). A wide variety of animals have been studied for their emotional vocalizations, but rats are the most commonly used preclinical animal model. Rats emit emotional vocalizations at ultrasonic frequencies, known as ultrasonic vocalizations (USVs), through a complex orchestration of respiratory, laryngeal, and vocal tract movements (Riede, 2018). A whistle-like vocalization is created by airflow passing through the tracheal tract, and augmented by air pressure within the subglottal space and by constriction of intrinsic laryngeal muscles (Riede, 2018, 2013, 2011; Håkansson et al., 2021; Kober, Datta, Goyal & Benecchi, 1994) as shown in **Fig.3**. The intrinsic laryngeal muscles regulate the subglottal space

by elongating its geometry and shortening or closing the vocal cords (Kobler et al., 1994). Ultrasonic vocalization subtypes are determined by the configuration of the vocal cords via laryngeal muscle constriction (Kobler et al., 1994). Although human speech takes place below the ultrasonic range, rat vocal cords are shown to be anatomically similar (Inagi, Schultz & Ford, 1998; Toya et al., 2014) and may exhibit similar sexual dimorphic differences (Tatlipinar et al., 2011; Feng, Zhang, Ralston & Ludlow, 2012; Lenell & Johnson, 2017; Kim et al., 2020).

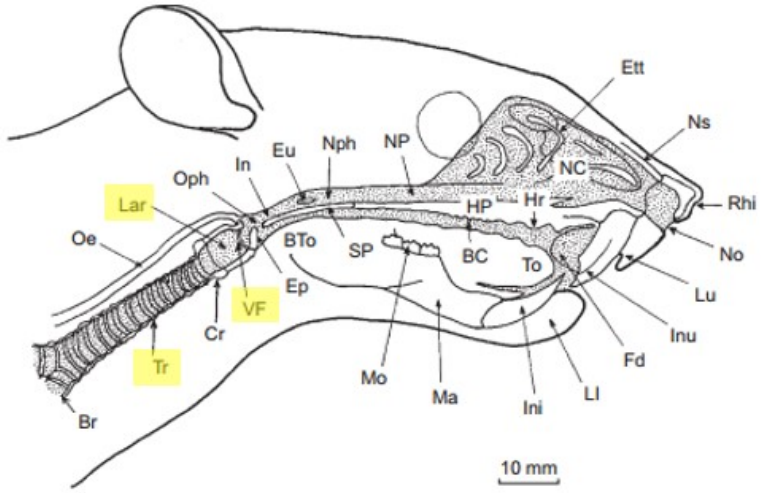

Fig.3. *Cross-section of an adult male Wistar rat demonstrating selected head and neck structures (stippled). Adapted from Brudzynski (2010). Abbreviations are highlighted in yellow: Lar: larynx; Tr: trachea; VF: vocal folds.*

Appetitive and aversive arousal can be objectively inferred by the main acoustic parameters of emitted USVs (Brudzynski, 2021; Burgdorf et al., 2019; reviewed in Burgdorf, J., Panksepp, J., & Moskal, 2018; Burgdorf, Kroes & Moskal, 2017). Adult USVs are classified into the two call categories of opposing affective valences, 50- and 22-kHz calls. These two call categories differ by 2-10 fold across acoustic parameters of frequency, duration, and bandwidth, i.e. frequency change (Brudzynski, 2007). Fifty kHz USVs range from 35-70 kHz (Wintink &

Brudzynski, 2001) and, on average, are shorter in call duration, 5 to 150 msec (Sales, 1972). The emission of 50-kHz USVs are produced by high amplitude bursts of intrinsic laryngeal muscle activity (Riede, 2011). Twenty-two kHz USVs range from 18-32-kHz (Sales & Pye, 1974) and have a varied call duration ranging from 10 to 3000 msec (Sales, 1972). Unlike 50-kHz USVs, 22-kHz USVs are the result of tonic intrinsic laryngeal muscle activity (Riede, 2011). Fifty kHz USVs show rich patterns of frequency change (3-50kHz; Wright, Gourdon, & Clarke, 2010) whereas these change patterns tend to be absent for most 22-kHz USVs (1-5 kHz; Sales & Pye, 1974). A zero overlap in sound frequency is evident between two call categories, while sound duration overlaps 0.75% and bandwidth overlaps 48%**.** Although sound frequency differences are sufficient to distinguish calls, accurate discrimination requires the combination of all acoustic parameter differences (Saito, Tachnibana & Okanoya, 2019; Brudzynski, 2007). The main acoustic parameters of 50- and 22-kHz calls are thereby distinct, and thus their associated valence of arousal is recognized unambiguously by the recipient and the emitter.

Acoustic parameters offer a clear indication of the arousal dimensions of affective states, but not their concurrent hedonic elements. Hedonic elements instead are believed to correspond to the syllabic features of USVs. Syllabic features are patterns of peak frequency changes in one or more spectral elements in a particular ultrasonic call. Call categories of 50- and 22-kHz differ in their syllabic features and can be further divided into subtypes as shown in **Fig.4**. Broad call categories correspond to opposing hedonic states, but their emission represents the concurrent shifts in arousal required for the induction of the immediate hedonic state and its associative learning (Brudzynski, 2021). Therefore, the emission patterns of USV subtypes can be used to identify the type of immediate hedonic state, e.g., social reward/aversion vs. sexual reward/aversion.

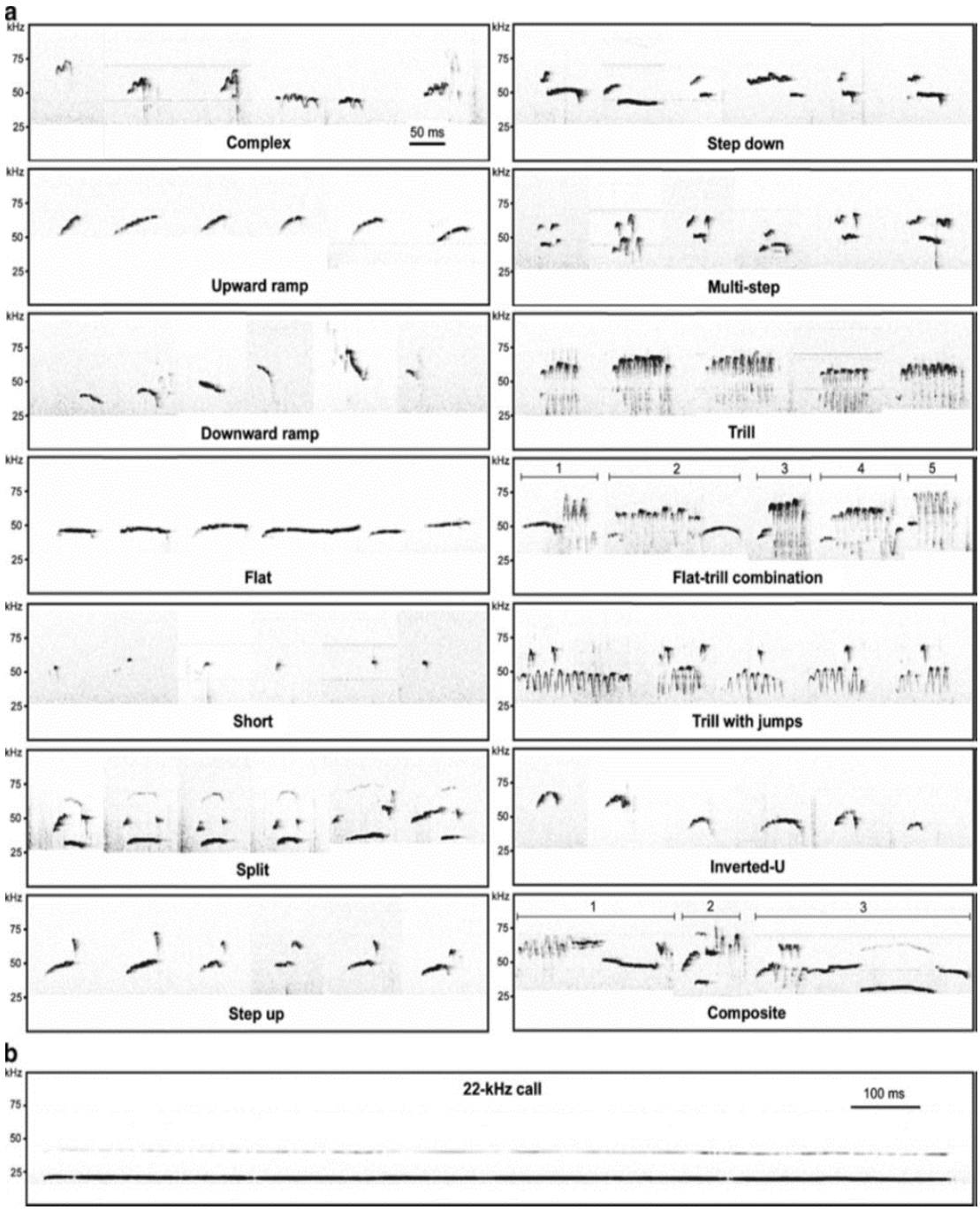

Fig.4. *Representative spectrograms for each of the 14 categories of 50-kHz USVs (a) and for 22-kHz USV (b). The top left panel shows the time scale for all 50-kHz calls.*

Fifty kHz USVs consist of frequency-constant (flat) and frequency-modulated (FM) syllabic features (Burgdorf, Panksepp, & Moskal, 2011; Wright, Gourdon, & Clarke, 2010). Flat

50-kHz calls are monotonic and do not possess distinct call subtypes. It has been proposed that flat 50-kHz USVs represent non-affective social calls that signal social coordination (Burke et al., 2017; Burgdorf et al., 2008; Wohr & Schwarting, 2008; Wohr, Houx, Schwarting & Spruijt, 2008) and social transmission of feeding behaviors (Monfils & Agee, 2019; Schweinfurth & Taborsky, 2018; Takahashi, Kashino & Hironaka, 2010). Rats are also reported to emit flat 50-kHz calls prior to aggressive interactions (Panksepp & Burgdorf, 2003; Burgdorf et al., 2008), and thereby suggested to signal social arousal (Burgdorf et al., 2008). Frequency-modulated 50-kHz calls, in contrast, have complex syllabic structures and can be further categorized into 14 distinct call subtypes Wright, Gourdon, & Clarke, 2010) as shown in **Fig.4.** Frequency modulated 50-kHz calls represent affective social calls that signal positive affect during rewarding social interactions, such as rough-and-tumble play, tickling and sexual behavior (Burgdorf et al., 2008). Rats also emit FM 50-kHz calls in response to natural (Burgdorf et al., 2018) and drug rewards (Taracha et al., 2014; Meyer, Ma & Robinson, 2012), and therefore this call type is seen as an indicator of hedonic reward. Of the 14 FM call subtypes, it is suggested that the trill subtypes as shown in **Fig.5**. indicate the induction of a hedonic reward state (Burgdorf et al., 2008, 2010; Wright et al., 2010, 2012). We and others have found that female rats emit these FM trill subtypes at a higher peak frequency than males (Gerson et al.,2019; Lenell and Johnson, 2017).

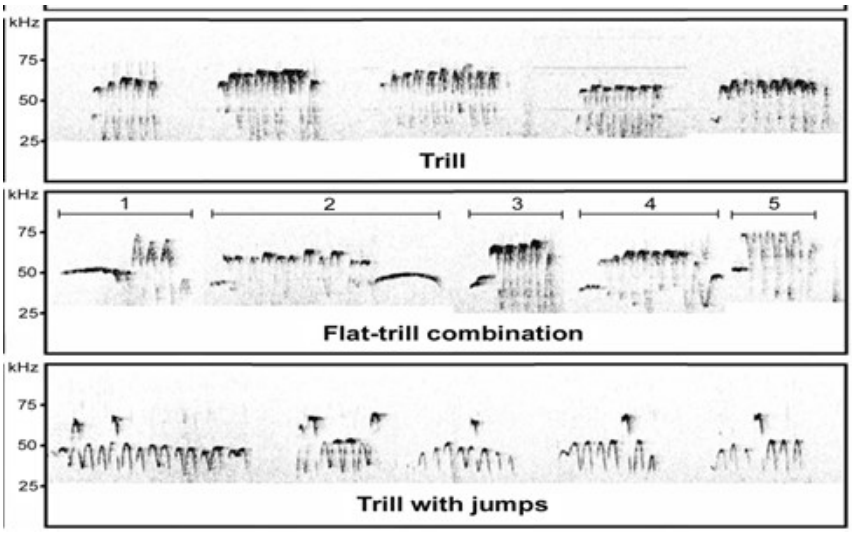

Fig. 5. *Spectrograms of FM 50-kHz of the trill subtypes.*

Unlike FM 50-kHz calls, 22-kHz USVs consist of discrete monotonic calls that signal social avoidance (Schweinfurth, 2020; Kisko, Wöhr, Pellis, Pellis, 2017; Assini.; Sirotin,& Laplagne, 2013; Vivian & Miczek, 1993a; Takahashi; Thomas, Barfield, 1983), social detachment (Bialy et al., 2016; Sachs & Biały, 2000; van der Poel & Miczek, 1991; McIntosh, Barfield & Thomas, 1984; Geyer & Barfield, 1980), and states of negative affect (Knutson et al., 2002a; Knutson et al., 2002b). Due to their monotonic call structure, 22-kHz USVs were previously categorized based on their duration length as short (sUSVs) or long (lUSVs). Short 22-kHz calls are 10-500 ms in duration (Simmons, Barker, & West, 2018; Barker et al., 2010) and are emitted in response to aversive situations/stimuli that pose no external threat to the wellbeing of the rat. In the absence of an external threat, short 22-kHz calls are assumed to reflect an internal dysphoric state, irritation, and/or displeasure (Simmons, Barker & West, 2018). It has also been suggested that short 22-kHz represent frustration-induced anxiety triggered by limited access to drugs of abuse or natural rewards (Biały et al., 2019; Taylor et al., 2019). Long 22-kHz calls are 300 to 3000 ms in duration (Brudzynski & Holland, 2005) and, in

contrast to short 22-kHz calls, are emitted when a direct external threat is present (Kisko, Wohr, Pellis & Pellis, 2017; Bali & Jaggi, 2015; Assini, Sirotin & Laplagne, 2013; Blanchard, Blanchard, & Griebel, 2005; Blanchard, Blanchard, Agullana & Weiss, 1991). One exception to this is the long 22-kHz call emitted during states of satiety such as the post-ejaculatory interval (Barfield & Geyer, 1972; Bialy et al., 2016). Post-ejaculatory calls often possess a downward deflection that likely signals behavioral inhibition rather than aversion (Burgdorf et al., 2008; Sach & Bialy, 2000; van der Poel & Miczek, 1991; Anisko, Suer, McClintock & Adler, 1978; Barfield & Geyer, 1972). Aside from post-ejaculatory calls, recent evidence shows that males in a state of sexual frustration will emit a variety of FM 22-kHz calls (Vivian and Miczek, 1993a; Vivian and Miczek, 1993b; Biały et al., 2019) as shown in **Fig.6**.

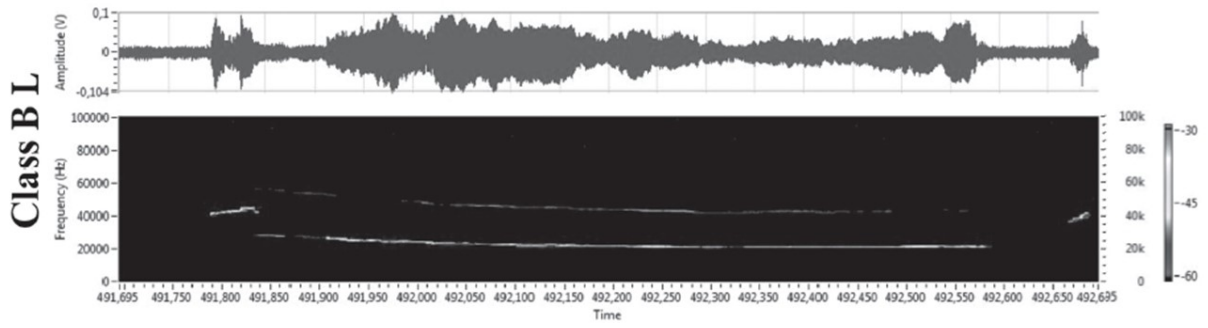

Fig. 6. *Spectrogram example of 22-kHz lUSV subtype, Class B L. Class B L calls consist of a long monotonic split with a flat and/or upward prefix. Class B L is associated with states of sexual frustration according to Bialy et al., 2019.*

` This FM subtype was shown to contain a high-frequency prefix and/or suffix to the monotonic portion of the call (Vivian and Miczek, 1993a; Vivian and Miczek, 1993b; Biały et al., 2019). Males tend to emit 22-kHz calls more consistently than females (Laine et al., 2022). However, 22-kHz distress calls[are emitted by female rats during Pavlovian fear learning and

cued fear extinction (Tryon et al., 2021; Laine et al., 2022). The phenomenon of females emitting lUSVs with a similar pattern as 22-kHz distress calls remains unexplored.

Taken together, 50- and 22-kHz USVs represent opposing states of affect in the rat. This hedonic hypothesis was challenged by non-hedonic interpretations of the available 50-kHz USV data (discussed in Burgdorf et al., 2011). Non-hedonic interpretations instead posited that 50-kHz emission instead represented a non-affective contact calls (Schwarting, Jegan & Wohr, 2007), a non-positive affective wanting state (Schwarting et al., 2007), and/or a non-positively valenced state of arousal (Bell, 1974). These interpretations were based on reports that adult rats emitted 50-kHz USVs prior to aggressive encounters (Berridge, 2003), and during drug extinction bursts (i.e., frustrative non-reward; Burgdorf et al., 2000) or in response to highly arousing stimuli (Bell, 1974). However, the increasing prevalence of call subtype analysis of USV data arguably supports the hedonic hypothesis. The majority of 50-kHz USVs emitted prior to aggression, for instance, were reported to be of the flat variety (Panksepp & Burgdorf, 2003; Burgdorf et al., 2008). Hedonic interpretations of USVs are also supported when call subtype analysis is used to examine arousal shifts, i.e. the rate of 50 kHz calls vs. 22-kHz calls. For example, during extinction bursts, frustration based appetitive behaviors decrease rates of 50-kHz USVs and increase rates of 22-kHz calls (Burgdorf et al., 2000). Highly arousing aversive stimuli invokes a similar USV pattern whereas rewarding stimuli are reported to increase 50-kHz USVs as 22-kHz calls decrease (Knutson et al., 2002a,b). The combination of call subtype and arousal shift analysis, therefore, should be continued to be utilized for the proper interpretation of USVs. This especially holds true for situations involving drug and natural reward as they arguably involve multiple arousal shifts, from anticipation to consummation to satiety. While a majority of drug studies have adopted this approach, call subtypes and/or arousal shifts have

been assessed by only a few USV studies assessing sexual behavior (Bogachi-Kchlik, Rolf, and Bialy, 2021), sexual reward (Gerson et al., 2019), and sexual aversion (Bialy et al., 2019).

Rats as an animal model of human sexual behavior

The rat remains an invaluable model for studying a variety of human behaviors and diseases, assuming that the right questions are asked (Pfaus, Coria-Avila, and Rodríguez-Manzo, 2023). Thus, rat models have undergone incremental refinements to their conceptualizations, behavioral paradigms, and experimentation techniques over the last decade. There has been a recent global change in scientific culture that normalizes the use of female rats in behavioral neuroscience and as preclinical models (Bangasser & Cuarenta, 2021; Shansky & Murphy, 2021; Rechlin et al., 2022). For most preclinical models, it is essential to determine which behavioral repertoires are biased by sex and whether these biases are accurately measured (Bangasser & Cuarenta, 2021; Shansky & Murphy, 2021; Rechlin et al., 2022). Such a determination, naturally, is crucial for preclinical models of female sexual function and dysfunction. Female rodent sexual behavior is often assessed using copulatory interactions as the basis for the preclinical model and has been shown to be influenced by the male's sexual vigor or sluggishness (Afonso & Pfaus, 2006; Beach, 1968). Similar to studying affective outcomes, behavioral outcomes can also be understood by clearly defining their parameters.

Sex is a cascade of behavioral events for all mammals, including humans, which can be separated into appetitive and consummatory phases (Beach, 1976; Pfaus, 1999). Appetitive behaviors bring animals into close proximity with goal incentives, e.g. potential sex partners, or goal objects, e.g. natural or drug rewards (Beach, 1976). Appetitive sexual behaviors in female rats also include solicitations that initiate and impose an interval between sexual interactions

with a male (Beach, 1976; Erskine, 1985; McClintock, 1984). Female rats' appetitive behaviors, i.e. solicitations, hops and darts, and ear wiggling, have been conceptualized by researchers as indicators of sexual motivation (Pfaus, 1999), and have been shown to accurately predict the effect of drugs on sexual desire in human females (Gelez et al., 2013; Pfaus Giulinao, and Gelez, 2007) . Consummatory behaviors, on the other hand, occur when the animal is in direct contact with the goal incentive or goal object (Beach, 1976; Pfaus, 1999). These tend to be sexually stereotypical and dimorphic compared to the behavioral flexibility of appetitive sexual behaviors. Lordosis, i.e. a postural reflex with dorsiflexion of the vertebral column, is a key consummatory behavior used to measure sexual receptivity (Beach, 1976) and sexual reward (Coria-Avila et al., 2008) in female rats.

Appetitive and consummatory behaviors of female rats are homologous to those that comprise the human sexual response as its distinct theoretical phases fits a similar appetitive and consummatory framework (Pfaus, 1999). Sexual desire, motivation to engage in sexual fantasy and/or sexual activity, initiates the human sexual response in both men and women (Kaplan, 1980; Masters & Johnson, 1966). Increases in sexual desire may elicit sexual excitement, increased physiological arousal and genital blood flow, followed by a plateau, or parasympathetic maintenance of blood in genital and other erogenous erectile tissues (Kaplan, 1980; Masters & Johnson, 1966). After receiving sufficient sexual stimulation, climax and orgasm (euphoric pleasure co-occurring with sympathetic activation) may occur, and the fleeting euphoric state transitions into a temporary state of resolution, a reduction in the salience of external and somatosensory sexual stimuli that characterize an inhibitory refractory period (Georgiadis, Kringelbach, and Pfaus, 2012; Kaplan, 1980; Masters & Johnson, 1966; Pfaus, 2009). Often the aforementioned phases are cyclical for women as they are strongly modulated

by internal motivational and affective states (Basson, 2005; Laan et al., 1994) as well as hormonal states (Nappi et al., 2003). Some women report experiencing spontaneous innate desire (induced by sex steroid fluctuations), activating behavioral receptivity and attentional focus on incentive sexual stimuli. In turn, this increases overall sexual arousal and responsive desire (induced by sexual incentive stimuli, physical interaction, and sexual touch; Basson 2005). Sensitization occurs if this is positively reinforced with sexual and emotional pleasure and satisfaction, increasing the willingness to engage in subsequent sexual activity in the presence of incentive sexual cues. This is akin to behavioral outcomes outlined in sexual incentive and arousal models in rats (Toates, 2009; Pfaus, 1999). Female rats, for instance, will frequently solicit and receive selective ejaculations from males bearing a positively reinforced odor (Coria-Avila et al., 2005, 2006; Parada et al., 2010) or somatosensory cue (Quintana et al., 2013).

Besides homologous sexual behavior, female rats are also anatomically similar to women with both species having regular estrous cycles, identical clitoral structure (consisting of an externally visible clitoral glans and an internal cruc and corpus cavernosum, two cylindrical and sponge-like erectile bodies (Martin-Alguacil, Pfaff, Shelley, & Schober, 2008), and a cervix that when stimulated produces analgesia and estrous termination (inhibition, satiety, and longer refractory periods (Crowley et al., 1976; Whipple and Komisaruk, 1985, 1988; Mac Cionnaith et al., submitted). The physiology of these erogenous structures in both female rats and humans are altered by their internal hormonal milieu (Hall, 1983; Min et al., 2001; Park et al., 2001; Yoon et al., 2001; reviewed in Giraldi et al., 2004). Estrogens and androgens are integral to the maintenance of clitoral structure and the circulation of regional blood flow (Korenchevsky, Dennison, and Hall, 1937; Park et al., 2001). Ovariectomy, i.e. removal of the ovaries which eliminates circulating estrogens, androgens, and progestins, leads to atrophy of genital and other

erogenous structures and decreases the production of vaginal lubrication (Min et al., 2001). A sufficient dose of estradiol or testosterone can, however, reverse this surgical menopausal state (Korenchevsky, Dennison, and Hall, 1937; Yoon et al., 2001).

Female rats and women differ in their dependence on hormonal states despite their anatomical similarities. Female rats engage in sexual behaviors during the periovulatory states of proestrus and estrus, whereas women engage in such behaviors all through their ovarian cycle. Fluctuations in estradiol and testosterone are, however, reported to increase the occurrence of spontaneous innate desire (Nappi et al., 2003; Stanislaw & Rice, 1988; Zuspan & Zuspan, 1979) and the frequency of orgasm in women (Cutler, Garcia, & McCoy, 1987; Dennerstein, Burrows, Wood, & Hyman, 1980; Matteo & Rissman, 1984; Puts et al., 2012; Udry & Morris, 1968, 1970, 1977). Women experience increases in both phases of their sexual response in the periovulatory period when levels of estradiol begin to fall from their peak, and the pulse of testosterone peaks (Nappi et al., 2003; Stanislaw & Rice, 1988; Cutler, Garcia, & McCoy, 1987; Dennerstein, Burrows, Wood, & Hyman, 1980; Matteo & Rissman, 1984; Pfaus et al., 2015; Puts et al., 2012;Zuspan & Zuspan, 1979; Udry & Morris, 1968, 1970, 1977). This positive correlation suggests that estradiol and testosterone set the stage for these appetitive aspects of sexual behavior in women to align sexual motivation with ovulation.

As mentioned above, sexual behaviors of female rats are dependent on hormonal control during the ovulatory cycle. Female rats ovulate every 4 to 5 days, and this occurs during the evening of proestrus and morning of estrus (Beach, 1976). Behavioral estrus is thus defined as a hormonally dependent state of sexual receptivity in which females will permit the male to copulate. In the absence of behavioral estrus, copulatory attempts by males will be antagonistic to females, resulting in displays of escape-related rejection responses, e.g. kicking, biting,

boxing, and fleeing (Barnett, 1963). The full expression of the female's sexual behavior repertoire, or estrus behaviors, depends on the fluctuations of ovarian sex steroids, estradiol, testosterone, and progesterone. Estradiol levels rise sharply during diestrus and peak the morning of proestrus, before ovulation. Testosterone is released from the ovaries as a pulse that coincides with ovulation. Once ovulation occurs, the ruptured follicle, known as the corpora luteum, secretes progesterone, and the combined actions of estradiol, testosterone, and progesterone lead to behavioral estrus 4 to 6 hours later. By administering estradiol benzoate and progesterone to OVX rats, the timing of the estradiol and progesterone peaks can be mimicked and induce full approach, solicitations, and lordosis as observed in gonadally intact females(Moreines & Powers, 1977; Pfaus, Smith, & Coopersmith, 1999; Powers, 1970; Schwartz & Talley, 1965; Södersten & Hansen, 1977). Hormone-induced estrus in OVX rats thus permits experimental control over the timing of estrous behaviors. Besides its practical applications, many preclinical models use this procedure to investigate how sex steroids affect sexual function and/or mimic various hormonal conditions prevalent in women with sexual dysfunction.

Female rat sexual behavior and vocalizations

The incentive value of female vocal behavior is commonly studied within the context of a male partner or conspecific. Thomas and Barfield (1985) first identified that female rats emit 50-kHz USVs during mating that are spectrally similar to those produced by their male partners. Prior to this study, it was generally believed that female rats vocalized less than male rats during copulation. The rate of female vocalization has yet been shown by several studies (Thomas and Barfield, 1985; Lenell and Johnson, 2021; Matochik, White, Barfield, 1992; Gerson et al., 2019) to be controlled by ovarian hormones, and in turn the estrus cycle. Vocalization rates and peak frequencies of 50-kHz USVs tend to be the highest during diestrus and proestrus and the lowest

during estrus and metestrus (Thomas and Barfield, 1985; Lenell and Johnson, 2021; Matochik, White, Barfield, 1992). During diestrus, proestrus, and estrus - phases of the estrus cycle when ovarian hormones and sexual receptivity peak - duration and frequency modulation of 50-kHz USVs also tend to be the greatest (Lenell and Johnson, 2021). When the estrus cycle is eliminated via OVX, fewer 50-kHz USVs are produced during mating (Thomas and Barfield, 1985; Lenell and Johnson, 2021; Matochik, White, Barfield, 1992), but their acoustics remain the same to those of age matched controls across the estrous cycle (Lenell and Johnson, 2021). Adequate hormonal priming with EB and P, but not EB or P alone, can counteract OVX-induced reductions in 50-kHz USV rates (Matochik, Barfield, and Nyby, 1992; McGinnis and Vakulenko, 2003). Estradiol or P alone, however, does not counteract this reduction (Matochik, Barfield, and Nyby, 1992).

Male partners pose a unique challenge to assessing the incentive value of female USVs directly during mating, in addition to those posed by hormonal influences. It has been shown that attempts to 'silence' one partner of a mating pair through devocalization affects the sexual performance of the other partner (Thomas, Howard, & Barfield, 1982; Thomas, Talalas, & Barfield, 1981; White and Barfield, 1990).Solicitations, lordosis, and the timing interval between them allow the females to control the rate of copulatory stimulation received from the male partner, i.e., clitoral (CLS), flank (FLS) and vaginocervical (VCS) stimulation (Paredes and Alonso, 1997; Paredes and Vazquez, 1999; Pfaus et al., 2012). Clitoral stimulation induces pleasure, FLS induces lordosis, and VCS results in a faster termination of estrus as well as the induction of reproductive neuroendocrine reflexes which result in nightly prolactin surges and the maintenance of pregnancy (if gonadally intact and inseminated) or pseudopregnancy (if

gonadally intact and not inseminated). All or any of these aspects of copulatory stimulation could thereby result in modulating female USV rates and acoustics.

Female solicitation behavior such as darting is also shown to be facilitated by male mating calls known as pre-ejaculatory 50-kHz USVs (Thomas, Howard, & Barfield, 1982; Thomas, Talalas, & Barfield, 1981). Vocally intact females darted less frequently near devocalized males (Thomas, Howard, & Barfield, 1982; Thomas, Talalas, & Barfield, 1981) and were less likely to remain immobile during their mounting attempts (White and Barfield, 1990). Compared to vocally intact females, devocalized females dart more during mating (White and Barfield, 1987; White and Barfield, 1989). Devocalization, according to some researchers (Agmo and Snoeren, 2015; Snoeren and Agmo, 2013), does not alter copulatory behaviors, thus providing no incentive to either mating partner. Yet, it seems reasonable to hypothesize that vocalizations serve as a proceptive cue that facilitate copulatory behavior of both the female (self-regulating) and the male (solicitation of sexual behavior) mating partner.

Distributed CLS and sexual reward

As mentioned above, CLS is normally experienced by female rats during copulation with males, as the pelvic thrusting during penile intromission results in direct contact with the external clitoral glans (Pfaff, Montgomery, & Lewis, 1977). If female rats are allowed to control or "pace" the initiation and rate of copulation, the CLS - and potentially also cervical stimulation - they receive is unambiguously rewarding and induces conditioned place and partner preferences (Coria-Avila et al, 2005; Martínez and Paredes, 2001; Parada et al., 2010; Paredes & Alonso, 1997). If females cannot pace the copulation, this stimulation no longer induces those preferences. External CLS can also be applied manually to the clitoral glans using a soft natural

fiber paint brush, i.e. camel hair, that mimics the paced (distributed) or unpaced (continuous) stimulation females receive during copulation in different contexts (Parada et al., 2010; 2011, 2013). Distributed CLS that mimics paced copulation induces both conditioned place and partner preferences (Parada et al., 2010; 2011).

A pilot study discussed in Pfaus et al. (2016) demonstrated that manual distributed CLS applied by a soft-bristle #4 camel hair paintbrush increased the emission of trill and flat-trill subtypes as shown in **Fig.7.** Trill subtypes, as previously mentioned, are associated with reward. Female FM USVs were found only when the females were primed with estradiol and progesterone in our pilot study. This suggests that soft-bristle distributed CLS may elicit calls that reflect sexual reward, and are a hormonally-dependent sexual response in females rats.

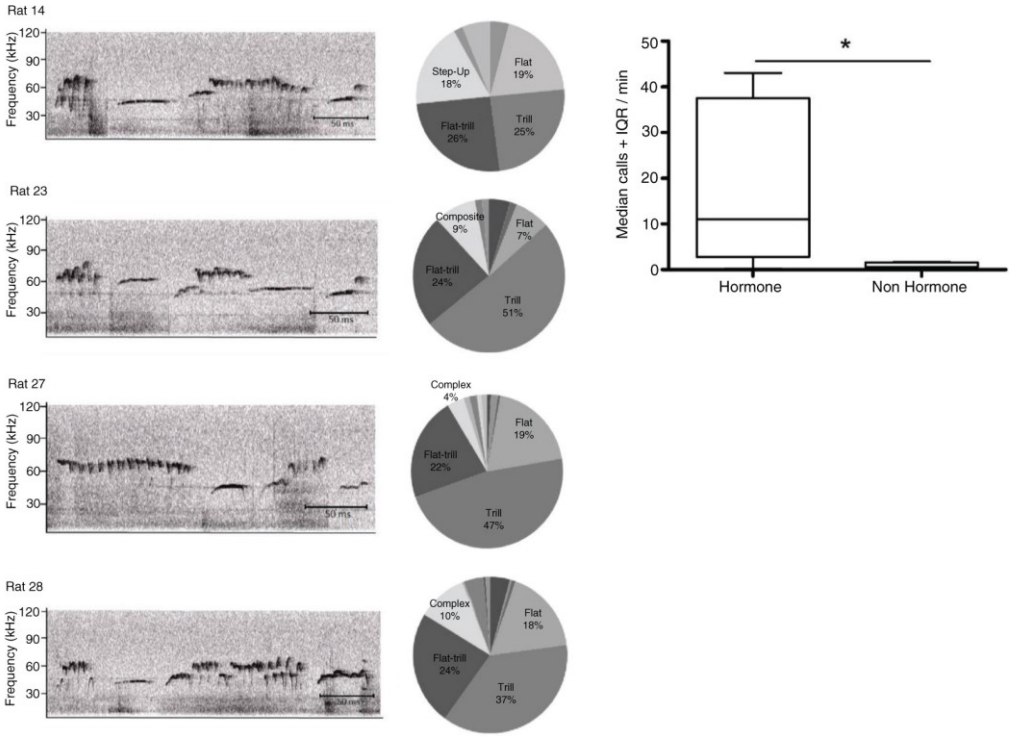

Fig.7. *Adapted from Pfaus et al. (2016). The ultrasonic vocalizations made by female rats in response to distributed CLS. Raw calls are shown on the left while the middle depicts the proportion of total calling during distributed CLS, and the right depicts median calls in females*

*that receive full hormone priming with estradiol benzoate and progesterone versus no hormone (oil vehicle). *P<0.01.*

Changing the tactile sensation of distributed CLS was later found to augment its rewarding properties. By switching paintbrush bristles from soft-camel hair to hard-hog hair, females were observed in a later pilot to display aggressive responses towards CLS despite its delivery in a distributed manner. These rejection responses were likely induced by clitoral pain from the hard bristle brush. Thus, this observation suggested that distributed CLS could be made sexually aversive and/or sexually frustrating through a simple change of its tactile sensation. Preliminary data from this later pilot indicated that hard-bristle distributed CLS augmented the emission of FM vocalizations and elicited a few 22-kHz lUSVs. Distributed CLS delivered with soft-bristle brushes is herein referred to as soft-CLS (S-CLS) and distributed CLS delivered with hard-bristle brushes is referred to as hard-CLS (H-CLS).

Dyspareunia in rat models: what can we anticipate?

Dyspareunia is genital pain typically expressed by women who find tactile stimulation of the vagina and related structures to be painful. It arises due to physical (e.g., lichens sclerosis) and/or psychosexual (anticipation of pain) causes. Acute onset of dyspareunia often suggests a psychosexual cause, whereas gradual pain symptoms indicate physical causes (Lee, Jakes, Llyod & Frodsham, 2018). Although dyspareunia can occur in men, it affects approximately 53% of adult women during their lifetime (Wu et al., 2014). Women experiencing dyspareunia may have localized pain in the vagina, clitoris, or labia, or generalized pain spread through their genito-pelvic region. Symptoms of dyspareunia can be categorized into three types: deep, vaginal, and superficial. Current rodent models in female rats have focused on modeling deep and vaginal

dyspareunia (Farmer, Binik & Mogli, 2009), while superficial (external, tactile) dyspareunia, like clitorodynia, has yet to be explored.

Female dyspareunia models continue to focus on the physiology of nociception (Farmer, 2018). The affective dimension of genital and non-genital pain, i.e. unpleasantness, has only been assessed by one mouse study in terms of sexual motivation (Farmer et al., 2014). Female sexual behavior, however, is shown to be subject to inaccurate interpretations based on standard behavioral paradigms of sexual motivation that are biased toward viewing male behavior (Heijkoop, Huijens & Snoeren, 2017; Pfaus et al., 2016). Heijkoop, Huijens, and Snoeren (2017) suggest that accuracy can be increased by measuring all aspects of the female's sexual behavioral repertoire, i.e., paracopulatory and copulatory. The ideal evaluation of this repertoire would be either independent of the male's performance (which has drawbacks in that the male's response to females often is indicative of her hormonal or motivational state; see Pfaus & Pinel, 1989; Pfaus, Smith & Coopersmith, 1999), or in situations that allow the female to regulate the initiation and rate of copulatory contact (Heijkoop, Huijens & Snoeren, 2017; Paredes & Alonso, 1997; Paredes & Vazquez, 1999; Pfaus, Smith, & Coopersmith, 1999). By the same token, USVs are an aspect of the rat's sexual behavioral repertoire as they signal shifts in arousal and hedonic reward throughout the copulatory period. Hence, when paired with appropriate behavioral paradigms, the analysis of USVs could also increase interpretation accuracy.

Learning is another aspect of dyspareunia that has yet to be properly incorporated into current animal models. Anticipatory models in women propose that the initial experience of pain and/or unpleasantness leads to pain-related anxiety, i.e., anticipation, in novel sexual situations. Pain anticipation has been shown to decrease sexual arousal and vaginal lubrication (Brauer, ter Kuile, Janssen & Laan, 2007) and to increase pelvic floor muscle tone and tightening of the

vaginal entrance (Van der Velde, Laan, & Everaerd, 2001; Both, van Lunsen, Weijenborg,, & Laan, 2012). Accordingly, the likelihood of pain is thereby increased during attempted penetration, which reinforces pain anticipation and avoidance behavior (Thomten & Linton, 2013).

Humans and rodents possess analogous neural systems of nociception to detect potential threat or injury (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). It seems likely that the capacity for adaptive associative learning had multiple fitness benefits for a variety of species from ancestral to modern humans and laboratory rodents, (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). However, natural selection seldom results in *optimal* solutions to problems of survival and reproduction. Through classical conditioning, maladaptive conditioned responses can sometimes be acquired along with potentially adaptive responses (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). Anticipation is an example of such an occurrence. As mentioned prior, anticipation refers to the tracking of certain internal/external stimuli within and/or around the organism during a situation. As a result, behavioral indicators of affect are generalized to novel situations. An anticipatory response is conditioned after repeated pairings of the US with neutral stimuli during sexual encounters. A once appetitive sexual stimulus, such as a caress from a partner, now elicits anticipatory defensive responses and decreases sexual arousal.

Could an analogous anticipatory response be modeled in the female rat? Recent preclinical studies utilizing Pavlovian fear learning and extinction offer insights into what an analogous anticipatory response might entail (Laine et al., 2022; Lovick & Zangrossi, 2021; Tryon et al., 2021; Machado Figueiredo et al., 2019). During Pavlovian fear learning, rodents are exposed to an aversive US, typically a noxious or a stressful stimulus (e.g., footshock), in conjunction with neutral stimulus -- one that initially elicits only an orientating response when

first encountered. The conditioning procedure involves repeated and paired presentations of the initially-neutral stimulus followed by the aversive US. As a result of such conditioning, the initially-neutral stimulus acquires the capacity to illicit new responses, which are referred to as conditioned responses (CRs). After conditioning, the previously-neutral stimulus is referred to as a conditioned stimulus (CSs). When conditioning involves an aversive US, conditioned responses such as freezing and active avoidance typically occur in response to presentation of a CS. As the neutral stimulus comes to predict the aversive US, the newly formed CS prompts rats to display defensive. Rats will also emit distress USVs alongside these CS elicited behaviors as well as during unconditioned behaviors (Nunes et al., 2005; Koo, Han, Kim, 2004; Sanchez, 2003; Brudzynski & Chiu, 1995; Miczek, Weerts, Vivian, Barros, 1995; Vivian, Farrell, Sapperstein & Miczek, 1994). Distress USVs have been suggested to reflect anxiety-like states, but this is based primarily on male vocal behavior. Females do not consistently emit distress USVs in response to noxious (Laine et al., 2022; Tryon et al., 2021) and non-noxious stimuli (Lovick & Zangrossi, 2021; Machado Figueiredo et al., 2019; Inagaki & Sato, 2016; Inagaki & Mori, 2015). In a recent preclinical study modeling post-traumatic stress disorder (PTSD), 45% of females did not emit distress USVs during cue fear acquisition, even after receiving intense foot shocks (Laine et al., 2022). Another preclinical PTSD study reported similar sex differences in distress USV emissions (Tryon et al., 2021). Interestingly, females' resistance to cued fear extinction was instead predicted by 50-kHz USV emissions (Tryon et al., 2021). Extinction resistant females tended to emit fewer 50-kHz USVs during fear acquisition as compared to extinction competent females (Tryon et al., 2021). This recent preclinical evidence therefore suggests that conditioned fear may be better modeled in females in terms of 50-kHz USV patterns rather than distress USVs within the context of the chosen behavioral paradigm. The

lUSVs are another possible vocal measure of anticipation as the call subtype reflects a frustration-like state, but little is known about whether lUSVs show consistent sex differences like distress USVs. The preliminary study with H-CLS suggested that both USV types may be emitted in anticipation of aversive CLS.

Together, S-CLS and H-CLS may provide an effective technique for assessing the fundamental and learning components of tactile dyspareunia such as clitorodynia. Clitorodynia is a type of tactile dyspareunia that shares similarities with premenstrual dysphoric disorder (PMDD) which has been successfully modeled in female rats using USVs. Similar to PMDD, clitorodynia also presents with disabling somatic, behavioral, and affective symptoms related to clitoral pain induced by direct touch or the anticipation of painful touch (reviewed in Parada et al., 2015; Farmer, 2018; Rowan & Goldstein, 2018). Behavioral paradigms and USV measures are combined in preclinical models of PMDD to operationalize its behavioral and affective components. Conditioned partner preference and USV emission in response to S-CLS and to H-CLS could therefore be used to quantify the behavioral-affective component of clitorodynia anticipation**.**

Aim of thesis

The present thesis is of an exploratory nature, and attempts to answer one of three basic phenomenological questions regarding CLS-induced USVs within each chapter. The first chapter addresses whether S-CLS reliably elicits appetitive 50-kHz USVs. This was examined in two studies which investigated the effect of ovarian hormones and the selective serotonin reuptake blocker fluoxetine on S-CLS induced USVs. The latter study also sought to examine whether S-CLS induced USVs correlate with paced copulatory behaviors and the induction of conditioned

partner preference, both of which are inhibited by chronic fluoxetine treatment. The experiments described in Chapter 2explored the nature of H-CLS in more depth. The aim was to examine whether H-CLS is sexually aversive and would be responded to with USVs and/or alter the display of a conditioned partner preference. The experiments described in Chapter 3addressed whether S-CLS and H- CLS are associated with differential activation of reward and aversion systems in the brain. This was examined in the brains of females receiving S-CLS and H-CLS directly, or in another group of females in response to an odor cue (almond) that was used as a CS to predict S-CLS or H-CLS, using immunohistochemical detection of Fos protein as a marker of neuronal activation.

The overarching aim of the research described in the present thesis was to assess whether CLS-induced USVs can be a reliable measure of appetitive and/or aversive sexual affect. Together, the new findings described in this thesis suggest that CLS-induced USVs may indeed provide a useful index of sexual affect in rats. Chapter 5 discusses implications for pre-clinical rat models of dyspareunia and clitorodynia.

CHAPTER TWO
FOUNDATIONAL CHARACTERISTICS OF S-CLS INDUCED USVS

Overview and rationale

Sexual function and dysfunction are distinct yet intertwined. In order to consider whether a phenomenon is related to sexual dysfunction, one must first determine whether the phenomenon is present during normal sexual function. Although ovarian hormones like E2 in combination with T or P4 can stimulate both appetitive and consummatory measures of sexual response in female rats and humans, hormonal suppression or overstimulation (due to negative feedback on gonadotropins) can inhibit sexual arousal and desire. Certain psychotropic agents, such as serotonin reuptake inhibitors (SSRIs), are also commonly associated with a lack of sexual desire and orgasm disorders in women (reviewed in Goldstien et al., 2004).

Aims of this chapter

To examine the role of appetitive USVs in clitoral based sexual function. By building upon the work done by Parada et al. (2010, 2011, 2012, 2013), the first experiment of this chapter explored the role of ovarian hormones on CLS induced USVs. The second experiment examined the impact of the SSRI fluoxetine. Together, these studies provide evidence that S-CLS induced USVs reflect a sexually appetitive state that results in sexually appetitive behavior.

Chapter 2.1

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

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Gerson, C. A., Mac Cionnaith, C. E., Quintana, G. R., & Pfaus, J. G. (2019). Effects of ovarian hormones on the emission of 50-kHz ultrasonic vocalizations during distributed clitoral stimulation in the rat. *Hormones and Behavior*, *109*, 1-9.

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 socioaffective 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 by male and female rats during sexual interaction is a form of socioaffective 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 sessions 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 Ultra-SoundGate 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 Gavg) 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 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, ε (3)=0.889, $p < 0.018$, thereby a Greenhouse-Geiser correction was used.

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

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

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.

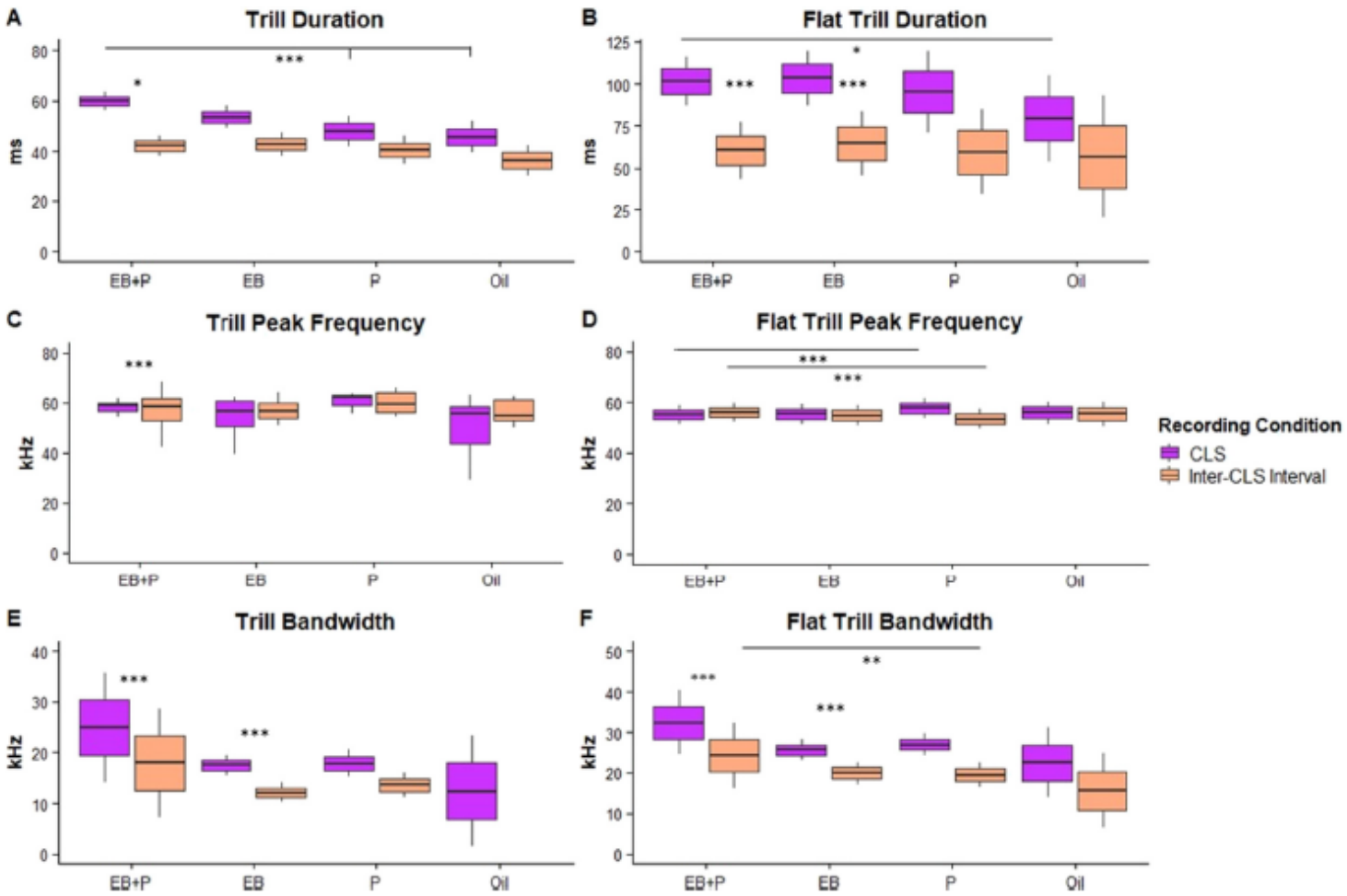

Fig. 1. Boxplots with the midline representing the estimated marginal means from the mixed linear models. The boxes represent ± 1 SEM, and the whiskers the 95% CIs of the estimated means. * < 0.05, ** < 0.01, *** < 0.005.

