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The temporal copulatory patterns of female rat sexual behavior

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ABSTRACT

Female sexual behavior is a naturally rewarding activity that plays an important role in reproduction and species survival. For female rats, regulating the timing of sexual interactions is essential for optimizing mating satisfaction and enhancing the physiological conditions needed for successful fertilization. So far, traditional research on female sexual behavior has relied on a limited set of behavioral parameters, which has certain shortcomings. To address this, our study aimed to develop a more detailed behavioral framework for assessing temporal copulatory patterns in female rats. We compared fully receptive females and less-receptive females, while also investigating the effects of (R)-(+)-8-OH-DPAT, a 5-HT_{1A} receptor agonist known for its inhibitory impact on female sexual behavior. Additionally, we examined how sexual experience and pacing conditions influence these copulatory patterns. Our results revealed that female rats engage in structured patterns of sexual bouts and time-outs, with higher receptivity leading to more sexual bouts and shorter time-outs. This suggests that sexual bouts can be viewed as an indicator of copulatory speed, while time-outs reflect motivation to continue mating. Sexual experience did not enhance sexual performance but did result in females receiving more copulatory events from males. Lastly, we found that the conditions under which mating occurs (paced vs. non-paced) may not significantly impact copulatory behavior in fully-receptive females, but could be more relevant for less-receptive females. Despite this, paced mating conditions remain preferable for studying female sexual behavior.

1. Introduction

Female sexual behavior is a natural rewarding behavior that plays an essential role in reproduction and the survival of the species. From observations in the field and more controlled experiments in semi-natural environments, we learned that rats often mate in groups, where one or multiple males and females interact simultaneously (Robitaille and Bovet, 1976; Calhoun, 1963; Chu and Ågmo, 2014; Hegstad et al., 2020). Repetitive patterns of paracopulatory behaviors (such as darts and hops) and reflexive lordosis responses to mounts, intromissions, or ejaculations represent the sexual behaviors of the female rat. In these natural settings, the presence of multiple receptive females and the availability of space for females to move away from sexually active males give them the ability to control, or "pace," their mating encounters. In the lab, however, the use of small copulation boxes limits females to express this kind of pacing activities, which is why this is referred to as non-paced mating. To overcome this limitation, a divider with small holes, that limit passage to the smaller sized female animals, can be placed inside the copulation box, allowing the female to withdraw to a separate compartment. In this paced mating set-up, the female regains control over the timing and number of sexual encounters with the male rat. Studies in the lab have shown that by managing the intervals between these interactions, female rats can optimize the reward derived from mating (Coria-Avila et al., 2005; Martinez and Paredes, 2001) and the physiological conditions necessary for successful fertilization (Coopersmith and Erskine, 1994; Erskine, 1989). Understanding these dynamics in behavior can provide important knowledge into how female-driven sexual behavior influences reproductive success and ensures the continuation of the species. Moreover, analyzing these complex patterns of behavior become essential when studying the neurobiological mechanisms underlying mating (Krakauer et al., 2017).

Traditionally, a limited amount of parameters has been used to analyze and interpret female sexual behavior. While the number of paracopulatory behaviors may reflect the level of sexual motivation, sexual receptivity is measured with a lordosis quotient and score (reviewed in (Heijkoop, Huijgens, and Snoeren, 2018; Ventura-Aquino and Paredes, 2023)). In a paced-mating setting, the withdrawal behavior can be analyzed by calculating the percentages of exits after mounts, intromissions and ejaculations and the time it takes for the female to resume copulation (also called contact return latency). These pacing parameters, as they are also called, are dependent on the stimulation intensity, with the largest percentages and longest return latencies after

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ejaculation, followed by intromissions and mounts (Krieger, Orr, and Perper, 1976; Meerts et al., 2014; Brandling-Bennett, Blasberg, and Clark, 1999; Zipse, Brandling-Bennett, and Clark, 2000; Erskine, 1985, 1989). While the percentages of exits may indicate the female rats' ability to discriminate sensory stimulation, contact return latencies (CRL) reflect their motivation to continue copulation (reviewed in (Heijkoop, Huijgens, and Snoeren, 2018)).

While percentages of exits and CRL offer interesting insights into the pacing behavior of female rats, they also have constraints. For instance, CRL can only be measured following an exit, which excludes latencies where the female immediately resumes copulation. There is a large variability in how different laboratories define an exit. Some labs set a specific time limit within which the female must have moved to the other compartment to be considered an exit, hereby assuring a causal relationship between the received stimulation and the withdrawal but decreasing the amount of data points for calculating the CRL. Others, on the other hand, define an exit without considering a withdrawal time. This could increase the power for calculating the CRL but opens the question what a CRL actually represents. Finally, as the females do not have control over the type of stimulation they receive, their pacing behavior is highly dependent on the behavior of the male. It has been shown, for example, that CRL after ejaculations, sometimes referred to as female post-ejaculatory intervals (PEI), are longer when the same male remains in the mating chamber than when a different male was introduced (Corlett et al., 2022).

Given the constraints of current parameters used to study female sexual behavior, an improved and complementary behavioral assessment tool could be beneficial. Additionally, a more detailed analysis of how females control the timing of sexual encounters beyond a simple withdrawal to another compartment, would do justice to the complexity of this phenomenon. An example can be taken from male rats, for which we recently employed an idea by (Sachs and Barfield, 1970), who demonstrated that male rat copulation is temporally organized in mount bouts. These bouts are defined as sequences "of mounts (one or more), with or without intromission, uninterrupted by any behavior (other than genital autogrooming) that is not oriented towards the female." The breaks between these mount bouts, in which the male performs behaviors that are not related to the female, are defined as "time-outs" (Huijgens et al., 2021a). Using these parameters in an extended behavioral analyses, we have been able to obtain valuable insights into the neural regulation of male rat sexual behavior, and specific roles for different brain regions have been revealed with the medial amygdala being involved in regulating copulatory sensitivity and ejaculatory threshold (Huijgens et al., 2021b) and the bed nucleus of stria terminalis in regulating motivation to continue copulation (Huijgens et al., 2024). A similar assessment tool has not yet been developed for female rats, but could potentially provide valuable information about female sexual behavior as