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

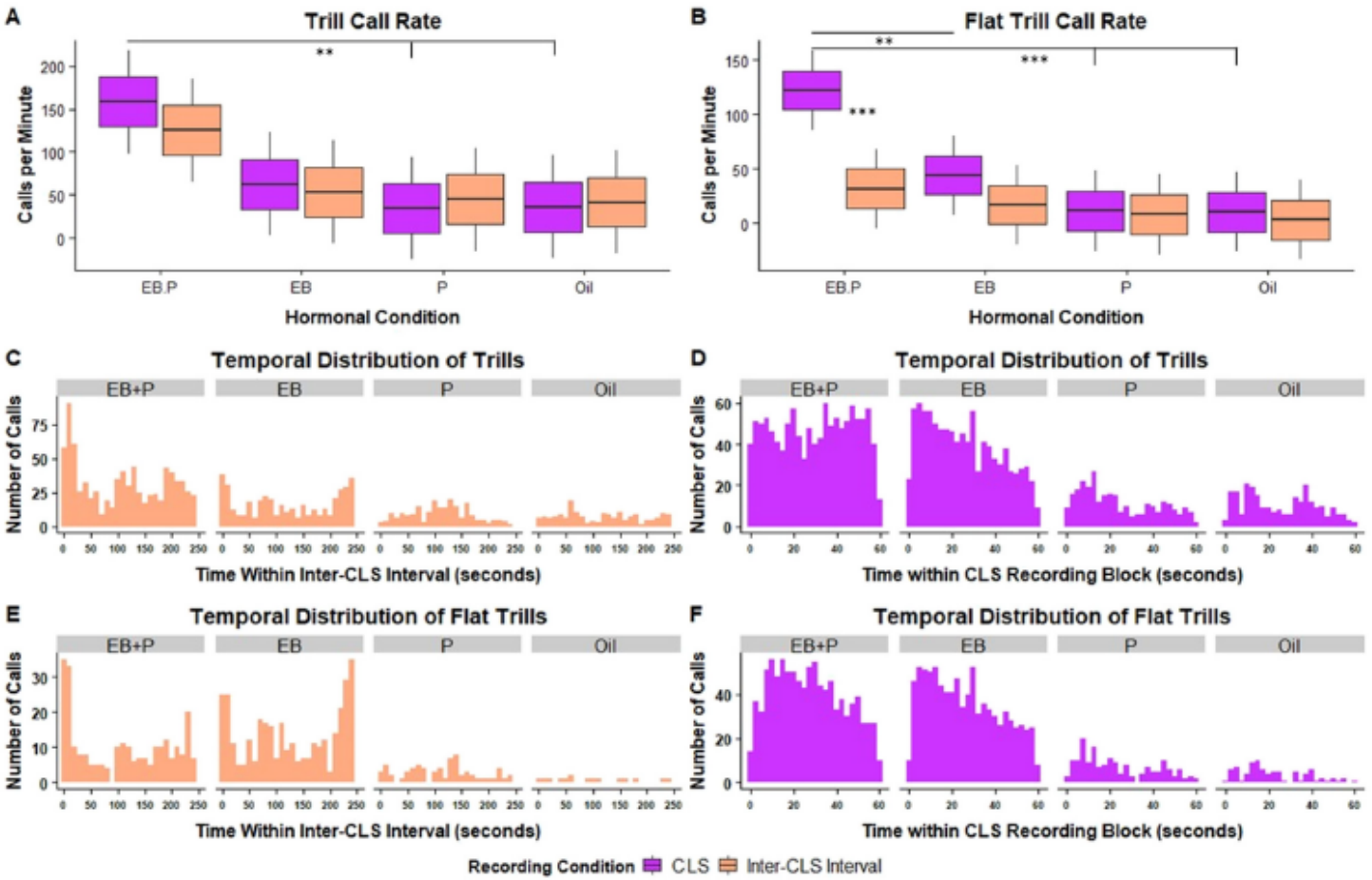

Fig. 2. Figs. A–B midlines in boxplot show the estimated marginal mean, boxes demonstrate ± 1 SEM, and the whiskers display the 95% Cis around the mean. A) demonstrates the trill call rate with no interaction between Hormonal Condition and recording block B) flat trill call rate is highest in females 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.

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 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, $\mathcal{E} (3)=0.395$, $p=0.018$, 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, 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 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, an 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 distributed 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.

Chapter 2.2.

Influence of chronic administration of fluoxetine on CLS elicited 50-kHz vocalizations and expression of female sexual behavior

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Abstract

Rationale: Fluoxetine (FLU) is a commonly prescribed selective serotonin reuptake inhibitor that causes anorgasmia and hypoactive sexual desire in women. In preclinical studies with female rats, chronic subcutaneous FLU administration decreased sexual solicitations and lordosis. A behavioral measure not included in these trials was the 50-kHz ultrasonic vocalizations (USV) which are thought to be indicative of positive affect and reward. Adult rats emit 50-kHz USVs as a response to reward stimuli including sexual interaction and genital stimulation. Previously, we reported that clitoral stimulation (CLS) administered with full hormonal priming increased the spectrotemporal parameters and call rates of 50-kHz USVs.

Objective: To examine the effects of chronic FLU (daily 10 mg/kg, i.p.) on CLS-related trills and flat-trill call profiles in sexually experienced female rats.

Methods: Eight female rats were ovariectomized and given six baseline acquisition tests of sexual behavior with sexually vigorous males prior to daily FLU administration for 36 days, encompassing another 9 tests of sexual behavior. At both the drug baseline and specific time points of the daily FLU administration, each female was recorded for USVs during CLS.

Results: At drug baseline, sexually experienced females emitted a similar spectrotemporal pattern of CLS induced 50-kHz trills and flat-trills, with the exception of bandwidth, that have been reported previously in sexually naïve females. Daily FLU administration decreased the 50-khz trills and flat-trills emissions progressively. Calls during the Inter-CLS interval were scarce, while calls during CLS recording blocks remained present but displayed reduced spectrotemporal parameters, call rate and profile.

Conclusions: Chronic FLU decreases vocalizations indicative of sexual anticipation and reward.

Key words: chronic fluoxetine, female sexual behavior, ultrasonic vocalizations

1. Introduction

Fluoxetine (FLU) is a commonly prescribed selective serotonin reuptake inhibitor (SSRI) shown to cause a loss of sexual desire and/or sexual arousal, and a loss of orgasm in women and men. Sexual side effects occur due to the main mechanisms of SSRIs, blocking presynaptic reuptake of serotonin (Pfaus, 2009; Stahl, 1998). This increases levels of serotonin (5-HT) within the synaptic cleft, activating both ascending and descending serotonergic pathways from the raphe nucleus (Stahl, 1998). Ascending pathway activation increases behavioral inhibition (Bari & Robbins, 2013) and inhibition of sexual arousal and sexual desire toward sexual cues (Pfaus, 2009). Descending 5-HT actions in the spinal cord are involved in the maintenance of parasympathetic genital engorgement, and inhibition of sympathetic outflow for orgasm (Normandin & Murphy, 2008; Pfaus, 2009). Along with its direct effects on serotonin, FLU also exhibits weak affinity for norepinephrine (NE) and dopamine (DA) reuptake proteins, and exerts a small but significant potentiation of DA transmission (Li et al., 2002; Wong et al., 1995). An acute peripheral injection of FLU increases extracellular NE and DA concentrations in mesocorticolimbic terminal regions (Li et al., 2002), which are critical for the activation of sexual arousal and desire (Pfaus, 2009). Sexual arousal and desire could also be diminished by chronic FLU as the increase of DA release initiates inhibitory feedback on mesolimbic DA neurons (Tanda et al., 1995). Thus, the sexual side effects of chronic FLU arise through direct excitatory effects on 5-HT and indirect inhibitory effects on DA and possibly also NE through negative feedback.

Like humans, male and female rats display appetitive and consummatory measures of sexual behavior that are disrupted by chronic FLU. For example, chronic daily subcutaneous (sc) injections of 10 mg/kg/ml FLU to male rats decreases ejaculations and appetitive level changes in

a bilevel chamber displayed in anticipation of a sexually receptive female rat (Cantor et al., 1999). Similarly, both solicitations and lordosis are reduced significantly by chronic daily sc injections of 10 mg/kg/ml of FLU (González Catuela et al., 2021; Matuszczyk et al.,1998; Sarkar et al., 2008; Uphouse et al., 2006). FLU-treated females also spend less time in the vicinity of male conspecifics during sexual motivation tests (Matuszczyk et al., 1998). Acute FLU (10 and 20 mg/kg) treatment also decreased lordosis and time spent with males (Sarkar et al., 2008).

The ovarian steroids estradiol (E2) and progesterone (P) reduce extracellular concentrations of 5-HT in the hypothalamus (Frankfurt et al., 1994; McQueen et al., 1997) along with mRNA for 5-HT reuptake protein expression (Zhou et al., 2002). Levels of extracellular 5-HT tend to be higher during diestrus and early proestrus, periods when female rats do not display sexual behaviors (Frankfurt et al., 1994; McQueen et al., 1997). Daily FLU treatment disrupts estrous cyclicity Uphouse et al. (2006) found that nearly 50% of naturally cycling female rats that received chronic FLU treatment (10 mg/kg) had estrous cycle disturbances during their first cycle with every rat failing to cycle by the second predicted estrous cycle. This effect however appeared to transient as estrous cyclicity returned after 15 to 16 days of daily FLU treatment (Uphouse et al., 2006). The return of estrous cyclicity may suggest that FLU may inhibit female sexual behaviors by blocking the hormonal events required for their expression.

One behavioral measure not included in these studies was the 50-kHz frequency modulated ultrasonic vocalization pattern (FM-USV). This pattern is indicative of a positive affective reward state (Burgdorf et al., 2008; Knutson et al. 2002; Pfaus et al., 2016). Fifty-kHz FM USVs are part of the female rats' sexual behavioral repertoire and are sensitive to ovarian steroid hormones (Gerson et al., 2019a; Matochik et al., 1992; Thomas & Barfield, 1985). Previous studies have shown that manual clitoral stimulation (CLS) induces FM-USV subtypes

associated with hedonic reward in OVX rats primed fully with estradiol benzoate (EB) and P (Gerson et al., 2019a). Relative to EB alone, or the oil vehicle, full priming with EB+P yielded significant increases in spectrotemporal parameters and call rate in that study.

Numerous studies have been conducted on the effects of SSRI administration (e.g., citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and zimeldine) on 40-kHz distress USV parameters and call rate in rat pups (Hodgson et al., 2008; Iijima & Chaki, 2005; Kehne et al., 2000; Komatsu et al., 2015; Mos & Olivier, 1989; Olivier et al., 1993, 1998; Starr et al., 2007; Zimmerberg & Germeyan, 2015). A few studies on adult rats have examined the inhibitory effects of SSRIs on 50-kHz USVs in response to chronic stress (Vares et al., 2018) and to acute footshock (Kassai & Gyertyan, 2012; Schrebier et al., 1998). These studies show a linear dose-response relationship in the reduction rate of USV calls following SSRI administration (Kassai & Gyertyan, 2012; Schrebier et al., 1998; Vares et al., 2018;). As of yet, no studies have examined whether chronic FLU administration leads to reductions in adult USV acoustics during a positive context, such as receiving sexual reward like CLS. As previously reported, sexually naive female rats that receive clitoral stimulation delivered with a soft bristle paint brush (S-CLS) emit 50-kHz trills and flat-trills (Gerson et al., 2019a). Parada et al. (2010) showed that S-CLS induced a conditioned place preference in sexually naïve females, whereas Parada et al. (2013) found that prior experience with paced copulation blocked the ability of CLS alone to induce a conditioned place preference. This effect suggests that sexually naïve females process CLS as a full reward, but that experience with copulatory stimulation from a male, including CLS and vaginocervical stimulation (VCS), reduces the perceived reward from stimulation of the glans clitoris alone. It is yet not known whether this difference in reward value could alter the USV pattern or call rate in

sexually experienced females given CLS. The present study examined whether chronic FLU disrupts USVs induced by CLS in females over the course of sexual experience.

Methods

Animals and surgery

Eight adult female Long-Evans rats (3–5 months, 250–400 g, Charles River, St- Constant, QC, Canada) received chronic FLU administration. At baseline, female rats were drug and sexually naive. Females were pair-housed in a colony room kept on a reverse 12 h:12 h light/dark cycle (lights on at 20:00) at 21 °C. Each pair-housed cage had access to tap water and Purina® rat chow ad libitum. Experimental procedures were approved by Concordia University's Animal Research Ethics Committee (protocol #30000300) and followed guidelines set by the Canadian Council on Animal Care.

Females were ovariectomized (OVX) prior to USV recording and sexual experience trials. Bilateral OVX were performed following the surgical procedure used in Gerson et al., 2019. Sexual receptivity was induced by giving each female sc injections of 10 μg estradiol benzoate (EB, 17β-diol 3-benzoate, ID E0970–000, Steraloids) 48 h prior each training session, and 500 μg of progesterone (P, 4-Pregnen-3, 20-dione, ID Q2600–000, Steraloids) 4 h prior to each training and test session. An injection volume of 0.1 ml of steroid solution was prepared by dissolving steroids in reagent grade sesame oil.

Drug preparation and administration

Fluoxetine hydrochloride was purchased from Sigma (St. Louis, Mo.). The dose of 10 mg/kg/ml was selected based on previous studies (e.g., Cantor et al., 1999; Dulawa et al., 2004; González Catuela et al., 2021). FLU solutions were made fresh daily by dissolving in distilled water and 0.9 M Tris buffered saline (v:v 50:50) then sonicated for at least 10 minutes. FLU has

been reported to produce taste aversions (Prendergast et al., 1996), which were avoided by injecting FLU prior to the rats' sleep cycle, between 19 h and 20 h. Weights were also monitored daily prior to each injection.

Clitoral stimulation and apparatus

Soft CLS was made by lightly brushing the clitoris with a No. 4 camel hair paintbrush. Following a 4 min recording block without S-CLS, i.e. Inter-CLS, CLS was applied every 5 sec for 1 minute repeated for 7 cycles for a total session duration of 35 minutes, as in Gerson et al. (2019a). In Gerson et al. (2019a), the inter-CLS interval was tailored to ensure that female rats would adequately return to baseline levels of sexual excitability (i.e., heightened locomotor activity in the anticipation of sexual stimulation; Pfaus et al. 2001). An apparatus similar to the one described in Gerson et al., 2019 was used for the application of S-CLS.

Sexual experience

Fully primed OVX rats received six baseline sexual experience sessions at 4-day intervals with sexually vigorous males in bilevel chambers to equate them for sexual experience (as in Pfaus et al., 1999). During each baseline sexual experience session, a single male was placed in the bilevel chamber and allowed to acclimate for 5 minutes prior to the introduction of a fully primed female. Each session lasted 30 min and were recorded using a GoPro Hero 9 camera with a 250 GB SD card and scored subsequently for female and male behavior using a computerized behavioral scoring program (Cabilio, 1996). The last baseline sexual experience session served as the USV and drug baseline for all females. Daily FLU administrations commenced that evening and females tested every 4 days for a total of 9 FLU tests. Following the training phase, eight females were randomly assigned to receive S-CLS during a USV recording session. Prior to maintenance sexual experience, which is similar to baseline sexual experience, USV recordings

were conducted at selected time points of interest. Selected time points of interest for USV recordings were drug baseline (BL), FLU test 1 (F1; day 4 of daily FLU administration), FLU test 6 (F6; day 24 of daily FLU administration), FLU test 7 (F7; day 28 of daily FLU administration), and FLU test 8 (F8; day 32 of daily FLU administration). We selected the time of the F1 to be within the range of the acute onset of sexual receptivity decline, which is approximately between 3 to 10 days, while the time of F6, F7, and F8 was chosen to be well past the expected return of the transient effects of daily FLU administration, i.e., approximately 15 to 16 days after administering the drug daily.

Ultrasonic vocalizations and statistical analysis

With the exception of call distribution, all vocalization recordings were analyzed according to the spectrotemporal parameters of trills and flat-trills outlined in Gerson et al. (2019). R software version 4.1.2 (R Development Core Team, 2021) was used for analysis of spectral-temporal data through RStudio: Integrated Development Environment for R (RStudio Team, 2023, version v1.4.1717-3). Subsequent analyses excluded all cases of missing data. Log10 transformations were applied to the duration, peak frequency, and bandwidth parameters to correct distributive skew. Throughout the results section, we refer to the estimated means as raw units despite using mixed linear models on log transformed data.

FLU test day and recording block interaction was tested for trill and flat-trill calls using the same model structure. Our model included: the interaction between FLU test day and recording block as a fixed factor and the crossed effects of FLU test day across subjects with uncorrelated random intercepts and slopes as a random effect. FLU test day was defined as a random effect in order to account for the variability in response to drug baseline and chronic daily FLU treatment time point. Similar to Gerson et al. (2019a), the recording block was not entered as

a random effect to prevent overfitting. The linear mixed model implemented variance components and maximum likelihood as repeated covariance structures.

Two-way repeated measures analyses of variance (ANOVAs) were used to examine trill and flat-trill rates, with FLU test day and recording blocks, and their interactions as within-subject factors. Calls made per minute of recording block, i.e. call rate, was the dependent variable in this analysis. Call profile was calculated as call type percentage (i.e., total number of subtype calls divided by total number of all calls), replacing the distribution calculation previously done in Gerson et al (2019). Generalized eta squared was used to calculate effect sizes for repeated measure ANOVA main effects and interactions, as it has been proposed to be more generalizable than partial eta squared (Olejnik & Algina, 2003).

3. Results

3.1. Trills

Across drug baseline and FLU test days, S-CLS increased the duration, lowered the peak frequency, and widened the bandwidth of the trill subtype compared to the Inter-CLS Interval. S-CLS call profiles were shown to consist of a high percentage of trills at drug baseline, which decreased at F1. Interestingly, at F6, this percentage increased, exceeding those at drug baseline. These call percentages however lowered on F7 and F8.

3.1.1. Overall effects

A significant interaction between FLU test day and recording block was observed for trill duration, $F(4,3106.52) = 11.86$, $p < .001$, trill peak frequency, $F(4,3623.26) = 9.83$, $p < .001$, and trill bandwidth, $F(4, 1817.39) = 8.25$, $p < .001$. The estimated marginal means (EMM) of trill spectrotemporal parameters and call rate are in **Table 1**. A simple contrast of each spectrotemporal parameter for each FLU test day x Recording Block group in **Table 3.**

An ANOVA revealed a significant main effect for recording block ($F(1,6) = 19.50$, $p = .004$, $\eta 2$ Generalized = .202) and a trend towards significance for the main effect of FLU test day treatment ($F(1.98, 11.87) = 3.58$, $p = .061$, $\eta 2$ Generalized = .233) and for trill call rates as shown in **Table 2.**

3.1.2 Trill Duration

Females emitted longer trills during the S-CLS recording block compared to the Inter-CLS interval at BL ($EMM_{S\text{-}CLS} = 52.500$, $SE_{S\text{-}CLS} = 2.069$, $EMM_{Inter\text{-}CLS} = 33.792$, $SE_{Inter\text{-}CLS} = 2.311$, $p < 0.0001$, $g = 2.851$), F1 ($EMM_{S\text{-}CLS} = 50.822$, $SE_{S\text{-}CLS} = 1.959$, $EMM_{Inter\text{-}CLS} = 38.908$, $SE_{Inter\text{-}CLS} = 4.500$, $p = 0.037$, $g = 1.148$), and F8 ($EMM_{S\text{-}CLS} = 57.504$, $SE_{S\text{-}CLS} = 3.696$, $EMM_{Inter\text{-}CLS} = 35.367$, $SE_{Inter\text{-}CLS} = 5.235$, $p <0.0001$, $g = 1.633$) as shown in **Fig.1**

Table 1.
Estimated marginal means for the Spectrotemporal Parameters and Call Rates of Trill and Flat-Trill Calls

			Duration (ms)	Peak Frequency (kHz)	Bandwidth (kHz)	Call Rate
Call Subtype	Recording Block	FLU test day	*EMM*	*EMM*	*EMM*	*EMM*
Trill						
	CLS					
		BL	52.5	60.1	29	135.42
		F1	50.82	55	38.4	73.28
		F6	49.8	49	43.3	40.71
		F7	58.7	41.6	45.3	15.95
		F8	57.5	53.5	34.9	12.21
	Inter-CLS Interval					
		BL	33.79	63.7	28.3	54.14
		F1	38.91	60.7	33.4	8.28
		F6	47.4	59.4	36.9	9.85
		F7	54.44	51.8	38	12.61
		F8	35.37	60.6	26.2	5.95
Flat-Trill						
	CLS					
		BL	77.5	52.7	24.8	118.85
		F1	65.2	50.2	36.3	52.14
		F6	74.6	46.2	45.5	24.71
		F7	78.9	45	41.8	41.42
		F8	90.3	50.8	34	30.42
	Inter-CLS Interval					
		BL	44.6	60.6	22.9	23
		F1	55.9	58.7	24.6	2.85
		F6	55.3	60.7	30	2
		F7	60.7	52.2	34.4	18.14
		F8	42.1	61	32.3	3.57

Table 2.
Model Statistics for Spectrotemporal Parameters of Trill Subtype Calls

	Trills												
Model Statistics	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	61.660	2959.480	<0.001	70.590	4057.58	<0.001	69.310	2212.120	<0.001	19.490	6	0.004	0.202
FLU test	12.510	13.180	<.0001	12.030	16.110	<0.001	18.610	17.720	<0.001	3.570	24	0.020	0.233
FLU test x Recording Block	11.860	316.52	<0.001	9.830	3623.26	<0.001	7.290	2575.630	<0.001	1.740	24	0.173	0.052
Fixed Effects													
	Estimate	t-value	p-value	β	t-value	p-value	β	t-value	p-value				
Intercept	1.630	175.943	<0.001	1.690	84.58	<0.001	1.487	232.312	<0.001				
Recording Block (CLS vs. no CLS)	0.042	7.853	<0.001	-0.490	-8.400	<0.001	0.042	8.325	<0.001				
BL vs. F1	-0.052	-3.275	<0.050	0.092	5.220	<0.001	-0.068	-5.501	<0.001				
BL vs. F6	-0.013	-0.998	0.318	0.032	1.507	0.168	0.011	0.699	0.494				
BL vs. F7	0.007	9.457	0.779	0.021	-0.634	0.542	0.048	3.247	0.009				
BL vs. F8	0.060	6.071	<0.001	0.107	-4.173	0.002	0.075	5.198	0.0002				
F1 x Recording Block	0.030	4.943	<0.001	0.035	4.845	<0.001	-0.031	-4.906	<0.001				
F6 x Recording Block	0.001	0.094	0.925	0.013	0.958	0.338	-0.007	-0.626	0.531				
F7 x Recording Block	-0.034	-2.986	0.002	-0.035	-2.822	0.004	0.005	0.783	0.433				
F8 x Recording Block	-0.826	-2.933	0.003	-0.023	-2.332	0.019	0.008	0.988	0.323				

Table 3. Post Hoc Comparisons of Treatment Day x Recording Block groups. P-values shown have undergone the Holm Adjustment.

Trills	Duration	Peak Frequency	Bandwidth
BL: CLS vs Inter-CLS Interval	<0.0001	<0.0001	0.302
F1: CLS vs Inter-CLS Interval	0.036	0.012	0.04
F6: CLS vs Inter-CLS Interval	0.553	<.0001	0.001
F7: CLS vs Inter-CLS Interval	0.289	<0.0001	<0.0001
F8: CLS vs Inter-CLS Interval	<0.0001	0.001	<0.0001
CLS Block: BL vs. F1	1	0.289	<0.0001
CLS Block: BL vs. F6	1	0.002	<0.0001
CLS Block: BL vs. F7	0.126	<0.0001	<0.0001
CLS Block: BL vs. F8	1	0.931	0.041
CLS Block: F1 vs. F6	1	0.689	0.006
CLS Block: F1 vs. F7	0.011	<0.0001	0.0002
CLS Block: F1 vs. F8	1	1	0.449
CLS Block: F6 vs. F7	0.199	0.339	0.736
CLS Block: F6 vs. F8	1	1	0.0002
CLS Block: F7 vs. F8	1	0.202	0.0002
Inter-CLS Interval: BL vs. F1	1	1	0.186
Inter-CLS Interval: BL vs. F6	0.099	1	0.0001
Inter-CLS Interval: BL vs. F7	<0.0001	0.0001	<0.0001
Inter-CLS Interval: BL vs. F8	1	1	0.864
Inter-CLS Interval: F1 vs. F6	1	1	0.736
Inter-CLS Interval: F1 vs. F7	0.051	0.197	0.449
Inter-CLS Interval: F1 vs. F8	1	1	0.184
Inter-CLS Interval: F6 vs. F7	1	0.564	0.864
Inter-CLS Interval: F6 vs. F8	1	1	0.002
Inter-CLS Interval: F7 vs. F8	0.023	0.931	0.002

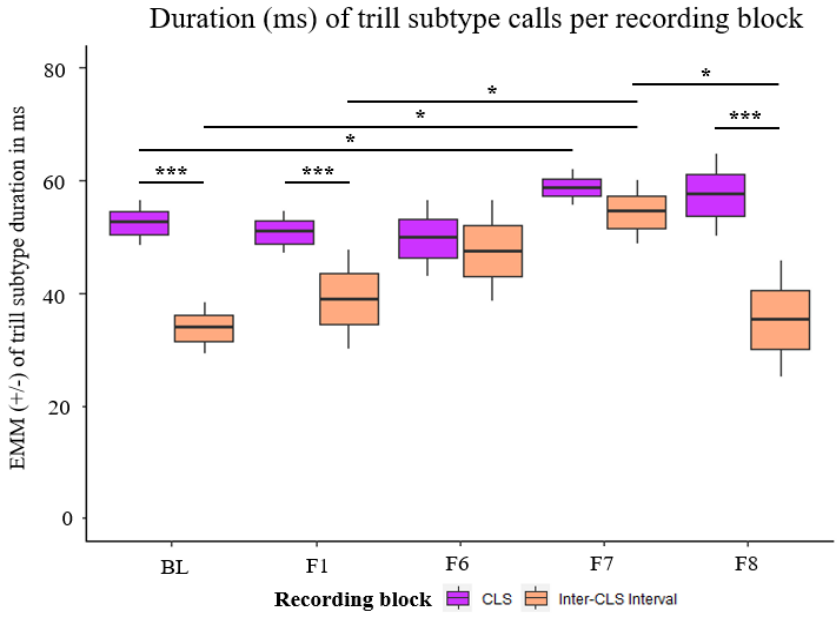

Fig 1. *Boxplots displaying the marginal means of mixed linear models for trill duration. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

F7 produced longer S-CLS trills compared to those emitted on F1, p = 0.011, g = 0.755. Inter-CLS trills were also longer on F7 ($EMM_{Inter\text{-}CLS}$ = 54.444, $SE_{Inter\text{-}CLS}$ = 2.871) when compared with drug baseline, p < 0.0001, g = 2.649), F1, p = 0.051, g = 1.376, and F8, p = 0.023, g = 1.510.

3.1.3 Trill Peak frequency

Drug baseline and FLU test days showed lower peak frequency trills during S-CLS recording blocks ($EMM_{baselineS\text{-}CLS}$ = 60.078 , $SE_{baselineS\text{-}CLS}$ = 1.895; $EMM_{F1S\text{-}CLS}$ = 54.950, $SE_{F1S\text{-}CLS}$ = 2.403; $EMM_{F6S\text{-}CLS}$ = 48.970, $SE_{F6\text{-}SCLS}$ = 3.021; $EMM_{F7S\text{-}CLS}$ = 41.587, $SE_{F7S\text{-}CLS}$ = 2.517; $EMM_{F7S\text{-}CLS}$ = 53.484, $SE_{F7S\text{-}CLS}$ = 3.904) than Inter-CLS recording blocks ($EMM_{baselineInter\text{-}CLS}$ = 63.688, $SE_{baselineInter\text{-}CLS}$ = 1.959, p < 0.0001, g = 0.626; $EMM_{F1Inter\text{-}CLS}$ = 60.673, $SE_{F1Inter\text{-}CLS}$ =

3.043, p = 0.012 , g = 0.698; $EMM_{F6Inter\text{-}CLS}$ = 59.362, $SE_{F6Inter\text{-}CLS}$ = 3.345, p < 0.001 , g = 1.090; $EMM_{F7Inter\text{-}CLS}$ = 51.841, $SE_{F7Inter\text{-}CLS}$ = 2.756 , p <0.001, g = 1.299; $EMM_{F8Inter\text{-}CLS}$ = 60.626, $SE_{F8Inter\text{-}CLS}$ = 4.263 , p = 0.0009 , g = 0.584) as shown in **Fig 2.**

F7 had significantly lower peak frequency of S-CLS trills than BL, p <0.0001, g = 2.774, and F1, p <0.0001, g = 1.815. On F6, S-CLS trills were also lower than at BL, p = 0.002, g = 1.472. Peak frequency was significantly higher at BL than at F7 during the Inter-CLS Interval, p = 0.0001, g = 1.656. For the Inter-CLS Interval, the mean trill peak frequency did not differ significantly between drug baseline and other treatment days.

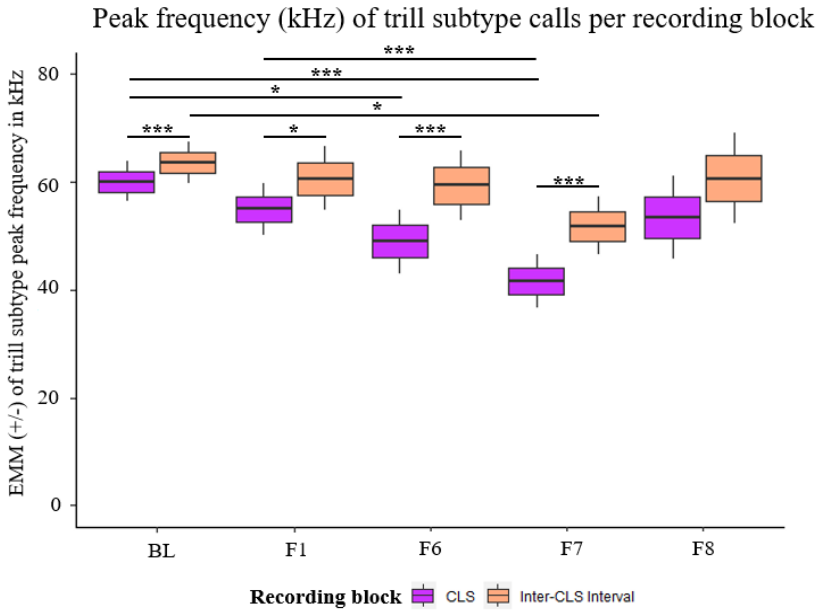

Fig 2. *Boxplots displaying the marginal means of mixed linear models for trill peak frequency. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.1.4 Trill Bandwidth

For all FLU test days, trill bandwidth was significantly wider during S-CLS recording block ($EMM_{F1S\text{-}CLS}$ = 38.449, $SE_{F1S\text{-}CLS}$ = 1.103; $EMM_{F6S\text{-}CLS}$ = 43.309, $SE_{F6S\text{-}CLS}$ = 1.082; $EMM_{F7S\text{-}CLS}$ = 45.346, SE_{F7CLS} = 1.272; $EMM_{F8S\text{-}CLS}$ = 34.880, $SE_{F8S\text{-}CLS}$ = 1.682) than the Inter-CLS Interval ($EMM_{F1Inter\text{-}CLS}$ = 33.407, $SE_{F1Inter\text{-}CLS}$ = 2.141, p = 0.040 , g = 0.990; $EMM_{F6Inter\text{-}CLS}$ = 36.929, $SE_{F6Inter\text{-}CLS}$ = 1.703, p = 0.001, g = 1.495; $EMM_{F7Inter\text{-}CLS}$ = 38.032, $SE_{F71Inter\text{-}CLS}$ = 1.679, p <0.0001, g = 1.641; $EMM_{F8Inter\text{-}CLS}$ = 26.159, $SE_{F8Inter\text{-}CLS}$ = 2.374, p <0.0001, g = 1.417) as shown in **Fig 3.**

S-CLS trills were narrower in bandwidth at drug baseline (EMM = 29.017, SE = 0.962) than those emitted on F1, p <0.0001, g = 3.05, F6, p <0.0001, g = 4.666, F7, p < 0.0001, g = 4.840, and F8, p = 0.041, g = 1.430. Bandwidth became sequentially wider each drug treatment day, with the exception of F8, with S-CLS and trills being narrower on F1 compared to F6, p = 0.006, g = 0.843, and to F7, p = 0.0002, g = 1.196. On F8, CLS trills became significantly narrower in bandwidth than during prior drug treatment F6, p = 0.0002, g = 1.461, and F7, p = 0.0002, g = 1.815. The bandwidth of the Inter-CLS Interval trills at drug baseline and at F8 was also narrower than those on F6, p_{bl} = 0.0001, g_{bl} = 1.499, p_{F8}= 0.002, g_{F8}= 1.868, and on F7, $p_{baseline}$ < 0.0001, $g_{baseline}$ = 1.690, p_{F8}= 0.002, g_{F8}= 2.059.

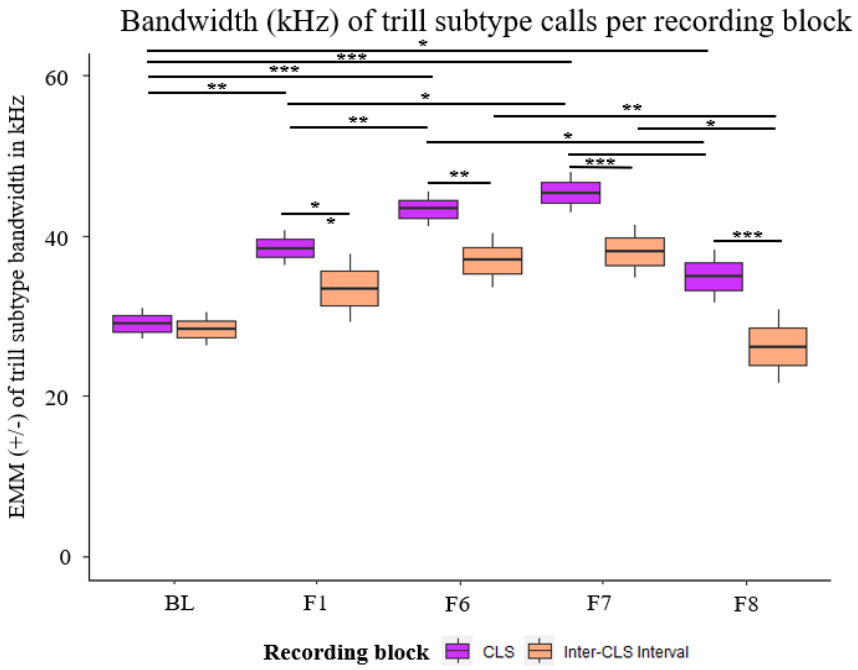

Fig 3. *Boxplots displaying the marginal means of mixed linear models for trill bandwidth. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.1.5 Call rate and call profile percentages

The combination of FLU test and recording block had no significant effect on trill call rates as shown in **Fig 4**. The S-CLS Recording Block call profile had a higher percentage of trill subtype calls than the Inter-CLS Interval call profile across drug baseline and treatment days. BL had the highest call rate (EMM = 135.428, SE = 40.863) and percentage of S-CLS trills (42.470 %) during the CLS recording block, with the exception of F1 (EMM = 73.285, SE = 22.658; 31.870 %), then F7 (EMM = 58.00, SE = 12.613; 31.359 %), F6 (EMM = 40.714, SE = 15.127; 46.309 %), and F8 (EMM = 39.285, SE = 12.217; 39.488 %). F1, however, had the lowest call rate (EMM = 8.285, SE = 3.509) and percentage of Inter-CLS Interval trills (19.219 %) than BL

(EMM = 54.142, SE = 24.532; 38.602 %), F6 (EMM = 9.857, SE = 4.881; 25.105 %), F7 (EMM = 30.428, SE = 12.613; 22.646 %), and F8 (EMM = 8.571, SE = 5.955; 32.696%).

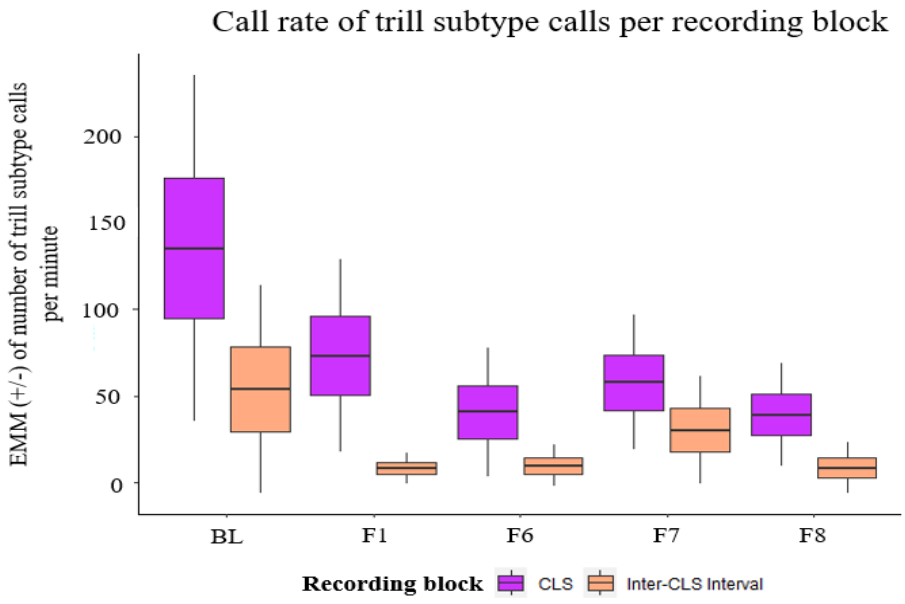

Fig 4. *Boxplots displaying the marginal means of mixed linear models for trill call rate. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.2. Flat-trills

Spectrotemporal parameters of flat-trills were found to resemble those of the trill call subtype. S-CLS call profile also demonstrated a similar pattern from FLU to across FLU test days.

3.2.1 Overall effects

FLU test days and recording block showed significant interactions for flat-trill duration ($F(4,3015.93) = 63.37$, $p < 0.001$), flat-trill peak frequency ($F(4,2947.88) = 4.94$, $p < 0.001$), and flat-trill bandwidth ($F(4,2890.09) = 9.48$, $p < 0.001$). **Table 1.** shows the EMMs of flat-trill

spectral-temporal parameters and call rate. Simple contrasts were also conducted on EMMs of each spectral-temporal parameter for each FLU test day x Recording Block group as shown in **Table 5**.

For flat-trill call rates, the ANOVA revealed a significant main effect of Recording Block ($F(1,6) = 8.67$, $p = 0.026$, $\eta 2$ Generalized = 0.179 and a trend towards significance for the main effect of FLU test day ($F(1.52,9.14) = 3.68$, $p = 0.076$, $\eta 2$ Generalized = 0.163) as shown in **Table 4**. The assumption of sphericity as indicated by the Mauchly's Test of Sphericity was violated for FLU test day, ε (24) =0.0134, p=0.035, thereby a Greenhouse-Geiser correction was used.

Table 4. Model Statistics for Spectrotemporal Parameters of Flat-Trill Subtype Calls

Trills													
Model Statistics	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	118.24	3010.16	<0.001	108.49	3022.190	<0.001	82.310	2892.610	<0.001	8.670	6	0.026	0.179
FLU test	3.79	11.31	0.034	4.410	10.970	0.023	53.580	8.120	<0.001	3.680	9.140	0.076	0.163
FLU test x Recording Block	8.48	2937.83	<0.001	4.94	2947.88	<.001	9.480	2890.03	<0.001	2.870	8.390	0.121	0.082
Fixed Effects													
	Estimate	t-value	p-value	β	t-value	p-value	β	t-value	p-value				
Intercept	1.755	97.821	<.001	1.706	104.939	<0.001	1.450	176.386	<0.001				
Recording Block (CLS vs. no CLS)	0.07	10.874	<.001	0.058	-10.416	<0.001	0.056	9.072	<0.001				
BL vs. F1	-0.022	-1.355	0.234	0.043	2.399	0.040	-0.010	-13.317	<0.001				
BL vs. F6	-0.007	-0.329	0.748	0.006	0.458	0.647	-0.034	-1.549	0.175				
BL vs. F7	0.007	0.222	0.829	0.002	-0.088	0.932	0.051	1.735	0.163				
BL vs. F8	0.041	3.236	0.001	-0.061	-3.219	0.012	0.075	3.377	0.008				
F1 x Recording Block	0.029	3.694	0.0002	0.024	3.619	0.0003	-0.033	-4.539	<0.001				
F6 x Recording Block	-0.042	-2.551	0.01	0.001	0.099	0.921	0.035	2.175	0.029				
F7 x Recording Block	-0.008	-0.562	0.574	0.037	-2.943	0.003	0.053	3.797	0.0001				
F8 x Recording Block	-0.002	-2.248	0.024	0.014	1.507	0.132	-0.013	-1.264	0.206				

Table 5. Post Hoc Comparisons of Treatment Day x Recording Block groups. P-values shown have undergone the Holm Adjustment.

Flat-Trills	Duration	Peak Frequency	Bandwidth
BL: CLS vs Inter-CLS Interval	<0.0001	<0.0001	0.027
F1: CLS vs Inter-CLS Interval	0.339	0.0008	0.0001
F6: CLS vs Inter-CLS Interval	0.035	<0.0001	<0.0001
F7: CLS vs Inter-CLS Interval	0.003	<0.0001	<0.0001
F8: CLS vs Inter-CLS Interval	<0.0001	<0.0001	0.330
CLS Block: BL vs. F1	1	0.289	<0.0001
CLS Block: BL vs. F6	1	0.002	<0.0001
CLS Block: BL vs. F7	0.126	<0.0001	<0.0001
CLS Block: BL vs. F8	1	0.931	0.041
CLS Block: F1 vs. F6	1	0.689	0.006
CLS Block: F1 vs. F7	0.011	<0.0001	0.0002
CLS Block: F1 vs. F8	1	1	0.449
CLS Block: F6 vs. F7	0.199	0.339	0.736
CLS Block: F6 vs. F8	1	1	0.0002
CLS Block: F7 vs. F8	1	0.202	0.0002
Inter-CLS Interval: BL vs. F1	1	1	0.186
Inter-CLS Interval: BL vs. F6	0.099	1	0.0001
Inter-CLS Interval: BL vs. F7	<0.0001	0.0001	<0.0001
Inter-CLS Interval: BL vs. F8	1	1	0.864
Inter-CLS Interval: F1 vs. F6	1	1	0.736
Inter-CLS Interval: F1 vs. F7	0.051	0.197	0.449
Inter-CLS Interval: F1 vs. F8	1	1	0.184
Inter-CLS Interval: F6 vs. F7	1	0.564	0.864
Inter-CLS Interval: F6 vs. F8	1	1	0.002
Inter-CLS Interval: F7 vs. F8	0.023	0.931	0.002

3.2.2 Duration

At BL and across FLU test days, except for F1, flat-trill duration was significantly longer during the CLS Recording Block ($EMM_{BLS\text{-}CLS}$ = 65.085, $SE_{BLS\text{-}CLS}$ = 8.842; $EMM_{F6S\text{-}CLS}$ = 73.153, $SE_{F6S\text{-}CLS}$ = 8.920; $EMM_{F7S\text{-}CLS}$ = 81.747, $SE_{F7S\text{-}CLS}$ = 6.628; $EMM_{F8S\text{-}CLS}$ = 88.881, $SE_{F8S\text{-}CLS}$ = 10.999) than the Inter-CLS Interval ($EMM_{BLInter\text{-}CLS}$ = 48.631, $SE_{BLInter\text{-}CLS}$ = 9.086, p < 0.0001, g = 0.614; $EMM_{F6Inter\text{-}CLS}$ = 54.554, $SE_{F6Inter\text{-}CLS}$ = 11.30, p = 0.039, g = 0.611; $EMM_{F7Inter\text{-}CLS}$ = 64.680, $SE_{F7Inter\text{-}CLS}$ = 8.044, p = 0.007, g = 0.774; $EMM_{F8Inter\text{-}CLS}$ = 41.174 , $SE_{F8Inter\text{-}CLS}$ = 12.040, p <0.001, g = 1.383) as shown in **Fig 5.** The duration of CLS flat-trills were found to only be significantly longer on F7 compared to F1, p = 0.0182, g =0.366. BL and FLU test days did not differ significantly in the duration of Inter-CLS flat-trills.

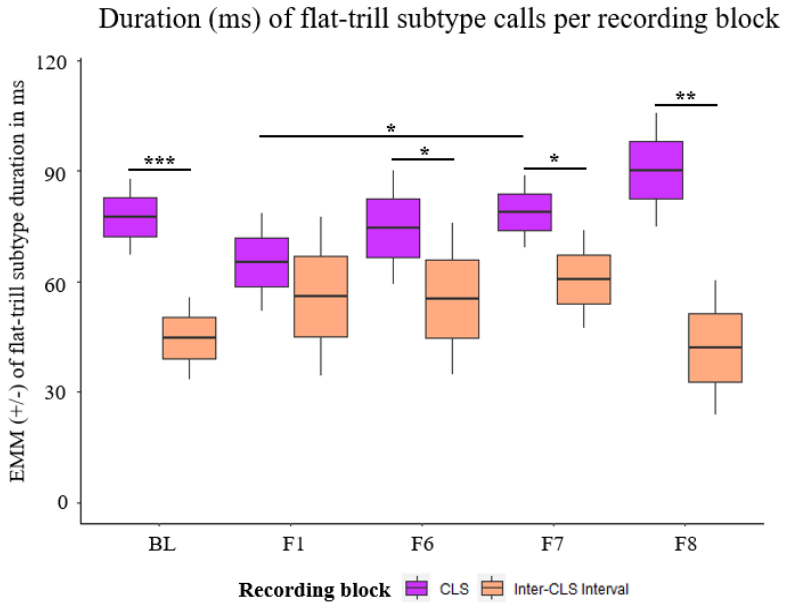

Fig 5. *Boxplots displaying the marginal means of mixed linear models for flat-trill duration. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.2.3 Peak frequency

S-CLS flat-trills emitted at the drug baseline (EMM_{BLCLS} = 52.724, SE_{BLCLS} = 2.123) and treatment days ($EMM_{F1S\text{-}CLS}$ = 50.224, $SE_{F1S\text{-}CLS}$ = 1.666; $EMM_{F6S\text{-}CLS}$ = 46.191, $SE_{F6S\text{-}CLS}$ = 2.292; $EMM_{F7S\text{-}CLS}$ = 44.951, $SE_{F7S\text{-}CLS}$ = 2.281; $EMM_{F8S\text{-}CLS}$ = 50.793, $SE_{F8S\text{-}CLS}$ = 3.174) were lower in peak frequency than those emitted during the Inter-CLS Interval ($EMM_{BLInter\text{-}CLS}$ = 60.620, $SE_{BLInter\text{-}CLS}$ = 2.935, $p < 0.001$, $g = 1.030$; $EMM_{F1Inter\text{-}CLS}$ = 58.675, $SE_{F11Inter\text{-}CLS}$ = 2.824, $p = 0.0008$, $g = 1.218$; $EMM_{F6Inter\text{-}CLS}$ = 60.678, $SE_{F6Inter\text{-}CLS}$ = 2.935, $p < 0.0001$, $g = 1.841$; $EMM_{F7nter\text{-}CLS}$ = 52.217, $SE_{F7Inter\text{-}CLS}$ = 2.584, $p < 0.0001$, $g = 0.997$; $EMM_{F8Inter\text{-}CLS}$ = 60.993 , $SE_{F8Inter\text{-}CLS}$ = 3.421, $p < 0.0001$, $g = 1.033$) as shown in **Fig 6.** At BL, S-CLS and Inter–CLS flat-trills were significantly higher in peak frequency only compared to F7, $p_{S\text{-}CLS}$ = 0.029, $g_{S\text{-}CLS}$ = 1.179, $p_{Inter\text{-}CLS}$= 0.048, $g_{Inter\text{-}CLS}$= 1.016. S-CLS and Inter-CLS flat-trill peak frequency did not differ significantly between BL and any other FLU test days.

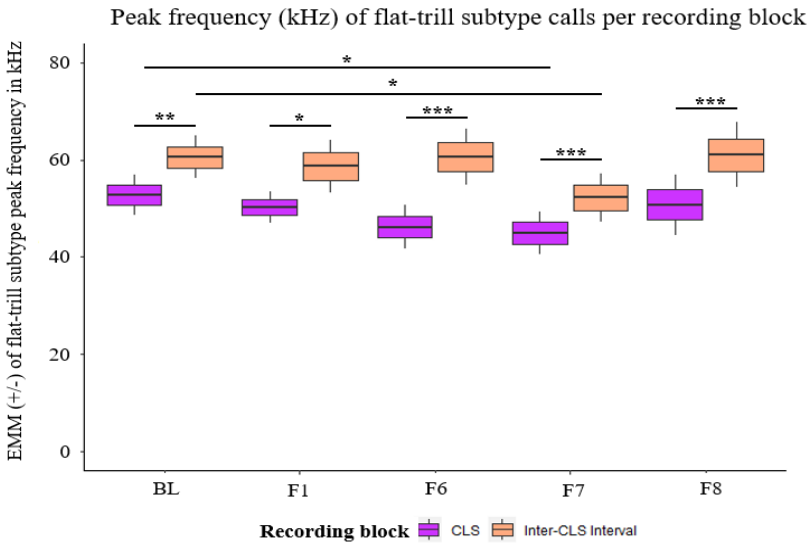

Fig 6. *Boxplots displaying the marginal means of mixed linear models for flat-trill peak frequency. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.2.4. Bandwidth

A significantly wider bandwidth was observed for S-CLS flat-trills than those emitted during the Inter-CLS Interval at BL (EMM_{CLS} = 24.800, SE_{CLS} = 0.579; $EMM_{Inter\text{-}CLS}$ = 22.900 $SE_{Inter\text{-}CLS}$ = 0.799; p = 0.027, g = 0.917), F1(EMM_{CLS} = 36.300, SE_{CLS} = 0.941; $EMM_{Inter\text{-}CLS}$ = 24.600, $SE_{Inter\text{-}CLS}$ = 2.674; p = 0.0001, g = 1.959), F6 (EMM_{CLS} = 45.500, SE_{CLS} = 2.739; $EMM_{Inter\text{-}CLS}$ = 30.000, $SE_{Inter\text{-}CLS}$ = 3.360; p < 0.0001, g = 1.691), and F7(EMM_{CLS} = 41.800, SE_{CLS} = 1.966; $EMM_{Inter\text{-}CLS}$ = 34.400, $SE_{Inter\text{-}CLS}$ = 2.330; p <0.0001, g = 1.62), but not F8 (EMM_{CLS} = 34.000, SE_{CLS} = 3.072; $EMM_{Inter\text{-}CLS}$ = 32.300, $SE_{Inter\text{-}CLS}$ = 3.369) as shown in **Fig 7.**

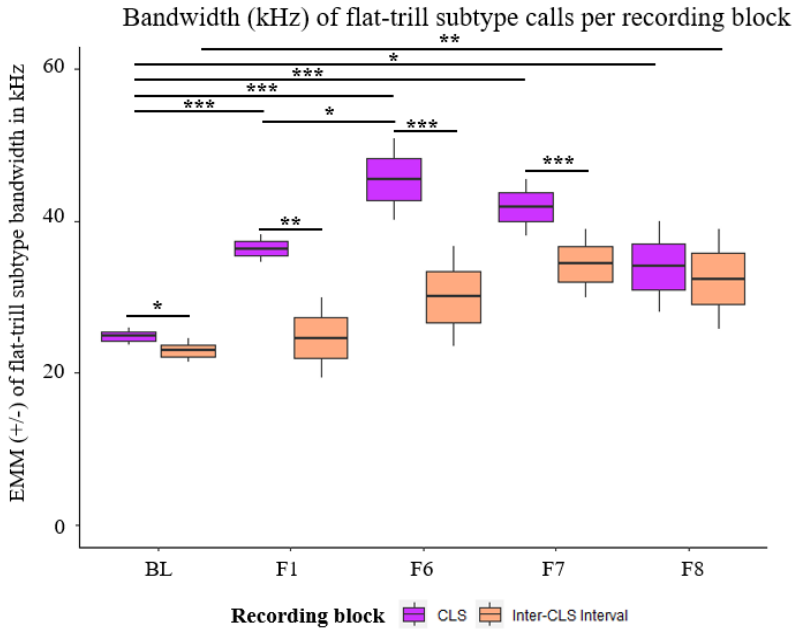

Fig 7. *Boxplots displaying the marginal means of mixed linear models for flat-trill bandwidth. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

S-CLS flat-trill bandwidth at BL was narrower than CLS flat-trill bandwidth at FLU (p_{F1} < 0.0001, g_{F1} = 4.929, p_{F6} < 0.0001, g_{F6} = 3.495, p_{F7} < 0.0001, g_{F7} = 4.008, p_{F8} = 0.042, g_{F8} = 1.388) test days. S-CLS flat-trill bandwidth at F1 was also narrower than S-CLS flat-trill

bandwidth at F6, p = 0.016, g = 1.498, but not F7 and F8. As with S-CLS flat-trills, Inter-CLS flat-trills had a narrower bandwidth at BL than on F8, p < 0.0001, g = 2.201.

3.2.5. Call rate and call profile percentages

FLU test day and Recording Block had no significant interaction on flat-trill call rates. Similar to the trill subtype. S-CLS Recording Block call profiles had a higher percentage of flat-trill subtype calls across drug baseline and treatment days than Inter-CLS Interval call profiles as shown in **Fig 8**.

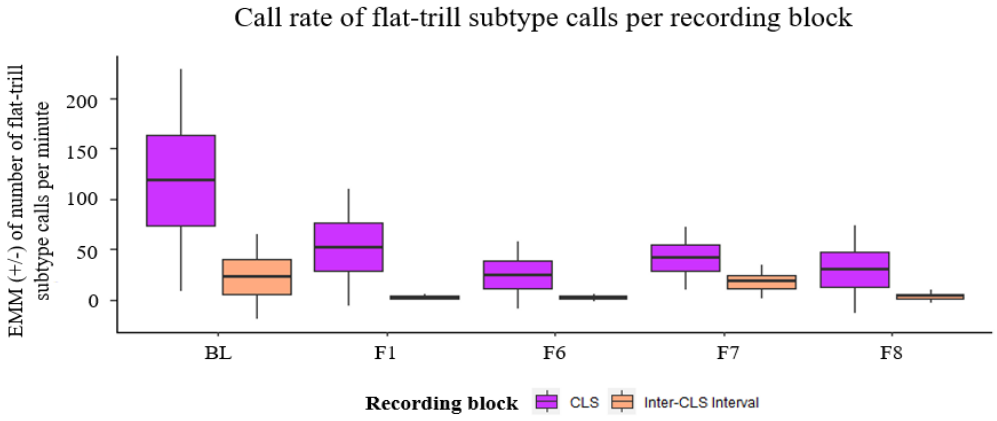

Fig 8. *Boxplots displaying the marginal means of ANOVA for flat-trill call rate. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

BL also had the highest call rate (EMM = 118.857, SE = 45.102) and percentage of S-CLS flat-trills (36.362 %) than F1 (EMM = 52.142, SE = 23.840; 20.755 %), F6 (EMM = 24.714, SE = 13.602; 31.208 %), F7 (EMM = 47.428, SE = 12.783; 29.80 %), and F8 (EMM = 30.428, SE = 17.765; 27.178 %). F6 had the lowest call rate (EMM = 2.00, SE = 1.527) but not percentage (28.452 %) followed by F1 (EMM = 2.857, SE = 1.010; 14.286 %) then F8 (EMM =

3.571, SE = 2.580; 18.047 %), F7 (EMM = 18.142, SE = 6.860; 13.004 %), then BL (EMM = 23.00, SE = 17.280; 30.161 %).

4. Discussion

This experiment examined the effect of chronic daily FLU administration on the spectral-temporal parameters and call rates of S-CLS induced trills and flat-trill subtype calls in female rats as a function of sexual experience. Chronic daily FLU was hypothesized to alter the acoustics of these 50-kHz subtype calls and attenuate their call rates. Furthermore, it was predicted that sexually experienced females would emit these 50-kHz subtype calls in response to S-CLS in a similar manner to sexually naive females, although with slight differences in their spectral-temporal characteristics. Soft-CLS evoked similar spectral-temporal characteristics, with the exception of bandwidth, and call rates at BL in sexually experienced female rats comparable to those of sexually naive females (Gerson et al., 2019a). Given the conditioned place preference data of Parada et al. (2010, 2013) this suggests that bandwidth may relate to the consummatory rewarding properties of S-CLS that are distinct from its appetitive properties, which remain following copulatory experience.

Across FLU test days, a different pattern for each spectrotemporal parameter for S-CLS and Inter-CLS trill subtypes emerged. Chronic FLU did not alter the duration of S-CLS trills and flat-trills but was found to progressively decrease their peak frequency and increase their bandwidth. There was also a progressive decrease in the duration difference between S-CLS and Inter-CLS across FLU test days. For peak frequency and bandwidth, chronic FLU progressively increased this difference for trill and flat-trill call subtypes. Trill and flat-trill call rates were shown to attenuate over chronic FLU treatment days, with Inter-CLS showing the greatest reduction. A prior report (Gerson et al., 2019a) proposed that trill subtype calls emitted during

the S-CLS recording block are indicative of sexual reward, while trill subtype calls emitted during the Inter-CLS recording block are indicative of sexual anticipation. Attenuation of trill subtypes during the S-CLS recording block may therefore indicate diminished sexual appetitive reward, while attenuation of trill subtypes during the Inter-CLS may indicate reduced sexual anticipation.

Trill subtype attenuation is consistent with behavioral observations that chronic FLU reduces consummatory and appetitive aspects of sexual behaviors in females, such as lordosis and solicitations, and in males, such as ejaculations (González Cautela et al., 2021). Acute caffeine administration (CAF; 10 or 20 mg/kg, ip), a potential on demand treatment for the side effects of FLU, restored these diminished aspects as the stimulant acts through adenosine receptor antagonism (reviewed in Fisone et al., 2004; Nehlig et al., 1992). Sympathetic outflow is inhibited by adenosine A1 and A2a receptors and their blockage by CAF increases central and peripheral NE and DA transmission (reviewed in Fisone et al., 2004; Nehlig et al., 1992). Several studies have shown that acute CAF administration modifies the acoustic characteristics, number, and subtype patterns of male 50-kHz USVs (Simola et al., 2010, 2012; Willadsen et al., 2018). These reports together suggest that 50-kHz USV emissions and copulatory behavior are modified by peripheral NE and DA transmission, which are crucial for female sexual arousal and motivation.

It has been suggested previously that FLU may disrupt sexual receptivity in female rats by disrupting the estrous cycle (Matuszczyk et al., 1998, Sarkar et al., 2008; Uphouse et al., 2006). In the initial stage of FLU's action, serotonin transporters are blocked, resulting in a rapid increase in extracellular 5-HT (Gobert et al., 1997; Perry & Fuller, 1992; Tao et al., 2002). Later actions of FLU lead to the desensitization of 5-HT1A somatodendritic autoreceptors in the dorsal

raphe (Hensler, 2003) and 5-HT1B terminal autoreceptors in the frontal cortex and hypothalamus (Newman et al., 2004). Within the hypothalamus, 5-HT modulates the secretion of gonadotropin-releasing hormone (Wada et al., 2006), which stimulates the pituitary to secrete gonadotropins, follicle-stimulating hormones (FSH) and luteinizing hormones (LH). Specifically, FSH secretion is stimulated by 5-HT binding to 5-HT2 receptors, while LH secretion is modified by 5-HT binding to both 5-HT1 and 5-HT2 receptors (Gouveia & Franci, 2004). The SSRI is reported to desensitize hypothalamic postsynaptic 5-HT1A signaling (Van de Kar et al., 2002). This leads to modifications of FSH and LH secretion, which in turn disrupt sexual function and behavior. FLUs inhibition of female sexual behavior has been attributed to SSRIs blocking hormonal events required for their expression in free cycling females (Sakar et al., 2008; Uphouse et al., 2006). However, for fully primed OVX females, FLU can still diminish sexually appetitive behaviors (Frye et al., 2003, González Cautela et al., 2021; Matuszczyk et al., 1998).

We have shown previously that the full expression of spectrotemporal parameters of S-CLS induced USVs depends on full hormone priming with EB+P (Gerson et al., 2019a). Soft CLS trills and flat-trills were emitted at drug baseline by hormonally primed OVX females with similar spectrotemporal patterns to those of our previous study. Although females received adequate hormonal priming, chronic FLU within the present study was shown to alter spectrotemporal parameters of S-CLS and Inter-CLS trills and flat-trills. As FLU is shown to inhibit the effects of ovarian hormones, it is possible these alterations were a result of the inhibition of exogenous hormonal priming. However, the diminishing effects of the SSRIs on ovarian hormones appear to be transient. A dramatic decrease was observed in the call profiles of the S-CLS recording block and the Inter-CLS interval at FLU Day 1 compared with drug baseline and other drug treatment days. On FLU Day 6, call profiles 'rebounded' for the CLS

recording block and for Inter-CLS Interval. This might indicate a transient suppression of the effects of hormonal priming, since vocal expression is hormone-dependent in females. Trills and flat-trills however displayed progressive decreases or increases in spectrotemporal parameters over chronic FLU treatment days. The call rates also declined despite this rebound in the call profile. Chronic FLU administration reduces USV emission during S-CLS and during Inter-CLS, which may indicate diminished sexual reward and sexual anticipation, respectively. These findings are consistent with earlier preclinical studies that showed FLU administration reduced sexual behaviors associated with sexual anticipation and reward in fully hormonally primed OVX females (Frye et al., 2003, González Cautela et al., 2021; Matuszczyk et al., 1998).

Conclusion

Chronic FLU administration diminished vocalizations indicative of appetitive sexual reward, i.e., S-CLS trills and flat-trills, as well as those indicative of sexual anticipation, i.e., Inter-CLS trills and flat-trills. As with our prior report, these results indicate that S-CLS-induced USVs are a key component of a female rat's sexual behavioral repertoire. Other characteristics of S-CLS, however, need to be explored before establishing these calls as a subjective measure of sexual reward in females. However, the fact that chronic FLU inhibited sexual solicitations and lordosis along with USVs is consistent with the hypothesis that USVs were tracking the emotional response to sexual stimulation.

CHAPTER THREE
VOCAL AND CONDITIONED BEHAVIORS IN RESPONSE TO S-CLS AND H-CLS

Overview and rationale

Chapter 2 found that that S-CLS, shown previously to induce both conditioned place and partner preferences, induced FM 50-kHz USV subtypes indicative of hedonic reward, namely trills and flat-trills (Burgdorf et al., 2008). We assessed the effects of two contributing factors in sexual function and sexual dysfunction, ovarian hormones and SSRIs, in *Chapter 2.1* and *Chapter 2.2*, respectively. EB + P priming facilitated spectrotemporal parameters of these call subtypes, while FLU reduced them in a regimen associated with a decrease in orgasm-like responses (OLRs), desire (solicitations), and reward (lordosis). The findings of *Chapter 2* suggest that distributed S-CLS reliably evokes 50-kHz USVs in the female rat, and these USVs are an integral part of their sexual responses. It also provides initial evidence that S-CLS induced USVs may signal an immediate affective state.

Aims of this chapter

If the USVs induced by S-CLS reflect an immediate affective state, a hedonic reward call profile should only be induced by rewarding CLS. Non-rewarding sexual stimulation should thereby induce a different call profile which includes 22-kHz USVs. A serendipitous occurrence found that use of a hard-bristle brush (H-CLS) resulted in an aversive response by EB + P primed OVX females towards distributed CLS using that brush. Experiments within *Chapter 3* were therefore aimed to assess the vocal and behavioral differences between H-CLS and S-CLS. *Chapter 3.1* investigated whether H-CLS was non-rewarding or aversive, and would result in a call subtype profile that contained 22-kHz lUSV calls. This investigation also sought to address the lack of studies on ovarian hormones and 22-kHz USVs through replicating and extending the findings of *Chapter 2.1*.

Chapter 3.1

Effect of aversive clitoral stimulation on female rat sexual behavior. I: Pattern of 50- and 22-kHz ultrasonic vocalizations

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Abstract

Adult rats are shown to emit frequency modulated (FM) 50-kHz and long 22-kHz USVs during sexual interaction as signals of sexual reward and sexual aggression respectively. Distributed clitoral stimulation (CLS) mimics stimulation females receive during mating and has

been shown to be either sexually rewarding or sexually aversive depending on the context of its delivery. It is unknown whether changing the tactile quality of CLS will also render its delivery to be sexually aversive to hormonally primed ovariectomized (OVX) female rats. To address this inquiry, eight OVX rats were treated with four ovarian hormonal treatments while receiving CLS delivered with a hard-bristle paintbrush (H-CLS) instead of one with soft bristles. Prior to ultrasonic vocalization recording of H-CLS, each female was randomly assigned to receive a treatment order of estradiol benzoate (EB) + progesterone (P), EB alone, P alone, and Oil vehicle over four ultrasonic recordings of H-CLS. A nine-day wash out period was conducted between each hormonal treatment to prevent carryover effects on spectrotemporal patterns and call profiles. Ovarian hormone treatment was found to not significantly modulate the duration, peak frequency, bandwidth, and call rate of FM 50-kHz trill and flat trill subtype calls during the H-CLS recording block. Rather, females responded to H-CLS by significantly increasing the proportion of long 22-kHz Class B subtype calls. When females received EB+P and EB alone, compared to other hormonal treatments, this subtype calls was emitted in a significantly higher proportion during the H-CLS recording block. This pattern of FM 50-kHz and long 22-kHz co-emission suggests that females find H-CLS to be sexually aversive.

Key words: Sexual aversion, ultrasonic vocalizations, female sexual behavior

1. Introduction

Rewarding and aversive situations induce ultrasonic vocalizations in adult rats, and are classified into two broad call categories, 50-kHz and 22-kHz. Fifty-kHz USVs (~32–92-kHz) consist of flat and frequency-modulated (FM) syllabic features. Flat 50-kHz USVs are frequency

constant and are posit to represent non-affective social calls that signal social coordination (Burgdorf et al., 2008; Burke et al., 2017; Wohr et al., 2008; Wohr & Schwarting, 2008), aggression (Burgdorf et al., 2008; Panksepp & Burgdorf, 2003), and ambivalence (Burgdorf et al., 2008). FM 50-kHz USV are complex in syllabic structure and are suggested to reflect positive affect during rewarding social interaction (Burgdorf et al., 2008) and in response to receipt of natural (Burgdorf et al., 2018) and drug reward (Meyer et al., 2012; Taracha et al., 2014). Of the 14 FM 50-kHz subtypes outlined by Wright et al. (2010), trills and the trill-flat combination are posited as indicators of hedonic reward states (Barker et al., 2010; Browning et al., 2011; Ma et al., 2010; Meyer et al., 2012; Willadsen et al., 2014; Wöhr & Schwarting, 2013).

Twenty-kHz USVs (~20–30-kHz), by contrast, are discrete monotonic calls that signal social avoidance (Kisko et al;. 2017; Schweinfurth, 2020; Assini et al., 2013; Thomas et al., 1983; Vivian & Miczek, 1993a), drug withdrawal (Covington III & Miczek, 2003; Vivian et al.,1994), negative affective states (Knutson et al., 2002a,b; Wöhr et al., 2005; Yee et al., 2012), but also the rewarding state of postejaculatory sexual satiety in male rats (Barfield & Geyer, 1972; Bialy et al., 2016. 2019). Because 22-kHz USVs are monotonic, they are further classified as short or long duration calls. Short 22-kHz calls are typically 10 to 300 ms (Barker et al., 2010; Simmons et al., 2018) and are emitted in response to aversive situations and/or stimuli (Simmons et al., 2018). Long 22-kHz are 300 to 3000 ms in duration and are emitted during direct external threat (Assini et al., 2013; Bali & Jaggi, 2015; Blanchard et al., 1991, 2005; Kisko et al. 2017) or during sexual satiety (Barfield & Geyer, 1972; Bialy et al., 2016) and/or sexual behavioral inhibition (Anisko et al., 1978; Barfield & Geyer, 1972; Burgdorf et al., 2008; Sach & Bialy, 2000; van der Poel & Miczek, 1991).

An integral part of the sexual repertoire of rats are FM 50-kHz and long 22-kHz. Despite some reports showing that males and females emit similar FM 50-kHz USV patterns during sexual interaction (Thomas & Barfield, 1985), others demonstrate that females tend to vocalize less often during sexual interaction (Inagaki & Mori, 2015). Sexual responsiveness nevertheless has been shown consistently to be enhanced by FM 50-kHz USVs emitted by both males and females during sexual interaction (Barfield et al., 1979; McGinnis & Vakulenko, 2003; White & Barfield, 1987, 1990). Females are more receptive toward males who emit more courtship calls (Thomas et al., 1981, 1982) compared to devocalized males (White & Barfield, 1990). The devocalization of the copulatory male partner, however, revealed that hormonally primed ovariectomized (OVX) females emit flat, trill, flat-trill, and composite FM 50-kHz subtype calls during copulation (White & Barfield, 1990). Gerson et al. (2019a) found that hormonally primed OVX females will also emit trill and flat-trill 50-kHz subtypes in response to mimicked paced copulation vis external CLS.

Both adult male and female rats emit FM 50-kHz USVs to signal sexual reward; however, adult male rats emit long 22-kHz USVs to signal sexual satiety (Barfield & Geyer, 1972; Bialy et al., 2016) and emotional relaxation from high to low sexual arousal (Bialy et al., 2019). Because they share a monotonic structure with alarm calls, some researchers suggest post-ejaculatory calls reflect aversion rather than sexual behavioral inhibition. Yet, post-ejaculatory calls end in a steep downward deflection, which is purposed to signals behavioral inhibition during a pause in the copulatory bout (Anisko et al., 1978; Barfield & Geyer, 1972; Burgdorf et al., 2008; Sach & Bialy, 2000; van der Poel & Miczek, 1991). Male rats will, however, emit syllabically distinct 22-kHz USVs during barrier non-contact tests. Barrier non-contact tests (NC) consist of presenting an inaccessible receptive mating partner behind a wire mesh screen.

According to Bialy et al. (2019), these tests are characterized by enhanced arousal (i,e., visual and chemosensory presence of a receptive mating partner) due to sexual frustration (i.e., inability to physically contact the receptive female). Indeed, when males were presented with an inaccessible female behind a wire mesh, they emitted long 22-kHz USVs that possessed either a flat prefix or an up-ramp suffix (Bialy et al., 2019). Bialy et al. (2019) found these long 22-kHz USVs, referred to as Class B lUSVs, to be syllabically distinct from long monotonic 22-kHz emitted when males were allowed to freely copulate to ejaculation with females. Interestingly, a few Class B lUSVs were emitted by hormonally primed females during the same NC tests. When sexually receptive female rats are given CLS in the presence of an inaccessible male behind a wire-mesh screen that bears a neutral odor like almond, they will subsequently avoid copulation with males bearing the same odor when given a choice on a final test to copulate with two males, one scented and one unscented (Parada et al., 2011). This is in contrast to females given CLS in the presence of the same odor alone on gauze, who subsequently solicit and take ejaculations selectively from the scented male relative to the unscented male during their first sexual experience (Parada et al., 2011).

Frequency modulated vocalizations during sexual interaction, and/or sexual stimulation, are dependent on steroid priming. Sex steroid priming, in particular, modulates spectrotemporal parameters of adult FM 50-kHz calls such as call rate, duration, peak frequency, and bandwidth (Chen et al. 2017; Fernandez-Vargas, 2017; Gerson et al., 2019a; Thomas & Barfield, 1985). Rates of FM 50-kHz USV are the highest during diestrus and proestrus and the lowest during estrus and metestrus in free cycling females (Matochik et al., 1992), and can be restored in OVX with sufficient priming with estradiol benzoate (EB) + P (Thomas & Barfield, 1985). When copulating with a devocalized male rat, OVX females primed with EB + P will vocalize more

than sesame oil-treated OVX females (Matochik et al., 1992). When receiving external CLS, OVX females primed with EB+P will also increase trill and flat-trill duration while lowering the peak frequency and narrowing the bandwidth of these calls (Gerson et al., 2019a). Gonadally intact males administered testosterone (T) or EB alone will decrease the duration, but not the bandwidth or peak frequency, of their FM 50-kHz USVs during copulation (Chen et al. 2017; Fernandez-Vargas, 2017). Castration without T replacement will gradual decrease FM 50-kHz (Matochik & Barfield, 1992), while high doses of T will restore vocalization rates to those of pre-castration (Harding & Velotta, 2010).

It is unclear whether sex steroids, particularly E2 and P, have an impact on 22-kHz USV production and acoustics in male and female rats (reviewed in Lenell et al., 2021). Inagaki and Mori (2015) reported that free-cycling female Wistar rats emit shorter stress induced 22-kHz USVs in response to an air puff during proestrus and diestrus than gonadally intact males. Stress induced 22-kHz USV emission however did not differ between proestrus and diestrus, indicating there is no relationship between cycling ovarian hormones and 22-kHz USV production. The same researchers found that T implants did not influence stress induced 22-kHz USV emission in OVX females but did so for castrated males. Combined, these studies suggest that stress-induced 22-kHz USVs emitted by females are less hormonally dependent than those emitted by males. While startle stimuli, such as air puffs, abrasive sound, or electric foot shocks, are a reliable way to induce the emission of 22-kHz USVs from rats, their interpretations cannot yet be fully extended to sexually induced 22-kHz USVs. It is therefore unknown how sex steroids impact sexually induced 22-kHz USVs.

Although CLS induced by a brush containing soft, natural camel-hair fibers (S-CLS) induced a state of sexual reward that was reflected in behavior (conditioned place and sexual

partner preference) and USV pattern (trills and flat-trills), CLS by a harder bristle brush containing natural hog-hair fibers (H-CLS) was found in preliminary work to induce avoidance behaviors commonly associated with aversive or painful stimulation (Gerson et al., 2019b). The present experiment was designed to assess the effect of H-CLS on the spectrotemporal pattern of USVs and to determine whether the USV patterns are altered by steroid hormone priming.

2. Materials and methods

2.1 Animals and surgery

Female Long-Evans rats (3–5 months, 250–400 g, Charles River, St-Constant, QC, Canada) were used as subjects. The females were drug- and sexually naive but had prior H-CLS experience. Females were housed in pairs in a colony room kept at 21°C in a 12 h:12 h light/dark cycle (lights on at 20:00). Each pair-housed cage was provided with ad libitum tap water and Purina® rat chow. Experimental procedures were approved by Concordia University's Animal Research Ethics Committee (protocol #30000300), and followed guidelines set by the Canadian Council on Animal Care.

All females underwent bilateral OVX prior to hormonal priming and testing. Bilateral OVXs were performed under ketamine/xylazine anesthesia following the surgical procedure used in Gerson et al. (2019a). Females were injected with 10 μg of estradiol benzoate (EB, 17β-diol 3-benzoate, ID E0970–000, Steraloids) 48 hours and 500 μg of progesterone (P, 4-Pregnen-3, 20-dione, ID Q2600–000, Steraloids) 4 h prior to each training session to induce full sexual receptivity. Steroids were dissolved in reagent grade sesame oil to prepare an injection volume of 0.1 ml steroid solution.

2.2. Apparatus and clitoral stimulation

Clitoral stimulations were made and recorded in a transparent Plexiglas chamber (38×60×38 cm) lined with a steel wire grid and beta chip at the bottom. The Plexiglas chamber featured two openings (13.5×13.5 cm) on either side of the front wall for experimenter access. H-CLS was delivered by lifting the base of the tail and gently brushing the clitoris with a #4 hog hair natural fiber paint brush coated with K-Y® Jelly, a non-toxic water-soluble lubricant. Distributed stimulations were performed as in Gerson et al. (2019a,b) and Parada et al. (2010; 2011) as three quick down strokes approximately every 5 s, during a one-minute period to mimic the CLS induced by male pelvic thrusting during mounts and intromissions. Each CLS session consisted of 4 minutes without any manipulation by the experimenter (i.e., inter-CLS interval), followed by 1 minute of CLS. This was repeated for 7 cycles for a total of 35 minutes per test.

2.3. Experimental procedure

Eight female rats with previous H-CLS experience (3 stimulation sessions lasting 5 minutes with 4 days between each session) were tested in a fully counterbalanced within-subject design. Treatment order was counterbalanced using a Williams design to control for carryover effects with females receiving EB+P, EB alone, P alone, and the oil vehicle. Hormonal treatments were administered on an injection schedule detailed in Gerson et al. (2019a) prior to testing. A 9-day washout period between each test day was also implemented to eliminate hormonal carryover effects that could sensitize the response to estradiol (Kow & Pfaff, 1973; Jones & Pfaus, 2014). Ultrasonic audio recordings were made for each H-CLS session on test days.

2.4. Analysis and classification of USVs

Above the transparent Plexiglas chamber, a microphone holder held a condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustic, Berlin, Germany) in the center of the long wall of the chamber. Females were recorded 15-30 cm away from the microphone. To ensure that vocalizations would be captured from all angles, the positioning of the microphone was tested using a Batty Ultrasound Generator (Goffin, 2012). Microphone signals were fed into an Ultra-SoundGate 416H data acquisition device and recorded at a sampling rate of 250 kHz with 16-bit resolution.

Avisoft SASLab Pro program (version 4.2, Avisoft Bioacoustics) was used to analyze the acoustics of rat USVs. Spectrograms were generated with 512 fast Fourier transform points and a 75% overlap (FlatTop window, 100% frame size), with a frequency resolution of 490 Hz and a time resolution of 0.5 ms. Manually selecting and labeling calls in these spectrograms was completed by an investigator blinded to the hormone treatments of the subjects. Several spectrographic criteria had to be met for each call, including 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). Identified 50-kHz USVs and 22-kHz lUSVs were classified based on their syllabic compositions, specifically those of the trills and flat-trill combination categories and of the Class B category (Wright et al. 2010; Bialy et al., 2019).

2.5. Call parameter measurements

Avisoft SASLab Pro software automatically measured the duration, bandwidth, and peak frequency of all trills and flat-trills. These automatic measurements were improved by setting a threshold of -50 dB ("Reject if peak amplitude<") and manually erasing background noise that overlapped with sound elements. Parameter analysis excluded elements of sound which were

overlaid by background noise. Bandwidth calculation was made by subtracting the maximum spectrum of the entire element by the minimum spectrum of the entire element, and a peak frequency was calculated by averaging these two spectrums. Call rates were calculated by dividing total calls for each subtype by overall recording block duration. Call rate percentages and call profile percentages were calculated by dividing total calls for each subtype by overall total of calls.

2.6. Statistical analysis

2.6.1. 50kHz USVs: Trills and flat-trills subtype calls

R software version 4.3.1 (R Development Core Team, 2023) was used to analyze spectrotemporal data of trills and flat-trills through RStudio: Integrated Development Environment for R (RStudio Team, 2023). The subsequent analyses excluded all missing data cases. Log10 transformations were applied to duration, peak frequency, and bandwidth to correct distributional skew. Although significance tests were conducted using mixed linear models using log-transformed data, contrasts and graphs use estimated marginal means in raw units. The call profile percentages of trills and flat-trill subtype calls were calculated by dividing the total number of each subtype call by the total number of all other subtype calls.

Trill and flat-trill subtype calls were tested using the same model structure to test for interaction effects between Hormonal Condition and Recording Block. The current model specified its fixed and random effects in a similar manner outlined in our prior report (Gerson et al., 2019). The current linear mixed model used repeated covariance structures and parameter estimations like our previous linear mixed model, and was fitted using Analysis of Factorial Experiments (afex) package (Singmann et al. 2018). Using Maximum Likelihood Estimation, the

mixed linear model fit was calculated, and the null hypothesis significance test was performed using Satterthwaite's approximation.

Two-way repeated measures Analyses of Variance (ANOVAs) were used to examine hormonal treatment and CLS recording blocks, and their interaction, on trill and on flat-trill call rates. H-CLS recording blocks and hormonal treatment were considered between-subject factors, while their interaction was considered within-subject. Calls made per minute of recording blocks, i.e. call rate, were the dependent variable. Within subjects' ANOVAs were also computed using the afex package (Singmann et al. 2018). The generalized eta squared was calculated to determine the effect sizes of the main and interaction effects of the repeated measure ANOVAs. Generalized eta was chosen over partial eta squared as the former provides greater generalizability (Olejnik & Algina, 2003).

Simple contrasts were performed to test interactions using the statistical packages emmeans: Estimated Marginal Means, aka Least-Squares Means (Lenth et al. 2018). For simple contrasts, marginal means were estimated using an asymptotic correction (Singmann & Kellen 2017). Multiple comparisons were adjusted for using the Holm adjustment to control for type 1 errors (Aickin & Gensler 1996). Hedge's G_{avg} were calculated as the effect size of these contrasts based on Laken (2013). All data was visualized using ggplot2: Elegant Graphics for Data Analysis (Wickham, 2016).

2.6.2. 22-kHz USVs: Class B subtype calls

JASP (JASP Team, 2019) was used to analyze the proportion of Class B subtype calls during the H-CLS recording block and the Inter-CLS Interval for each hormonal treatment. Phi φ were calculated as the effect size of all significant chi-square independence tests. For the class B

call profile percentages, the number of class B calls was divided by the number of other subtype calls, similar to the trill and flat-trill calls.

3. Results

3.1. Qualitative observations of behavioral responses to H-CLS

OVX females in the present study were observed to exhibit partial lordosis and less pacing during H-CLS recording blocks, even after EB+P was administered. Hard CLS delivery by the experimenter was often obstructed as OVX females would either grasp the bottom wire mesh to preventing lifting of the tail or hide their anogenital areas in the corners of the CLS chamber. It was noticed that failed attempted to thwart H-CLS delivery overall lead to increased displays of aggressive behaviors towards the hard-bristled paintbrush as such biting, kicking, and audible vocalizations. In several instances, the hard-bristle paintbrush was successfully bitten from the experimenters' hands after the H-CLS session ended.

3.2. Trills

Hormonal treatment did not have an overall significant effect on the duration, bandwidth, or call rate of H-CLS-induced trills, nor trills emitted during the Inter-CLS Interval. When females were hormonally primed, however, trill duration was found to significantly longer during the H-CLS recording block than the Inter-CLS Interval. Trill bandwidth was also significantly narrower during the H-CLS recording block than the Inter-CLS Interval when females received P alone, but not other hormonal treatments. H-CLS-induced bandwidth was narrower when females received P alone treatment when compared to other hormonal treatments Across hormonal treatments, there was a greater percentage of trills emitted during the Inter-CLS Interval than during the H-CLS recording block.

3.2.1. Overall effects

A significant main effect of Recording Block, but not Hormonal Condition, was found for trill duration ($F(1,1393.77) = 11.68$, $p < .001$) and trill peak frequency ($F(1,1897.46) = 29.33$, $p < 0.001$), but not for trill bandwidth. A significant interaction was however found between Hormonal Condition and Recording Block on trill duration ($F(3, 1105.80) = 5.26$, $p = 0.001$) and on trill peak frequency ($F(3,851.54) = 22.88$, $p < .001$), but not trill bandwidth. An overview of trill spectrotemporal parameters and their estimated marginal means (EMM) is given in **Table 1** while trill model parameters is given in **Table 2**. For each spectrotemporal parameter, simple contrasts were conducted on the estimated marginal means of Hormonal Conditions x Recording block groups as given in **Table 3.**

Unlike the spectrotemporal parameters of trills, the repeated measures ANOVA detected a significant main effect of Hormonal condition on the call rate of trill calls, $F(1.86, 13.02) = 5.15$, $p = 0.024$, $\eta 2$ Generalized = 0.103. A Greenhouse-Geiser correction was applied since the assumption of sphericity as indicated by the Mauchly's Test of Sphericity was violated for hormonal treatment, $\mathcal{E}(3) = 0.620$, $p < 0.024$.

Table 1.

Estimated marginal means for the Spectrotemporal Parameters and Call Rates of Trill and Flat-Trill Calls

			Duration (ms)	Peak Frequency (kHz)	*Bandwidth (kHz)*	Call Rate
Call Subtype	Recording Block	Hormonal Treatment	*EMM*	*EMM*	*EMM*	*EMM*
Trill						
	CLS					
		EB + P	57.988	58.293	34.480	52.5
		EB Only	57.112	58.071	28.726	41.625
		P Only	49.691	54.131	33.847	11.875
		Oil Vehicle	52.887	58.220	35.960	12.75
	Inter-CLS Interval					
		EB + P	50.954	57.771	36.896	112.25
		EB Only	53.724	57.555	28.034	84.25
		P Only	51.810	59.394	38.467	46.5
		Oil Vehicle	49.315	57.106	36.276	44.625
Flat-Trill						
	CLS					
		EB + P	85.456	52.302	52.3023	45.25
		EB Only	92.033	52.889	52.88859	32.625
		P Only	83.799	43.080	43.07961	5.125
		Oil Vehicle	86.709	56.544	56.54427	7.5
	Inter-CLS Interval					
		EB + P	78.413	54.161	54.16107	38
		EB Only	77.785	54.696	54.69605	43.375
		P Only	67.741	59.325	59.32494	11.5
		Oil Vehicle	68.068	55.532	55.53239	30.5

Table 2. **Model Statistics for Spectrotemporal Parameters of Trill Subtype Calls**

	Trills												
Model Statistics	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	11.680	1393.770	<0.001	29.330	1897.460	<0.001	3.370	3406.730	0.066	2.490	7	0.158	0.089
Hormonal Treatment	1.820	10.130	0.207	1.420	9.440	0.296	3.520	9.990	0.057	5.150	13.02	0.024	0.103
Hormonal Treatment x Recording Block	5.260	1105.80	0.001	22.880	851.540	<0.001	1.890	3441.020	0.128	1.550	10.69	0.253	0.006
Fixed Effects													
	Estimate	t-value	p-value	β	t-value	p-value	β	t-value	p-value				
Intercept	1.693	103.149	<0.001	1.754	320.87	<0.001	1.496	88.737	<0.001				
Recording Block (CLS vs. no CLS)	0.013	3.418	0.0006	-0.011	-5.416	<0.001	-0.006	-1.836	0.066				
EB + P vs. EB only	0.014	1.915	0.106	0.003	0.668	0.518	0.091	0.487	0.639				
EB + P vs. P only	0.0217	1.179	0.271	0.007	1.698	0.140	-0.068	-3.205	0.012				
EB + P vs. Oil Vehicle	-0.024	-1.027	0.332	-0.013	-1.318	0.215	0.014	0.295	0.775				
EB only x Recording Block	0.015	3.132	0.001	0.008	2.931	0.003	-0.006	-0.390	0.696				
P only x Recording Block	-0.0006	-0.106	0.915	0.012	4.278	<0.001	0.009	1.882	0.059				
Oil Vehicle x Recording Block	-0.024	-3.013	0.002	-0.035	-8.151	<0.001	-0.012	-1.700	0.089				

Table 3. Post Hoc Comparisons of Hormonal Condition x Recording Block groups. P-values shown have undergone the Holm Adjustment.

Trills			
	Duration	Peak Frequency	Bandwidth
EB+P: CLS vs Inter-CLS Interval	0.003	0.060	<0.0001
EB: CLS vs Inter-CLS Interval	0.003	0.535	0.625
CLS Block: EB+P vs EB	0.491	1	1
CLS Block: EB+P vs P	1	0.0002	1
CLS Block: EB+P vs Oil	1	0.739	1
Inter-CLS Interval: EB+P vs EB	1	1	1
Inter-CLS Interval: EB+P vs P	0.006	0.061	1
Inter-CLS Interval: EB+P vs Oil	0.110	1	1
Flat Trills			
	Duration	Peak Frequency	Bandwidth
EB+P: CLS vs Inter-CLS Interval	0.003	0.059	<0.0001
EB: CLS vs Inter-CLS Interval	0.003	0.535	0.403
CLS Block: EB+P vs EB	0.491	1	1
CLS Block: EB+P vs P	1	<0.0001	1
CLS Block: EB+P vs Oil	1	1	1
Inter-CLS Interval: EB+P vs EB	1	1	1
Inter-CLS Interval: EB+P vs P	0.006	0.0122	1
Inter-CLS Interval: EB+P vs Oil	0.110	1	1

3.1.2. Duration

Females treated with EB + P emitted significantly longer trills during the H-CLS recording block (EMM = 57.987, SE= 2.062) than during the Inter-CLS interval (EMM = 50.954, SE = 1.949), $p < 0.001$, $g = 1.172$, as shown in **Fig 1**. There were no significant differences in mean trill duration during the H-CLS recording and during the Inter-CLS Interval when comparing EB + P to EB alone, P alone, or Oil treatment.

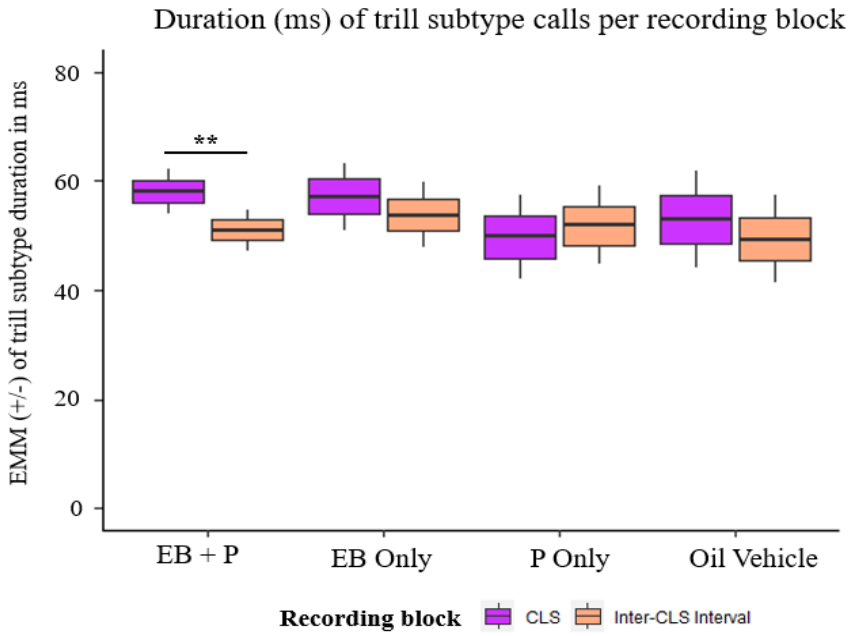

Fig 1. *Boxplots displaying the marginal means of mixed linear models for trill duration. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.2.3. Peak frequency

Trills emitted during the H-CLS block (EMM = 54.131, SE = 1.179) were lower in peak frequency than those emitted during the Inter-CLS block (EMM = 59.394, SE = 1.083), $p <$

0.001, g = 1.554, when females were treated with P alone. EB + P (EMM = 58.293, SE = 0.735) and EB alone treatment (EMM = 58.071, SE = 0.811) significantly increased trill peak frequency during the H-CLS recording block when compared with P alone, p_{EB+P} = 0.001, g_{EB+P} = 1.416, $p_{EBalone}$ = 0.005, $g_{EBalone}$ = 1.302, as shown in **Fig 2**. Among other hormonal treatments, the mean trill peak frequency did not differ significantly between CLS and Inter-CLS Intervals.

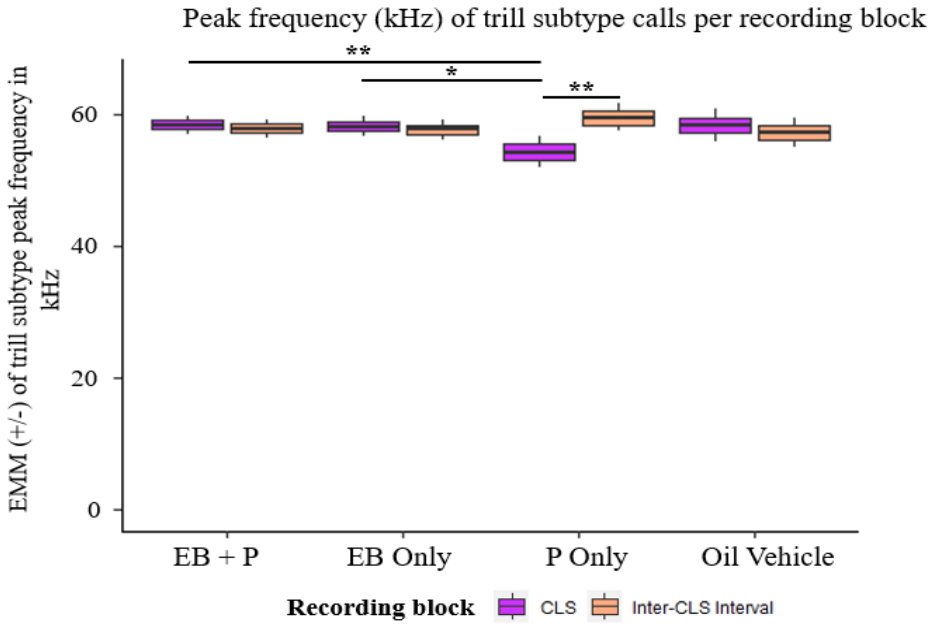

Fig 2. *Boxplots displaying the marginal means of mixed linear models for trill peak frequency. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005.***

3.2.4. Bandwidth

Log10 trill bandwidth mixed linear model results showed that Recording Block and Hormonal Condition, as well as their interaction term, had no significant effects as shown in **Fig 3**. Therefore, no simple contrasts were performed using EMMs of trill bandwidth.

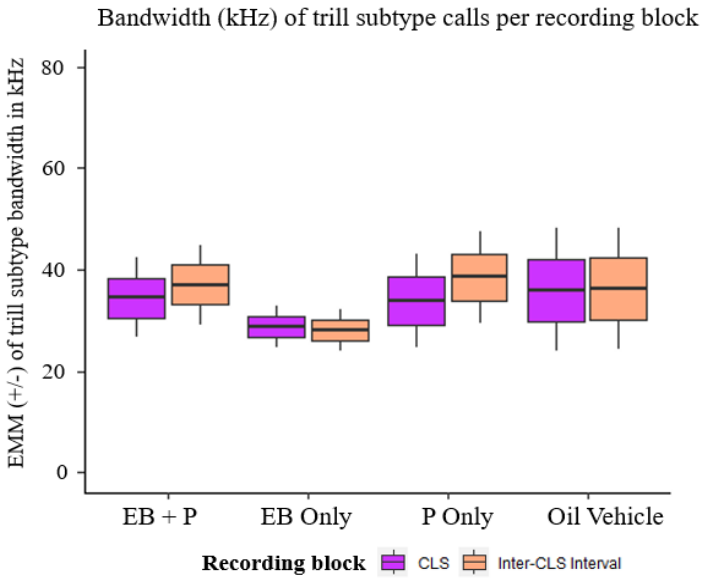

Fig 3. *Boxplots displaying the marginal means of mixed linear models for trill bandwidth. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.*

3.2.5. Call rate and call profile percentages

Trill call rates were not significantly affected by the interaction of hormonal condition and recording block as shown in **Fig 4**. There was a lower percentage of trill subtype calls in the CLS recording block call profiles compared to the Inter-CLS Interval call profiles across hormonal treatments. EB + P treatment (15%), followed by Oil treatment (14%), had the highest percentage of CLS trills than EB alone (13.6%) and P alone (9.6%). EB + P (27%), EB only (27.8%), and P (26.9%) only had high percentages of Inter-CLS Interval trills compared to Oil treatment (17.5%).

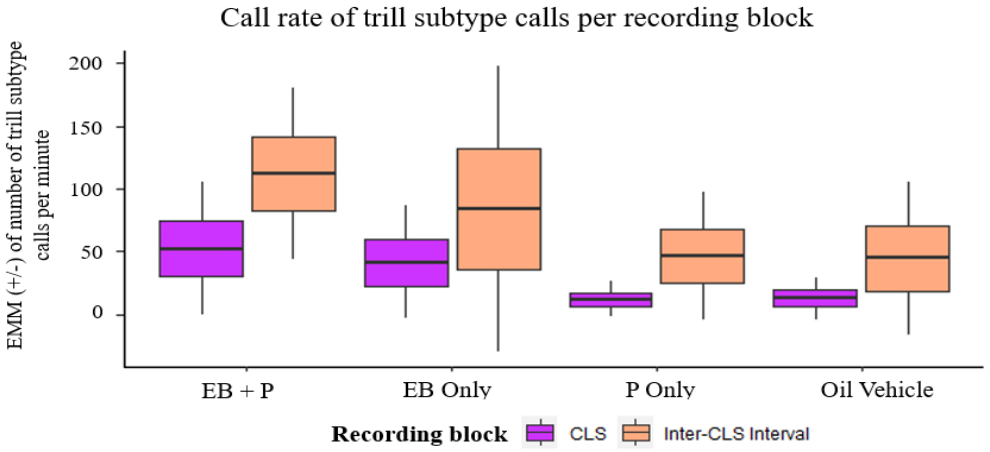

Fig 4. *Boxplots displaying the marginal means of ANOVA for trill call rate. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.*

3.3. Flat-trills

H-CLS flat-trills demonstrated similar spectrotemporal and temporal properties to H-CLS trills with the exception of call duration. In contrast to H-CLS trills, the H-CLS recording block call profile had a higher percentage of H-CLS flat-trills across hormonal treatments compared to the Inter-CLS Interval.

3.3.1. Overall effects

The mixed linear model for flat-trills revealed a significant main effect of Recording Block, but not Hormonal Condition, on duration ($F(1, 706.51) = 7.29$, $p < 0.001$), peak frequency ($F(1, 904.83) = 52.72$, $p < 0.001$), and bandwidth ($F(1, 962.01) = 4.13$, $p = 0.042$). There was also a significant interaction of Hormonal Condition x Recording Block on flat-trill peak frequency ($F(3, 265.76) = 39.78$, $p < 0.001$), and flat-trill bandwidth ($F(3, 1050.79) = 3.48$, $p = 0.016$), but not flat-trill duration. **Table 1** also summarizes estimated marginal means and call rates for flat-trill spectrotemporal parameters while **Table 4** summarizes the parameters of the flat-trill mixed linear model. Again, comparisons were conducted using estimated marginal

means of the Hormonal Condition×Recording block groups for each spectrotemporal parameter of flat-trill subtype calls as shown in **Table 3**.

A repeated measures ANOVA for flat-trill call rate revealed a trend towards significance for the main effect of Hormonal Condition, $F(1.49, 10.45) = 4.36$, $p = 0.051$, $\eta 2$ Generalized = 0.088. A Greenhouse-Geiser correction was again used for Hormonal Condition since Mauchly's Test of Sphericity was violated, $\varepsilon(3) = 0.498$, $p = 0.05$.

Table 4. Model Statistics for Spectrotemporal Parameters of Flat-Trill Subtype Calls

	Trills												
Model Statistics	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	47.290	706.510	<0.001	52.720	904.830	<0.001	4.130	962.010	0.042	0.870	7	0.383	0.009
Hormonal Treatment	2.450	9.150	0.129	0.950	5.470	0.480	0.180	13.410	0.905	4.360	10.450	0.051	0.088
Hormonal Treatment x Recording Block	2.500	156.770	0.062	39.780	265.760	<0.001	3.480	1050.790	0.016	0.560	8.310	0.502	0.560
Fixed Effects													
	Estimate	t-value	p-value	β	t-value	p-value	β	t-value	p-value				
Intercept	1.881	214.264	<0.001	1.718	168.527	<0.001	1.510	116.756	<0.001				
Recording Block (CLS vs. no CLS)	0.039	6.876	<0.001	-0.033	-7.261	<0.001	-0.010	-2.033	0.042				
EB + P vs. EB only	0.007	0.974	0.332	-0.00008	-0.010	0.990	0.006	0.252	0.807				
EB + P vs. P only	0.0260	2.288	0.076	0.008	0.765	0.496	-0.016	-0.548	0.605				
EB + P vs. Oil Vehicle	-0.020	-1.570	0.175	-0.032	-1.597	0.144	0.018	0.467	0.651				
EB only x Recording Block	-0.018	-2.637	0.008	0.023	4.293	<0.001	-0.013	-2.094	0.036				
P only x Recording Block	-0.004	-0.467	0.642	0.024	3.138	0.002	0.021	2.317	0.020				
Oil Vehicle x Recording Block	0.009	0.939	0.348	-0.086	-10.604	<0.001	-0.015	-1.720	0.085				

3.3.2. Duration

Flat-trill duration was significantly longer during the H-CLS Recording Block ($EMM_{EB+PCLS}$ = 85.456, $SE_{EB+PCLS}$ = 2.259; $EMM_{EBaloneCLS}$ = 92.033, $SE_{EBaloneCLS}$ = 3.529; $EMM_{PaloneCLS}$ = 83.80, $SE_{PaloneCLS}$ = 4.808; EMM_{OilCLS} = 86.709, SE_{OilCLS} = 5.099) compared to the Inter-CLS Interval ($EMM_{EB+PInterCLS}$ = 78.413, $SE_{EB+PInterCLS}$ = 2.509, p = 0.003, g = 0.986; $EMM_{EBaloneInterCLS}$ = 77.784, $SE_{EBaloneInterCLS}$ = 3.229, p = 0.003, g = 1.408; $EMM_{PaloneInterCLS}$ = 67.740, $SE_{PaloneInterCLS}$ = 3.095, p = 0.003 , g = 1.328; $EMM_{OilInterCLS}$ = 68.068, SE_{OilCLS} = 4.019, p = 0.003, g = 1.357) for all Hormonal Conditions as shown in **Fig 5**. Flat-trill duration did not differ significantly during the H-CLS Recording Block and the Inter-CLS Interval when comparing EB+P to EB alone, P alone, and Oil treatments

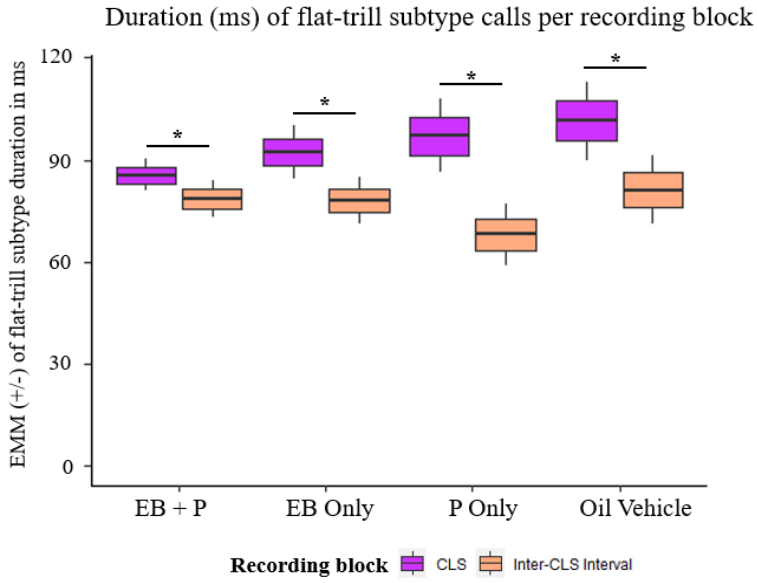

Fig 5. *Boxplots displaying the marginal means of mixed linear models for flat-trill duration. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005***

3.3.3. Peak frequency

P alone treatment significantly reduced flat-trill peak frequency during the H-CLS recording block (EMM = 43.079, SE = 2.291) compared to the Inter-CLS recording block (EMM = 59.324, SE = 2.059), $p < 0.001$, $g = 2.493$, as shown in **Fig 6**.

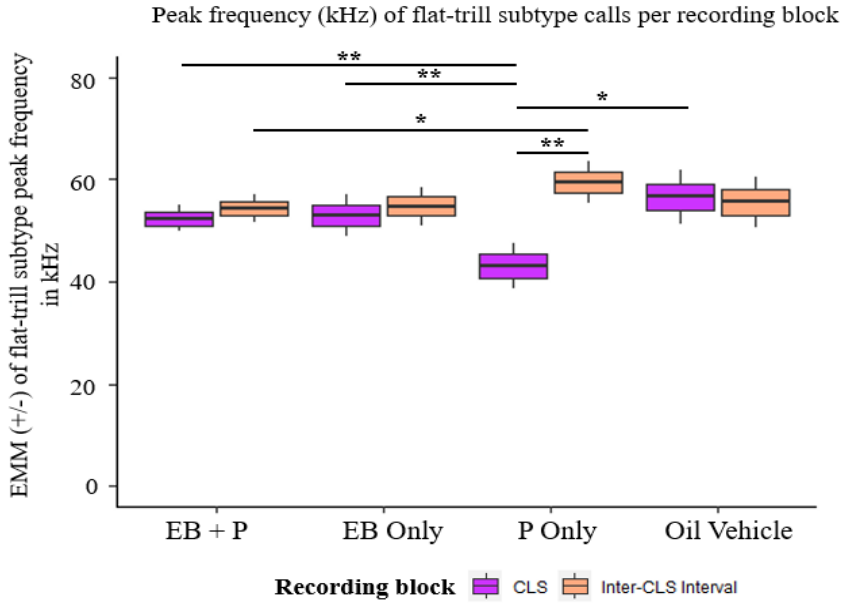

Fig 6. *Boxplots displaying the marginal means of mixed linear models for flat-trill peak frequency. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005***

Flat-trill peak frequency was significantly lower during the H-CLS recording block when females received P alone treatment compared to when they received EB + P (EMM = 52.302, SE = 1.319), $p < 0.001$, $g = 1.649$, and EB alone (EMM = 52.888, SE = 2.026), $p < 0.001$, $g = 1.516$. Interestingly, when females received Oil treatment (EMM = 56.544, SE = 2.666), they emitted higher flat-trill peak frequencies during the H-CLS recording block than when they received P alone, $p = 0.0004$, $g = 1.811$. During the Inter-CLS Interval, flat-trill peak frequency was significantly higher when females received P alone (EMM = 59.324, SE = 2.060) compared

to EB + P treatment (EMM = 54.161, SE = 1.363), p = 0.012, g = 0.988. Peak frequency of flat-trills did not differ significantly between females treated with EB+P in H-CLS recording block and the Inter-CLS Interval, compared to other hormonal treatments.

3.3.4. Bandwidth

When females were treated with EB+P, the flat-trill bandwidth was significantly narrower during the H-CLS recording block (EMM = 52.302, SE = 1.319) than during the inter-CLS interval (EMM = 54.161 , SE = 1.363), p < 0.0001, g = 0.463, as shown in **Fig 7**.

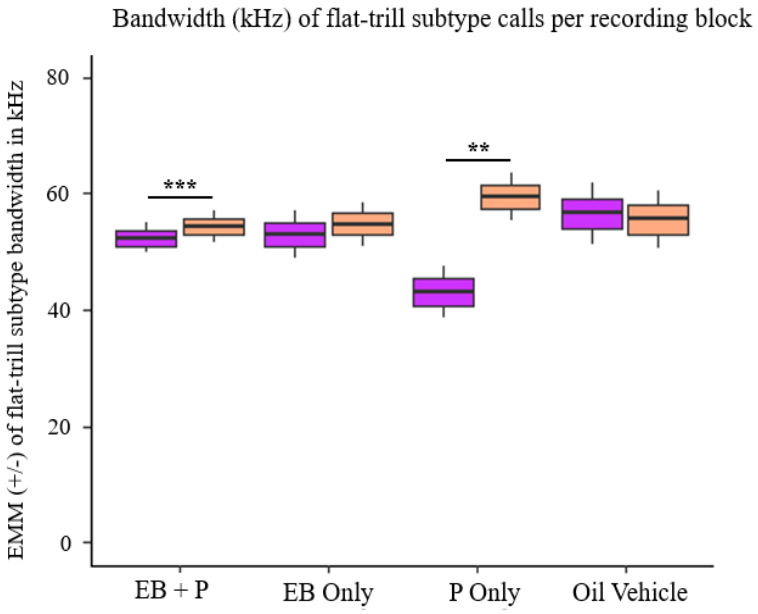

Fig 7. *Boxplots displaying the marginal means of mixed linear models for flat-trill bandwidth. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.* ****<0.05, **<0.01, ***<0.005***

Similarly, when females received P alone, the flat-trill bandwidth was significantly narrower during the H-CLS recording block (EMM = 43.079, SE = 2.291) than during the inter-CLS interval (EMM = 59.325, SE = 2.059), p = 0.0008, g = 2.493. There were no significant

differences in flat-trill bandwidth during the H-CLS recording block when comparing EB+P to the EB alone and Oil treatments. A comparison of hormonal treatments did not reveal any significant differences in flat-trill bandwidth during the Inter-CLS Interval.

3.3.5. Call rate and call profile percentages

Flat-trill call rates were also not significantly affected by the interaction between hormonal condition and recording block as shown in **Fig 8**. Unlike the trill subtype, the CLS recording block call profiles had a higher percentage of flat-trill calls than the Inter-CLS Internal call profiles. EB + P (10.5%) and EB alone (10.8%) treatments had the highest percentage of CLS trills compared to P alone (3.7%) and Oil (6.9%) treatments. There were low percentages of flat-trills occurring during Inter-CLS Intervals when females received EB + P (6.1%), EB alone (7.0%), P alone (4.7%), and Oil (6.7%) treatments.

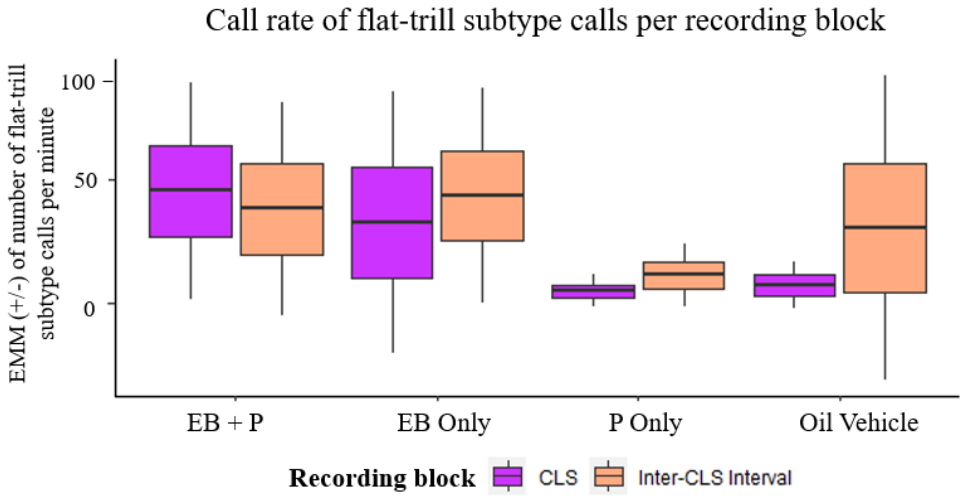

Fig 8. *Boxplots displaying the marginal means of ANOVA for flat-trill call rate. The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means.*

3.4. lUSVs

3.4.1. Proportion of Class B lUSVs between recording blocks

OVX females overall emitted a greater proportion of Class B subtype calls during the H-CLS recording block compared to the Inter-CLS interval for each hormonal treatment as shown in **Fig 9**. When primed with EB + P, OVX females emitted more Class B subtype calls during the H-CLS recording block (93.056%) than the Inter-CLS Interval (6.444%), χ^2 (1) = 53.389, p < 0.001. When OVX females received EB alone, a greater proportion of Class B subtype calls were emitted during the H-CLS recording block (92.308%) compared to the Inter-CLS Interval (7.692%), χ^2 (1) = 37.231, p < 0.001. When OVX females received EB alone, a greater proportion of Class B subtype calls were emitted during the H-CLS recording block (85.417%) compared to the Inter-CLS Interval (14.583%), χ^2 (1) = 24.083, p < 0.001. Finally, when OVX females received Oil Vehicle, a greater proportion of Class B subtype calls were emitted during the H-CLS recording block (76.923%) compared to the Inter-CLS Interval (23.077%), χ^2 (1) = 11.38, p < 0.001.

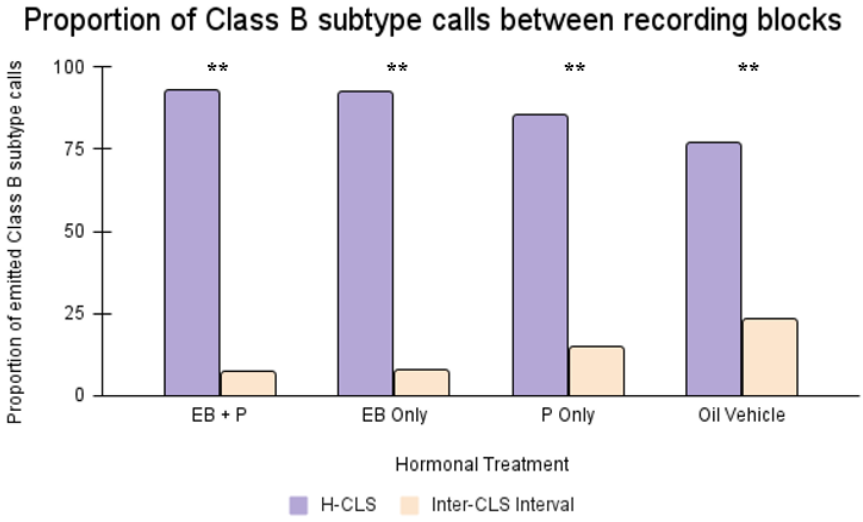

Fig 9. *Proportion of Class B subtype calls emitted between recording blocks. ** p <0.0013.3.2. Proportion of Class B lUSVs between hormonal treatments*

3.4.2. Proportion of Class B lUSVs between hormonal treatments

During the H-CLS recording block, when OVX females received EB + P or EB alone, more Class B calls were emitted than when P alone or Oil Vehicle were administered as shown in **Fig 8** and **Fig 9.** For the Inter-CLS Interval, the Class B lUSV proportions did not differ significantly between hormonal treatments. OVX females emitted a greater proportion of Class B subtype calls in response to H-CLS when they were fully hormonally primed with EB +P (62.037%) than when they received P only (37.936%), χ^2 (1) = 6.259, p = 0.012. A greater proportion of Class B subtype calls were emitted by OVX females after they were fully hormonally primed with EB +P (69%.072%) than after receiving Oil Vehicle (30.928%). After receiving EB alone (61.538%), OVX females emitted more Class B subtype calls than after receiving Oil Vehicle (38.462%), χ^2 (1) = 64.154, p = 0.042.

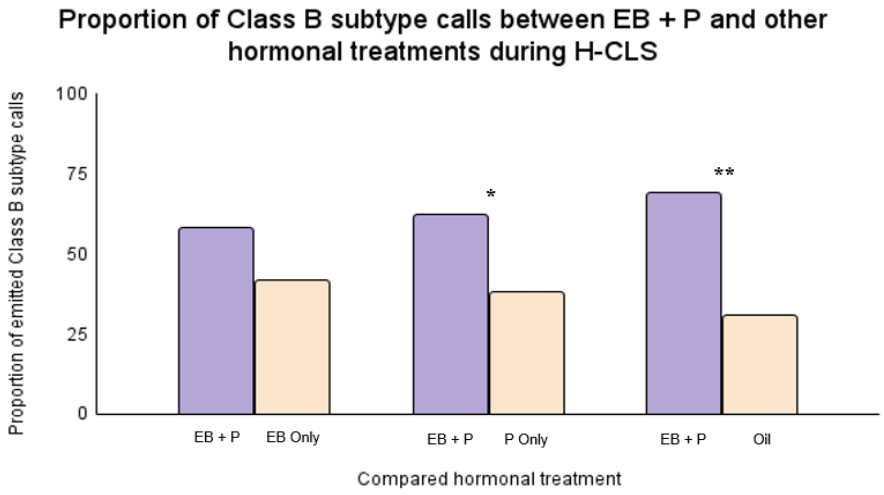

Fig 10. *Proportion of Class B subtype calls emitted between EB + P and other hormonal treatments. * p < 0.005, ** p <0.001.*

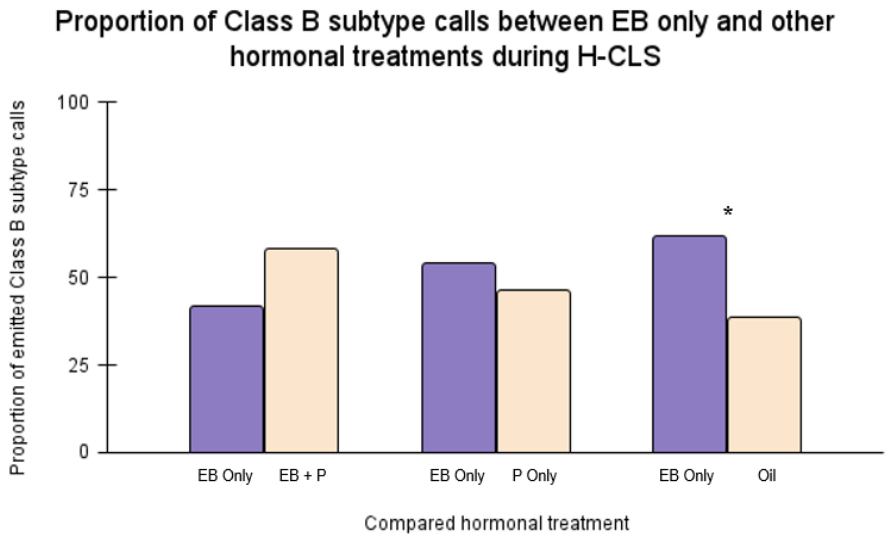

Fig 11. *Proportion of Class B subtype calls emitted between EB only and other hormonal treatments. * $p < 0.005$.*

4. **Discussion**

The present study investigated whether H-CLS induced a similar spectrotemporal pattern to S-CLS induced trills and flat-trills, and whether H-CLS induced trill subtype calls were also modulated by steroid hormonal priming. The comparison of the H-CLS recording blocks and the Inter-CLS intervals revealed that H-CLS did alter spectrotemporal parameters for trill and flat-trill call subtypes. The trill call subtype however decreased in call rate and in call profile percentage during H-CLS compared to Inter-CLS. An opposite yet non-significant trend was observed for the comparison between H-CLS and Inter-CLS flat-trill calls. Despite spectrotemporal alterations via H-CLS in both trill subtypes, steroid hormone priming did not modulate these alterations significantly, with the exception of call rate.

In addition to examining trill call subtypes, the present study evaluated whether H-CLS might influence the emission of long 22-kHz USVs, particularly those of Class B lUSV subtype. Class B lUSVs were the focus of our long 22-kHz USV analysis because the subtype is

indicative of sexual frustration or aversion. We examined whether H-CLS influence on Class B lUSV subtype was hormonally influenced as it is in the case of trill and flat-trill subtype calls, despite constituting about 3% of H-CLS and 0.39 % of Inter-CLS call profiles. For all hormonal treatments, OVX females emitted more Class B subtype calls during the H-CLS recording block than during the Inter-CLS Interval. A greater proportion of Class B subtype calls were emitted during the H-CLS recording block when females were primed with EB + P than compared to P alone and Oil Vehicle, but not EB alone. When females received EB alone, the proportion of Class B subtype calls during the H-CLS recording block was only greater compared to Oil Vehicle. As Class B subtype calls were greater in proportion during the H-CLS recording block when females were sufficiently hormonally primed, we hypothesize that these lUSVs may reflect sexual frustration or aversion during high sexual receptivity states, as suggested by the observed hormonal differences. If so, then this is the first study to demonstrate that ovarian hormones influence sexually induced 22-kHz USVs unlike stress induced 22-kHz USVs, which are reported to be hormonally independent (Lenell et al., 2021; Inagaki & Mori, 2015).

It has consistently been reported that sex steroids modulate female FM-50 kHz USV emissions and their call spectrotemporal parameters during copulatory behavior (Gerson et al., 2019a; Matochik et al., 1992; Thomas & Barfield, 1985). Matochik et al. (1992) found that USV call rates of free cycling females increase during the evening of proestrus when sexual receptivity is at its peak. OVX females primed with EB + P vocalize at a higher rate when copulating with a devocalized male rat compared to OVX females treated with sesame oil (Matochik et al, 1992b). We showed previously that trills and flat-trills were emitted at a significantly higher rate during S-CLS when OVX females were primed with EB + P relative to priming with EB alone, P alone, or Oil treatment (Gerson et al., 2019a). Bogacki-Rychlik et al.

(2022) reported a similar pattern of female vocalizations during behavioral estrus, characterized by highly modulated 50-kHz frequencies combined in clusters. H-CLS and Inter-CLS trills, but not H-CLS flat-trills, were significantly modulated by steroid hormonal priming in the current study. A greater proportion of Inter-CLS calls were emitted at a higher rate across steroid hormonal priming compared to that of H-CLS calls. The lower call rate for trills and flat-trills during H-CLS recording blocks may indicate hormone-primed females found H-CLS less rewarding as both subtypes are thought to indicate reward.

We previously demonstrated that hormone priming modulated spectrotemporal parameters such as duration, peak frequency, and bandwidth of S-CLS trills and of S-CLS flat-trills (Gerson et al., 2019a). When OVX females received EB + P, S-CLS trills and flat-trills were longer in duration, lower in peak frequency, and narrower in bandwidth. However, in the present study, hormone priming did not modulate the spectrotemporal parameters of H-CLS trills and flat-trills significantly. The rewarding properties of sexual behaviors are often measured using a conditioned place preference (CPP) paradigm, in which a rodent spends more time in one side of the CPP box previously paired with a rewarding stimulus, such as S-CLS. For example, Parada et al. (2010) showed that S-CLS facilitated the acquisition of CPP in females primed with EB+P, and that the acquisition of CLS-CPP was hormonally dependent while the expression of its reward value after conditioning did not require hormonal priming. These data suggest that ovarian hormones control sexual behaviors and postures necessary for the female to experience rewarding CLS. It has been observed that S-CLS induces solicitation, runways, hopping, and darting in sexually experienced OVX females primed with EB + P. Likewise, EB + P primed OVX females were observed exhibiting a pre-lordosis “crouch” , or presenting posture, when undergoing S-CLS. This presenting posture typically becomes a full lordosis during rewarding

paced copulation, as it allows the male to mount the female and palpate her flanks. During S-CLS, a pre-lordosis crouch enables the experimenter to lift the female's tail with ease. In contrast, a number of defensive behaviors were observed in the present study, including biting, kicking, running in circles, and hiding the anogenital area in the corners of the CLS chamber. These behavioral displays are similar to those of sexually non-receptive females during paced copulation. OVX rats, by comparison, rarely demonstrate a pre-lordosis crouch during H-CLS, instead adopting a plantarflexion posture by grasping the bottom wire mesh, making CLS delivery difficult. Perhaps this plantarflexion posture contributed to the lack of modulatory influence of steroidal priming on H-CLS induced trill subtype calls.

Based upon prior spectrotemporal evidence (Gerson et al., 2019b) and the present behavioral observations, H-CLS induced FM 50-kHz USVs are non-hormonally dependent due to being non-sexually appetitive. This is further evidenced by the greatest co-emissions of Class B subtype calls and the greatest displays of agnostic behaviors occurring the H-CLS recording when OVX females were sexually receptive via EB + P. Several studies have reported that FM-50 kHz USVs are emitted during play fights (Bekoff, 1975; Himmler et al., 2014; Kisko et al., 2017; Palagi et al., 2015) while one report shows that social FM-50 kHz USVs of OVX females are not dependent on prior hormonal priming (Garcia et al., 2017). Social FM-50 kHz USVs are suggested by Palagi et al. (2015) to prevent rougher play from escalating into aggression during bouts of play-fighting. Himmler et al. (2015) found that juvenile male and female rats emit more vocalizations during defensive interactions. Trills accounted for 77% of USVs during interaction while flat-trill combinations and shorts accounted for only 2% and 5% (Himmler et al., 2015). Both male and females emitted significantly more trills and short calls following a complete rotation (i.e., on-back wrestling position) or evasion (i.e., withdrawal) defense (Himmler et al.,

2015). Pfaus et al. (2016) reported in a pilot study that S-CLS paired with EB + P priming resulted in trills and flat-trills making up 75% of total call proportion followed by flat and complex subtype calls. H-CLS within the current study resulted in a lower portion of trills and flat-trills of total calls, e.g. approximately 25.5%, with 3% of total call proportion consisting of short and Class B type calls. The total call proportion of H-CLS thereby shares more commonalities to those of defensive play compared to responses typically observed with S-CLS and perhaps other sexual responses. While the total proportion of trills is less, H-CLS results in a less prominent proportion of flat-trills within the call profile like defensive play with the same proportion of shorts.

Comparing the H-CLS recording block to the Inter-CLS recording block, we observed that Class B USVs account for 3% of the total number of H-CLS calls. Class B USVs may indicate sexual frustration or aversion during sexual interaction. During sexual frustration tests, Bialy et al. (2019) found that primed OVX females emitted significantly fewer lUSV calls than their male mating partners. Although we observed that primed OVX emitted more Class B lUSVs during the H-CLS recording block, we believe that the inconsistent emission of lUSVs across steroid hormonal priming is consistent with Bialy et al. (2019) findings. According to other recent reports, males and females appear to vary in their emission of 22-kHz USV subtypes in response to unpleasant startle stimuli (Laine et al., 2022; Tryon et al., 2021), which are assumed to reliably elicit USV emission. Laine et al. (2022) reported that intact male rats, but not free cycling female rats, will consistently emit 22-kHz USVs during cued fear acquisition of a neutral tone paired with foot shock. Approximately 25% of males and 45% of females were reported emitting no 22-kHz during cued fear acquisition. Male non-alarm callers demonstrated significant levels of freezing towards the tone cue whereas female non-alarm callers performed

no differently than their alarm caller counterparts. A prior Pavlovian fear conditioning study conducted by Tryon et al. (2021) reported a similar finding that free cycling females emit few 22-kHz during fear learning with no differences between extinction competent and extinction resistant phenotypes. However, USV emissions of 50 kHz during fear learning were more predictive of resistance to fear extinction and of generalization to novel cues. Based on these reports, females demonstrate sex and individual differences in the emission of 22-kHz USVs in response to unpleasant situations, and it is possible that this varies across their estrous cycle. The absence of 22-kHz by non-callers, when compared to callers, during unpleasant situations are not suggestive of the absence of fear or aversion. During such situations, 50-kHz USVs may not reflect positive affect, as indicated by the co-emission of 22-kHz, but rather general arousal to facilitate predictive learning of unpleasant cues. We speculate that the observed co-emission of a few 22-kHz with 50-kHz USVs within the current study is therefore reflective of general arousal during unpleasant sexual stimulation by H-CLS.

Limitations

During H-CLS recording blocks, aggressive and sexual behaviors could not be quantified to corroborate with the vocal measures in this study. Video scoring of H-CLS sessions proved to be challenging due to the nature of delivering distributed CLS. Clear observation of aggressive and sexual behaviors via video recording were either obscured by the position of experimenter and/or the female, as attempted camera angles (e.g., top downward angle, eye level, etc.) did not properly cover all the CLS chamber dimensions. Attempts to score H-CLS sessions in real time also proved to be difficult as the subtleties of aggressive and sexual behaviors occurred too quickly to be accurately scored by the naked eye alone. Such challenges and/or difficulties, thereby lead to the present qualitative observations. The ability to accurately capture sexual

behaviors during vocalizations in response to S-CLS and H-CLS would nevertheless provide further evidence of their aversive and/or appetitive qualities.

Conclusion

Distributed CLS delivered with a hard-bristle paint brush in the current study was shown to induce trills, flat-trills, and Class B lUSVs. Spectrotemporal patterns of trills and of flat-trills during H-CLS recording block were not modulated by steroid hormonal priming, suggesting that H-CLS trill call subtypes are hormonally independent. The presence of Class B lUSVs was observed when OVX rats were primed with EB + P and EB alone during H-CLS, suggesting that females found it more sexually aversive when sexual receptivity was induced (which could also increase the sensitivity of the clitoris, as estradiol does in humans; Kim, 2009). Results of the current study demonstrate that H-CLS induced USVs differ from S-CLS induced USVs and other courtship USVs, which are modulated by sex steroids. Emissions of Class B lUSVs were however consistent with overall findings that females emit fewer lUSVs in response to unpleasant situations and/or stimuli. To our knowledge, this is the first study to report the co-emission of 22-kHz and 50-kHz USVs in response to aversive sexual stimulation in the female rat.

Chapter 3.2.

Effect of aversive clitoral stimulation on female rat sexual behavior. 2: Conditioned partner preference and conditioned partner aversion

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Abstract

Distributed clitoral stimulation (CLS) delivered with a soft bristle paint brush (S-CLS) induces conditioned partner preference (CPaP) and 50-kHz ultrasonic vocalizations (USVs), both indicative of reward. When CLS is delivered with a hard-bristle paint brush (H-CLS), females emit 22-kHz USVs, indicating aversion. The present study tested whether H-CLS could be utilized to induce a conditioned partner aversion (CPaA) and compared it to S-CLS induced CPaP based upon vocal and sexual behaviors. Seventy-two OVX, hormonally-primed females were assigned randomly to one of six conditioning groups (n = 12/group): (1) *scented* H-CLS (+); (2) *unscented* H-CLS(-); (3) *scented* S-CLS (+); (4) *unscented* S-CLS (-); (5) *scented aversive CLS differentiation* - H-CLS (+)/S-CLS (-); (6) *scented rewarding CLS differentiation*- S-CLS(+)/H-CLS(-). All females underwent 12 counterbalanced conditioning trials (6 in each condition) in which they received CLS. The final two conditioning trial days were recorded and analyzed for USVs. Four days after the last conditioning trial, females were tested in an open field partner preference test with two sexually vigorous males, one scented (Sc) and the other unscented (UnSc). A conditioned preference to receive ejaculations selectively from the Sc male was found for females assigned to S-CLS (+), replicating our previous findings. Females assigned to H-CLS (+) showed partner aversion to the Sc male, whereas females assigned to CLS differentiation groups exhibited no preference. Compared to females assigned to S-CLS (+), females also emitted more 22-kHz USVs during S-CLS than during H-CLS when the two were paired during conditioning, suggesting that S-CLS was made aversive by being counter conditioned within the same context as H-CLS.

Keywords: Clitoral Stimulation, Vocalization, Sexual Aversion

1. Introduction

Clitoral stimulation (CLS) is a powerful sexual reward in female rats. It induces both conditioned place and partner preferences in sexually naïve female rats (Parada et al., 2010, 2011) following repeated pairing of the reward state induced by CLS with either a novel environment or neutral odour cue (e.g., almond) presented on gauze when females receive CLS. When sexually naïve females have their first sexual experiences with male rats in an open field, one scented (Sc) with the same odour and the other unscented (UnSc), females in the group that had CLS paired with the odour solicit more the Sc male and receive more ejaculations from that male, compared to the UnSc male. In contrast, females in an unpaired group either do not show a preference for the ScM, or if the scent is paired with sham CLS they display a preference for an UscM (Parada et al., 2011). The neutral odour, or its absence, essentially acts as a discrete cue during conditioning that predicts the sexual reward state induced by CLS. Thus, when the odour is placed on a male during the female's first copulatory experience, it generates a significant conditioned partner preference (CPaP).

The reward value associated with CLS for sexually naive females is context-dependent (Parada et al., 2011), and it is linked to the activation of excitatory brain regions such as the medial preoptic area (mPOA) and medial amygdala (MEA; Parada et al., 2010), two regions associated with genitosensory input (Aguilar-Moreno et al., 2022; Marson, 1995), sexual incentive salience (Quintana et al., 2019), sexual desire (Pfaus, 2009) and reward (Martz, Vasquez, and Dominguez, 2023; Parada et al., 2010). In contrast, CLS induces a state of sexual frustration when delivered to sexually naïve females in the presence of an inaccessible scented or unscented male behind a wire mesh (Parada et al., 2011). Although sexual desire is stimulated by CLS, the male's inaccessibility prevents the female from interacting sexually, which created a negative association with the odour. When given a choice between a Sc male and an UnSc male

during their first copulatory experience, females with this conditioning history solicited and received ejaculations selectively from the UscM (Parada et al., 2011).

Analysis of ultrasonic vocalizations has also been used to assess the reward value of distributed CLS. In a pilot study involving distributed S-CLS, Pfaus et al. (2016) found that female 50-kHz USVs were primarily trill and flat-trill subtype calls, comprising approximately 75% of the CLS induced call profile. Trill and flat-trill subtype calls are posited to be indicators of hedonic reward state (Barker et al., 2010;Browning et al., 2011; Ma et al., 2010; Meyer et al., 2012; Willadsen, Seffer et al., 2014; Wöhr and Schwarting, 2013). Later studies by Gerson et al. (2019a; Chapter 2.*1*) showed that estradiol benzoate (EB) and progesterone (P) priming facilitated spectrotemporal parameters of these call subtypes of ovariectomized (OVX) females in response to distributed CLS, whereas chronic fluoxetine (FLU) lowered them in a regimen associated with a decrease in orgasm-like responses, desire, and reward (Chapter 2.2). Taken together, these findings provide initial evidence that distributed CLS reliably evokes 50-kHz USVs as a part of the sexual behavioral repertoire of the female rat and is a signal of an immediate affective state of pleasure during sexual interaction.

We found that a serendipitous swapping of a soft-bristle paintbrush (S) with one with hard-bristles (H) resulted in an opposite effect in EB + P primed OVX females towards distributed CLS, even when delivered in a rewarding distributed manner. Specifically, H-CLS was found to induce avoidance behaviour as well as the co-emission of trill call subtypes with long 22-kHz USVs (lUSVs; Chapter 3.1), indicative of an immediate aversive state. Compared to call profiles reported in Pfaus et al (2016), H-CLS call profiles did not exhibit trill and flat-trill dominance like those in response to S-CLS (Chapter 3.1). In addition, unlike S-CLS induced trills and flat-trills reported in Gerson et al. (2019a), EB + P priming of OVX rats did not

facilitate spectrotemporal parameters and call rates of H-CLS induced trills or of H-CLS induced flat-trills (Chapter 3.1). However, H-CLS evoke the emission of 22-kHz lUSVs of the Class B call subtype, comprising approximately 3% of the H-CLS call profile. Class B class lUSVs have been posited to indicate sexual frustration (Bialy et al., 2019) and females have been consistently shown to emit few 22-kHz USVs in response to aversive situations and/or stimuli (Bialy et al., 2019; Laine et al., 2022; Tryon et al., 2021). The high emission of FM USVs at 50 kHz combined with a low emission of 22 kHz USVs by female rats is similar to reports by experiments that used aversive Pavlovian conditioning (Laine et al., 2022; Tryon et al., 2021). These reports found that FM-kHz USVs emissions were more likely to predict aversive learning outcomes for female rats rather than 22-kHz USVs emission (Laine et al., 2022).Hard-CLS induced USVs therefore differ from those induced by S-CLS as they signal aversion rather than hedonic reward.

Although S-CLS was sufficient to induce a CPaP, it is not known what impact distributed H-CLS paired with a neutral odour might have on the sexually naive female rat's first sexual experience with scented or unscented male partners, and whether it would yield a negative association with the discrete odour cue, i.e. a conditioned partner aversion or CPaA. Previous studies have shown that pairing a neutral stimulus with sexual non-reward induces a CPaA in male and female rats. This has been done using odour or a rodent tethering jacket as the CS and either exposure to an inaccessible or sexually nonresponsive partner as the inducer of the UCS (Kippin et al., 1998; Parada et al., 2011; Quintana et al., 2019). It is possible that the aversive state induced by H-CLS would also create a CPaA for a sexually vigorous male bearing the odour. The present study examined this possibility by comparing it to the effects of S-CLS. First, we replicated the S-CLS induction of CPaP reported by Parada et al. (2011) by differential

conditioning in which S-CLS was paired with a neutral almond odour and Sham CLS with no odour. We then assessed whether H-CLS would induce either a CPaA or no conditioning by differential pairing with the neutral odour versus Sham CLS paired with no odour. We also examined whether this would occur if females had experience with H-CLS paired with the odour and S-CLS paired with no odour, or vice versa. Conditioned partner preference was predicted to occur when scented S-CLS was differentially conditioned with unscented Sham CLS, while CPaA was predicted to occur when scented H-CLS was differentially conditioned with unscented Sham CLS. Although it was not immediately obvious what effect the differential pairing of scented H-CLS and unscented S-CLS (or vice-versa) might have, we predicted that either no preference would be displayed, or that females would avoid whichever CS condition (scented or unscented) was associated with H-CLS

2. Methods

2.1. Animals

Seventy-two sexually naive Long-Evans female rats (3-5 months, 250-400g, Charles River Canada, Inc, St Constant, QC) were used in the experiment. Sixty sexually vigorous Long-Evans were used as sexual stimuli. A colony room with a 12-hour light/dark cycle (lights on at 20:00) kept at 21°C was used for housing the females in pairs and for housing the males in groups of 3 to 4 in gang cages. Tap water and Purina® rat chow were provided ad libitum to each cage. All animal procedures were approved by the Concordia University Animal Research Ethics Committee (protocol #30000300) and were in accordance with guidelines set by the Canadian Council on Animal Care.

2.2. Surgery and hormonal replacement

Each female underwent bilateral OVX prior to odour conditioning trials. Following the surgical procedure used in Gerson et al. (2019), bilateral OVXs were performed under ketamine/xylazine anesthesia. To induce sexual receptivity, OVX females were injected with 10 μg of estradiol benzoate (EB, 17β-diol 3-benzoate, Steraloids) 48 hours and 500 μg of progesterone (P, 4-Pregnen-3, 20-dione, Steraloids) 4 hours prior to each conditioning trial. An injection volume of 0.1 ml of steroid solution was prepared by dissolving steroids in reagent grade sesame oil.

2.3. Clitoral stimulation

S-CLS was made by lightly brushing the clitoris with a No. 4 soft bristle paint brush while H-CLS was using similar motion using a No. 4 hard bristle paint brush. S-CLS was applied every 5 sec for 1 min after a 2 min inter-CLS interval and this was repeated for 5 cycles for a total session duration of 15 minutes. Sham CLS was made by lifting the base of the tail but not touching the clitoris. H-CLS and sham CLS were delivered using similar timing as S-CLS. A 4-min interval between CLS was previously used in Gerson et al. (2019, Chapter 2.1) to ensure that female rats would return to baseline levels of sexual excitability (i.e., heightened locomotor activity in anticipation of sexual stimulation; Pfaus et al. 2001). To facilitate associative learning, the inter-CLS interval was shortened to 2 minutes to keep females in a state of sexual excitement and/or frustration, as demonstrated by Parada et al. (2010; 2011). CLS took place in a modified unilevel pacing chamber (38 x 60 x 38 cm) with two openings (13.5 x 13.5 cm) on either side of the front wall. This allowed for the experimenter to access the rat in the chamber to apply distributed CLS.

2.4. Odour Conditioning Procedure and Odour Cues

As in previous research, a neutral almond odour was used as the CS. Cotton gauze soaked in Club House Pure Almond Extract (McCormack Canada, London, ON) served as the scented cue (Sc) while gauze soaked in water served as the unscented control (USc). The almond cue was placed in the USV recording and conditioning chamber 5 min before placement of the female into the chamber. Trials of scented conditioning and unscented conditioning were run on alternate days in order to prevent cross-odour exposure. Following the completion of each conditioning trial, the CLS/conditioning chambers were cleaned with Lysol wipes and beta chips were replaced. Sparkleen was used to clean paintbrushes.

A week after OVX recovery, females were randomly assigned to one of six CLS-odour conditions: (1) *scented* H-CLS (+); (2) *unscented* H-CLS (-); (3) *scented* S-CLS (+); (4) *unscented* S-CLS (-); (5) *scented aversive CLS differentiation* - H-CLS(+)/S-CLS (-); (6) *scented rewarding CLS differentiation* - S-CLS (+)/H-CLS (-). Before the final open field test, each female underwent 12 odour-CLS conditioning sessions, which consisted of 6 CLS-odour sessions and 6 discrimination sessions with either sham CLS or the other sensory condition (H-CLS vs. S-CLS). For each conditioning group, the order of sessions was counterbalanced to control for order effects.

2.5. Male Sex Training

To ensure sexual vigor, stimulus males were given 10 copulation training sessions before open field trials. Each copulation training session was 30-min in duration and took place in unilevel pacing chambers (60 L x 40 W x 40 H cm) with a sexually receptive female. Sexually receptive females used for copulation training sessions were not subject to odour conditioning. In the training sessions, males who mounted females within 15 seconds were deemed good copulators.

2.6. Final Open Field Test

Open field tests were conducted as in Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus (2005) and Coria-Avila & Pfaus (2007). Females were placed in large open fields (123 cm x 123 cm x 46 cm) lined with beta chip bedding four days after the final odour conditioning trial. Two sexually vigorous males were tethered in diagonal corners of the open field with a rat tethering jacket connected to a 30 cm length spring. This allowed the males to roam within a 45-cm radius. After the males were tethered to their jackets, one was randomly scented with the neutral almond odour applied with a Q-tip to the back of the neck and anogenital area. Before introducing the female, the males were given 5 min to adjust to the jacket and scent. Females were placed in the center of the field and allowed to freely interact with both males for 30 minutes. All open field tests were video recorded using a GoPro (*HERO4 Silver*) and later scored for the proportions of females that chose either scented or unscented males for their first solicitation, mount, intromission, and ejaculation, and the number of mounts, intromissions, and ejaculations received from each male, using a behavioral scoring program (Cabilio, 1996). The final analysis excluded videos where one or both males were sexually sluggish. It is common for females to increase solicitations in order to elicit sexual engagement out of sexually sluggish males (Afonso & Pfaus, 2006; Beach, 1968). This aggravates the female, as indicated by kicking and mounting, causing the female to either only copulate with the remaining sexually vigorous male or with no males.

2.7. Operationalization of CPaP and CPaA

We defined CPaP as a preference to either solicit and/or receive ejaculations preferentially from the scented male bearing the odour associated with S-CLS. We defined a conditioned partner avoidance (CPaA) two ways: 1) as a preference to either solicit and/or receive ejaculations

preferentially from the UnSc male if the odour was previously paired with H-CLS; and/or 2) as the display of rejection responses and avoidance of copulation with either male, despite full hormone priming. Finally, a lack of conditioning was assumed if the female copulated but showed no preference for one male over the other.

2.8. Data Analysis

Open field data were analyzed with the IBM SPSS software version 26 (2019). A researcher blind to the odour-CLS conditioning group of each female scored video recordings of open field partner preference tests. The female's choice of male for first solicitation, intromission, and ejaculation was calculated using $\chi 2$ tests. Wilcoxon rank tests were used to determine the number of ejaculations, solicitations, intromissions, and visit duration for each odour-CLS conditioning group while Mann-Whitney U tests were used to assess these behavioral outcomes amongst rewarding CLS, aversive CLS, and CLS differentiation odour-CLS conditioning groups. Effect size estimates for significant $\chi 2$, Wilcoxon rank, and Mann-Whitney U tests were calculated by hand using formula for φ ($\sqrt{\chi^2}/n$) , r ($Z\sqrt{n}$), and r ((1-(2U))/(n1-n2)), respectively.

Ultrasonic vocalizations were analyzed using call parameters outlined in Gerson et al. (2019) for 50-kHz trill and flat-trill subtype calls and in Gerson et al. (Chapter 1.2 and Chapter 2.1) for Class B lUSVs. Statistical analysis of USV data was preceded by correction for skewness, resulting in log10 transformations of USV parameters of duration, peak frequency, and bandwidth. Trill and flat-trill subtype calls were analyzed using three mixed-design, between-within 2 x 2 analysis of variance (ANOVA). Each USV parameter towards CLS-odour pairing on the final odour conditioning days was treated as a within subject condition. The odour group was a between subject condition in mixed ANOVA, with two levels, scented and

unscented. All ANOVA models met the homogeneous variance assumption. Frequency of Class B lUSVs emission the final odour conditioning trials were also calculated and analyzed by three χ^2 tests. Effect sizes for significant χ^2 tests were also calculated using φ.

Estimated marginal means (EMM) for ANOVA models were calculated by SPSS software. While significance tests were performed on log transformed data, we refer to EMM in raw units throughout the results section. Planned comparisons were used to compare EMM of USV parameters made during the CLS recording blocks of the final odour conditioning trials, i.e. day 11 and 12, between Sc (+) and UnSc (-) conditioning groups. Comparisons were thereby made as follows: 1) rewarding CLS: S-CLS (+) and S-CLS (-); 2) aversive CLS: H-CLS (+) and H-CLS (-); 3) differential CLS: S-CLS (+)/ H-CLS (-) and H-CLS (+) vs S-CLS (-). For all statistical analysis, a p-value between 0.099 and 0.05 was considered to be "trending" toward significance accompanied with either a moderate or large effect size.

4. Results

4.1. Rewarding CLS: S-CLS(+) and S-CLS (-)

4.1.1 Open field tests

4.1.1.1. First choice

First ejaculation was found to be trending towards significance, $\chi^2(1) = 3.393$, $p = 0.065$, yielding a medium effect size of $\varphi = 0.361$, as shown in **Fig. 1.A**. More females in the S-CLS (+) group choose to receive their first ejaculation from the Sc male (60.0 %) than from the UnSc male (10.0 %) or no ejaculation (30.00%). Females in the S-CLS (-) group, by comparison, choose to receive their first ejaculation equally between Sc male (45.5 %) and UnSc male (45.5 %) with a few receiving no ejaculation (25.0 %). No significant differences were found for first

solicitation and for first intromission received between S-CLS and Sham groups, as shown in **Fig 1.B.** and F**ig.1.C.**

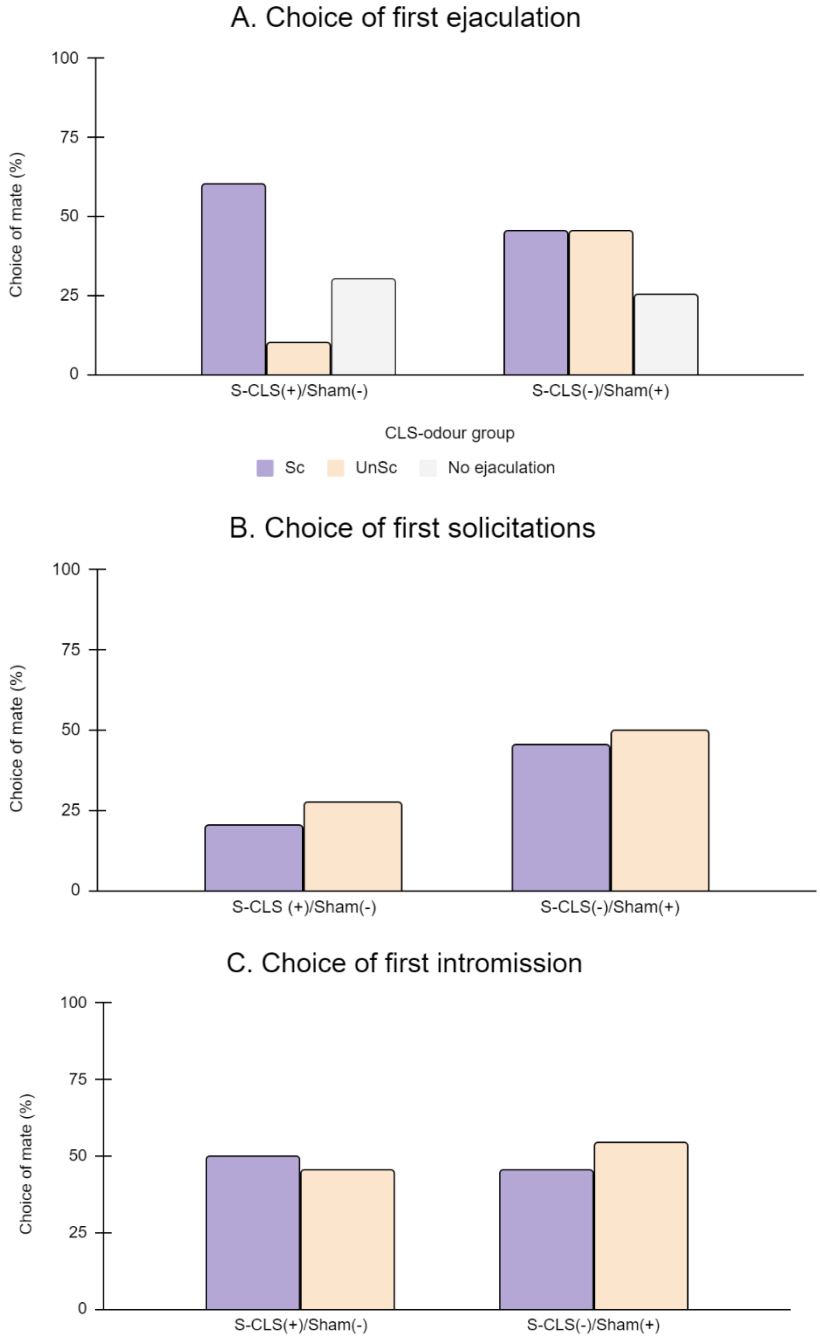

Fig. 1. *Proportion of first choice of ejaculation (A), solicitation (B), and intromission (C) between S-CLS(+)/Sham(-) and S-CLS(-)/Sham(+) conditioning groups. # indicates a trend at p <0.06.*

4.1.1.2. Frequency and total duration

Females in the S-CLS (+) group received a significantly more ejaculations from the Sc male (Mdn = 1.000) than the UnSc male (Mdn = 0.000), z = -2.070, p = 0.038, r = -3.874. as shown in **Fig.2.A**. For intromissions, solicitations, and total visit duration, there was no significant difference between Sc male and UnSc male for these females as shown in **Fig.2.B. to D**. Females in the S-CLS(-) group showed no significant differences in all behavioral outcomes towards either the Sc male and UnSc male as shown in **Fig2.A to D**.

Mann-Whitney U tests revealed no significant differences in all behavioral outcomes towards the Sc male when the odour signaled S-CLS (+) compared to when it signaled Sham (+).

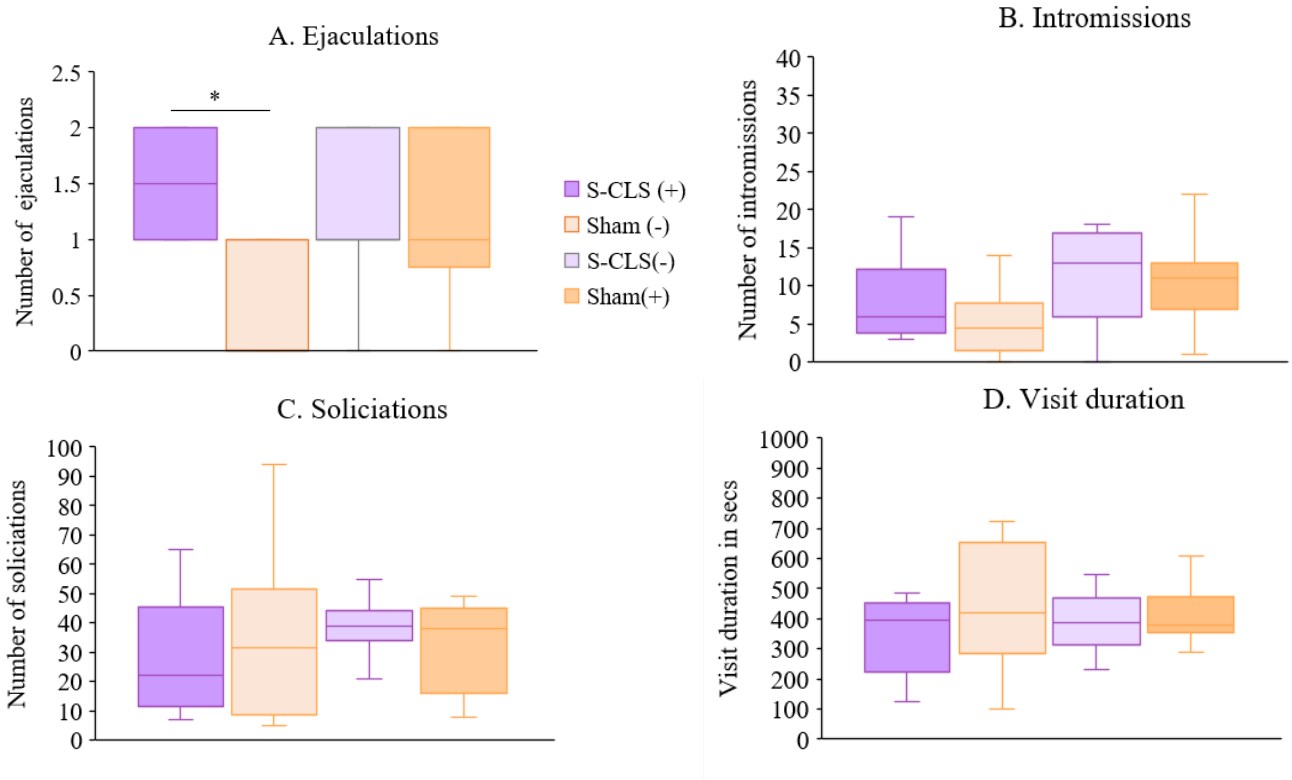

Fig 2. *Boxplots representing the number of ejaculations (A), intromissions (B), solications (C), and visit duration (D) for rewarding CLS conditioning groups. Boxes represent interquartile range while the solid line represents the median and the whiskers represent the maxium and minimum for each conditioning group. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 2.A*** ** $p < 0.05$.*

4.1.2. USV call subtype analysis

There was a significant interaction between CLS-odour pairing and odour conditioning group on the duration of trill subtype calls, $F(1,18) = 30.864$, $p < 0.001$, $\eta^2_g = 0.633$, and of flat-trill subtype calls, $F(1,18) = 14.156$, $p = 0.001$, $\eta^2_g = 0.446$, as shown in **Fig 3 A and Fig 4A**. No significant main and interaction effects were found for peak frequency and for bandwidth of both trill (**Fig 3 A to C**) and flat-trill subtype calls (**Fig 4 A to C**). Class B subtype emissions were rarely observed during the final S-CLS (+) and S-CLS (-) conditioning trial as shown in **Fig 5**.

4.1.2.1. 50-kHz USVs

Pairwise comparisons revealed significant differences for trill and flat-trill duration between CLS-odour pairings. Females emitted longer trills subtype calls during the final S-CLS(+) trial (EMM = 88.439, SEM = 7.129) compared to the final Sham (-) trial (EMM = 51.501, SEM = 7.129), $p = .023$. Trills were also longer in duration during the final S-CLS (-) trial (EMM = 73.295, SEM = 4.171) compared to the final Sham (-) trial (EMM = 63.587, SEM = 4.171), $p = .002$. Soft CLS (+) induced trills were significantly longer than Sham (-) induced trills, $p < 0.001$, but not Sham (+) induced trills. Females emitted longer flat-trill subtype calls on the final S-CLS (-) (EMM = 97.111, SEM = 6.022) trial compared to the final Sham (-) trial (EMM = 76.40, SEM = 4.087), $p = 0.003$. Soft CLS (+) flat-trills (EMM = 98.818, SEM = 6.022) were found to be significantly longer than Sham (-) induced flat-trills, $p = 0.001$.

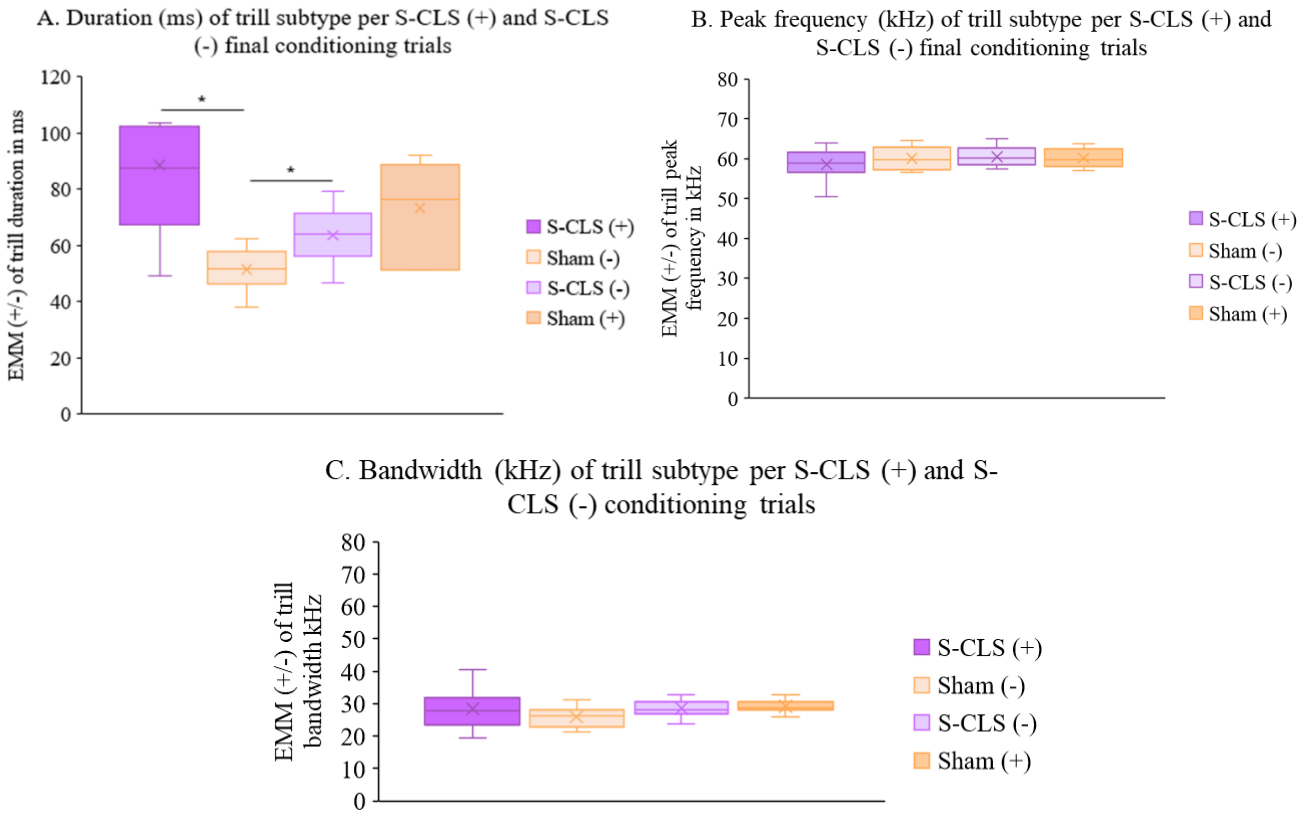

Fig 3. *Boxplots displaying the marginal means of ANOVA for trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 3.A.*** ** $p < 0.05$.*

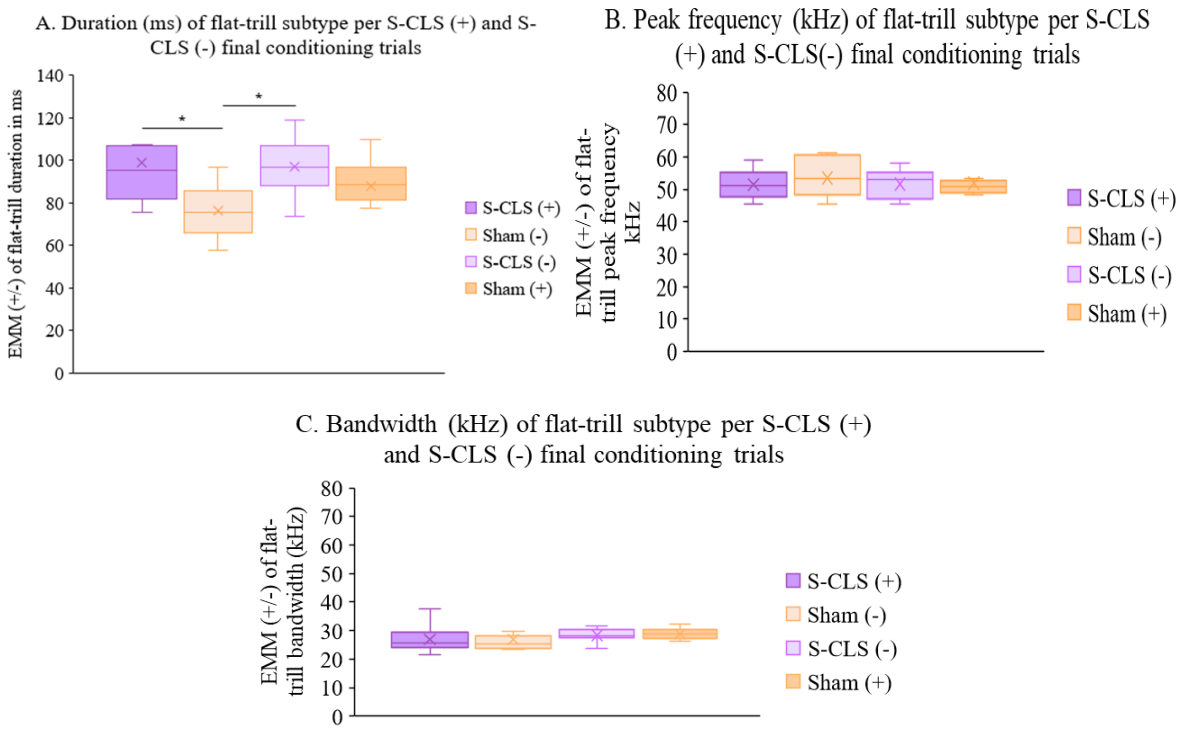

Fig 4. *Boxplots displaying the marginal means of ANOVA for flat-trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 4.A.*** ** $p < 0.005$.*

4.1.2.2. 22-kHz USVs

A greater proportion of Class B subtype calls were emitted during the Sham (-) final conditioning trial (100.000%) than the S-CLS (+) final conditioning trial (0.000%), but this proportion was found to be non-significant $\chi^2(1) = 1.000$, $p = 0.317$. Class B subtypes were also emitted in a greater proportion during the Sham (+) final conditioning trial (100.000%) than the S-CLS (-) final conditioning trial (0.000%), but this was also found to be non-significant, $\chi^2(1) = 1.000$, $p = 0.317$.

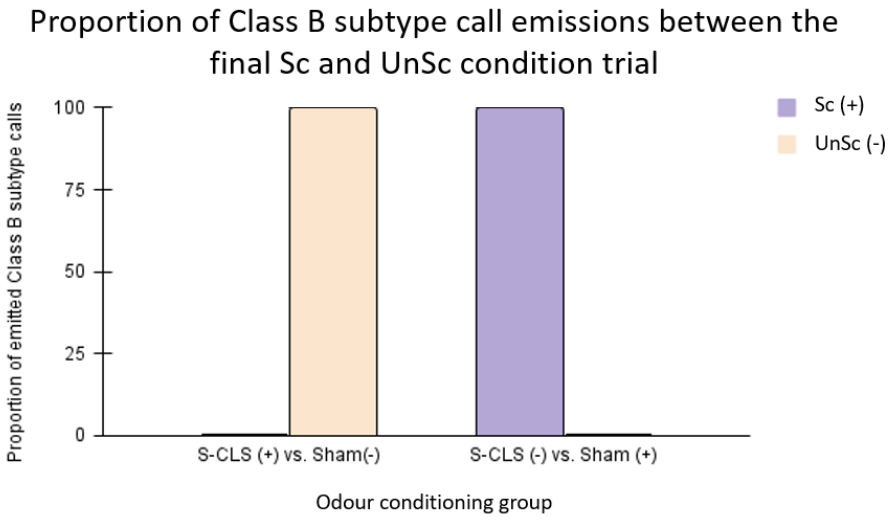

Fig 5. *Proportion of Class B subtype calls emitted between the Sc (+) and UnSc (-) final conditioning trial for S-CLS (+) and S-CLS (-) conditioning groups.*

4.2. Aversive CLS: H-CLS (+) and H-CLS (-)

4.2.1 Open field tests

4.1.2.1. First choice

While behavioural proportions were higher for Sham overall, no significant differences were found between H-CLS and Sham for first ejaculation, first solicitation, and first intromission received as shown in **Fig. 6.A. to C**.

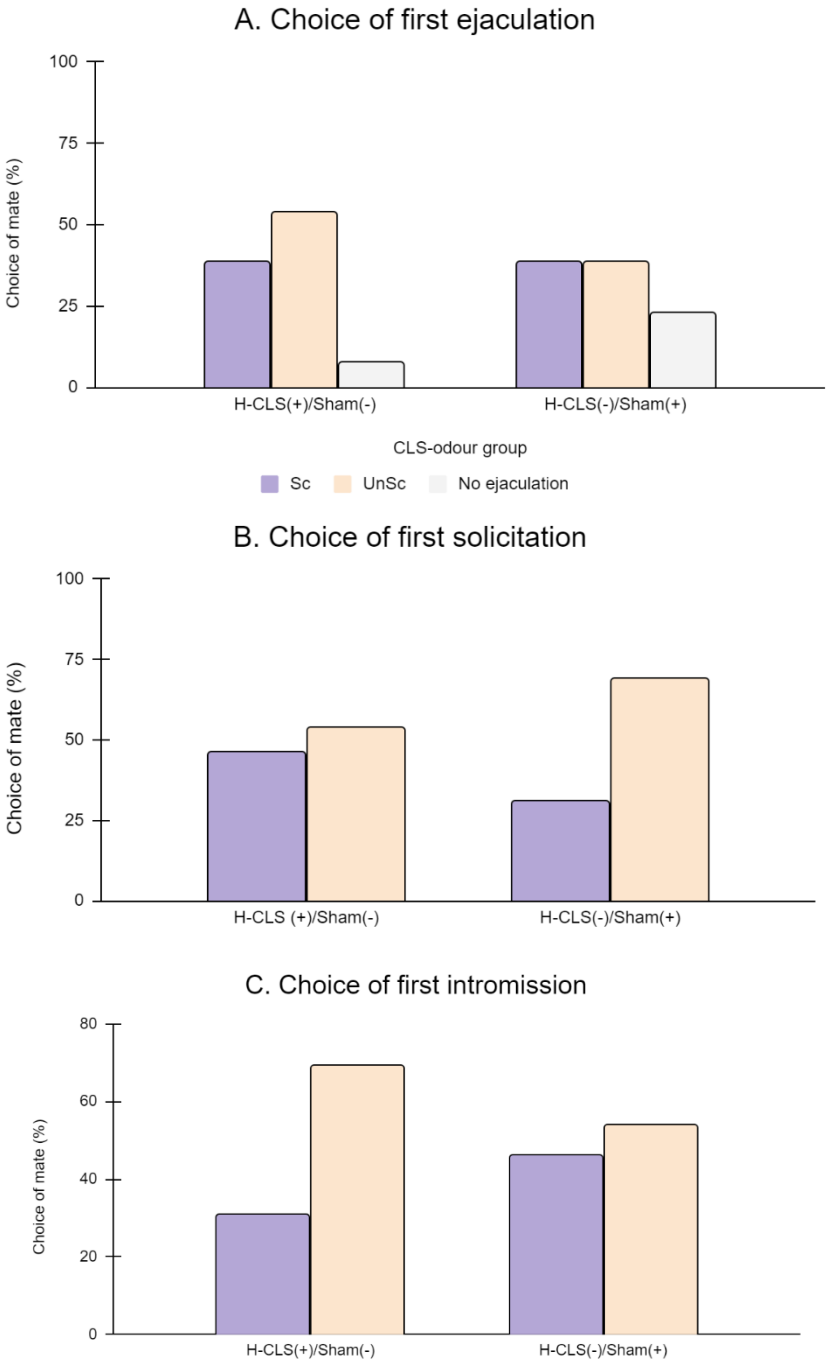

***Fig. 6**. Proportion of first choice of ejaculation (A), solicitation (B), and intromission (C) between H-CLS(+)/Sham(-) and H-CLS(-)/Sham(+) conditioning groups.*

4.2.1.2. Frequency and total duration

There was a trend towards significance for females in the H-CLS (+) group to receive fewer intromissions from the Sc males (Mdn = 10.000) than the UnSc males (Mdn = 12.000), z = 1.820, p = 0.069, r = -6.562, as shown in **Fig 7.B**. These females also spent significantly less time visiting the Sc males (Mdn = 259.050 secs) compared to the UnSc males (Mdn = 396.450 secs), z = -2.794, p = 0.005, r = -10.454, as shown in **Fig 7. D.** For ejaculations and solicitations, there was no significant difference between Sc males and UnSc males for these females as shown in **Fig 7.A. and Fig 7.C**, respectively. Females in the H-CLS(-) group showed no significant differences in all behavioral outcomes towards either the Sc males and UnSc males as shown in **Fig 7.A. to D**.

Mann-Whitney U tests revealed differences for intromissions received (**Fig 7.B.**) and total visit duration (**Fig 7.D**.), but not ejaculations (**Fig 7.A.**) or solicitations (**Fig 7.C**), towards the Sc males when the odour signaled H-CLS (+) compared to when it signaled Sham (+). A trend towards significance was found for intromissions, as females received less intromissions from the Sc males when the odour signaled H-CLS (+; Mdn = 10.000) compared to when it signaled Sham (+; Mdn = 14.000), U = 47.000, p = 0.057, r = -0.550. Females were found to spend significantly less time with the Sc males when the odour signaled H-CLS (+ ; Mdn = 249.500 secs) than when it signaled Sham (+; Mdn = 470.900 sec), U = 37.00, p = 0.014, r = -0.432.

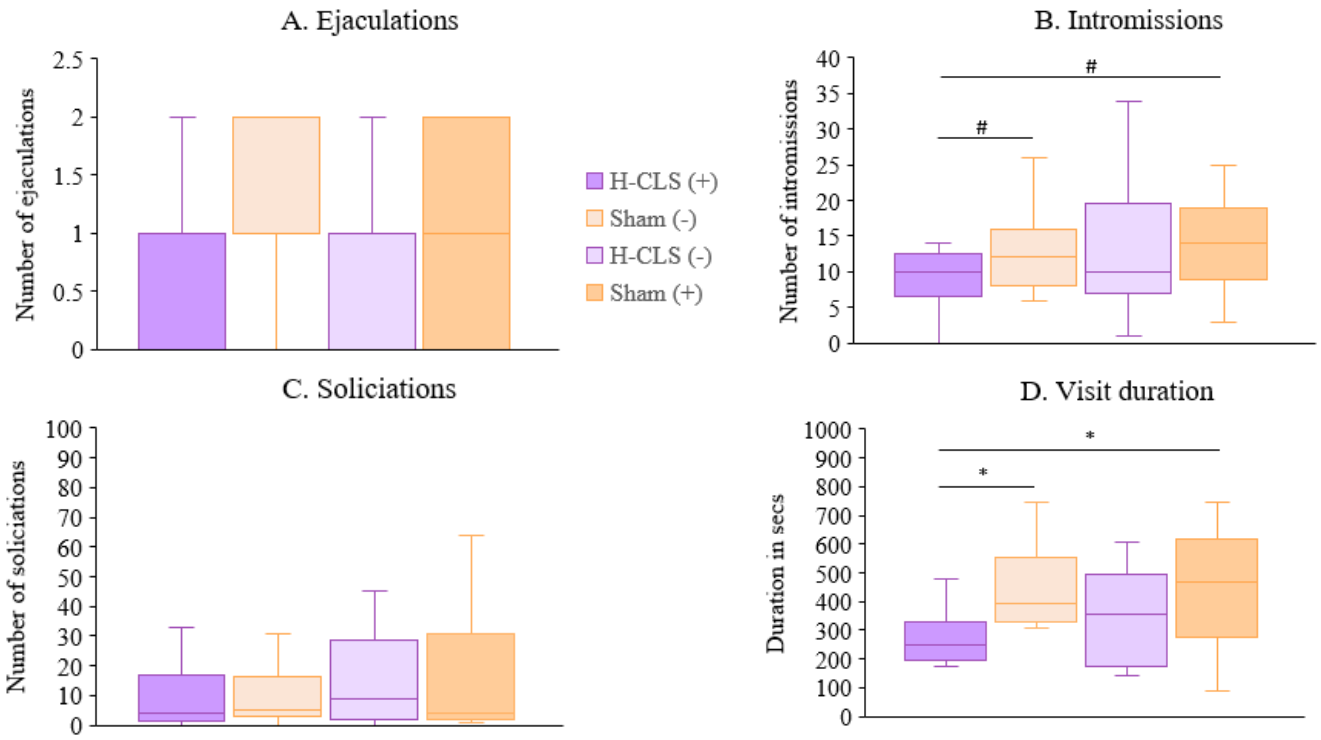

Fig 7. *Boxplots representing the number of ejaculations (A), intromissions (B), solications (C), and visit duration (D) for aversive CLS conditioning groups. Boxes represent interquartile range while the solid line represents the median and the whiskers represent the maxium and minimum for each conditioning group. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 7.A. # trend towards significance at*** *$p < 0.06$,* $p < 0.05$.*

4.1.2. USV call subtype analysis

No significant main and interaction effects were found for duration, peak frequency, and for bandwidth of trill (**Fig 8. A to C**) and of flat-trill subtype calls (**Fig 9 A to C**). Therefore, no post hoc comparisons were conducted. Class B subtype call emission was observed for aversive CLS conditioning groups and therefore a chi square analysis was conducted as shown in **Fig 10**.

4.1.2.2.1 50-kHz USVs

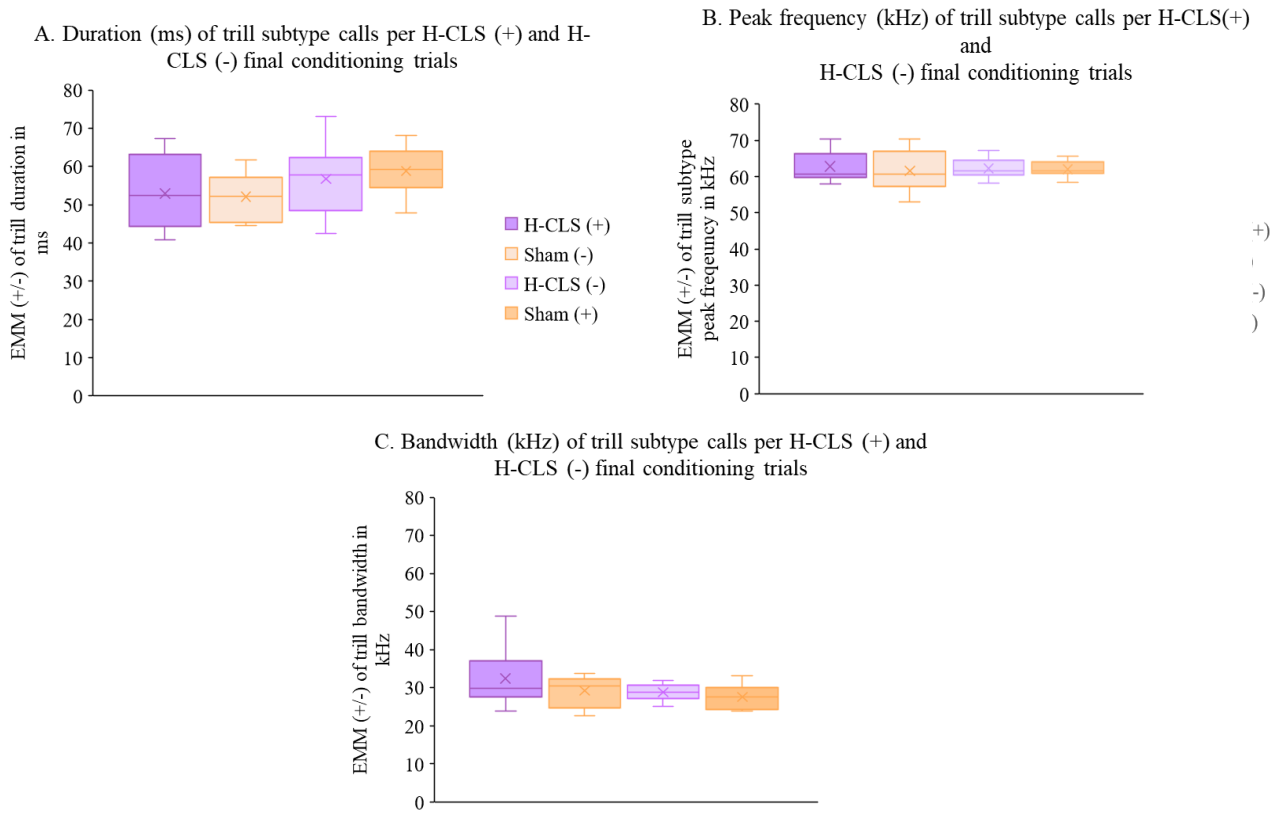

Fig 8. *Boxplots displaying the marginal means of ANOVA for trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 8.A.***

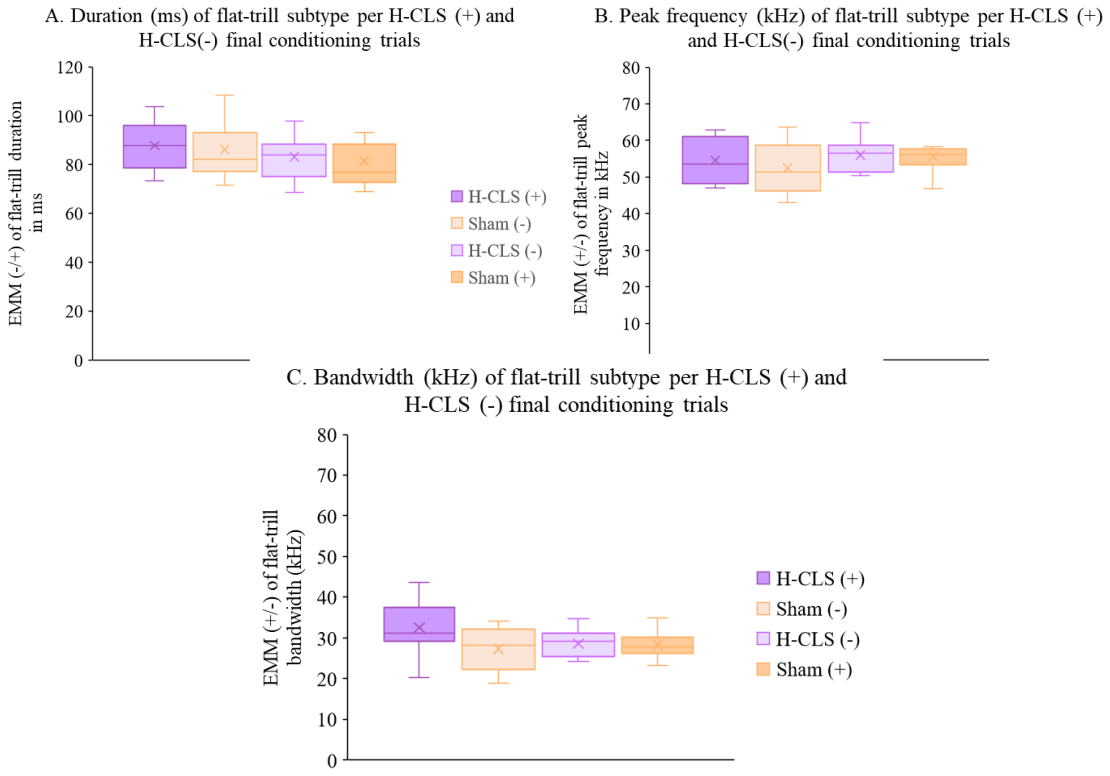

Fig 9. *Boxplots displaying the marginal means of ANOVA for flat-trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 9.A.***

4.1.2.2.2. 22-kHz USVs

A greater proportion of Class B subtype calls were emitted during the H-CLS (+) final conditioning trial (81.720%) than the Sham (-) final conditioning trial (18.280%), $\chi^2(1) = 74.860$, $p < 0.001$, $\varphi = 0.634$. Class B subtypes were also emitted in a greater proportion during the H-CLS (-) final conditioning trial (61.667%) than the Sham (+) final conditioning trial (38.333%), $\chi^2(1) = 13.067$, $p < 0.001$, $\varphi = 0.233$.

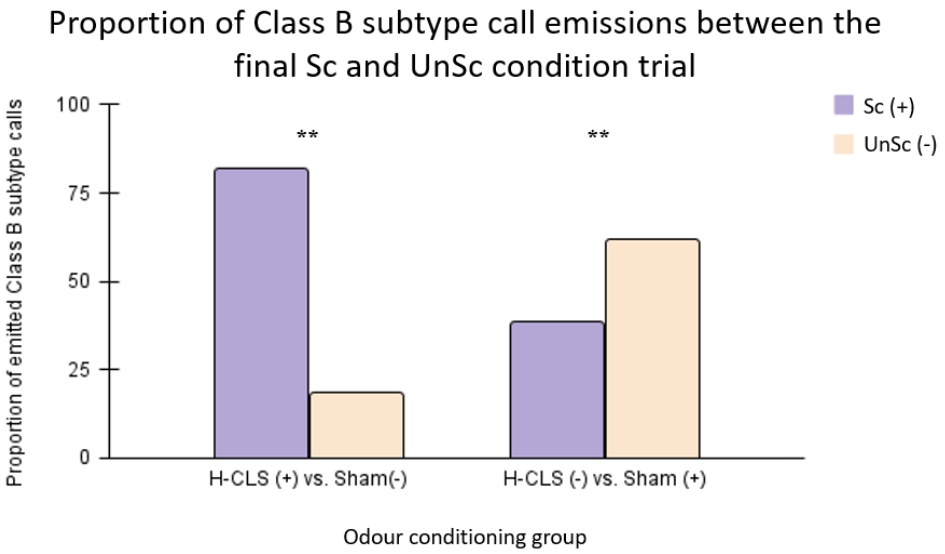

Fig 10. *Proportion of Class B subtype calls emitted between the Sc (+) and UnSc (-) final conditioning trial for H-CLS (+) and H-CLS (-) conditioning groups. ** p <0.001*

4.3. CLS Differentiation: S-CLS (+)/H-CLS(-) and H-CLS (+)/S-CLS (-)

4.3.1 Open field tests

4.3.1.1. First choice

Overall, behavioral proportions were higher for H-CLS groups, but there were no significant differences for first ejaculation, first solicitation, and first intromission between H-CLS vs. S-CLS groups as shown in **Fig. 11.A to C.**

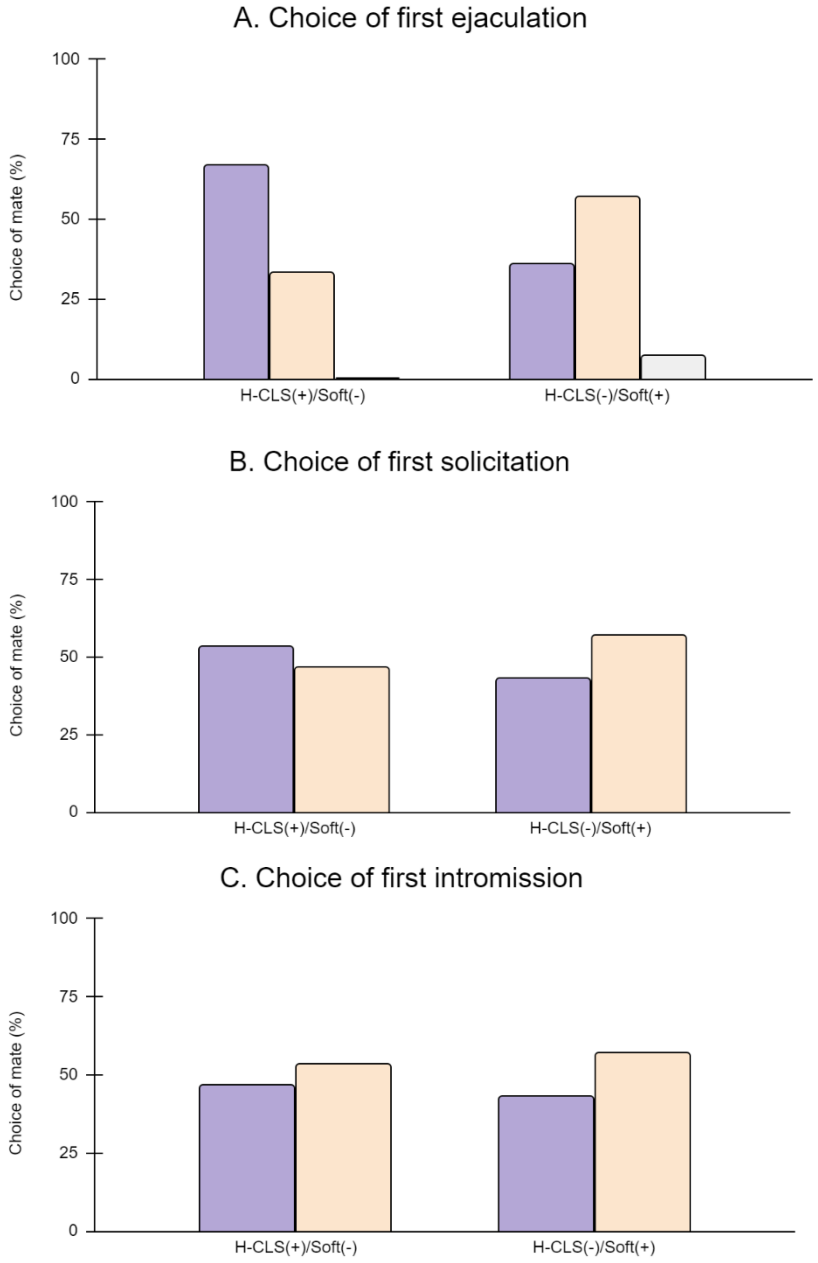

Fig. 11. *Proportion of first choice of ejaculation (A), solicitation (B), and intromission (C) between H-CLS(+)/Soft(-) and H-CLS(-)/Soft(+) conditioning groups.*

4.3.1.2. Frequency and total duration

Females in the Rough(+)/Soft(-) group showed no significant differences in all behavioral outcomes towards either the Sc males and the UnSc males as shown in **Fig 12.A. to D**. Females in the Rough(-)/Soft(+) groups also showed no significant differences in all behavioral outcomes

towards either the Sc males and the UnSc males with the exception of total visit duration. These females spent significantly less time the Sc males (Mdn = 339.050 secs) compared to the UnSc males (Mdn = 460.650), z = -2.103, p = 0.035, r = -7.689.

Mann-Whitney U tests, however, revealed no significant differences on any of the behavioral outcomes towards the Sc males when the odour signaled H-CLS(+) compared to when it signaled S-CLS(+).

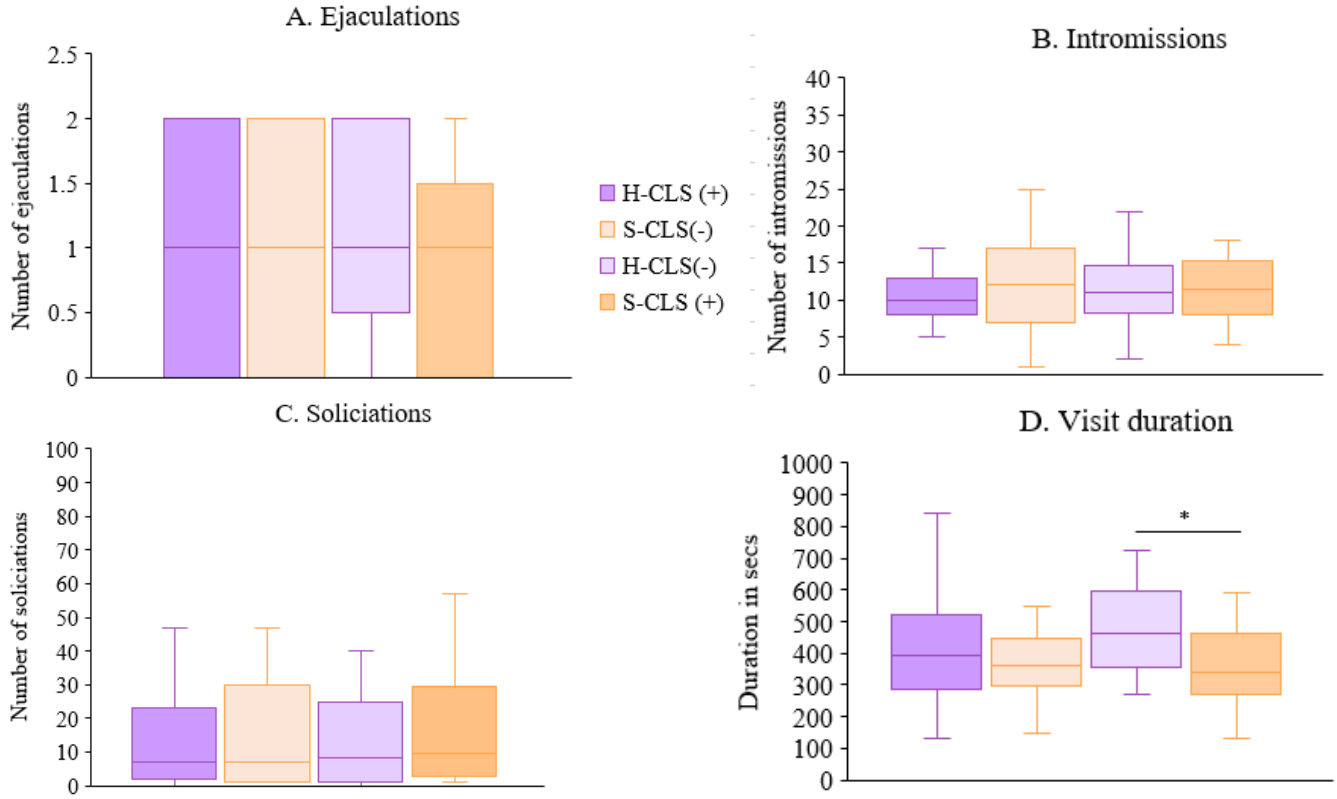

Fig 12. *Boxplots representing the number of ejaculations (A), intromissions (B), solications (C), and visit duration (D) for CLS differentiation conditioning groups. Boxes represent interquartile range while the solid line represents the median and the whiskers represent the maxium and minimum for each conditioning group. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 12.A*** *p < 0.06,* p < 0.05.*

4.3.2. USV call subtype analysis

There were trends towards a significant interaction between CLS-odour pairing and odour conditioning group of trill subtype calls for duration, $F(1,19) = 4.275$, $p = 0.053$, $\eta^2_g = 0.184$, (**Fig. 13.A**) and for bandwidth, $F(1,19) = 3.385$, $p = 0.081$, $\eta^2_g = 0.151$, (**Fig. 13.C**). No significant main and interaction effects were found for the duration and bandwidth of flat-trill subtypes (**Fig. 14.A and C**) and for the peak frequency of both trill (**Fig.13.B**) and flat-trill subtype calls (**Fig.14.B**). Class B call emission was observed similarly to H-CLS (+), so a chi square analysis was conducted as shown in **Fig. 15.**

4.1.3.2.1 50-kHz USVs

Based on pairwise comparisons, there were significant differences between CLS and odour pairings for trill duration and bandwidth. Trill duration was found to significantly differ between H-CLS (-) (EMM = 68.518, SEM = 3.899) and S-CLS (-) (EMM = 53.146, SEM = 3.713), $p = 0.011$, but not H-CLS (+) and S-CLS (+). Hard CLS (-) induced trills were longer in duration than S-CLS (+) induced trills (EMM = 57.263, SEM = 2.542), $p = 0.005$. Hard CLS (+) trills (EMM = 23.814, SEM = 1.582) were found to trend towards being narrower in bandwidth compared to S-CLS (+) trills (EMM = 28.261, SEM = 2.231), $p = 0.098$, and were found to be significantly narrower than S-CLS (-) trills (EMM = 30.436, SEM = 2.128), $p = 0.007$.

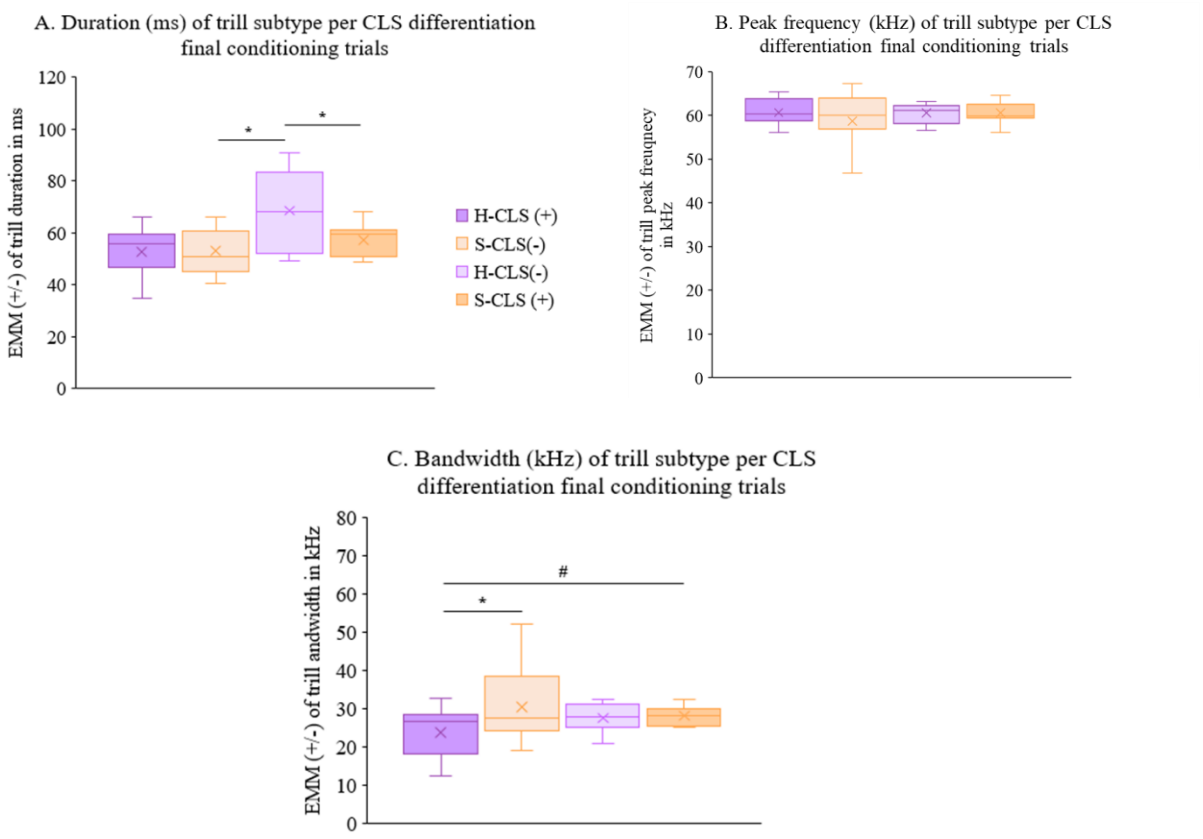

Fig 13. *Boxplots displaying the marginal means of ANOVA for trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 13.A***

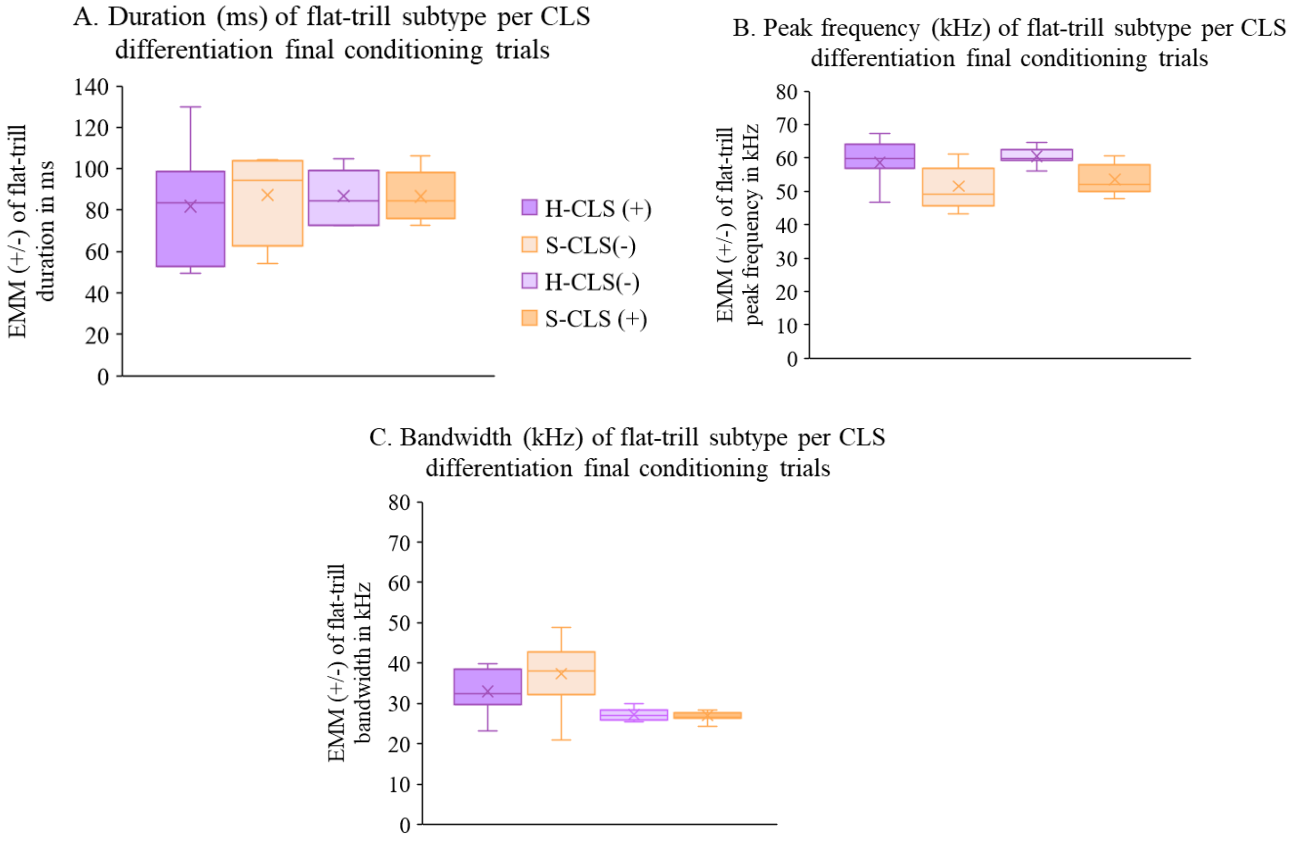

Fig 14. *Boxplots displaying the marginal means of ANOVA for trill duration (A), peak frequency (B), and bandwidth (C). The boxes represent ±1 SEM, and the whiskers the 95% CIs of the estimated means. X-axis represent CLS-odour pairing as indicated in legend in* ***Fig 14.A***

4.1.3.2.2. 22-kHz USVs

It was found that in the S-CLS (-) final conditioning trial, a greater proportion of Class B subtype calls (54.545%) were emitted than in the H-CLS (+) final conditioning trial (45.455%), but this proportion was found to be non-significant, $\chi^2(1) = 1.364$, $p = 0.243$. Class B subtypes were also emitted in a greater proportion during the S-CLS (+) final conditioning trial (44.809 %) than the H-CLS (-) final conditioning trial (55.191%), but again this proportion was found to be non-significant, $\chi^2(1) = 1.973$, $p = 0.160$.

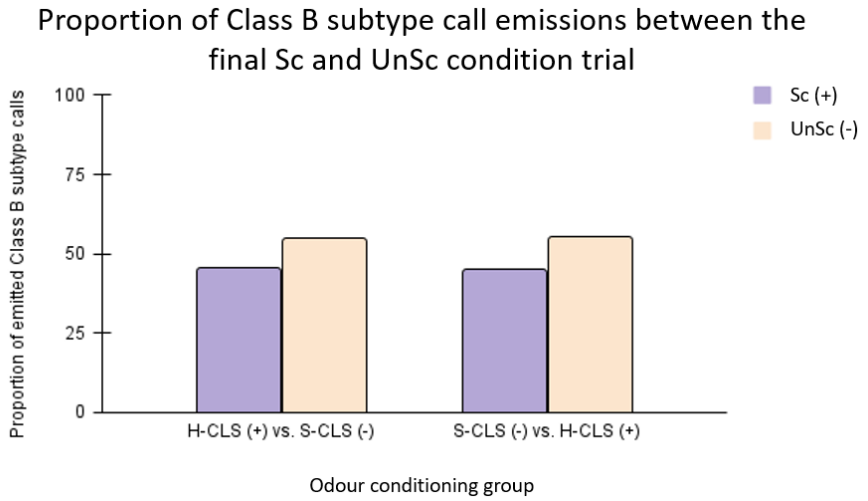

Fig 15. *Proportion of Class B subtype calls emitted between the Sc (+) and Usc (-) final conditioning trial for H-CLS (+)/S-CLS (-) and H-CLS (-)/S-CLS (+) conditioning groups.*

5. Discussion

The present experiment examined whether H-CLS could induce a CPaA relative to S-CLS induced CPaP and S-CLS induced USVs. By first replicating Parada et al. 2011, we compared H-CLS induced sexual behaviors and USVs to those differentially conditioned with Sham and/or S-CLS. Ejaculatory preference, i.e., significant mate choice and number of ejaculations received, was found for the Sc males when S-CLS (+) pairings were differentially conditioned with Sham (-), but not H-CLS (-). In contrast, an ejaculatory preference for Sc males was not found when H-CLS(+) pairings were differentiated from Sham (-)or with S-CLS (-). The first solicitation and the first intromission received did not differ significantly across all odour conditioning groups. Females in the H-CLS(+)/Sham(-) group, however, were shown to receive fewer intromissions from the Sc males, and spent less time with them compared to the UnSc males. Females also spent significantly less time with the Sc males when S-CLS(+) was differentially conditioned with H-CLS(-), but not with Sham (-). All other behavioral outcomes did not significantly differ between CLS differential conditioning groups.

Indicators of sexual and non-sexual reward during the final open field test were found to be consistent with the vocal behaviors recorded during the final USV recordings trials. Trill and flat-trill call subtypes were longer in duration for the S-CLS(+) final trial compared to those emitted during the final Sham(-) trial. Hard-CLS (+) and H-CLS (-), by comparison, did not significantly alter spectrotemporal parameters of both trill and flat-trill subtype calls when differentially conditioned with Sham (-) and Sham (+), respectively. Hard-CLS significantly altered both trill duration and trill bandwidth when differentially conditioned with S-CLS. No Class B emissions were observed in rewarding CLS conditioning groups (S-CLS vs. Sham), but were observed in the final USV recording trials for aversive CLS and CLS differentiation conditioning groups. Class B emissions were significantly higher during H-CLS when differentially conditioned with Sham, but not with S-CLS. CLS differentiation groups exhibited higher Class B emissions during the final S-CLS USV recording trial than the final H-CLS recording trial. The proportion of USV emissions, however, between both the final CLS differential trials and groups were not significant.

We have shown previously that female rats develop CPaP for Sc males when S-CLS (+) is contrasted with Sham (-) (Parada et al. 2011). S-CLS has also been shown to elicit the emission of trills and flat-trills subtype calls (Pfaus et al., 2016) as well as alter the spectrotemporal parameters of their emission (Gerson et al., 2019). Pfaus et al (2016) reported that trill and flat-trill calls, Class B 22-kHz lUSVs, were dominantly evoked by S-CLS. We found in the current experiment that S-CLS (+) /Sham (-) resulted in ejaculatory preference for the Sc males. It was found that S-CLS (+) and S-CLS (-) produced longer trills and flat trills than Sham (-) and Sham (+), respectively, with S-CLS (+) resulting in the longest trill subtype call durations. Neither S-CLS and Sham, when differentially conditioned together, evoked the

emission of Class B 22-kHz lUSVs. Our current findings on S-CLS/Sham induced sexual and vocal behaviors are thereby consistent with our previous findings on S-CLS.

This is the first study to experiment with partner preference conditioning using a non-rewarding or aversive distributed CLS. We hypothesized that H-CLS should induce CPaA, which we defined as an ejaculatory preference for the UnSc male when the olfactory cue is previously paired with H-CLS. When differentially conditioned with Sham, we found that females chose to receive their first ejaculations as well as more ejaculations from males bearing odour cues associated with Sham. While this aligns the first part of our definition of CPaA (see introduction), the difference in the total ejaculations received between H-CLS(+)/Sham (-) were found to be statistically non-significant. Females in the H-CLS(+)/Sham(-) group, however, spent significantly less time and received fewer intromissions from the Sc males. Thereby, we argue that these behavioral outcomes together indicate an avoidance of copulation, which is consistent with the latter part of our definition of CPaA. Our previous study showed that H-CLS modulates the spectrotemporal parameter of trills, but not flat-trills, while evoking the emission of Class B 22-kHz lUSVs, and that this modulation is not dependent on steroid hormone priming (Chapter3.1). Unlike S-CLS/Sham, our current experiment found no difference between H-CLS and Sham in terms of spectrotemporal parameters of trills. In keeping with the findings in Chapter 3.1, H-CLS within the current study was found to reliably evoke Class B 22-kHz lUSVs at a higher frequency compared to a baseline such as Sham.

Although these findings demonstrate that S-CLS is sexually rewarding and H-CLS is sexually aversive, they also shows that reward value of S-CLS is potentially made aversive when differentially conditioned with H-CLS. The proportion of females that chose to receive their first ejaculation from the Sc males associated with H-CLS (+) was greater than those received from

the UnSc males associated with S-CLS (-), and vice versa. While this may seem to indicate that females prefer H-CLS to S-CLS, we argue that this interpretation does not consider the present open field data, which shows ejaculatory preference for S-CLS(+), but not H-CLS(+), relative to Sham(-). If H-CLS was either more or equally as rewarding as S-CLS, then pairing it with the odour (+) and differentially conditioning it with no-odour Sham (-) should have induced an ejaculatory preference like S-CLS(+). Females in the H-CLS(-)/Soft(+) group were also found to spent significantly less time with the Sc male associated with S-CLS (+) compared to the UnSc male associated with H-CLS (-). This is similar to decreases in visit duration for the Sc males observed in the H-CLS(+)/Sham(-) group, but not in the S-CLS(+)/Sham(-) group. We suggest that this potential (but non-significant) preference for H-CLS over S-CLS is instead due to competing salience of internal and external cues. While copulatory preference depends on the integration of multisensory cues (Hoglen & Manoli, 2022; Lenschow, Mendes, Lima, 2022; Ronald, Fernadez-Juricic & Lucas, 2018), it also relies on the salience of cues associated with the context of sexual interaction and the copulatory partner (discussed in Pfaus et al., 2012).

Like sexual desire, sexual frustration is context-sensitive. A no contact test, in which a wire mesh is placed inside a Plexiglas testing chamber, can induce context-sensitive sexual frustration. The wire mesh prevents direct sexual interaction but allows for visual and olfactory partner cues. During no contact tests, males who previously acquired sexual experience in the same chamber showed vocalizations and behaviors indicating increased levels of frustration (Bialy et al., 2019). When delivered in the presence of an inaccessible male behind a wire mesh, S-CLS becomes sexually frustrating, as females in the subsequent open field test will choose males bearing cues associated with Sham CLS condition (Parada et al., 2011). There was no inaccessible male present during the delivery of CLS in the present experiment, but both types of

CLS were delivered within the same Plexiglas chamber. Thus, the concurrent sexual experience of H-CLS and S-CLS within the same Plexiglas chamber could have led to sexual frustration or confusion regarding rewarding S-CLS. As Parada et al. (2011) found, the females' sexual frustration towards rewarding S-CLS transfers to their first sexual experience with males, and in the present case, resulting in either a small preference, or less aversion, towards the differentially conditioned H-CLS.

One mitigating factor that must be considered is the type of sexual stimulation the female receives during copulation. Vaginocervical stimulation (VCS) received from the male could potentially override real or anticipated genital pain as it activates analgesia (Komisaruk &Wallman, 1977; Crowley et al., 1976; Crowley, Rodriguez-Sierra, and Komisaruk, 1976; Komisaruk & Larson, 1971). Graded pressure upon female rats' uterine cervix via manual VCS produces graded inhibition of thalamic neuronal responses (Komisaruk & Wallman, 1977; Crowley et al., 1976; Crowley, Rodriguez-Sierra, & Komisaruk, 1976) and of flexion-withdrawal reflexes (Komisaruk & Larson, 1971) to noxious stimuli. Estradiol promotes VCS induced analgesia in OVX females, but this is blocked by co-administered with progesterone (Crowley, Rodriguez-Sierra, & Komisaruk, 1976). Combined with sexually frustrating S-CLS via differential conditioning, VCS induced analgesia via the male conspecific could explain females' preference towards discrete cues associated with H-CLS. Similarly, local anesthesia with lidocaine is often used as an immediate treatment for women with provoked vestibulodynia, vulvodynia, and/or clitorodynia (Bohm-Starke et al., 2023; Silverstein et al., 2023).

Trill subtype calls were evoked and modulated by H-CLS and S-CLS (Gerson et al., 2019a; Chapter 3.1). The duration of trills emitted in response to H-CLS (-) was found within the present experiment to be significantly longer than those emitted in response to S-CLS (+). The

result was not the same for H-CLS(+)/S-CLS (-), as trills during the H-CLS final recording trial (+) were also found to be narrower in bandwidth than those emitted during S-CLS (+) and S-CLS(-). It is possible that the appetitive rewarding properties of CLS may be reflected by duration, whereas bandwidth may relate to its consummatory rewarding properties. Trills associated with reward tend to be longer in duration and wider in bandwidth compared to trills associated with non-reward, which tend to be shorter and narrower. Shorter trills during the final S-CLS(+) trial of the S-CLS(+)/H-CLS(-) group may indicate a diminishing of the appetitive reward of distributed CLS. Conversely, narrower bandwidth during the final H-CLS(+) trial may indicate that the consummatory reward of distributed CLS properties are diminished for H-CLS. Together, these results suggest that the appetitive and consummatory rewarding properties of S-CLS are diminished when differentially conditioned with H-CLS.

Twenty-two kHz Class B subtype calls have previously been shown to be co-emitted with 50-kHz trill subtype calls in response to H-CLS (Chapter 3.1), but not in response to S-CLS (Pfaus et al., 2016). In the present experiment, however, females in the CLS differentiation conditioning groups responded more frequently to S-CLS than H-CLS with calls with Class B subtype. Based on increased emission of Class B calls, we believe that this indicates that S-CLS has been made 'aversive' like Parada et al. (2011) showed with sexual nonreward, but in this case due to its differential conditioning with H-CLS within the same context. While both 22-kHz and 50-kHz vocal behaviors indicate dampening of S-CLS rewards within the present experiment, it remains to be determined whether this is solely due to context. A further study is needed to examine how the salience of discrete, contextual, and partner cues interact and influence differential conditioning of S-CLS reward against H-CLS aversion.

Conclusion

Soft-CLS (+), but not H-CLS (+), induced an ejaculatory preference for Sc males when differentially conditioned with Sham (-). Spectrotemporal and emission patterns of 50- and 22-kHz USVs were in accordance with our prior reports regarding S-CLS and H-CLS when these CLS types were contrasted to Sham. Differential conditioning of S-CLS with H-CLS, however, resulted in the dampening of S-CLS reward as indicated by the absence of S-CLS induced CPaP, displays of avoidance of copulation (i.e., less time spent with the Sc male), and the co-emission of 50- and 22-kHz USVs. We believe that the results of the present experiment may have clinical relevance for dyspareunia. Women experiencing dyspareunia are reported to exhibit anticipatory anxiety and avoidance behaviors toward penetrative sexual encounters (Linton, 2013). Both humans and rodents possess similar nociception systems to detect potential threats and/or injuries (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). Through adaptive associative learning, these analogous systems have developed the ability to predict potential threats and injuries (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). This learned prediction or anticipation, however, can become "maladaptive" through Pavlovian conditioning (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). A painful and/or unpleasant stimulus like H-CLS, or S-CLS when differentially conditioned with H-CLS, acts as a US that evokes anticipation of pain. Repeated pairings of the US with neutral stimuli like an odour or contextual cue during sexual interaction condition an anticipatory response in the presence of the now conditioned cue CS. In turn, the conditioned stimulus now predicts pain during future sexual interaction. Sexual stimuli that were once appetitive, such as S-CLS, now may evoke defensive responses and reduce sexual arousal.

In contrast, it is possible that VCS-induced analgesia during penetrative sex diminishes the real or anticipated pain, and may – even during the female's first sexual experience – lead to new learning that diminishes the anticipation. Just as paced copulation induces conditioned place

and partner preferences, VCS itself induces a reward state in rats that leads to conditioned place preference (Meerts & Clark, 2009), and a conditioned preference for objects associated with the stimulation (Guterl et al., 2015). Thus, in addition to its analgesic effects, the reward state induced by VCS, either through intromissions or artificial probing with a glass rod, can reduce the pain. This is similar to reports of women with vulvodynia who sometimes experience pleasurable stimulation of the vulva, clitoris, and vagina during masturbation or partnered sex. The mixing of sexual pleasure on a background of vulvar pain leads to lower pain anxiety and fear, less catastrophizing, and higher scores on the Female Sexual Function Index total and all subscales (Mautz et al., 2023).

CHAPTER FOUR

COMPARING FOS ACTIVATION BY S-CLS AND H-CLS

Overview and rationale

Chapter 2 investigated whether S-CLS evoked hedonic USVs while *Chapter 3* investigated whether H-CLS evoked aversive USVs and conditioned partner aversion. The aim of both Chapters was to determine whether the acoustic properties of CLS-induced USVs could be used to infer the induction of an immediate hedonic and/or aversive affective state. Since female sexual receptivity is hormonal dependent, we examined in both Chapters whether the ovarian hormones estradiol and progesterone could modulate acoustic properties of S-CLS (*Chapter 2.1*) and H-CLS-induced USVs (*Chapter 3.1*). We found that hormonal treatment modulated the production and acoustics of hedonic, but not aversive, call subtypes. The subsequent studies within both chapters then aimed to also examine whether CLS-induced USVs corresponded to the expression of sexual behaviors. We found that vocalization behavior closely matches appetitive and consummatory behaviors during open field tests (*Chapter 3.2)*. Together, these results provide foundational evidence that S-CLS induced USVs signal an immediate reward while H-CLS induced USVs signal an immediate aversion during sexual stimulation.

Aim of this chapter

S-CLS has been shown previously to activate nuclear Fos-IR in neurons within regions that process sexual stimulation and reward, including the medial preoptic area (mPOA) and posterior dorsal region of the medial amygdala (MEApd; Parada et al., 2010) The activation of neurons in these regions correlate with the expression of appetitive female sexual behaviors. The mPOA in particular is a vital integrative site for genital and extra-genital stimuli (e.g., olfactory stimuli) to activate sexual behavior and reproductive function. Moreover, sexual reward and appetitive sexual responses are linked in this region via genitosensory integration (Parades, 2010). The pattern of Fos induction by H-CLS is not known, although it is likely that it differs from the induction by S-CLS given previous evidence by Parada et al. (2010) and the

experiments of this thesis showing aversive vs. rewarding behaviors and vocalizations associated with each type of stimulus. Accordingly, *Chapter Four* investigated the pattern of Fos induction in response to H-CLS, and whether it aligns with the aversive vocalizations and sexual responses observed previously. Fos induction was first examined in response to CLS type (i.e., H-CLS, S-CLS, and Sham CLS) then in response to CLS-paired odour cues (i.e., S-CLS, H-CLS, or Sham CLS) or CLS-paired odor cues (*scented* H-CLS (+); *unscented* H-CLS (-); *scented* S-CLS (+); *unscented* S-CLS (-); *scented aversive CLS differentiation* - H-CLS(+)/S-CLS (-); *scented rewarding CLS differentiation* - S-CLS (+)/H-CLS (-)). Regions of the brain associated with sexual arousal and reward, aversion and pain, and sensory processing of genital and extragenital sexual stimulation were examined.

Chapter 4.

Effect of aversive clitoral stimulation on female rat sexual behavior. 3: Fos activation in response to rewarding or aversive clitoral stimulation and paired odor cues

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Abstract

Distributed clitoral stimulation (CLS) delivered with a soft bristle paint-brush (S-CLS) induces Fos-like immunoreactivity (Fos-IR) within sexual reward regions throughout the female

rat brain. In contrast, female rats find CLS delivered with a hard-bristle paint brush (H-CLS) to be aversive, as indicated increased emission of 22-kHz USVs and display of defensive behaviors as well as the induction of conditioned partner aversion. The present study examined Fos-IR within regions of attention, reward, and aversion (i.e., nucleus accumbens, ventral tegmental area, anterior cingulate, lateral septum, medial amygdala, and periaqueductal gray), and sexual arousal and desire (i.e., medial preoptic area, bed nucleus of the stria terminalis, ventromedial hypothalamus, and arcuate nucleus) in response to S- vs. H-CLS, and in response to a CLS-paired odour cue (almond). Ovariectomized, hormone-primed Long-Evans rats were randomly reassigned after a final open field test to investigate Fos-IR to CLS type (S-CLS, H-CLS, or Sham CLS) or CLS-paired odor cues (scented H-CLS (+); unscented H-CLS (-); scented S-CLS (+); unscented S-CLS (-); scented aversive CLS differentiation - H-CLS(+)/S-CLS (-); scented rewarding CLS differentiation - S-CLS (+)/H-CLS (-)). The pattern of Fos-IR in response to H-CLS alone was distinct from S-CLS and Sham CLS: H-CLS reduced the number of Fos-IR cells in reward regions, but increased it in regions associated with aversion. The paired odour cues were found to induce similar numbers of Fos-IR cells relative to their paired CLS type, with the exception of odours that differentiated H-CLS from S-CLS, in which the odour cues alone were found to induce a pattern of Fos-IR indicative of aversion rather than reward. These data are consistent with our prior findings on vocal and sexual behaviors distinct for each CLS type.

Key Words: Clitoral Stimulation, Sexual Pleasure and Aversion, Fos expression

1. Introduction

The clitoral complex is a critical component of genitosensory pleasure during copulation or self-stimulation in females (Foldes & Buisson, 2009; Pfaus et al., 2016). Neural pathways from

the external clitoral glans and internal clitoral, urethral, and vaginal complex (CUV; formerly known as the "G-spot") travel via pudendal, hypogastric, and pelvic nerves into sacral, lumbar, and lower thoracic regions of the spinal cord. From there, this innervation projects to regions of the brain associated with genitosensory stimulation and arousal (e.g., paracentral lobule of primary sensory cortex, medial preoptic area (mPOA), ventromedial hypothalamus (VMH), lateral hypothalamus (LH), paraventricular nucleus of the hypothalamus (PVN), central grey, medial and posterior Raphé, nucleus paragigantocellularis (nPGi) and nucleus of the solitary tract (Jannini, Buisson, & Rubio-Casillas, 2014; Komisaruk et al., 2011; Marson & McKenna, 1996; Marson & Foley, 2004; Parada et al., 2010). From these regions, associated activation of limbic structures involved in sexual pleasure and incentive motivation (e.g., nucleus accumbens (NAc) and ventral tegmental area (VTA) can be activated by direct pleasurable clitoral stimulation (CLS; Pfaus et al., 2016).

In addition to neuroanatomical connections, pleasurable CLS in rats has been studied in terms of shaping female sexual behaviors (Pfaus et al., 2015; Gerson et al., 2019; Chapter 1.2 and 2.1), sexual learning, and sexual partner preference (Parada et al., 2010, 2011; Pfaus et al., 2012). Female rats experience CLS and vaginocervical stimulation (VCS) during paced copulation with male conspecifics (Pfaff et al., 1977). The male's pelvic thrusting during penile intromission causes direct and distributed contact with the female's external clitoral glans (Pfaff et al.,1977). CLS received during paced copulation can be mimicked with distributed stimulation of the external clitoral glans directly using the bristles of a soft, natural fiber paintbrush (Parada et al., 2011; 2010; Gerson et al., 2019a; Chapter 2.1 and 3.2). The ability of manual distributed CLS to induce a sexual reward state that underlies conditioned place preference (CPP) and conditioned partner preference (CPaP), requires that the paintbrush stimulation itself is

pleasurable (e.g., Gerson et al., 2019a; Parada et al., 2011; 2010) and not aversive (Chapter 3.1 and 3.2). Distributed CLS with a soft paintbrush (S-CLS) is a powerful sexual reward for female rats. In response to S-CLS, sexually naïve and experienced female rats emit hedonic 50-kHz ultrasonic vocalizations (USVs; Gerson et al, 2019a; Chapter 2.1; Pfaus et al. 2016). Approximately 75% of the S-CLS induced call profile is comprised of trill and flat-trill 50-kHz USV subtype calls (Gerson et al., 2019a) which are posited to be indicators of a hedonic reward state (Willadsen et al., 2014; Wöhr & Schwarting, 2013; Meyer et al., 2012; Browning et al., 2011; Barker et al., 2010; Ma et al., 2010).

S-CLS induces both CPP and CPaP in sexually naive females rats through repeated pairings of the S-CLS induced reward state with either a novel environment (Parada et al., 2010) or neutral almond odour cue presented on gauze when females receive S-CLS (Parada et al., 2011; Chapter 3.2). During their first sexual experience in an open field with two male rats, one scented with almond (Sc) and the other unscented (UnSc), sexually naive female rats that experienced S-CLS in the presence of the almond odour solicit and receive more ejaculations from the Sc male than the UnSc male (Parada et al., 2011; Chapter 3.2). Females in unpaired groups, however, either show no preference for the Sc male or, if the almond odour was previously paired with sham CLS, they show a preference for the UnSc male (Parada et al., 2011). During conditioning, the neutral almond odour acts as a discrete cue that predicts the sexual reward state. Hence, when the paired almond odour (now the CS) is placed on a male during the female's first copulatory experience, it generates a significant CPaP.

In contrast, distributed CLS with a hard-bristle paintbrush (H-CLS) is a potent source of sexual aversion for sexually naïve female rats (Chapters 3.1 and 3.2). Sexually naïve females show avoidance responses to H-CLS and co-emit trill and flat-trill call subtypes with long 22-

kHz USVs (lUSVs) of the Class B subtype (Chapter 3.1). While Class B subtype calls comprised 3% of the H-CLS induced call profile, they were not shown by females receiving S-CLS (Chapters 3.1 and 3.2) and they have been reported to be indicative of an immediate aversive state (e.g., Bialy et al., 2019). H-CLS induces conditioned partner avoidance (CPaA) in sexually naïve females through repeated pairings of the H-CLS induced aversive state with a neutral almond odour presented on gauze when females receive H-CLS during training (Chapter 3.2). When paired females subsequently received their first copulatory trial in an open field with two males, one Sc and one UnSc, they spent less time with the Sc male and received significantly fewer intromissions and ejaculations from him relative to the UnSc male (Chapter 3.2). Moreover, when H-CLS was differentially conditioned with S-CLS (Chapter 3.2), i.e., H-CLS (+)/S-CLS(-) vs. H-CLS(-)/S-CLS(+), the sexual and vocal behaviors of the females indicated that the reward value of S-CLS was reduced and in some cases made aversive (Chapter 2.2). Specifically, sexually naïve female rats choose to receive their first ejaculation from Sc males associated with H-CLS (+) rather than UnSc males associated with S-CLS (-). Sexually naive females were also found to spend significantly less time with the Sc male associated with S-CLS (+) compared to the UnSc male associated with H-CLS (-). In both CLS differentiation groups, the sexually naïve females emitted more Class B subtype calls in response to S-CLS compared to H-CLS during odour conditioning (Chapter 3.2). The presence of an inaccessible Sc or UnSc male behind a wire mesh screen is another condition that attenuates S-CLS induced reward (Parada et al., 2011). Sexual desire stimulated by S-CLS is in conflict with the female's inability to interact with the male conspecific, resulting in a negative association between the neutral odour and the S-CLS. During their first copulatory experience, females with this conditioning history selectively solicited and received ejaculations from the UnSc male (Parada et al., 2011).

CLS reward value is therefore context-dependent for sexually naive females (Chapter 3.2; Parada et al., 2011). Excitatory brain regions, such as the medial preoptic area (mPOA) and the posteriorventral aspect of the medial amygdala (MeApv), are linked to this context dependence (Parada et al., 2010). Both regions are associated with genitosensory input (Aguilar-Moreno et al., 2022; Marson, 1995), sexual incentive salience (Quintana et al., 2019), sexual desire (Pfaus, 2009) and reward (Martz, Vasquez, and Dominguez, 2023; Parada et al., 2010). Parada et al. (2010) examined Fos activation within both these regions as well as other hypothalamic and limbic structures associated with CLS reward, such as the NAc, LS, bed nucleus of the stria terminalis (BNST), VMH, basolateral amygdala (BLA), arcuate nucleus (Arc), and VTA. It was found that S-CLS, but not sham CLS, significantly increased Fos activation of the mPOA and the MeApv.

H-CLS has been studied in terms of its effects on vocal behavior (Chapter 3.1 and Chapter 3.2) and on conditioned sexual behavior (Chapter 3.2) but Fos activation in response to it has not yet been examined relative to Fos activation with S-CLS or Sham CLS. It is unknown whether H-CLS alone, or an odour paired with H-CLS, might induce a different pattern of Fos activation relative to S-CLS alone and S-CLS paired odour cues. For example, H-CLS might induce Fos within neural regions associated with aversion or pain, such as the periaqueductal gray (PAG), and/or with inhibition, such as the anterior cingulate (Cg1 and Cg2), while diminishing Fos activation in regions associated with rewarding genitosensory stimulation. It is also unknown whether differentiating H-CLS from S-CLS will alter the Fos response to S-CLS in these brain regions.

Accordingly, the present study examined patterns of Fos activation within the regions mentioned above within two separate experiments following the open field partner preference

test in Chapter 3.2. The first experiment was designed to extend Parada et al. 's (2010) report of Fos induction in neural regions of sexual reward in response to S-CLS alone. It also evaluated Fos induced by H-CLS alone and compared the regions activated and the number of Fos positive cells in those regions to Fos induced by S-CLS alone or Sham CLS alone. The second experiment examined Fos activation by the CS odour associated with rewarding CLS, i.e., S-CLS (+) vs. S-CLS (-), and aversive CLS, i.e., H-CLS (+) vs. H-CLS(-), and CLS differentiation. It was predicted that H-CLS alone and the CS odour associated with H-CLS would result in a different pattern of Fos activation compared to the activation by S-CLS alone and the CS odour associated with it. In particular, S-CLS alone and the CS odour associated with it was expected to increase the number of Fos-positive cells in reward-related regions, while H-CLS and the CS odour associated with it should increase the number of Fos positive cells in aversive regions. CS odours associated with CLS differentiation were also predicted to result in Fos activation of both reward and aversive regions.

2. Methods

2.1. Animals

Sexually naïve female Long-Evans rats (3-5 months, 250-400g, Charles River Canada, Inc, St Constant, QC) were used to investigate patterns of Fos activation in the brain. The first experiment assessed c-FOS activation in response to CLS alone (n = 15) while the second experiment assessed c-FOS activation in response to a CLS paired odor cue (n = 30). Females were drug, CLS, and sexually-naive prior to CLS conditioning were randomly selected for c-FOS induction after the final partner preference test outlined in Chapter 2.2. A colony room with a 12-hour light/dark cycle (lights on at 20:00) kept at 21°C was used for housing the females in pairs and for housing the males in gang cages. Tap water and Purina® rat chow were provided ad

libitum to each pair-housed cage. Animal Research Ethics Committee at Concordia University approved all experimental procedures (protocol #30000300), which followed guidelines set by the Canadian Council on Animal Care.

2.2. Surgery and hormonal replacement

Odour conditioning trials were conducted on each female following bilateral ovariectomy (OVX). Bilateral OVX was performed under ketamine (50 mg/mL; Ketaset©, Wyeth Canada) and xylazine (4 mg/mL; Rompum©, Bayer Healthcare) anesthesia following the procedure described by Gerson et al. (2019). All females were allowed one week of recovery prior to CLS conditioning. OVX females were injected with 10 μg of estradiol benzoate (EB, 17β-diol 3-benzoate, Steraloids) 48 hours and 500 μg of progesterone (P, 4-Pregnen-3, 20-dione, Steraloids) 4 hours prior to each conditioning trial in order to induce sexual receptivity. Reagent grade sesame oil was used to prepare an injection volume of 0.1 ml of steroid solution for each rat.

2.3. Clitoral stimulation

S-CLS consisted of lifting the base of the tail and then lightly brushing the clitoris with a No.4 soft bristle paintbrush, which was dabbed with K-Y® Jelly, a water-soluble and non-toxic lubricant. S-CLS was applied as quick three down strokes approximately every 5 s, during a one-minute period. H-CLS was delivered in a similar manner to S-CLS, with the exception of using a No. 4 hard bristle paintbrush. During a 15-minute session, S-CLS was applied every 5 seconds for 1 minute after a 2-minute inter-CLS interval and this was repeated for five cycles. For Sham CLS, the base of the tail was lifted but the clitoris was not touched. Similar timing was used for H-CLS and Sham CLS. In Gerson et al. (2019a, Chapter 2.1), a 4-min interval between CLS was used to ensure that female rats returned to baseline levels of sexual excitability (i.e., heightened locomotor activity anticipating sexual stimulation; Pfaus et al. 2001). In order to facilitate

associative learning, the inter-CLS interval was shortened to 2 minutes to keep females in a state of sexual excitement, as demonstrated by Parada et al., (2011; 2010) and/or frustration, as demonstrated by Gerson et al. (2019b, Chapter 3.1 and 3.2). A modified unilevel pacing chamber (38 x 60 x 38 cm) with two openings (13.5 x 13.5 cm) on either side of the front wall was used for CLS. The openings allowed for the experimenter to access the rat in the chamber to apply distributed CLS.

2.4. Odour cues and odour conditioning procedure

The CS was the same as in previous research: a neutral almond odor. The scented cue (Sc) was cotton gauze soaked in Club House Pure Almond Extract (McCormack Canada, London, ON), while the unscented control (UnSc) was cotton gauze soaked in water. Five minutes were allowed for the almond cue to rest in the USV recording and conditioning chamber before the female was placed inside. Cross-odour exposure was prevented by using alternate days for Sc conditioning and UnSc conditioning. Immediately following every conditioning trial, the CLS/conditioning chambers were cleaned with Lysol wipes and beta chips were replaced. The paintbrushes were cleaned with Sparkleen.

Following OVX recovery, females were randomly assigned to one of six CLS-odour groups: (1) *scented* H-CLS (+); (2) *unscented* H-CLS (-); (3) *scented* S-CLS (+); (4) *unscented* S-CLS (-); (5) *scented aversive CLS differentiation* - H-CLS(+)/S-CLS (-); (6) *scented rewarding CLS differentiation* - S-CLS (+)/H-CLS (-). A total of 12 odour-CLS conditioning sessions were performed on every female before the final open field test with a sexually vigorous male conspecific. Specifically, odour-CLS conditioning sessions consisted of 6 CLS-odour sessions and 6 discrimination sessions with either sham CLS or another sensory condition (H-

CLS or S-CLS). To control order effects, the order of sessions was counterbalanced for each conditioning group.

2.5. Male Sex Training

Ten copulation training sessions were conducted before open field trials to ensure that stimulus males demonstrated sexual vigor. Training sessions were conducted in unilevel pacing chambers (60 L x 40 W x 40 H cm) with sexually receptive females over a period of 30 minutes. No odor conditioning was performed on sexually receptive females used in copulation training sessions. Males who mounted females within 15 seconds were considered good copulators in the training sessions.

2.6. Final Open Field Test and Fos induction assignment

Open field tests were conducted as described in Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus (2005) and Coria-Avila & Pfaus (2007). Four days after the final odour conditioning trial, females were placed in large open fields lined with beta chip bedding. In diagonal corners of the open field, two sexually vigorous males were tethered with rat tethering jackets attached to springs 30 cm in length. They were able to roam within a 45-cm radius as a result of this. One of the males was randomly scented with the neutral almond odour by applying it with a Q-tip to the back of his neck and anogenital area after he was tethered to his jacket. The males were given 5 minutes to adjust to the jacket and scent before the female was introduced. The females were placed in the center of the field for 30 minutes and allowed to freely interact with both males. All open field tests were video recorded using a GoPro (*HERO4 Silver*) and later scored for the proportions of females that chose either scented or unscented males for their first solicitation, mount, intromission, and ejaculation, and the number of mounts, intromissions, and ejaculations received from each male, using a behavioral scoring program (Cabilio, 1996). Video recordings

containing one or both sluggish males were excluded from the final analysis. Increasing solicitation is common among females in order to stimulate sexual engagement in sexually sluggish males (Afonso & Pfaus, 2006; Beach, 1968). Females become aggravated by this, kicking and mounting as a result, and they will either copulate with the remaining sexually vigorous male or not copulate at all.

After the final partner preference test, 45 females were randomly selected for inclusion in one of two Fos induction experiments. The first experiment assessed Fos induction to S- or H-CLS alone while the second experiment assessed Fos induction to S- or H-CLS-paired odour cues.

2.7. Procedure for Fos induction by CLS alone

One week after the final partner preference test, fifteen females were randomly selected to investigate c-FOS induction to CLS alone. Females were then randomly reassigned to receive S-CLS (n = 5), H-CLS (n = 5), or Sham CLS (n = 5). The one-week washout period was designed to eliminate previous effects of odour conditioning on the activation of Fos by CLS alone. OVX females received their assigned CLS in a similar manner to what was described previously, after being primed with EB + P. The females remained in the uni-level chamber after receiving their assigned CLS, then were sacrificed 1 hour after CLS initiation and perfused to prepare tissue for immunocytochemistry.

2.8. Procedure for Fos induction to CLS paired odour cues

Four days after the final partner preference test, thirty females were randomly selected to examine Fos induction in response to CLS-paired odour cues. Females were then given two reconditioning sessions to re-establish the association between olfactory cue and CLS pairings,

which were conducted identically to the previously described conditioning trials. The following comparisons of CLS-paired odour cues were assessed: rewarding CLS – scented S-CLS (n= 4) vs. unscented S-CLS (n = 5), aversive CLS – scented H-CLS (n = 5) vs. unscented H-CLS (n = 5), and CLS differentiation – scented aversive CLS differentiation (n = 5) vs. scented rewarding CLS differentiation (n = 4).

2.9. Histology and immunohistochemistry

All females were sacrificed by a sodium pentobarbital overdose (120 mg/kg, i.p.) and perfused intracardially with a phosphate buffered saline solution (250 ml) followed by 4% paraformaldehyde in 0.1M phosphate buffer (250 ml). Brains were extracted and placed into fresh 4% paraformaldehyde for 4 hours and then in 30% sucrose overnight at 4°C.

From each frozen brain, coronal sections (40 μm) were cut on a cryostat. After washing in cold Tris-buffered saline (TBS), coronal brain sections were first incubated with 30% hydrogen peroxide (H_2O_2) in TBS for 30 minutes at room temperature. For 90 minutes following H2O2 incubation, sections were incubated with 3% Normal Goat Serum (NGS). Sections were then incubated with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 hours, followed by biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200) for 1 hour. All NGS, Fos ab5, and anti-rabbit IgG incubations were diluted in 0.05% Trition-TBS with 3% NGS and kept at 4 °C. Following this, sections were incubated with avidin-biotinylated-peroxidase complex (Vectastain *ELITE* ABC Kit, Vector Laboratories Canada; diluted 1:55) for 2 h at 4 °C. Sections were washed in TBS (3 × 5 min) between each incubation.

Section were stained using 3,3′-diaminobenzidine (DAB) to react the peroxidase, and nickel chloride to turn the nuclear reaction product blue-black. Section immunoreactions were

stained sequentially as follows: 50 mM Tris for 10 minutes; 3,3′-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 minutes; 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer + H2O2) for 10 minutes. The reaction of the final step was stopped by rising in cold TBS. After staining, sections were mounted onto gel-coated slides then allowed to air dry. Once dry, mounted sections were washed with a 1-minute wash in nanopure distilled water for 1-minute then dehydrated in alcohols (70%, 90% and 100%, 10 min each, respectively). Dehydration was followed by 2 hours in Xyelines then cover slipped using Permount (Fisher Scientific, SP15-500).

2.10. Microscopy and Histology Statistical Analyses

Photomicrographs of all brain regions of interest were captured using an Olympus light microscope at 20× magnification using QCapture Pro software. On average, 3 to 5 bilateral sections of each brain region of interest per rat were counted for Fos IR cells, which were identified by a dark-brown/black nuclear stain. ImageJ software was used to count the number of FOS-IR cells in each region of interest. Regions of interest were defined by using the atlas of Paxinos and Watson (2006): Cg1 and Cg2 (B 1.32 to 1.08 mm), NAc core and shell (B 1.32 to 1.08 mm), mPOA (B 0.00 to -0.36 mm), BNST (B 0.00 to -0.36 mm), LS (B 0.00 to -0.36 mm), VMHvll (B -2.64 to -2.92 mm), VMHvlm (B -2.64 to -2.92 mm), VMHdm (B -2.64 to -2.92 mm), ARH (B -2.64 to -2.92 mm), MeApv (B -2.64 to -2.92 mm), MeApd (B -2.64 to -2.92 mm), BLA (B -2.64 to -2.92 mm), CeA (B -2.64 to -2.92 mm), VTA (B -6.60 to -6.84 mm), DMPAG (B -6.60 to -6.84 mm), DLPAG (B -6.60 to -6.84 mm), LPAG (B -6.60 to -6.84 mm). Counting Fos cells using ImageJ first consisted of adjusting the brightness and contrast on the first counted section and noting the contrast value. This value was then applied to all subsequent images of the region. Next, all cells subjectively identified as immunopositive, blind to

experimental group, were manually captured using the threshold tool. Circularity was set between 0.3-1, and pixel size was between 2 to 40 for all images. A mean was calculated for each area for each rat from the 3 bilateral sections per area, and statistical analyses were conducted for 4 to 5 rats in each CLS alone and CLS-odour pairing group. This yielded approximately 15 sections per group for each brain area as damaged tissue was not subject to analysis.

One between-subjects analysis of variance (ANOVA) was performed for each brain area of interest to assess differences in Fos induction for each experiment. Three ANOVAs were run for the first experiment to evaluate Fos induction for S-CLS alone, H-CLS alone, and S-CLS alone vs. H-CLS alone. An additionally three ANOVAs were run for the second experiment to evaluate Fos induction for rewarding CLS paired odour cues, aversive CLS paired odour cues, and CLS differentiation paired odour cues. Post hoc analysis of mean differences were run for each significant ANOVA using least significant difference (LSD) method, $p< 0.05$. Type 1 error inflation is a high risk when using the LSD method as the alpha level of each comparison is not corrected for multiple comparisons (Willams & Abdi, 2010). Type 2 error inflation however occurs due to correcting for multiple comparisons, which can impede exploratory analysis (McDonald, 2014). The LSD method was therefore chosen over correcting multiple comparisons due to the exploratory nature of the present statistical analysis.

3. Results

3.1. CLS alone

3.1.1. S-CLS

S-CLS was found, relative to Sham CLS, to significantly increase Fos induction in the Cg1 ($F(1, 8) = 10.167$, $p = 0.013$, $\eta^2_g = 0.560$), NAc shell ($F(1,8) = 182.060$, $p <0.001$, $\eta^2_g =$

0.958), LSv ($F(1,8) = 5.961$, $p = 0.040$, $\eta^2_g = 0.427$), MeApv ($F(1,8) = 84.203$, $p < 0.001$, $\eta^2_g = 0.913$), VMHvll ($F(1,8) = 186.872$, $p < 0.000$, $\eta^2_g = 0.959$), and the DLPAG ($F(1,8) = 27.621$, $p < 0.001$, $\eta^2_g = 0.775$). A trend towards significance for Fos induction was found for the mPOA ($F(1,8) = 4.509$, $p = 0.066$, $\eta^2_g = 0.360$).

S-CLS was found to significantly decrease Fos induction in the LSv ($F(1,8) = 5.961$, $p = 0.040$, $\eta^2_g = 0.427$), with a trend towards significance for the BLA $F(1,8) = 3.570$, $p = 0.096$, $\eta^2_g = 0.309$).

Fos induction between Sham-CLS and S-CLS did not differ significantly in the Cg2 ($p = 0.343$, $\eta^2_g = 0.112$), NAc core ($p = 0.273$, $\eta^2_g = 0.148$), BNST ($p = 0.801$, $\eta^2_g = 0.008$), CeA ($p = 0.631$, $\eta^2_g = 0.030$), MeApd ($p = 0.131$, $\eta^2_g = 0.261$), VMHvlm ($p = 0.287$, $\eta^2_g = 0.140$), VMHdm ($p = 0.807$, $\eta^2_g = 0.008$), ARC ($p = 0.538$, $\eta^2_g = 0.049$), DMPAG ($p = 0.502$, $\eta^2_g = 0.058$), LPAG ($p = 0.887$, $\eta^2_g = 0.003$), and the VTA ($p = 0.152$, $\eta^2_g = 0.239$). This overall induction pattern by S-CLS alone is summarized in **Fig 1.** All post hoc comparisons are summarized in **Table 1.**

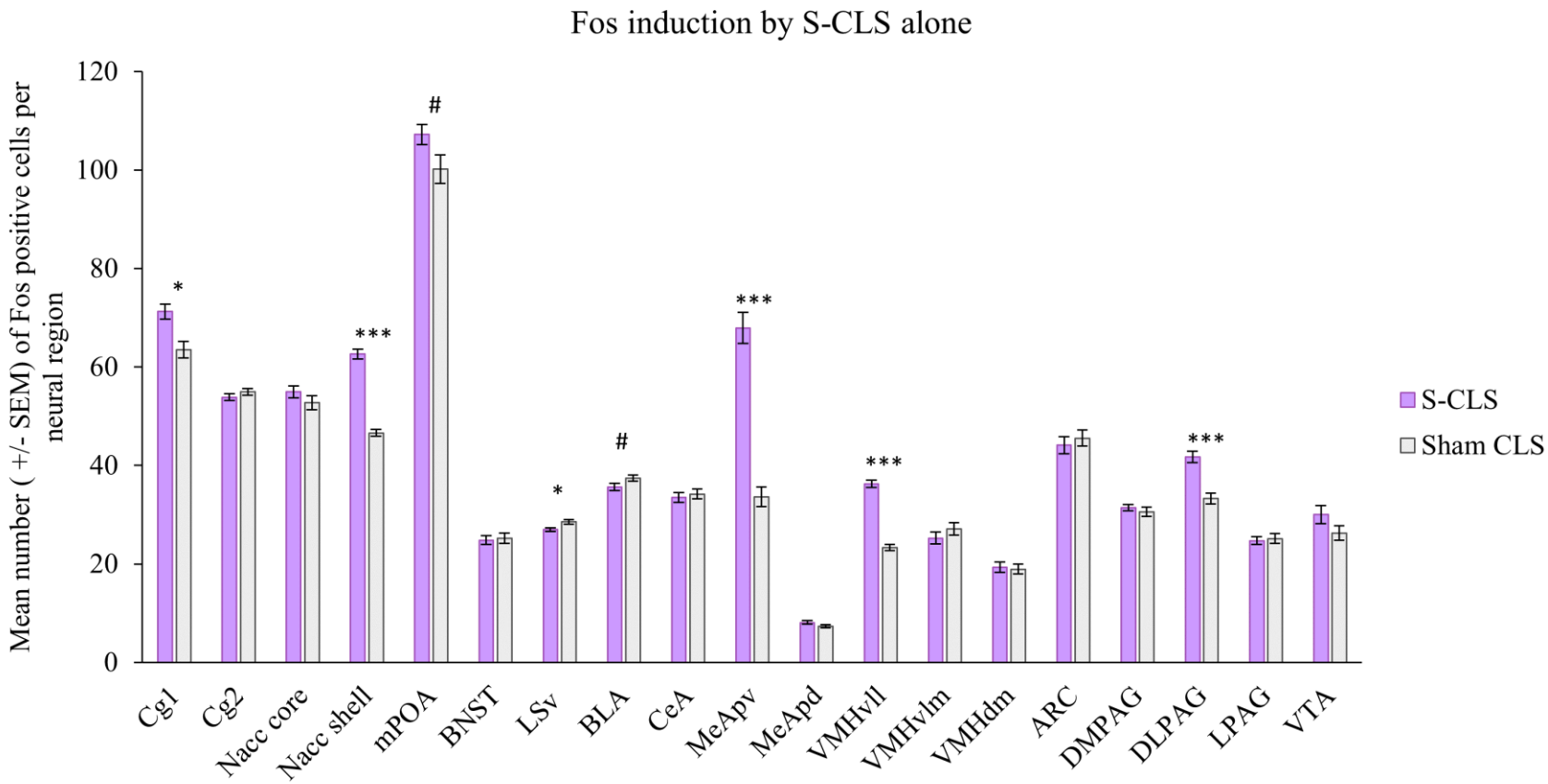

Fig 1. *Neural regions of female rats that expressed significant differences of Fos positive cells following S-CLS alone. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired and unpaired groups.*

Table 1.

Post hoc comparisons of Fos induction by S-CLS alone

Area	*S-CLS*	*Sham CLS*	*t-value*	*Cohen's d*
Cg1	71.242 ± 1.500	63.533 ± 1.682	3.189*	2.017
Cg2	53.867 ± 0.659	54.933 ± 0.684	n.s	
Nacc core	54.967 ± 1.208	52.733 ± 1.461	n.s	
Nacc shell	62.633 ± 0.988	46.617 ± 0.658	1.187***	8.534
mPOA	107.250 ± 2.063	100.200 ± 2.895	2.124#	1.343
BSNT	24.833 ± 0.929	25.200 ± 1.055	n.s	
LSv	26.950 ± 0.385	28.533 ± 0.523	2.441*	-1.544
BLA	35.633 ± 0.690	37.400 ± 0.631	1.889#	-1.195
CeA	33.475 ± 0.988	34.183 ± 1.015	n.s	
MeApv	67.900 ± 3.160	33.633 ± 1.989	9.176***	5.804
MeApd	8.117 ± 0.334	7.367 ± 0.295	n.s	
VMHvll	36.233 ± 0.750	23.333 ± 0.640	13.670***	8.646
VMHvlm	25.242 ± 1.200	27.100 ± 1.231	n.s	
VMHdm	19.300 ± 1.027	18.950 ± 1.038	n.s	
ARC	44.100 ± 1.715	45.567 ± 1.625	n.s	
DMPAG	31.400 ± 0.638	30.600 ± 0.944	n.s	
DLPAG	41.750 ± 1.161	33.267 ± 1.121	5.256***	3.324
LPAG	24.708 ± 0.789	25.150 ± 0.977	n.s	
VTA	30.000 ± 1.817	26.267 ± 1.503	n.s	

*p< 0.05, *** p <0.001, # trend towards significance

3.1.2. H-CLS

Relative to Sham-CLS, H-CLS was found to significantly increase Fos induction in the Cg1 ($F(1, 8) = 5.874$, $p = 0.042$, $\eta^2_g = 0.423$), LSv ($F(1, 8) = 14.772$, $p = 0.005$, $\eta^2_g = 0.649$), BLA ($F(1, 8) = 238.809$, $p < 0.001$, $\eta^2_g = 0.968$), CeA ($F(1, 8) = 53.254$, $p < 0.001$, $\eta^2_g = 0.869$), MeApv ($F(1, 8) = 156.621$, $p < 0.001$, $\eta^2_g = 0.951$), VMHvlm ($F(1, 8) = 61.043$, $p < 0.001$, $\eta^2_g = 0.884$), DMPAG ($F(1, 8) = 101.978$, $p < 0.001$, $\eta^2_g = 0.927$), , DLPAG ($F(1, 8) = 393.064$, $p < 0.001$, $\eta^2_g = 0.980$), and LPAG ($F(1, 8) = 35.797$, $p < 0.001$, $\eta^2_g = 0.817$).

H-CLS was found to be significantly decrease Fos induction in the Cg2 ($F(1, 8) = 170.961$, $p < 0.001$, $\eta2g = 0.955$), NAc core ($F(1, 8) = 18.492$, $p = 0.003$, $\eta2g = 0.698$), mPOA ($F(1, 8) = 7.651$, $p = 0.024$, $\eta2g = 0.489$), and ARC ($F(1, 8) = 30.488$, $p < 0.001$, $\eta2g = 0.792$).

There was no significant induction of Fos in the NAc shell ($p = 0.106$, $\eta^2_g = 0.293$), BSNT ($p = 0.304$, $\eta^2_g = 0.131$), MeApd ($p = 1.000$, $\eta^2_g = 0.000$), VMHvll ($p = 0.124$, $\eta^2_g = 0.269$), VMHdm ($p = 0.927$, $\eta^2_g = 0.001$), and the VTA ($p = 0.210$, $\eta^2_g = 0.189$). This overall induction pattern by H-CLS alone is summarized in **Fig 2** while all post hoc comparisons are summarized in **Table 2.**

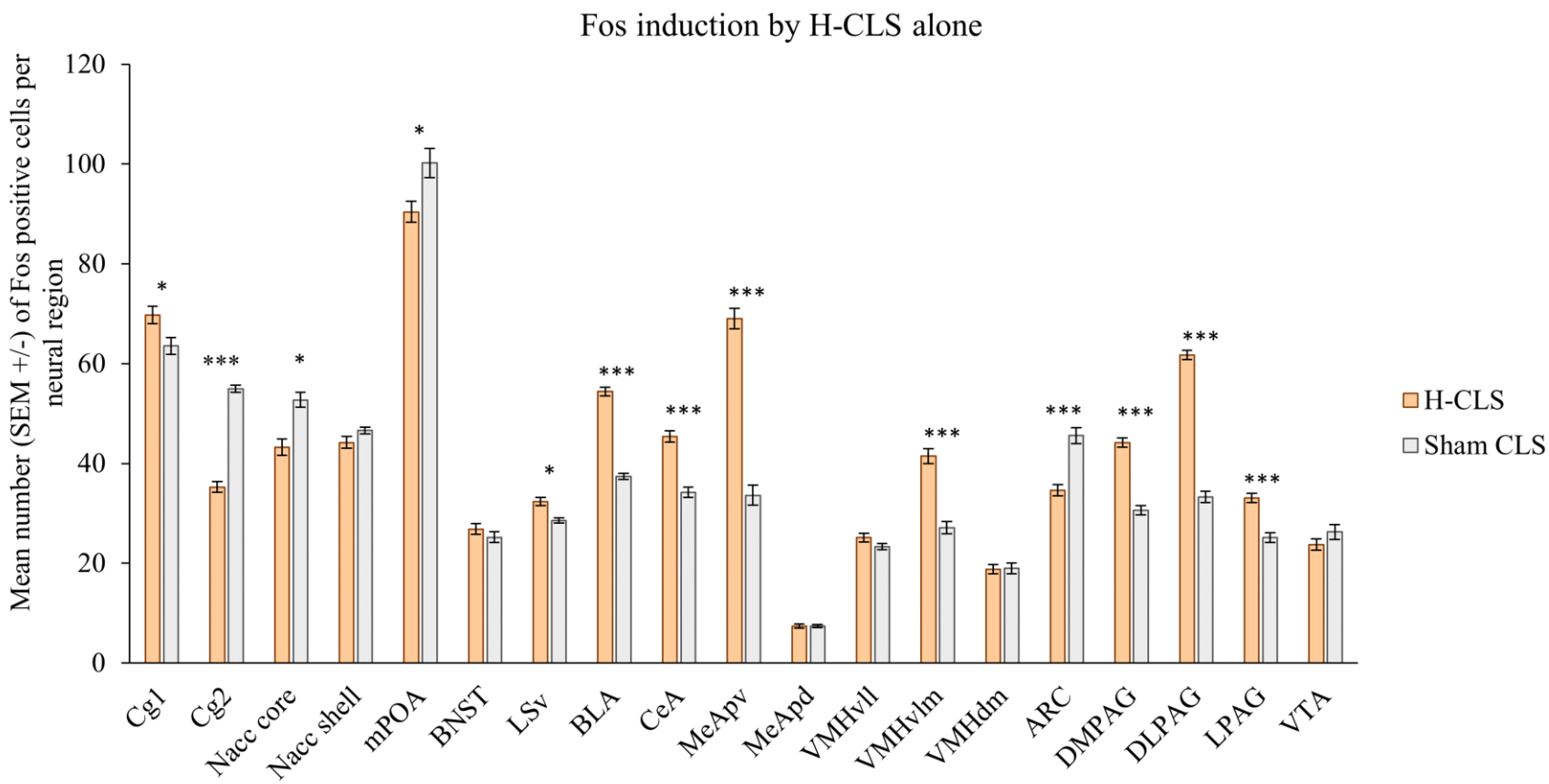

Fig 2. *Neural regions of female rats that expressed significant differences of Fos positive cells following H-CLS alone. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired and unpaired groups.*

Table 2.

Post hoc comparisons of Fos induction by H-CLS alone

Area	*H-CLS*	*Sham CLS*	*t-value*	*Cohen's d*
Cg1	69.750 ± 1.728	63.533 ± 1.682	2.424*	1.533
Cg2	35.233 ± 1.081	54.933 ± 0.684	13.075***	-8.269
Nacc core	43.217 ± 1.662	52.733 ± 1.461	4.300*	-2.72
Nacc shell	44.208 ± 1.149	46.617 ± 0.658	n.s	
mPOA	90.427 ± 2.117	100.200 ± 2.895	2.766*	-1.749
BSNT	26.850 ± 1.067	25.200 ± 1.055	n.s	
LSv	32.367 ± 0.850	28.533 ± 0.523	n.s	
BLA	54.383 ± 0.900	37.400 ± 0.631	15.453**	9.774
CeA	45.367 ± 1.148	34.183 ± 1.015	7.298***	4.615
MeApv	69.017 ± 2.009	33.633 ± 1.989	12.515***	7.915
MeApd	7.367 ± 0.416	7.367 ± 0.295	n.s	
VMHvll	25.117 ± 0.866	23.333 ± 0.640	n.s	
VMHvlm	18.825 ± 0.933	27.100 ± 1.231	7.813***	4.941
VMHdm	41.458 ± 1.471	18.950 ± 1.038	n.s	
ARC	44.167 ± 0.956	45.567 ± 1.625	5.522***	-3.492
DMPAG	61.750 ± 0.898	30.600 ± 0.944	10.098***	6.387
DLPAG	33.067 ± 0.892	33.267 ± 1.121	19.826***	12.539
LPAG	34.600 ± 1.142	25.150 ± 0.977	5.983***	3.784
VTA	23.700 ± 1.133	26.267 ± 1.503	n.s	

*p< 0.05, *** p <0.001, # trend towards significance

3.1.3. H-CLS vs. S-CLS

Differential CLS, i.e., S-CLS vs. H-CLS , significantly affected Fos induction in the Cg2 ($F(1, 8) = 216.650$, $p < 0.001$, $\eta^2_g = 0.964$), NAcc core ($F(1, 8) = 32.701$, $p < 0.001$, $\eta^2_g = 0.803$), NAcc shell ($F(1, 8) = 147.859$, $p < 0.001$, $\eta^2_g = 0.949$), mPOA ($F(1, 8) = 32.390$, $p < 0.001$, $\eta^2_g = 0.802$), LSv ($F(1, 8) = 33.696$, $p < 0.001$, $\eta^2_g = 0.808$), BLA ($F(1, 8) = 273.444$, $p < 0.001$, $\eta^2_g = 0.972$), CeA ($F(1, 8) = 61.658$, $p < 0.001$, $\eta^2_g = 0.885$), ARC ($F(1, 8) = 23.321$, $p = 0.001$, $\eta^2_g = 0.745$), VMHvll ($F(1, 8) = 94.103$, $p < 0.001$, $\eta^2_g = 0.922$), VMHvlm ($F(1, 8) = 72.962$, $p < 0.001$, $\eta^2_g = 0.901$), DMPAG ($F(1, 8) = 123.341$, $p < 0.001$, $\eta^2_g = 0.939$), DLPAG ($F(1, 8) = 185.578$, $p < 0.001$, $\eta^2_g = 0.959$), LPAG ($F(1, 8) = 42.167$, $p < 0.001$, $\eta^2_g = 0.841$), and the VTA ($F(1, 8) = 8.658$, $p = 0.019$, $\eta^2_g = 0.520$). Induction of Fos was not significant in the Cg1 ($p = 0.533$, $\eta^2_g = 0.050$), BSNT ($p = 0.192$, $\eta^2_g = 0.202$), MeApv ($p = 0.773$, $\eta^2_g = 0.011$), MeApd ($p = 0.198$, $\eta^2_g = 0.198$), and the VMHdm ($p = 0.741$, $\eta^2_g = 0.014$). Overall induction patterns by S-CLS alone compared to H-CLS alone is summarized in **Fig 3.**

3.1.3.2. Fos induction within significant neural regions

A comparison of females who last received S-CLS prior to being anesthetized with those who last received H-CLS revealed significant increase in Fos induction in the Cg1, Cg2, NAc core, NAc shell, mPOA, ARC, VMHvll, and the VTA in response to S-CLS than H-CLS. This comparison also revealed significant increased FOS in the LSv, BLA, CeA, VMHvlm, DMPAG, DLPAG, and the LPAG in response to H-CLS than S-CLS. All post hoc comparisons are summarized in **Table 3**.

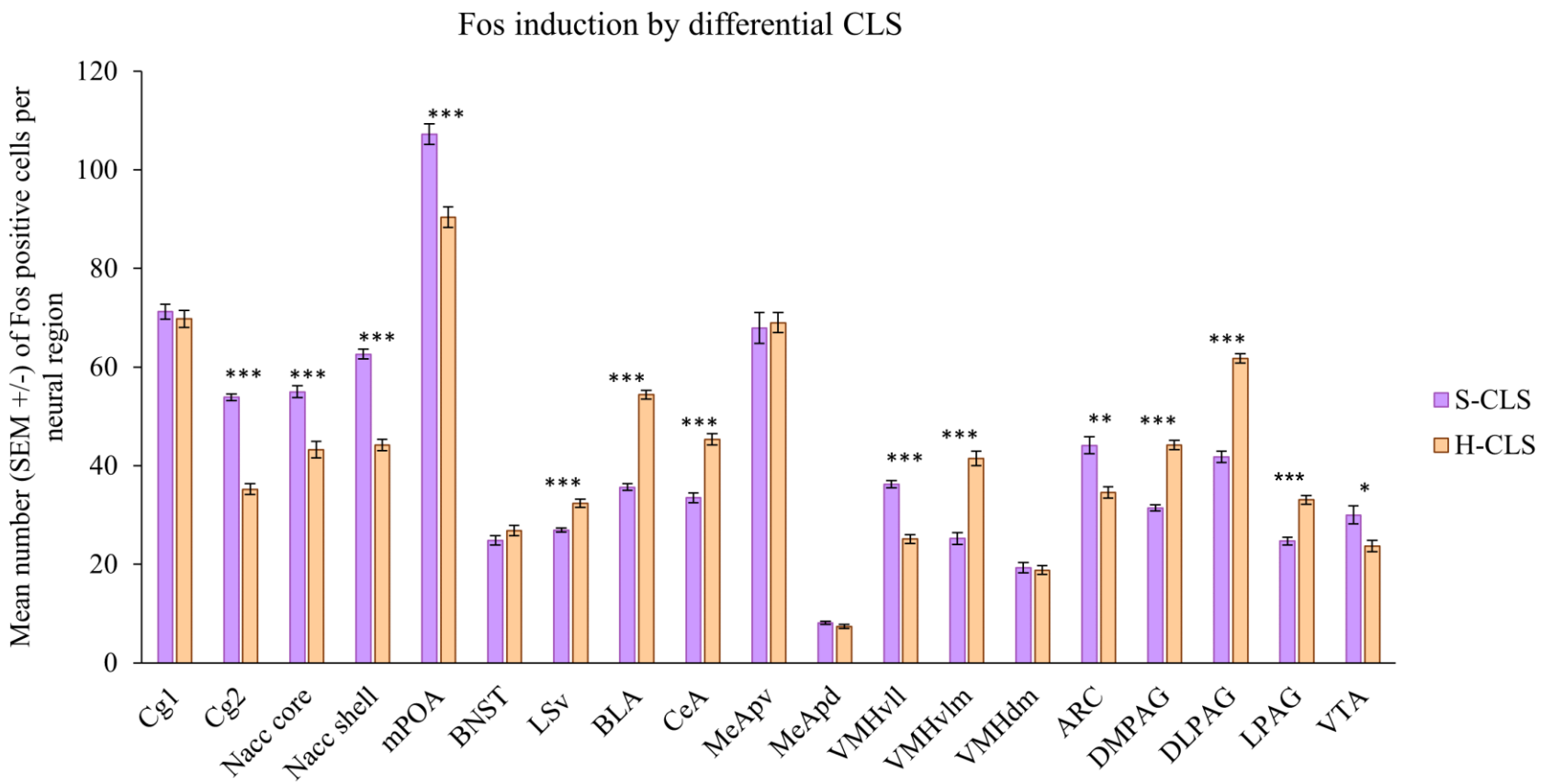

Fig 3. *Neural regions of female rats that expressed significant differences of Fos positive cells between S-CLS alone and H-CLS alone. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired groups.*

Table 3.

Post hoc comparisons of Fos induction by S-CLS alone compared to H-CLS alone

Area	*S-CLS*	*H-CLS*	*t-value*	*Cohen's d*
Cg1	71.242 ± 1.500	69.750 ± 1.728	n.s	
Cg2	53.867 ± 0.659	35.233 ± 1.081	14.719***	-9.309
Nacc core	54.967 ± 1.208	43.217 ± 1.662	5.718**	-3.617
Nacc shell	62.633 ± 0.988	44.208 ± 1.149	12.160***	-7.690
mPOA	107.250 ± 2.063	90.427 ± 2.117	5.691***	-3.599
BSNT	24.833 ± 0.929	26.850 ± 1.067	n.s	
LSv	26.950 ± 0.385	32.367 ± 0.850	5.805***	3.671
BLA	35.633 ± 0.690	54.383 ± 0.900	16.536***	10.458
CeA	33.475 ± 0.988	45.367 ± 1.148	7.852***	4.966
MeApv	67.900 ± 3.160	69.017 ± 2.009	n.s	
MeApd	8.117 ± 0.334	7.367 ± 0.416	n.s	
VMHvll	36.233 ± 0.750	25.117 ± 0.866	9.701***	-6.135
VMHvlm	25.242 ± 1.200	18.825 ± 0.933	8.542***	5.402
VMHdm	19.300 ± 1.027	41.458 ± 1.471	n.s	
ARC	44.100 ± 1.715	44.167 ± 0.956	4.829*	-3.054
DMPAG	31.400 ± 0.638	61.750 ± 0.898	11.106***	7.024
DLPAG	41.750 ± 1.161	33.067 ± 0.892	13.623***	8.616
LPAG	24.708 ± 0.789	34.600 ± 1.142	6.494***	4.107
VTA	30.000 ± 1.817	23.700 ± 1.133	2.942*	-1.861

*p< 0.05, *** p <0.001, # trend towards significance

3.2. CLS-paired odour cues

3.2.1. Rewarding CLS: S-CLS (+) and S-CLS (-)

Relative to S-CLS(-), S-CLS (+) was found to significantly increase Fos induction in the NAc core ($F(1,7) = 42.618$, $p < 0.001$, $\eta^2_g = 0.859$), mPOA ($F(1,7) = 18.172$, $p = 0.004$, $\eta^2_g = 0.722$), MeApv ($F(1,7) = 19.750$, $p = 0.003$, $\eta^2_g = 0.738$), MeApd ($F(1,7) = 5.640$, $p = 0.049$, $\eta^2_g = 0.446$), VMHvll ($F(1,7) = 489.921$, $p < 0.001$, $\eta^2_g = 0.986$), VMHdm ($F(1,7) = 11.369$, $p = 0.012$, $\eta^2_g = 0.619$), DMPAG ($F(1,7) = 13.129$, $p = 0.008$, $\eta^2_g = 0.652$), DLPAG ($F(1,7) = 127.217$, $p < 0.001$, $\eta^2_g = 0.948$), and the VTA ($F(1,7) = 43.749$, $p < 0.001$, $\eta^2_g = 0.862$).

No significant Fos induction was found in the Cg1 ($p = 0.677$, $\eta^2_g = 0.026$), Cg2 ($p = 0.516$, $\eta^2_g = 0.063$), NAc shell ($p = 0.694$, $\eta^2_g = 0.023$), BSNT ($p = 0.353$, $\eta^2_g = 0.124$), LSv ($p = 0.695$, $\eta^2_g = 0.023$), BLA ($p = 0.595$, $\eta^2_g = 0.042$), CeA ($p = 0.871$, $\eta^2_g = 0.004$), VMHvlm ($p = 0.115$, $\eta^2_g = 0.316$), ARC ($p = 0.661$, $\eta^2_g = 0.029$), and the LPAG ($p = 0.415$, $\eta^2_g = 0.097$). Overall induction patterns by S-CLS (+) and by S-CLS (-) are summarized in **Fig 4.** All post hoc comparisons are summarized in **Table 4**.

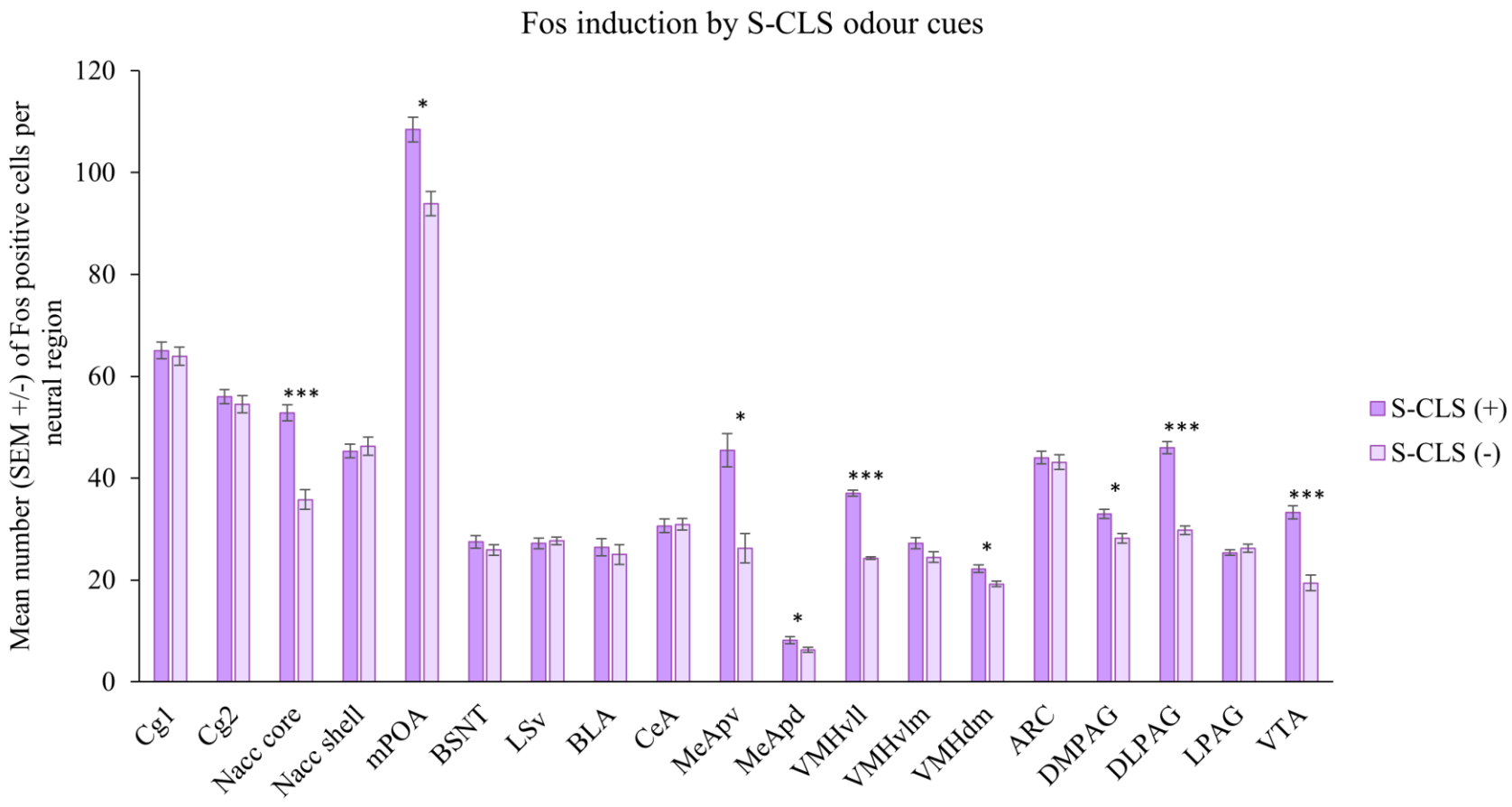

Fig 4. *Neural regions of female rats that expressed significant differences of Fos positive cells following exposure to S-CLS odour cues. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired and unpaired groups.*

Table 4.
Post hoc comparisons of Fos induction in response to S-CLS (+) and S-CLS(-) odour cues

	S-CLS			
Area	(+)	(-)	t-value	Cohen's d
Cg1	65.042 ± 1.636	63.950 ± 1.816	n.s	
Cg2	56.042 ± 1.395	54.517 ± 1.641	n.s	
Nacc core	52.833 ± 1.629	35.783 ± 1.927	6.528***	4.379
Nacc shell	45.333 ± 1.307	46.283 ± 1.775	n.s	
mPOA	108.458 ± 2.412	93.900 ± 2.363	4.263*	2.86
BNST	27.500 ± 1.221	25.900 ± 1.059	n.s	
LSv	27.188 ± 1.040	27.700 ± 0.757	n.s	
BLA	26.458 ± 1.691	25.000 ± 1.900	n.s	
CeA	30.646 ± 1.310	30.933 ± 1.113	n.s	
MeApv	45.479 ± 3.284	26.233 ± 2.849	4.444*	2.981
MeApd	8.188 ± 0.672	6.267 ± 0.488	2.375*	1.593
VMHvll	37.042 ± 0.563	24.300 ± 0.260	22.134***	14.848
VMHvlm	27.208 ± 1.092	24.483 ± 1.036	n.s	
VMHdm	22.208 ± 0.731	19.200 ± 0.546	3.372*	2.262
ARC	44.042 ± 1.257	43.150 ± 1.416	n.s	
DMPAG	33.000 ± 0.869	28.200 ± 0.955	3.623*	2.431
DLPAG	45.958 ± 1.210	29.767 ± 0.851	11.279***	7.566
LPAG	25.375 ± 0.520	26.233 ± 0.776	n.s	
VTA	33.292 ± 1.277	19.433 ± 1.560	6.614***	4.437

*p< 0.05, *** p <0.001, # trend towards significance

3.2.2. Aversive CLS: H-CLS (+) and H-CLS (-)

Relative to H-CLS(-),H-CLS(+) induced significantly greater numbers of Fos-IR cells in the BLA ($F(1,7) = 11.310$, $p = 0.012$, $\eta^2_g = 0.618$), CeA ($F(1,7) = 23.966$, $p = 0.002$, $\eta^2_g = 0.774$), MeApv ($F(1,7) = 19.752$, $p = 0.003$, $\eta^2_g = 0.738$), ARC ($F(1,7) = 17.923$, $p = 0.004$, $\eta^2_g = 0.719$), VMHvlm ($F(1,7) = 45.526$, $p < 0.001$, $\eta^2_g = 0.851$), DMPAG ($F(1,7) = 138.638$, $p < 0.001$, $\eta^2_g = 0.959$), DLPAG ($F(1,7) = 80.306$, $p < 0.001$, $\eta^2_g = 0.930$), and LPAG ($F(1,7) = 28.441$, $p = 0.002$, $\eta^2_g = 0.826$).

This comparison also revealed significantly less Fos in the Cg2 ($F(1,7) = 37.072$, $p < 0.001$, $\eta^2_g = 0.841$), and VTA ($F(1,7) = 9.179$, $p = 0.019$, $\eta^2_g = 0.567$) of females last exposed to H-CLS (+) than those last exposed to H-CLS (-). There was a trend towards significance for the H-CLS(+) odour to induce Fos in the BSNT ($F(1,7) = 3.911$, $p = 0.088$, $\eta^2_g = 0.358$).

No significant Fos induction was found in the Cg1 ($p = 0.753$, $\eta^2_g = 0.015$), NAc core ($p = 0.149$, $\eta^2_g = 0.273$), NAc shell ($p = 0.326$, $\eta^2_g = 0.137$), mPOA ($p = 0.604$, $\eta^2_g = 0.040$), LSv ($p = 0.886$, $\eta^2_g = 0.003$), MeApd ($p = 0.400$, $\eta^2_g = 0.103$), VMHvll ($p = 0.233$, $\eta^2_g = 0.173$), and the VMHdm ($p = 0.200$, $\eta^2_g = 0.196$). Overall induction patterns by H-CLS (+) and by H-CLS (-) are summarized in **Fig 5.** All post hoc comparisons are summarized in **Table 5**.

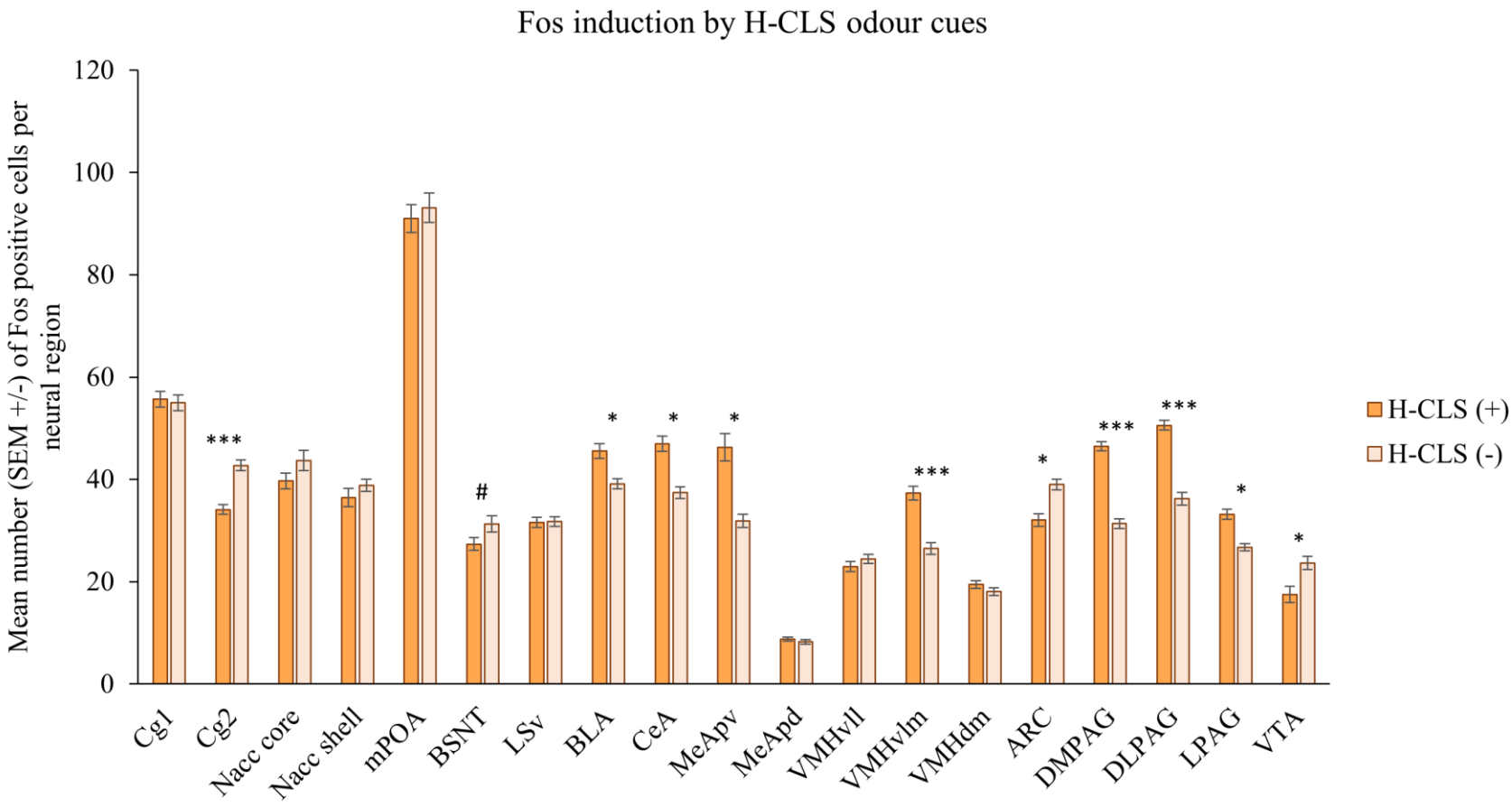

Fig 5. *Neural regions of female rats that expressed significant differences of Fos positive cells following exposure to H-CLS odour cues. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired and unpaired groups.*

Table 5.

Post hoc comparisons of Fos induction in response to H-CLS (+) and H-CLS(-) odour cues

	H-CLS			
Area	(+)	(-)	t-value	Cohen's d
Cg1	55.683 ± 1.525	54.958 ± 1.573	n.s	
Cg2	34.100 ± 0.942	42.750 ± 1.066	6.089***	-4.084
Nacc core	39.700 ± 1.558	43.729 ± 1.991	n.s	
Nacc shell	36.433 ± 1.771	38.833 ± 1.215	n.s	
mPOA	90.983 ± 2.697	93.133 ± 2.868	n.s	
BNST	27.350 ± 1.236	31.292 ± 1.614	1.978#	-1.327
LSv	31.567 ± 0.984	31.771 ± 0.921	n.s	
BLA	45.533 ± 1.478	39.167 ± 1.002	3.363*	2.256
CeA	46.950 ± 1.482	37.417 ± 1.120	4.895*	3.284
MeApv	46.300 ± 2.687	31.875 ± 1.275	4.444*	2.981
MeApd	8.767 ± 0.379	8.250 ± 0.438	n.s	
VMHvll	22.967 ± 0.945	24.467 ± 0.892	n.s	
VMHvlm	37.342 ± 1.332	26.500 ± 1.127	6.747***	4.267
VMHdm	19.467 ± 0.738	18.100 ± 0.741	n.s	
ARC	32.042 ± 1.266	39.000 ± 1.067	4.234*	-2.840
DMPAG	46.458 ± 0.875	31.354 ± 0.938	11.774***	8.326
DLPAG	50.583 ± 0.963	36.208 ± 1.283	8.961***	6.337
LPAG	33.208 ± 0.970	26.711 ± 0.718	5.333*	3.771
VTA	17.542 ± 1.577	23.667 ± 1.299	3.030*	-2.032

*p< 0.05, *** p <0.001, # trend towards significance

3.2.3. CLS differentiation: S-CLS(+)/H-CLS (-) and H-CLS (+)/S-CLS(-)

A comparison of CLS differentiation odour cues, i.e., S-CLS(+)/H-CLS (-) vs. H-CLS (+)/S-CLS(-), revealed significantly greater Fos induction in the BLA ($F(1,8) = 5.871$, $p = 0.042$, $\eta^2_g = 0.423$), CeA ($F(1,8) = 75.042$, $p < 0.001$, $\eta^2_g = 0.904$), MeApv ($F(1,8) = 8.392$, $p = 0.020$, $\eta^2_g = 0.512$), VMHvlm ($F(1,8) = 20.618$, $p = 0.002$, $\eta^2_g = 0.720$), DMPAG ($F(1,8) = 302.915$, $p < 0.001$, $\eta^2_g = 0.974$), DLPAG ($F(1,8) = 350.062$, $p < 0.001$, $\eta^2_g = 0.869$), and LPAG ($F(1,8) = 17.749$, $p = 0.003$, $\eta^2_g = 0.689$) of females last exposed to H-CLS (+)/S-CLS(-) compared to those last exposed to S-CLS(+)/H-CLS (-).

This comparison also revealed significantly greater Fos induction in the Cg2 ($F(1,7) = 134.438$, $p < 0.001$, $\eta^2_g = 0.944$), NAcc core ($F(1,8) = 23.317$, $p = 0.001$, $\eta^2_g = 0.745$), mPOA ($F(1,8) = 16.122$, $p = 0.004$, $\eta^2_g = 0.668$), ARC $F(1,8) = 360.000$, $p < 0.001$, $\eta^2_g = 0.832$), and the VTA ($F(1,8) = 6.000$, $p = 0.040$, $\eta^2_g = 0.429$) of females last exposed to S-CLS(+)/H-CLS (-) compared to those last exposed to H-CLS (+)/S-CLS(-).There was a trend towards significance for H-CLS odour cues in the induction of Fos in the VMHdm ($F(1,8) = 5.011$, $p = 0.056$, $\eta^2_g = 0.385$).

No significant Fos induction, however, was found in the Cg1 ($p = 0.182$, $\eta^2_g = 0.211$), NAcc shell ($p = 0.705$, $\eta^2_g = 0.019$), BSNT ($p = 0.204$, $\eta^2_g = 0.193$), LSv ($p = 0.588$, $\eta^2_g = 0.038$), MeApd ($p = 0.935$, $\eta^2_g = 0.001$), and the VMHvll ($p = 0.118$, $\eta^2_g = 0.277$). Overall induction patterns by S-CLS (+)/H-CLS(-) and by S-CLS(-)/H-CLS (+) are summarized in **Fig 6.** All post hoc comparisons are summarized in **Table 6**.

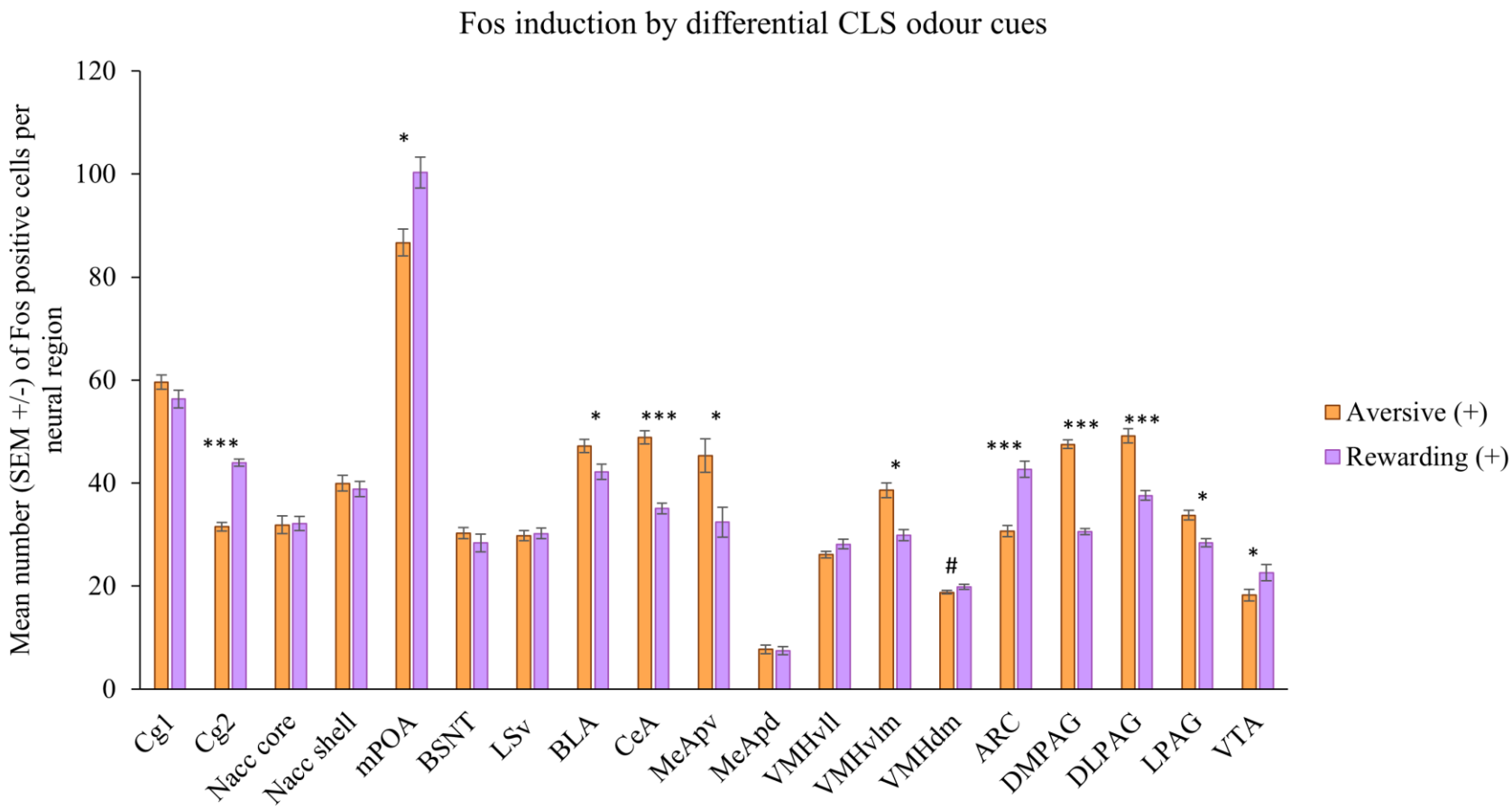

Fig 6. *Neural regions of female rats that expressed significant differences of Fos positive cells following exposure to rewarding CLS compared to aversive CLS odour cues. The bars represent the mean ± SEM. *** $p < 0.001$, * $p < 0.05$, # trend towards significance, between paired groups.*

Table 6.

Post hoc comparisons of Fos induction in response to Aversive (+) and Rewarding (+) CLS differentiation odour cues

	CLS differentiation			
Area	Aversive (+)	Rewarding (+)	t-value	Cohen's d
Cg1	59.597 ± 1.394	56.333 ± 1.717	n.s	
Cg2	31.550 ± 0.841	43.967 ± 0.663	11.595***	-7.333
Nacc core	31.867 ± 1.711	32.125 ± 1.353	4.829*	-3.054
Nacc shell	39.933 ± 1.524	38.833 ± 1.476	n.s	
mPOA	86.683 ± 2.570	100.270 ± 2.993	4.015*	-2.539
BNST	30.300 ± 1.059	28.375 ± 1.726	n.s	
LSv	29.800 ± 0.982	30.208 ±1.020	n.s	
BLA	47.200 ± 1.276	42.208 ± 1.479	2.423*	1.532
CeA	48.850 ± 1.250	35.052 ± 1.038	8.663***	5.479
MeApv	45.317 ± 3.268	32.417 ± 2.879	2.897*	1.832
MeApd	7.700 ± 0.867	7.458 ± 0.812	n.s	
VMHvll	26.100 ± 0.642	28.146 ± 0.921	n.s	
VMHvlm	38.617 ± 1.441	29.917 ± 1.092	4.541*	2.872
VMHdm	18.800 ± 0.355	19.875 ± 0.515	2.239#	-1.416
ARC	30.667 ± 1.070	42.708 ± 1.575	6.302***	-3.986
DMPAG	47.533 ± 0.798	30.542 ± 0.602	17.404***	11.008
DLPAG	49.167 ± 1.334	37.583 ± 0.925	7.288***	4.609
LPAG	33.733 ± 0.929	28.417 ± 0.825	4.213*	2.665
VTA	18.233 ± 1.174	22.583 ± 1.552	2.449*	-1.549

*p< 0.05, *** p <0.001, # trend towards significance

4. Discussion

The present study examined patterns of Fos activation in several brain regions associated with sexual arousal, reward, attention, and aversion. S-CLS alone and Sham CLS alone was compared in the first experiment to replicate and extend Parada et al. 's (2010) report of Fos induction in the mPOA and MEApv (***Figs. 7-10***). H-CLS alone was also evaluated by comparing the number of Fos positive cells in activated regions to those induced by Sham CLS alone (***Fig. 7-10***). The CS odour was evaluated in the second experiment by comparing Fos activation patterns of rewarding CLS, i.e. S-CLS (+) vs. S-CLS (-), aversive CLS, i.e., H-CLS (+) vs. H-CLS(-), and CLS differentiation, i.e., H-CLS (+)/S-CLS(-) vs. H-CLS(-)/S-CLS(+), separately. CLS alone and CS odours were overall found to induce significant Fos activation patterns in several key reward and aversive neural regions.

The CLS alone experiment revealed that the number of cells expressing Fos (***Figs. 7-10***) in response to S-CLS alone was significantly greater than Sham CLS alone in the Cg1 (***Figs. 7A***), NAcc shell, mPOA, MeApv, VMHvll, and DLPAG. In response to S-CLS alone, when compared to Sham CLS alone, the LSv and BLA were also found to express significantly fewer Fos cells. H-CLS alone compared to Sham CLS alone was also found to significantly increase the number of Fos expressing cells (***Fig. 7-10***) in the Cg1, MeApv, and the DLPAG, as well as the BLA, CeA, VMHvlm, DMPAG and LPAG. Fos cell expression was however shown to be significantly reduced in the Cg2, NAcc core, NAcc shell, mPOA, and ARC between H-CLS alone and Sham CLS alone. When S-CLS alone was compared with H-CLS alone, the number of cells expressing Fos (***Fig. 7-10***) was significantly greater in the Cg1, Cg2, NAc core, NAc shell, mPOA, ARC, VMHvll, and VTA in response to S-CLS alone than to H-CLS alone. Conversely,

Fos cell expression was significantly greater (***Fig. 7-10***) in the LSv, BLA, CeA, VMHvlm, DMPAG, DLPAG, and the LPAG in response to H-CLS alone relative to S-CLS alone.

Both S-CLS alone and H-CLS alone induced significant Fos activation in the Cg1 (***Fig 7.A***), a neural region involved in sensory discrimination (Bussey, Everitt, & Robbins, 1997; Parkinson, Willoughby, et al., 2000; Powell et al., 1994; Bussey, Muir, et al., 1997; Gabriel, 1993), as well as the DLPAG (***Fig. 10B***) and MeApv (***Fig. 8E***), neural regions involved in tracking genitosensory stimulation (Parada et al., 2010; Klop et al., 2005). Aside from this shared activation pattern, S-CLS alone and H-CLS alone overall activate reward and aversive regions respectively. S-CLS alone shows primary activation of the sexual reward and genitosensory input regions while H-CLS alone show primary activation of aversive regions as well as regions associated with affective-motivational reactions (Cg2; ***Fig. 7B***) and aggressive behaviors (VMHvlm; ***Fig. 9C***). H-CLS alone also shows attenuation of Fos expression in regions associated with reward (Nacc; ***Figs. 7C-D***) and sexual receptivity (ARC; ***Fig. 9E***). It must be noted, however, that the shared activation pattern in a given brain region does not mean that the same cells were activated. It is likely that both excitatory and inhibitory subsystems within these regions exist (e.g., Graham et al., in preparation; Tobiansky et al., 2016).

The CS odour experiment revealed that S-CLS (+) exposure led to a significantly greater number of cells expressing Fos in the NAc core, mPOA , MeApv, MeApd, VMHvll, VMHdm, DMPAG, and VTA compared to S-CLS(-) exposure. H-CLS (+) exposure led to a significant increase in Fos cell expression in the BLA, CeA, MeApv, VMHvlm, DMPAG, DLPAG, and LPAG compared to Fos cell expression in response to H-CLS (-) exposure. H-CLS (+) exposure, compared to H-CLS (-) exposure, also led to significant decrease in Fos expression in the Cg2, BNST, and VTA. For CLS differentiation, the number of Fos cells increased significantly

following S-CLS(+)/H-CLS(-) or rewarding CLS differentiation exposure rather than H-CLS(+)/S-CLS(-) or aversive CLS differentiation exposure in Cg2, NAc core, mPOA, ARC, VMHdm, and VTA. There were also significantly less cells expressing Fos in the BLA, CeA, MeApv, VMHvlm, DMPAG, DLPAG, and the LPAG following S-CLS(+)/H-CLS(-) exposure.

It appears that the type of CLS differentially conditioned with an odour CS influences Fos cell expression of select regions associated with affective-motivational reactions, sexual receptivity, and genitosensory input. Exposure to S-CLS (+) and to H-CLS (+), when differentially conditioned with Sham (-), showed Fos activation patterns similar to those induced by S-CLS alone and H-CLS alone, respectively. Rewarding CLS differentiation exposure also demonstrated an overall activation of sexual reward regions like S-CLS alone whereas aversive CLS differentiation exposure demonstrated an overall activation of aversive regions like H-CLS alone. Rewarding CLS differentiation exposure, however, was found to increase Fos cell expression within the VMHvlm (aggressive behaviors) while Fos cell expression was attenuated in the Nac shell (affective-motivational responding to reward) and VMHvll (lordosis). H-CLS alone, rather than S-CLS alone, evoked similar activation patterns within this subset of neural regions. It seems that differential conditioning S-CLS (+) with H-CLS (-) diminishes affective-motivational responding and consummatory aspects of S-CLS reward. Sexual reward regions still remain able to track S-CLS reward, despite the attenuation of regions associated with affective-motivational responding and lordosis, as indicated by overall Fos activation patterns across these regions. On a neural level, S-CLS alone and H-CLS alone are rewarding and aversive which can be predicted by their associated CS odour cues.

Parada at al. (2010) showed that a greater number of Fos expressing cells were induced in hypothalamic and limbic structures linked to sexual reward following distributed S-CLS (i.e., 1

CLS stimulation per 5 secs). This Fos activation pattern was distinct from those induced by massed S-CLS (i.e., 1 CLS stimulation per sec) and Sham S-CLS. In particular, distributed S-CLS was associated with significantly more Fos positive cells than massed S-CLS or Sham S-CLS in the mPOA. Both distributed and massed CLS were found to induce significantly more Fos positive cells in the MeApv than Sham CLS, suggesting the neural regions role in tracking incoming genitosensory stimulation. Statistical trends toward activation via distributed S-CLS were found for the NAc (core and shell), LS, BSNT, and ARC, but not the VMH and MeApd. Within the present study, S-CLS alone was found to significantly induce a greater number of Fos positive cells within the NAc shell, mPOA, LS, MeApv, VMH, and ARC, but not the BSNT and MeApd, which showed less Fos positive cells or non-significant Fos induction, respectively (***Figs. 7-10***).

A higher number of Fos positive cells were detected in the VMH (***Fig. 9B-D***) when S-CLS alone was administered in the present study than those reported in Parada et al. (2010). The discrepancy between the present study and Parada et al. (2010) is due to differences in Fos analysis. Specifically, Parada et al. assessed the VMH as a whole while the present study assessed the VMH based on its subnuclei, i.e. VMHvll, VMHvlm, and VMHdm. The present study focused on subnuclei because while the VMH is critical for lordosis, several reports indicate that systems within the VMH also inhibits it by mediating aggression (Lenschow & Lima, 2020; Micevych & Meisel, 2017; Hashikawa et al., 2018, 2017a). These contradictory behavioral responses are due to the competing functions of VMH subnuclei, specifically those within its ventrolateral portion (VMHvl; Lenschow & Lima, 2020; Micevych & Meisel, 2017; Hashikawa et al.,2018, 2017a). The lateral subdivision of VMHvl (VMHvll) is shown to mediate lordosis in female mice (Lenschow & Lima, 2020; Hashikawa et al., 2017a ; Micevych &

Meisel, 2017) while the medial subdivision of the VMHvl (VMHvlm) is shown to promote aggressive behaviors (Hashikawa et al., 2018, 2017a). The present study found significant Fos activation of the VMHvll in response to S-CLS alone (***Fig. 9B***), which are align with other prior reports stating the subnuclei role in facilitating female sexual behavior (Lenschow & Lima, 2020; Hashikawa et al., 2017a ; Micevych & Meisel, 2017). It is also the case that pharmacological inhibition of a glutamate subsystem in the VMHvl potentiates lordosis and solicitations (Georgescu & Pfaus, 2006a,b; Georgescu et al., 2009; Kow et al., 1985; McCarthy et al., 1991), and that activation of this subsystem by VCS leads to the inhibition of lordosis and solicitations (Georgescu et al., 2009). However, the overall patterns of Fos activation in response to S-CLS alone in this present study are generally in accordance with those previously described by Parada et al. (2010).

The present study extended the results of Parada et al. (2010) by examining Fos activation in response to H-CLS alone and in response to an odour CS previously paired with S-CLS, H-CLS, or Sham CLS. H-CLS alone was found to significantly increase the number of Fos positive cells across regions integral to aversive learning, such as the BLA (***Fig. 8C***; McCall et al., 2017) and PAG (***Figs. 10A-C***; Barcomb et al., 2022; Lopes et al., 2016; Mantyh, 1983; Ennis et al., 1991), aggression (VMHvlm; ***Fig. 9B***), and genitosensory stimulation, such as the MeApv (***Fig. 8E;*** Parada et al., 2010). Significantly fewer fos positive cells were also found in response to H-CLS within the Cg2 (***Fig. 7B***), NAcc core (***Fig. 7C***), and mPOA (***Fig. 7E***). As mentioned above, Fos activation in response to the odour CS was consistent with those induced by their associated CLS type with the exception of rewarding CLS differentiation.

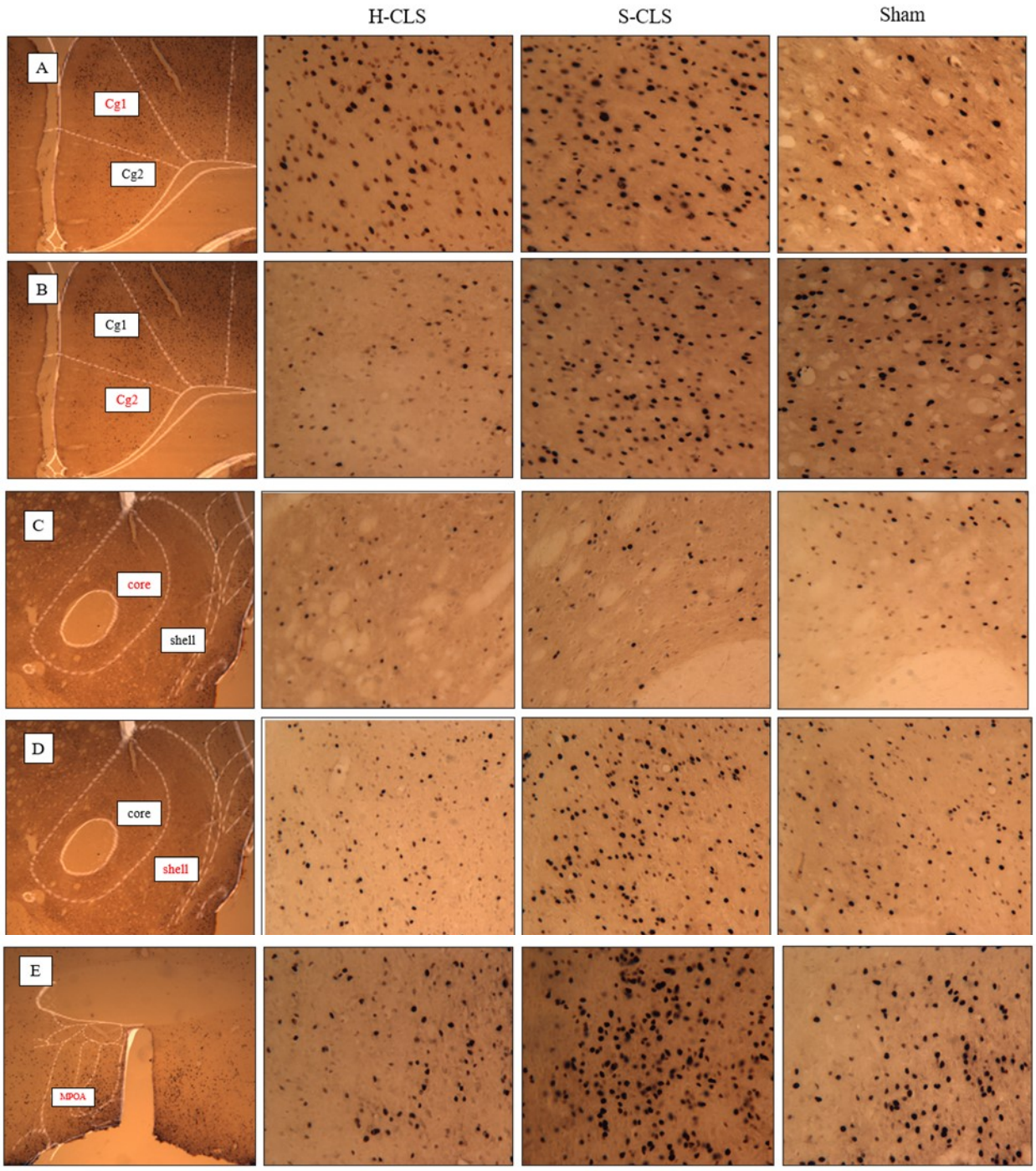

Fig 7. *Representative photomicrographs 40x (left) of Cg1 (A), Cg2 (B), NAcc core (C), NAcc shell (D), and MPOA (E) with magnified inserts (right) showing the number of Fos positive cells induced by H-CLS, S-CLS, and Sham CLS. Red indicates the area of interest.*

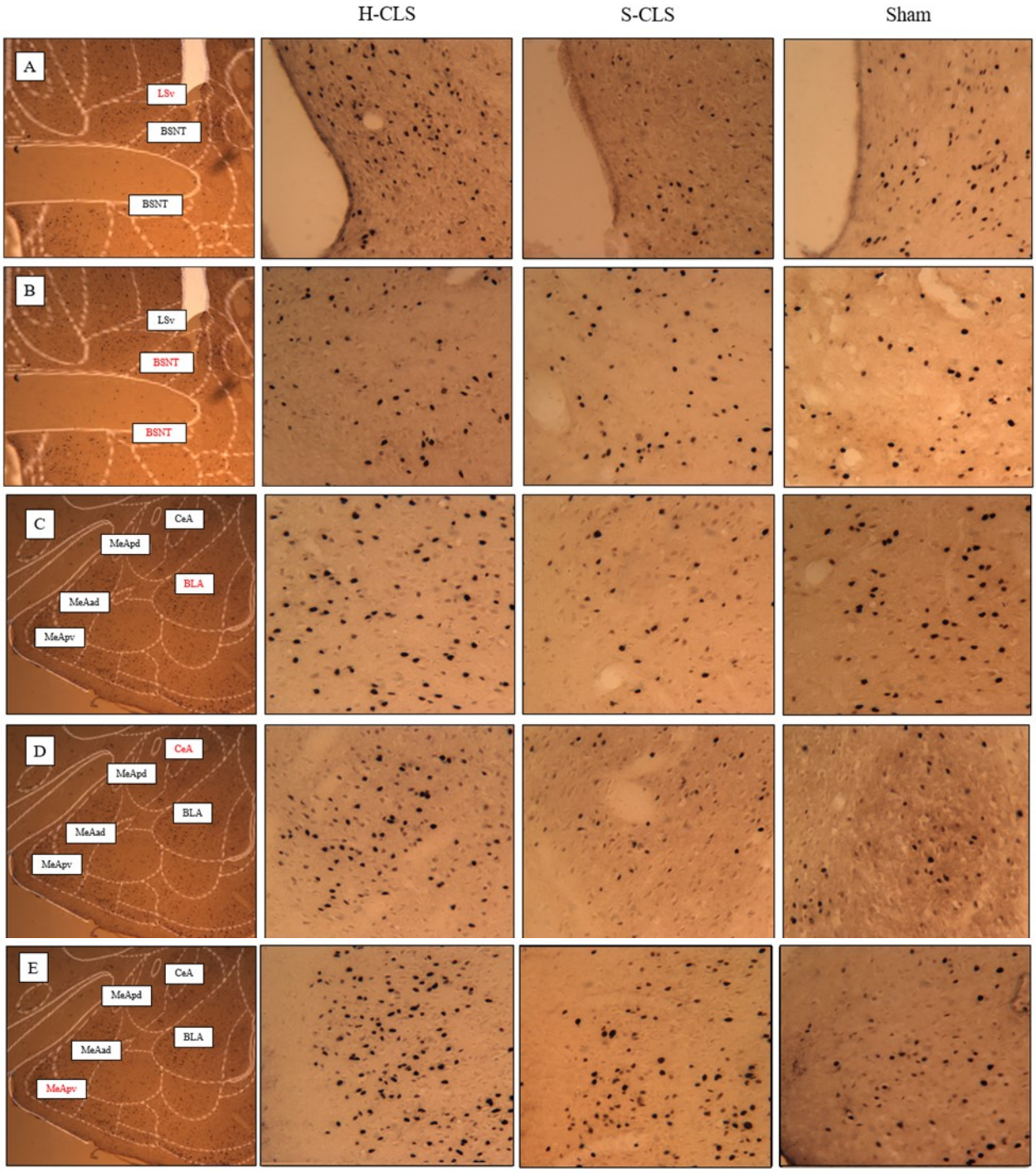

Fig 8. *Representative photomicrographs 40x (left) of LSv (A), BNST (B), BLA (C), CeA(D), and MeApv (E) with magnified inserts (right) showing the number of Fos positive cells induced by H-CLS, S-CLS, and Sham CLS. Red indicates the area of interest.*

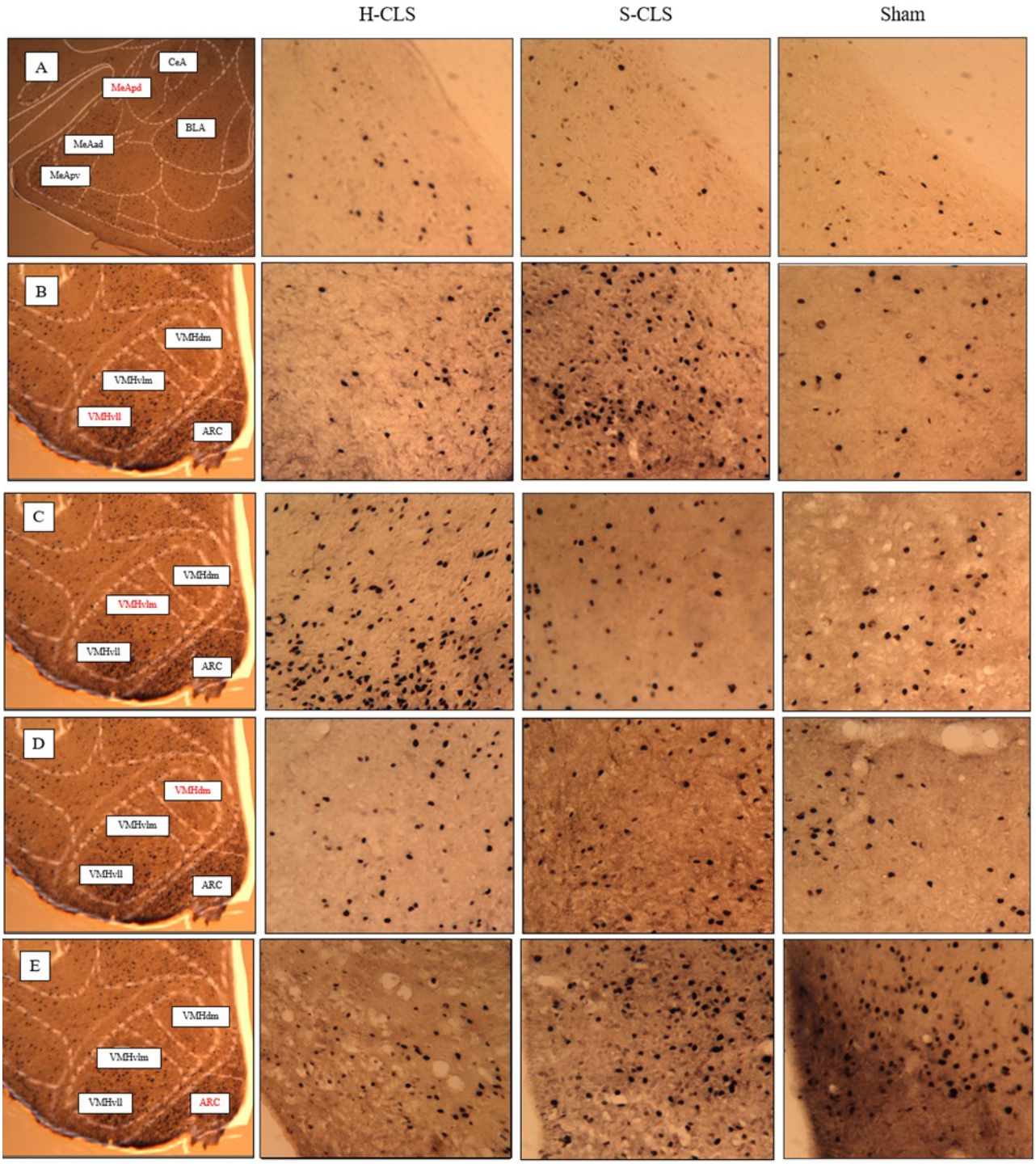

Fig 9. *Representative photomicrographs 40x (left) of MeApd (A), VMHvll (B), VMHvlm (C), VMHdm(D), and Arc (E) with magnified inserts (right) showing the number of Fos positive cells induced by H-CLS, S-CLS, and Sham CLS. Red indicates the area of interest.*

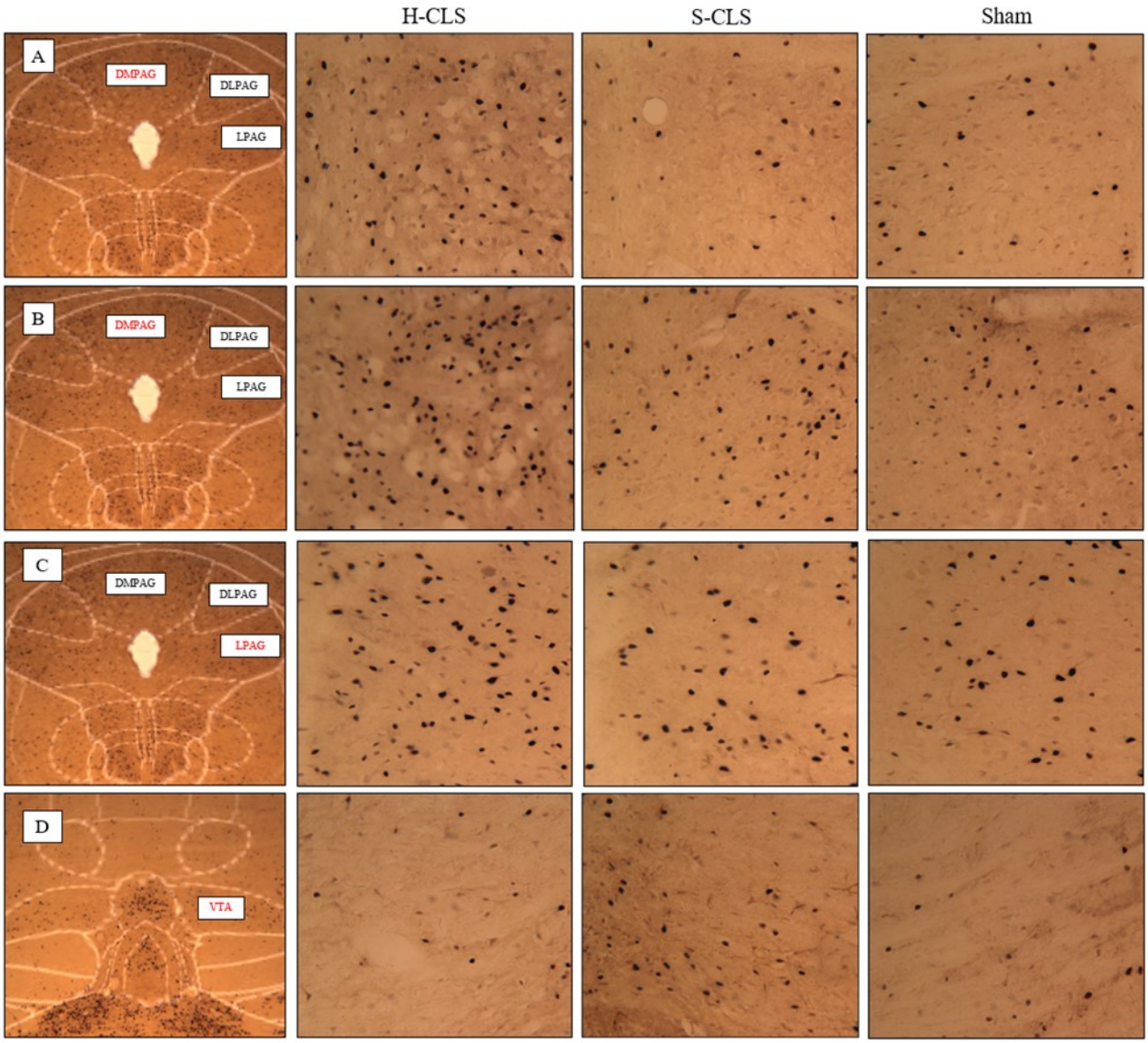

Fig 10. *Representative photomicrographs 40x (left) of DMPAG (A), DLPAG (B), LPAG (C), and VTA (D) with magnified inserts (right) showing the number of Fos positive cells induced by H-CLS, S-CLS, and Sham CLS. Red indicates the area of interest.*

Male rats have been studied using various aversive learning paradigms to examine Fos activation in the Cg, BLA, and PAG, and its correlation to anxiety-like responses such as startle (Veening et al., 2009), avoidance (De Andrade et al., 2013), and panic/defense (Johnson et al., 2011). These studies show that the number of Fos positive cells within the Cg, BLA, and PAG is

significantly greater in response to noxious (i.e., unpleasant) stimuli. These increases were correlated with increased displays of anxious and/or defensive reactions. By using H-CLS alone as a noxious stimulus, as opposed to classically implemented shock or startle stimuli, the present study investigated aversive Fos activation in female rats. H-CLS alone significantly increased the number of Fos-positive cells in the Cg1 while reducing Fos-positive cells in the Cg2. The odour CS associated with H-CLS reduced the number of Fos-positive cells in the Cg2 with no significant effects on Fos activation on Cg1. Both H-CLS and the associated odour CS were found to induce a greater number of Fos cells in the BLA and in all PAG subregions.

Greater Fos activation of the BLA and all PAG regions within the present study is consistent with prior aversive learning studies, whereas the pattern of Cg1 and Cg2 Fos activation are not consistent with these prior studies. A possible explanation for this discrepancy is the role Cg plays in stimulus discrimination and that S-CLS and H-CLS as contrasting stimuli only differ in terms of their tactile type (soft and smooth vs. hard and prickly). Aside from the regulation of affective-motivational reactions, the Cg is shown in appetitive and aversive tasks to be critical in discriminating between two or more similar stimuli based on their conditioned association (Cardinal et al., 2003; Bussey, Everitt, & Robbins, 1997; Bussey, Muir, et al., 1997; Gabriel, Kubota, et al., 1991; Parkinson, Willoughby, et al., 2000; Powell et al., 1994). Cg lesioned rats fail to discriminate between reward- and/or punishment-associated stimuli (Bussey, Everitt, & Robbins, 1997; Parkinson, Willoughby, et al., 2000; Powell et al., 1994; Bussey, Muir, et al., 1997; Gabriel, 1993), especially when the compared stimuli differ in terms of sensory modality (Cardinal et al., 1991). As S-CLS alone and H-CLS alone share temporal qualities, but not tactile qualities, we argue that the observed Fos activation in Cg1 and Cg2 may be reflective stimuli discrimination as opposed to affective-motivational reactions, which is instead indicated

by BLA and overall PAG Fos activation. There is evidence that both the BLA and PAG are involved in the production of 22-kHz USVs during innate and learned fear responses towards noxious stimuli such as electrical footshock (Kim et al., 2013). As mentioned previously, females emit 22-kHz USVs in response to H-CLS alone (Chapter 3.1 and 3.2) but not in response to S-CLS alone (Chapter 3.2). This study provides preliminary evidence that the neural systems activated by H-CLS underlie aversion. However, it remains unclear what exact mechanisms are involved to form this representation in response to H-CLS. A further study on how aversive, genitosensory, and stimulus discrimination regions interact to produce H-CLS aversion on a neural level is needed, as well as how this interaction could potentially be augmented by discrete, contextual, and partner-related cues.

5. Conclusion

CLS alone was shown in two separate experiments to induce distinct Fos activation patterns in neural regions integral in sexual reward and aversive learning, as well as key regions involved in genitosensory stimulation and stimulus discrimination. CS odour cues were shown to also induce similar Fos activation to the previously paired CLS type. The exception to this was CS odour cues associated with rewarding CLS differentiation which demonstrated overall Fos activation of sexual reward regions marked by attenuation of regions associated with affective-motivational responding and lordosis while activating region associated aggressive behavior. We argue that the results of the present experiment corroborate with our prior findings on the vocal and sexual behavioral characteristics of each CLS type. We have previously shown that S-CLS elicits hedonic 50-kHz ultrasonic vocalizations (Gerson et al., 2019a; Chapter 2.1 and 3.2) as well as induces significant CPP (Parada et al., 2010) and CPaP (Chapter 3.2; Parada et al., 2011). Conversely, we showed that H-CLS elicits the co-emission of non-hedonic 50-kHz with aversive

22-kHz USVs (Chapter 3.1 and 3.2) as well as induces CPaA (Chapter 3.2). Females, however, will co-emit 50 and 22-kHz USVs in response to S-CLS when it is differentially conditioned with H-CLS, i.e, S-CLS(+)/H-CLS. Additionally, females do not develop CPaP for males bearing CS odour cues under rewarding CLS differentiation conditioning. The underlying neural representation of S-CLS and of H-CLS, and associated CS odour cues, are thereby consistent with the vocal and sexual behavioral characteristics of each CLS type.

CHAPTER FIVE

GENERAL DISCUSSION

This thesis examined the basis of CLS-induced USVs through experiments that utilized novel call subtype analysis and well-established behavior paradigms of sexual reward and learning, CPaP and CPaA. CLS-induced USVs corresponded to behavioral changes that infer emotional affect, ranging from immediate awareness to long-term affective states. The changes in USVs in response to S- and H-CLS demonstrate evidence of augmentation as well as predictive judgement or anticipation. Augmentation refers to endogenous (e.g., hormones and drugs) and/or exogenous (e.g., CLS tactile qualities) stimuli that change behavioral indicators of affect. When ovaries are removed (OVX), for example, circulating ovarian sex steroids are reduced in the female rats hormonal milieu, thereby diminishing the display of copulatory behaviors (Blaustein & Feder, 1979; Walker & Feder, 1979; Blaustein & Wade, 1977) and the emission of courtship USVs (McGinnis & Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985). Both behaviors are then restored with sufficient hormonal priming (McGinnis & Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985; Blaustein & Feder, 1979; Walker & Feder, 1979; Blaustein & Wade, 1977). Anticipation refers to tracking certain internal/external stimuli within and/or around the organism during a situation, which generalizes behavioral indicators of affect to novel situations. Dogs with separation anxiety, for instance, behave cautiously in different ambiguous situations, exhibiting more 'pessimistic' and/or avoidance behaviors (Mendl et al., 2010b).While vocal and behavioral indication, endogenous/exogenous augmentation, and predictive judgement are fundamental to emotional affect, they are also integral aspects to the appropriate inference of clitoral pleasure and aversion as studied in the present thesis.

Vocal changes, such as acoustic parameters (i.e., duration, peak frequency, and bandwidth) and emission patterns (i.e., call rate and profile) of CLS-induced USVs were shown in the present thesis to coincide with their underlying subjective states (i.e., sexual pleasure and sexual aversion) and their associated hedonic valence Specifically, the present thesis focused on analyzing changes in acoustic parameters and emission patterns of 50-kHz trills and flat-trills subtype calls, and of 22-kHz lUSVs of the Class B call subtypes in response to different types of distributed CLS. Fifty-kHz trill and flat-trills subtype calls are positively correlated with appetitive behaviors, including rough and tumble play (Knutson et al., 1998; Webber et al., 2012), drug and natural reward (Burgdorf et al., 2001; Thompson et al., 2006; Wright et al., 2010), and sexual behavior (Burgdorf et al., 2008; Sales, 1972b; Thomas and Barfield, 1985). Class B lUSVs subtype calls, by contrast, are responses made to sexual aversion and/or frustration (Bialy et al., 2019). Although broad call categories, and more specifically certain call subtypes, correspond to opposing hedonic states, their emission represents the shift in arousal required for induction of the immediate hedonic state and its contribution to associative learning about cues that predict positive or negative states (Brudzynski, 2021). As a result, acoustic parameters, and emission patterns of CLS-induced USVs can reliably indicate the type of immediate affective state that has been induced - sexual reward or sexual aversion.

Pfaus et al. (2016) contend that short-term behavioral changes, like 50-kHz USVs, are indicative of an immediate awareness of a hedonic reward state during and/or after an orgasm-like response (OLR). Other short-term behavioral indictors of hedonic reward include rejection of further genitosensory stimulation during refractoriness or sexual satiety. It is through the immediate awareness of the hedonic reward state via an OLR that long-term behavioral changes result, such as the strengthening of sexual arousal and desire patterns in subsequent copulations,

as well as the induction of CPP and CPaP in response to contextual and partner-related cues (Pfaus et al., 2016). While copulatory interactions are often rewarding, they can in certain instances be unpleasant. Sexual aversion is another integral, yet opposite, state of affect that can occur during copulatory interaction. Much like hedonic reward, its subjective awareness is inferred through short-term behavioral changes. An immediate awareness of an aversive sexual state can be inferred from 22-kHz USVs, or other non-hedonic USVs, in tandem with displays of aggressive behaviors as such biting, kicking, and audible vocalizations, as well as defensive responses that prevent genital stimulation during copulatory interaction. Long-term behavioral changes that rise from immediate awareness of an aversive sexual state, by contrast, would be the attenuation of sexual arousal and desire patterns in subsequent copulations as well as the induction of CPaA in response to contextual and partner-related cues.

Females appear aware of distributed CLS as they respond to its delivery with short-term behaviors that infer an immediate awareness of sexual reward and/or sexual aversion, and long-term behaviors that infer an anticipation of such states. The tactile quality of the paintbrush, i.e., soft bristled camel hair (S-CLS) vs. hard bristled hog hair (H-CLS), utilized to deliver distributed CLS was shown in the present thesis to change its hedonic value. This change in reward and/or aversive value of distributed CLS is objectively based on USVs acoustics and emission patterns, CPaP and CPaA induction, and associated pattern of Fos expression. Data derived from the present thesis therefore contribute to the growing body of evidence that female USVs reflect a self-awareness of her emotional state induced either directly by external stimulation or by the anticipation of such stimulation. These data contribute to our prior findings on clitoral pleasure as well as provide the basis for developing an anticipatory-based model of the aversive state induced by painful clitoral stimulation, known as *clitorodynia*.

Ovarian sex steroids and serotonin

Females find S-CLS to be a powerful sexual reward as its distributed delivery induces both a significant CPP and CPaP (Parada et al., 2010; 2011). Pfaus et al. (2016) found in a pilot study that sexually naïve females emit 50-kHz USVs in response to S-CLS delivery. Of the emitted 50-kHz USVs, 75% of calls were of the trill and flat-trill subtype**.** Following up on the findings of this pilot study, it was examined in the first chapter whether S-CLS reliably evokes trills and flat-trills, 50-kHz subtype calls posited to signal hedonic reward. Two separate manipulations were implemented in the first chapter: ovarian sex steroids (*Chapter 2.1*) and FLU (*Chapter 2.2*). As mentioned previously, EB + P, are responsible for the full expression of female rat sexual behaviors, which was found to include CLS-induced USVs indicative of pleasure. FLU, on the other hand, is a commonly prescribed SSRI that disrupts the expression of sexual behaviors in sexually experienced female rats. These manipulations thereby provided behavioral evidence of endogenous augmentation of CLS-induced USVs.

While EB + P determine the full expression of female rat vocalizations and sexual behaviors, concurrent changes in respiratory and reproductive physiology can strengthen or weaken these vocal and sexual behaviors. OVX results in the anatomical remodeling (shrinking) of both the clitoris (Comeglio et al. 2016; Korenchevsky & Hall, 1937) and female vocal folds (Lenell et al., 2021; Kim et al., 2020; Tatlipinar et al., 2011; Oyarzún et al., 2011). Although evidence of vocal fold structure restoration is limited (Lenell et al., 2021), several studies have shown that adequate EB + P priming completely reverses diminished USV emission via OVX (McGinnis & Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985). Acoustic parameters of 50-kHz courtship USVs are also restored by EB + P priming (McGinnis & Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985). Possibly in

tandem with the restoration of USV emissions, EB + P priming reverses atrophy of clitoral structure as well as its diminished functionality (Comeglio et al. 2016; Korenchevsky & Hall, 1937).

The experiment in *Chapter 2.1* found that S-CLS elicited trill and flat-trill subtype calls from OVX, sexually naïve rats that were modulated by EB + P priming. Moreover, treatment with EB + P increased both call duration and rate, lowered the peak frequency, and widened the bandwidth of S-CLS-induced trills. A similar pattern was observed for S-CLS induced flat-trills with the exception of call duration, which was increased by S-CLS alone. Emission of trill and flat-trill subtype calls decreased steadily after each bout of S-CLS then rapidly increased prior to the next CLS bout, a pattern which reflects an anticipation of the stimulation. During each CLS bout, trills and flat-trills subtype calls were emitted continuously, a pattern which reflect consummatory reward. The findings from *Chapter 2.1* are consistent with prior reports that hormonal priming modulates the acoustic parameters of courtship 50-kHz USVs (McGinnis & Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985). As with other sexually induced 50-kHz USVs, the ability of EP+P priming to enhance the acoustic characteristics of S-CLS induced 50-kHz USVs suggests that these vocalizations are indicative sexual motivation and/or arousal. As such, it is the first study to report the modulation of 50-kHz USVs via hormonal priming without using a courtship procedure with a devocalized male partner.

Central serotonergic activity, like ovarian sex steroids, can also either facilitate or inhibit sexual behavior in the female rat (Gelez et al., 2013; Siddiqui et al., 2007; Rössler et al., 2006; Gonzalez et al., 1994; Gereau IV, Kedzie, & Renner,1993; Gorzalka, Mendelson & Watson 1990). The impact of serotonergic activity on sexual function is commonly assessed following

SSRI administration, resulting in sexual side effects such as diminished sexual desire and arousal, and anorgasmia in both men and women (Pfaus, 2009; Stahl, 1998). SSRIs, nevertheless, have been examined in fewer studies than ovarian sex steroids in adult rats in terms of 50-kHz USVs acoustics (Vares et al., 2018; Kassai & Gyertyan, 2012; Schrebier et al., 1998). Inhibitory effects on 50-kHz USV acoustics have only been examined in response to chronic stress (Vares et al., 2018) and acute footshock (Kassai & Gyertyan, 2012; Schrebier et al., 1998). Despite sexual dysfunction (anorgasmia and reduced sexual desire) being a common side effect of most SSRI treatments, no studies to our knowledge have assessed these inhibitory effects on USV acoustics within the context of sexual interaction. In those studies, SSRI treatment led to a linear, dose-response reduction in the rate of USV calls (Vares et al., 2018; Kassai & Gyertyan, 2012; Schrebier et al., 1998). Both acute and chronic administration of SSRIs, however, has been shown to reduce both appetitive and consummatory female sexual behaviors (González Cautela et al., 2021; Uphouse et al., 2015; Vega et al., 1998).

The experiment in *Chapter 2.2* found that at drug baseline, sexually-experienced females emitted S-CLS induced trills and flat-trills in a relatively similar manner to those emitted by sexually naive females receiving S-CLS in *Chapter 2.1*. S-CLS induced trill and flat-trill bandwidth was found to be the exception compared to other acoustic parameters. Despite sufficient sex steroid priming, it was found that chronic FLU did not affect the duration of S-CLS induced trills or S-CLS induced flat-trills over the course of drug treatment but was found to progressively decrease peak frequency while increasing the bandwidth of both trill subtype calls. Call rates for trills and flat-trills were also found to decrease over chronic FLU treatment days, with trills and flat-trills emitted during Inter-CLS intervals showing the greatest reduction. The attenuation of S-CLS induced trills and flat-trills suggests that chronic FLU diminishes sexual

reward, whereas in the attenuation of Inter-CLS interval trills and flat-trills indicates reduced sexual anticipation. The findings from *Chapter 2.2* of attenuated 50-kHz USVs strongly suggest that these vocalizations act as a monitoring mechanism for emotional responses to sexual stimulation, since chronic FLU inhibits other appetitive and consummatory female sexual behaviors such as solicitations and lordosis (González Cautela et al., 2021).

Tactile quality of distributed CLS

Unlike S-CLS, females find H-CLS to be sexually aversive. Gerson et al (2019b) found that switching from a soft-camel hair paint brush (S-CLS) to a hard-hog hair paint brush (H-CLS) disrupted the rewarding properties of distributed CLS. Sexually naïve females in this later pilot exhibited aggressive responses towards H-CLS despite its distributed delivery, which had been reported previously to be rewarding using S-CLS (Parada et al., 2010; 2011), similar to the imposition of delays between intromissions imposed by female rats with males during paced copulation (Paredes & Alonso, 1997; Paredes & Vazquez, 1999). Gerson et al (2019b) also found that H-CLS diminished the emission of 50-kHz trills and flat-trills in an aversive manner as well as causing an increase in 22-kHz Class B subtype calls, further corroborating the notion that females found H-CLS to be aversive. The display of rejection responses and vocalization patterns are likely due to clitoral pain, not suboptimal timing of CLS delivery, thereby suggesting that a simple change in the type of tactile sensation induced by H-CLS made it sexually aversive. This is reminiscent of the previous finding of Parada et al. (2011) where the reward value of S-CLS diminished due to the context of its delivery. When delivered in the presence of an inaccessible and distinctively scented male behind a wire mesh, S-CLS induced sexual frustration in sexually naive females (Parada et al., 2011). The scented male's inaccessibility prevented the female from interacting sexually, creating a negative association with the CS

paired odour cue despite S-CLS eliciting sexual desire. Females with this conditioning history chose to receive significantly more ejaculations from the unscented male, compared to the scented male, during their first copulatory experience (Parada et al., 2011).

Based upon the pilot study by Gerson et al. 2019b and Parada et al. 2011, a deeper exploration of whether CLS induced USVs could function as an emotional tracking signal of sexual stimulation was conducted in *Chapter 3*. We hypothesized that if CLS-USVs are indeed a reflection of an immediate affective state, then rewarding CLS should reliably elicit a hedonic reward call profile. On the other hand, if CLS was made aversive, then it should elicit aversive call subtypes as well as induce long-term behavioral changes indicative of aversion. The aversive qualities of H-CLS were therefore assessed in two experiments in *Chapter 3.1* and *Chapter 3.2*. A 22-kHz USV-based replication and extension of *Chapter 2.1* was conducted in the first H-CLS experiment in *Chapter 3.1* while the second H-CLS experiment in *Chapter 3.2* examined whether H-CLS could induce CPaA compared to S-CLS-induced CPaP and USVs. These H-CLS experiments together provide behavioral evidence of exogenous augmentation of CLS-induced USVs.

Long 22- kHz USVs are just as integral to the sexual behavioral repertoire of adult rats as FM 50-kHz USVs. FM 50-kHz USVs are emitted by adult rats as a signal of sexual reward, while long 22-kHz USVs are emitted by adult male rats as a signal of satiety (Barfield & Geyer, 1972; Bialy et al., 2016) and emotional relaxation (Bialy et al., 2019). Male and female rats also emit syllabically distinct 22-kHz USVs, known as Class B lUSVs, during barrier non-contact tests, which are thought to be signals of frustration or aversion (Bialy et al., 2019). Although 22-kHz lUSVs play an important role in sexual interaction, most studies have focused on demonstrating that FM courtship 50-kHz USVs are hormonally sensitive (McGinnis &

Vakulenko, 2003; Matochik, Barfield, & Nyby, 1992; Thomas & Barfield, 1985), while, to the authors' knowledge, no studies have considered how sex steroids affect 22-kHz USV emissions during sexual interaction. A report by Inagaki and Mori (2015) has, however, examined the impact of sex steroids on the production of stress induced 22-kHz USVs in response to startle stimuli (i.e., air puffs, abrasive sound, or electric foot shocks). Free-cycling female rats emitted shorter stress-induced 22-kHz USVs in response to an air puff during proestrus and diestrus compared to those emitted by gonadally intact males. No significant difference between stress-induced USV emission in proestrus and diestrus was found, indicating that cycling ovarian hormones do not affect 22-kHz USV production. Testosterone implants affect stress induced-22-kHz USV emission of castrated males, but not those of OVX females. This suggested that the emission of stress induced-22-kHz USV of males are affected more by testosterone than in females. Based upon Inagaki and Mori's (2015) report, it was unknown whether CLS induced Class B lUSVs would demonstrate hormonal independence for females similar to stress-induced 22-kHz USVs.

The first H-CLS experiment in *Chapter 3.1* revealed that females primed with EB + P or EB alone emitted more Class B subtype calls compared to females given the oil vehicle. The increased proportion of lUSVs emitted in response to H-CLS suggests an immediate sexually aversive state, as OVX rats are normally made sexually receptivity via sufficient EB + P priming. OVX rats were also found to emit trills and flat-trills in response to H-CLS. It was revealed that H-CLS, but not hormonal priming, altered acoustic parameters of these trill and flat-trill call subtypes. The finding that H-CLS induced trills and flat-trills that were not hormonally dependent suggests that these calls are not sexually appetitive. As further evidence, the highest co-emissions of Class B subtype calls and the highest displays of agnostic behaviors

(i.e., biting, kicking, and audible vocalizations) were observed when OVX rats were made sexually receptive via EB + P. As in women, estradiol could have increased clitoral sensitivity (Kim, 2009), contributing to this co-emission pattern.

While this study is the first to report the co-emission of 50- and 22-kHz USVs in response to aversive sexual stimulation, adult rats have been previously shown to co-emit 50-and 22-kHz during aversive Pavlovian conditioning (Tryon et al., 2021). Tryon et al. (2021) found that free cycling females emit fewer 22-kHz during aversive Pavlovian conditioning, with no difference between extinction competent and extinction resistant individuals. During fear acquisition, the co-emission of 50 kHz USV were instead found to be more predictive of fear extinction and generalization to novel stimuli. This suggests that co-emitted 50-kHz USVs might not reflect positive affect alone, but rather general arousal that facilitates predictive learning of unpleasant cues. Aversive sexual conditioning with H-CLS that resulted in CPaA was unknown prior to this thesis relative to rewarding sexual conditioning with S-CLS that induced CPaP (Parada et al., 2011; 2010). It was also unknown whether a co-emission pattern of 50- and 22-kHz USVs, like those reported by Tryon et al. (2021), would be demonstrated in response to aversive CLS conditioning, compared to rewarding CLS conditioning.

The second H-CLS experiment in *Chapter 3.2* found that H-CLS (+) induced a conditioned avoidance of the Sc male relative to Sham (-). Females in the H-CLS(+)/Sham(-) group received fewer intromissions from, as well as spent less time with, the Sc males than the UnSc males. As in previous studies (Parada et al., 2011), S-CLS (+) induced an ejaculatory preference for the Sc male relative to the UnSc male . This was in contrast to a lack of an ejaculatory preference for Sc males when S-CLS(+) was differentially conditioned with H-CLS (-), or vice versa. Females in the S-CLS(+)/H-CLS(-) group spent significantly less time with the

Sc males than UnSc males. Vocal behaviors recorded during the final USV trials were consistent with these indicators of aversion and sexual reward during the final open field test. Neither H-CLS (+) nor H-CLS (-) was shown to significantly alter spectrotemporal parameters of trill and flat-trill subtype calls when differentially conditioned with Sham (-) and Sham (+). S-CLS(+) final trial trill and flat-trill call subtypes were longer than those emitted during Sham(-) final trial calls. H-CLS did, however, significantly alter trill duration and bandwidth when conditioned differentially with S-CLS. Class B emissions were also observed in the final USV recording trial for aversive CLS (H-CLS vs. Sham) and CLS differentiation (H-CLS vs. S-CLS), but not rewarding CLS (S-CLS vs. Sham), conditioning groups. Specifically, H-CLS produced significantly higher emissions of Class B compared to Sham. CLS differentiation groups also exhibited higher Class B emissions during the final S-CLS USV recording trial as opposed to the final H-CLS recording trial, suggesting that S-CLS was made aversive due to differential conditioning with H-CLS. Taken together, these findings suggest that H-CLS and S-CLS can be utilized to condition sexual aversion and sexual reward, respectively. These findings also suggest that S-CLS can be made aversive to females when it is differentially conditioned with H-CLS.

Neural correlates of S-CLS and H-CLS

Predictive judgment, also known as anticipation, is an important function of affect. As mentioned previously, anticipation allows for the generalization to novel situations through tracking the occurrence of certain known or predicted internal/external stimuli within an organism's body and/or its immediate environment during situational events. Brain activation states, i.e., reward or aversion, are triggered by these tracked internal/external stimuli, which results in the organism responding to such stimuli with either approach/reward acquisition or avoidance of punishment or pain (Medel et al., 2010; Ekman, 1992; Frijda, 1986). There have

been several studies that link 50- and 22-kHz USV emission with the induction of reward and aversive brain states, respectively (reviewed in Brudzynski, 2015; Burgdorf et al., 2007; Kroes et al., 2007. While 50- and 22-kHz USVs in response to CLS has been assessed (Gerson et al., 2019a,b; Pfaus et al., 2016), the technique has only been assessed in terms of its ability to activate rewarding brain states (Parada et al., 2010).

Sexual stimulation, however, is organized within the brain into an evolutionarily conserved set of neural pathways, which heavily rely on value-based processing (Pfaus and Scepkowski, 2005; Pfaus, 2009). Among these conserved pathways are limbic and hypothalamic structures which integrate endogenous sex "drive" (e.g., hormonal priming) with autonomic arousal and the intensity and valence of incentive sexual stimuli, as well as higher cortical regions, which evaluate the context of sexual interactions (reviewed in Pfaus, Jones, Flanagan-Cato, & Blaustien, 2014). The clitoris, much like the cervix, transmits genitosensory information to these conserved neural pathways that then determine the reward value of sexual interactions. In turn, this shapes motivational responses for future sexual interactions as indicated by vocal expression and sexual learning outcomes, such as CPP, CPaP, and CPaA. Anticipation is thereby further demonstrated by the pattern of Fos expression in the brain in response to conditioned cues implemented during sexual learning, and should, therefore, coincide with the sexual learning outcomes it predicts.

As mentioned above, sexually-naive females respond differently to CLS reward depending on the context in which it is delivered (*Chapter 3.2*; Parada et al., 2011), and these context-dependent responses are associated with the activation of hypothalamic/limbic brain regions like the mPOA and MeA (Parada et al., 2010). Genitosensory input (Aguilar-Moreno et al., 2022; Marson, 1995), sexual incentive salience (Quintana et al., 2019), desire (Pfaus, 2009),

and reward (Martz, Vasquez, and Dominguez, 2023; Parada et al., 2010) also involve the activation of both of these hypothalamic/limbic brain regions. Parada et al. (2010) examined the number of Fos positive cells within the mPOA and MeA, as well as other hypothalamic/limbic structures involved in sexual reward, in response to distributed S-CLS. Structures specifically included in Parada et al.'s analysis were the NAc core and shell, LS, BNST, VMH, BLA, ARC, and VTA. A significant increase in Fos-positive cells was found in the mPOA and MeApv, but not other hypothalamic/limbic structures, following the delivery of S-CLS compared with Sham-CLS (Parada et al., 2010). A significant CPP was also found to be induced by distributed S-CLS within this same study (Parada et al., 2010).

Based upon Parada et al.'s report, *Chapter 4* sought to examine in two separate experiments the pattern of Fos expression within neural regions associated with reward, aversion, and genitosensory processing in response to CLS administration, i.e., CLS alone (H-CLS alone, S-CLS alone, and Sham CLS alone), and to CLS-paired odour cues (H-CLS (+), H-CLS (-), S-CLS(+), S-CLS(-), scented aversive CLS differentiation, and scented rewarding CLS differentiation). S-CLS induced a greater number of Fos-positive cells within the mPOA and MeApd, neural regions involved in processing sexual stimulation and sexual reward (Parada et al., 2010). Appetitive female sexual behaviors correlate with an activation of neurons in these regions (Parada et al., 2010). We therefore hypothesized that Fos induction by H-CLS would show a different pattern from the induction by S-CLS, as H-CLS would activate aversive-related neural regions compared to the activation of reward-related neural regions via S-CLS.

It was found in the CLS alone experiment of *Chapter 4* that both S-CLS and H-CLS induced Fos activation in neural regions associated with sensory discrimination and tracking genitosensory stimulation (***Fig 1***.). S-CLS alone and H-CLS alone respectively, activated

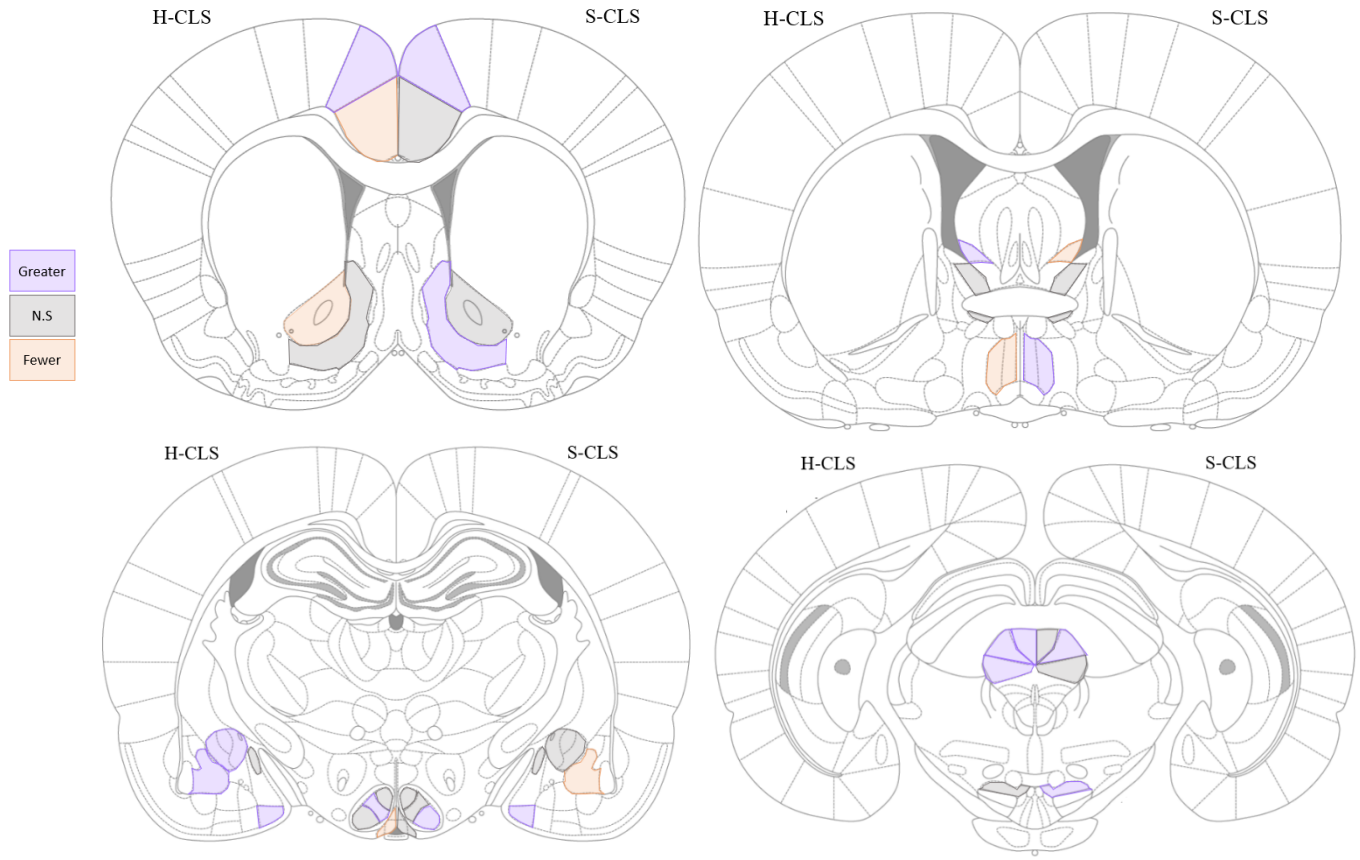

Fig 1. *Fos activation patterns in response to H-CLS (left) and S-CLS (right). Purple represents regions where the number of Fos-positive cells was significantly greater than Sham-CLS. Gray represents regions where the number of Fos-positive cells was not significantly different from Sham-CLS. Orange represents regions where the number of Fos-positive cells was significantly less than Sham-CLS.*

reward- and aversive-related regions apart from this shared activation pattern (***Fig 2***.). S-CLS alone activated genitosensory input and reward regions, while H-CLS alone activated regions associated with aversive and aggressive reactions, pain, and affective-motivational regions. Fos expression in response to H-CLS was also found to be decreased in regions associated with

reward and sexual receptivity. These Fos activation patterns thereby indicate that S-CLS alone and H-CLS alone induce distinct patterns of brain activation.

Findings from the CS odour experiment of *Chapter 4* revealed that the differences in Fos activation in areas associated with affective-motivational reactions, sexual receptivity, and genitosensory input depended on the type of CLS differentially conditioned with CS odor cues (***Fig 2.).*** When differentially conditioned with Sham (-), S-CLS (+) and H-CLS (+) induced similar Fos activation patterns as S-CLS alone and H-CLS alone, respectively. S-CLS(+)/H-CLS(-) exposure also showed an overall activation pattern similar to S-CLS alone, while H-CLS(-)/S-CLS(-) showed an overall activation pattern similar to H-CLS alone. S-CLS(+)/H-CLS(-) exposure, however, increased the number of Fos positive cells in the VMHvlm (associated with aggressive behaviors) while decreasing the number of Fos positive cells in the NAc shell (associated with affective-motivational responding to reward) and VMHvll (lordosis). Similar Fos activation patterns within these regions were observed in response to H-CLS alone compared to S-CLS alone. Affective-motivational reactions and consummatory aspects of S-CLS reward seem to diminish when S-CLS (+) is differentially conditioned with H-CLS (-). Although the activation of regions associated with affective-motivational responding and lordosis were attenuated by S-CLS (+) and H-CLS (-) differential conditioning, sexual reward regions were still able to track S-CLS reward signaled by the CS odour cue. This indicates that the CS odours that predict S-CLS and H-CLS neural representations of reward and aversion are consistent with the vocal and sexual learning outcomes predicted by these CS odours in Chapter 3.2. We therefore argue that this consistency between vocal expression and sexual learning outcomes reported in *Chapter 3.2* and Fos expression reported in *Chapter 4* indicate vocal, behavioral, and neural evidence of CLS anticipation as an appetitive or aversive stimulus.

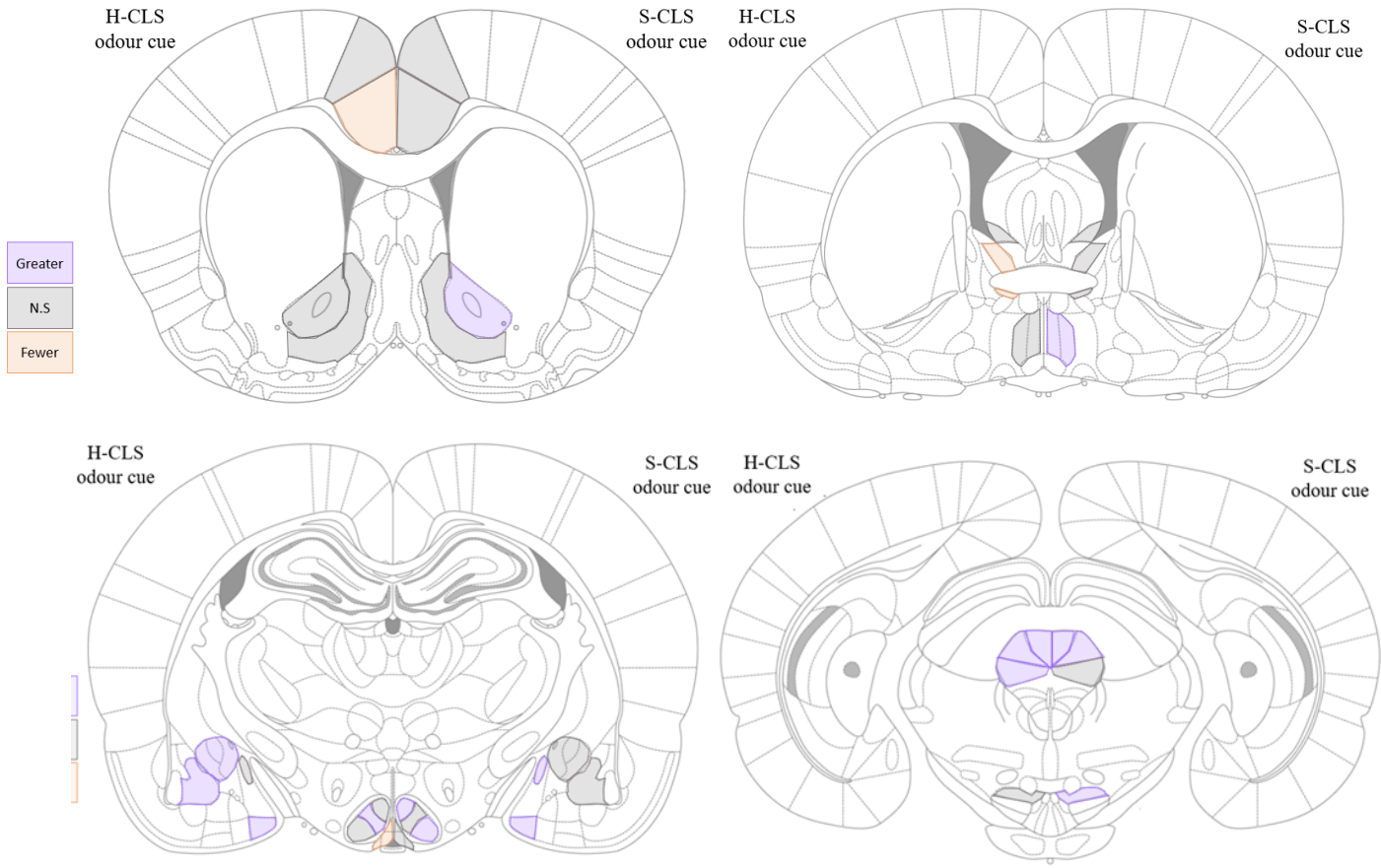

Fig 2. *Fos activation patterns in response to the H-CLS paired odour (left) or the S-CLS paired odour (right). Purple represents regions where the number of Fos-positive cells was significantly greater than Sham-CLS. Gray represents regions where the number of Fos-positive cells was not significantly different from Sham-CLS. Orange represents regions where the number of Fos-positive cells was significantly less than Sham-CLS.*

Prevalence and psychosocial burden of clitorodynia

Clitorodynia refers to clitoral pain experienced by women that persists for > 3 months and is classified as a type of localized dyspareunia (DSM-5, 2022; ICD, 2019; reviewed in

Parada et al., 2015), which is estimated to impact approximately 8% of women of all ages and ethnicities (Dunford et al., 2019; Wayal et al., 2017; Nyguen et al., 2015; Harlow et al., 2014; Reed et al., 2012; Reed & Cantor, 2008;). This persistent clitoral pain occurs due to biomedical causes, e.g., physical pain resulting from lichens sclerosis and low estrogen levels increasing vaginal innervation, and/or by psychosexual causes, e.g. anticipation of pain and degree of pain catastrophizing and low self-efficacy (van der Meijden et al., 2022; Bergeron et al., 2020; Parada et al., 2015; Farmer & Meston, 2005; Bergeron et al.,1997; Meana et al., 1997). Taken together, these data show that women and their partners' quality of life and intimate relationships are negatively affected as the anticipation of pain (and no pleasure) during sexual interaction. This can erode sexual intimacy and communication and can induce anxiety and/or to depression (van der Meijden et al., 2022; Bergeron et al., 2020; Parada et al., 2015; Farmer & Meston, 2005; Bergeron et al.,1997; Meana et al., 1997). Because of these deleterious consequences, women suffering from dyspareunia (whether clitoral, labial, or vaginal) carry a heavier psychological burden compared to other pain disorders (Desrochers et al., 2008). Frequently, women with this condition report feeling ashamed, inadequate as partners, have less affection for their bodies, and less self-esteem (Desrochers et al., 2008). Despite this burden, 40% of afflicted women are reported to never receive a diagnosis (Harlow et al., 2014; Harlow & Steward, 2003; Harlow, Wise, & Stewart, 2001), while the remaining 60% report feeling stigmatized by their physicians when they seek help (Nguyen et al., 2013; Donaldson et al., 2011). These statistics suggest that treatment for afflicted women and their partners is currently suboptimal despite advancements of medical treatments and psychosocial interventions.

Current models of dyspareunia and clitorodynia

The cause of dyspareunia has traditionally been conceptualized in a dualistic manner with

either a physical or a psychological cause. While contrary research and hypotheses suggest that dyspareunia should be approached from both biomedical and psychological perspectives, this dualistic conceptualization persisted until only recently. The focus has now shifted to examining how biopsychosocial factors give rise to, and maintain, dyspareunia and its associated psychosocial conditions, such as hypoactive sexual desire and/or mood disorders. As such, the onset, chronicity, and exacerbation of pain, loss of sexual desire, and anxiety/depression are believed to result from both medical and psychosocial mechanisms (Bergeron, Rosen, & Morin, 2011). . For instance, of the 99 studies published on vulvodynia since 2020, approximately 67% of these studies discussed the importance of new or concurrent anxiety-like symptoms, e.g., pain catastrophizing, hypervigilance (anticipation), and sexual distress, in the development and/or maintenance of the condition.

Although numerous *in vivo* female rodent models have contributed to our current understanding of human genital pain and sexual behavior (desire, arousal, motivation, and orgasm), these models continue to focus on the dualistic aspects of dyspareunia despite recent theoretical shifts within the human literature. Specifically, *in vivo* female rodent models of genital pain traditionally have employed simpler biomechanical paradigms, e.g., monitoring pelvic organ distension and/or surgical interventions, compared to *in vivo* models of sexual behavior which often utilize a combination of behavioral and learning paradigms, e.g., measuring appetitive and consummatory behaviors during open field tests and CPaP or CpaA. Recent preclinical rodent studies have mostly kept with this tradition in their assessment of biomechanical mechanisms contributing to human dyspareunia , such as early life stress and injury (Pierce et al., 2015; Peirce et al., 2014) and pro-inflammatory of genitopelvic area (Barry, Matusica, & Haberberger, 2019; Chakrabarty et al., 2018; Sharma et al., 2018; Farmer et al.,

2014). Although these studies have provided new insights into how vaginal hyperinnervation, nociceptor sensitization, and inflammatory agents impact genital pain and sexual physiology, only one of the aforementioned studies measured psychosocial factors in relation to these biomedical factors (Pierce et al., 2014). Also, these studies mostly examine genital pain and sexual physiology in relation to vaginal sensitivity, thus no assessment of clitoral sensitivity per se has been available.

Pierce et al. (2014) examined the impact of early life stress on vaginal sensitivity in adulthood. Female mice were exposed to neonatal maternal separation (NMS) for either two (NMS14) or three (NMS21) weeks following their birth to test this effect. NMS and naïve females as adults underwent vaginal balloon distension (VBD), an objective measure of vaginal sensitivity as well as an acute stressor. Both NMS and naïve females were assessed at baseline and after VBD for anxiety-like behavior (i.e., sensorimotor activity in an open field), hindpaw sensitivity (i.e., thermal and mechanical pain), and changes in HPA axis function (i.e., mRNA and protein expression). For NMS14 females, when compared to their naïve counterparts, hindpaw mechanical and thermal sensitivity increased following VBD, but anxiety-like behaviors did not change from baseline. For NMS21 females, however, both vaginal sensitivity and anxiety-like behavior were heightened following VBD as well as marked decreases in negative regulation of the HPA axis. While Pierce et al. focused on simple sensorimotor indicators of anxiety rather than learnt and/or vocal indicators of anxiety, their results show that experience augments future sexual physiology and sociopsychological behavioral responses. Although appetitive and consummatory sexual behaviors were not assessed within Pierce et al's study, these findings are aligned with current preclinical rodent models of sexual behavior that

demonstrate that early experiences shape adult sexual responding (reviewed in Pfaus et al., 2012) and its underlying sexual physiology (Davis et al., 2020).

Incorporating learning into current dyspareunia models

Sexual responses are shaped by experience, whether it is through early life and/or through later learning. Preclinical rodent models of sexual function suggest this, yet learning has not been fully incorporated into current preclinical models of dyspareunia. As mentioned above, anticipation is a function of affect that acts as a predictive judgment, which is acquired through learning about one's internal and external environment. For women suffering from dyspareunia, the experience of pain and/or unpleasantness during a novel sexual situation often leads to the development of an anticipation of genital pain. These women often exhibit anticipation in the form of both pain-related anxiety and avoidance behaviors toward future penetrative sexual encounters (Linton, 2013). Behavioral indicators of anticipation are further reinforced by the increased likelihood of pain during attempted penetration (Thomten & Linton, 2013). As humans and rodents share homologous genitopelvic structures (Martin-Alguacil, Pfaff, Shelley, & Schober, 2008), they also share homologous neural systems for nociception to detect potential injuries inflicted by internal/external noxious stimuli (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). In view of this, it makes sense for current preclinical models of dyspareunia to place more important consideration on the physiology of nociception than on the affective dimension, such as unpleasantness, of genital pain. Yet, this ignores the fact that adaptive associative learning is necessary for the brain to predict potential threats or injuries (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). Anticipation thereby arises when the learned prediction becomes “maladaptive” through classical conditioning (Vlaeyen, Crombez & Linton, 2016; Vlaeyen, 2015). When a painful or unpleasant stimulus elicits anticipation, this initial

stimulus is unconditioned (UCS). A sexual anticipatory response can then be conditioned through repeated pairings of the UCS with neutral stimuli during sexual interaction, which now becomes a conditioned cue CS. Impending pain is now predicted by the conditioned stimulus which now elicits anticipatory clenching of the pelvic floor, tightening of the vagina, both of which continue to make penetration painful, along with defensive responses and decreases in sexual arousal and desire.

The present thesis is built upon previous preclinical studies utilizing Pavlovian fear learning and extinction, whose collective findings provided insight into how an anticipation of clitoral pain/unpleasantness could be conditioned (Laine et al., 2022; Lovick & Zangrossi, 2021; Tryon et al., 2021; Machado Figueiredo et al., 2019). During aversive sexual learning within the present thesis, females were exposed to an aversive UCS, e.g., H-CLS, or rewarding CLS differentiation, paired with a neutral odour cue. The neural odour cue came to predict H-CLS or rewarding CLS differentiation, which was shown to induce CPaA or failed to induce a CPaP, respectively. Females were found to co-emit 50- and 22-kHz USVs, a vocal pattern we established as indicating sexual aversion, prior to CS-elicited behaviors on the final odour condition trials. CS odour cues associated with H-CLS or rewarding CLS differentiation, in addition, were found to activate neural systems associated with pain and aversion. Aversive sexual learning within the present thesis was conducted in contrast to rewarding sexual learning, which we argue creates an opposite type of anticipation – that of clitoral pleasure. During rewarding sexual learning within the present thesis, females were exposed to a rewarding UCS, e.g., S-CLS, paired with a neutral odour cue. The neutral odour cue came to predict S-CLS, which was shown to induce CPaP in a similar manner to that previously reported by Parada et al. (2011). Females emitted 50-kHz USVs in a vocal pattern that we established as indicating sexual

reward, prior to CS elicited behaviors on the final odour condition trials. CS odour cues associated with S-CLS, were found to elicit neural expression of sexual reward. These findings are the first to show that the fundamental learning components of clitorodynia can be effectively assessed through S-CLS and H-CLS and their respective USV patterns. Taken together, these results form a foundation for an anticipatory-based model of clitorodynia and clitoral pleasure.

Conclusions and future directions

The present thesis in total shows the development of rat model of clitorodynia and clitoral pleasure using vocal and neural indictors of sexual reward/aversion combined with well-established paradigms of conditioned sexual interaction. S-CLS and S-CLS induced USVs were specifically shown throughout this thesis to be associated with learning outcomes and neural expressions of sexual reward. By contrast, H-CLS and H-CLS induced USVs were shown throughout the present thesis to be associated with learning outcomes and neural expression of sexual aversion. These notions were further corroborated by evidence of anticipation as indicated by CS odours eliciting S-CLS and H-CLS neural representations of reward and aversion. It is, however, the nature of any scientific endeavor that the present results raise more questions than they answer. Yet, the present thesis findings serve as a foundation for a preclinical model of clitorodynia and clitoral pleasure in which future studies can and should explore pre-clinical applications of S-CLS and H-CLS, and their respective USVs.

Pharmacological manipulations are a hallmark for most pre-clinical animal models. Analgesics and/or libido enhancing drugs are one avenue of exploration for future studies, as these drugs reported to ameliorate the dampening effects of pain anticipation on female copulatory behavior. In a study by Farmer et al. (2014), inflammatory pain was shown to

diminished behaviors indicative of sexual motivation in female, but not male, mice in response to inflammogens zymosan A (0.5 mg/ml) or λ-carrageenan (2%) injections. Sexual motivational behaviors were restored by an analgesic, pregabalin (an α2δ ligand), and by libido-enhancing drugs, apomorphine (nonselective dopamine D1/D2 receptor agonist) and melanotan-II (prodrug converted to a melanocortin-3/melanocortin-4 receptor agonist known as bremelanotide, which also stimulates sexual solicitations in female rats; Pfaus et al., 2004; Pfaus, Giuliano, & Gelez, 2007; Kingsburg, Clayton, & Pfaus, 2015). These findings suggest that aversive impact of H-CLS on sexual learning could be ameliorated by pre-treatment with an analgesic drug, i.e., by dampening the discomfort of the physical application of H-CLS, and/or libido-enhancing drugs, i.e., by counteracting the pain anticipation of H-CLS.

The pattern of S- and H-CLS-induced USVs throughout the present thesis showed evidence of both immediate and long-term affect awareness. Changes to acoustic parameters and emission patterns demonstrate that S- and H-CLS-induced USVs are subject to augmentation by endogenous and exogenous stimuli, which revealed their differences in hedonic reward value. The emission of S-CLS induced 50-kHz USVs showed the same hormonal dependence and SSRI attenuation as the expression of other sexually rewarding behaviors. The emission of H-CLS induced 22-kHz USVs, but not 50-kHz USVs, instead demonstrated hormonal independence. The impact of SSRI administration of the co-emission of 50- and 22-kHz H-CLS induced USVs was, however, not assessed within the present thesis. Future studies could explore the contribution of other sex steroids, such as testosterone, or the potential disruption of endocrine disrupting chemicals, such as bisphenol-A, on augmentation of acoustic parameters and emission patterns of S-CLS and H-CLS induced USVs. Furthermore, it would be of great interest to investigate the impact of other anxiety/depression medications, as well as drugs of abuse on

CLS-induced USVs. Aside from pharmacological manipulations, the impact of changes to CLS qualities and/or CS context on vocal and sexual learning outcomes should also be explored. It is still unknown what the impact of early life stress/injury, nociceptor sensitization, and inflammatory agents on clitoral sensitivity, and in turn sexual motivation, has on CLS-USVs and learning outcomes.

In summary, sexual function and dysfunction experienced by humans involves a cascade of physiological and behavioral events that lead to anticipation. Vocal behaviors and well-established behavioral paradigms can be used to objectively measure and infer subjective aspects of this experience, such as affect. Distributed clitoral stimulation that mimics the kind of genital stimulation females impose on males during paced copulation, was found throughout this thesis to be a powerful sexual reward or sexual punishment for female rats, based on vocal and behavioral affective measures in response to rewarding versus aversive CLS. We can, with such measures, mirror and/or model the experiences of women suffering from clitorodynia and/or dyspareunia, where a once pleasurable genital caress from a lover has now become a source of physical pain and impending anxiety/distress. In addition to increasing our knowledge of the role of learning and anticipation in sexual behavior in general, it is hoped that the experiments of the present thesis help expand the current models of dyspareunia, and aid in the development of effective treatments for this debilitating condition.

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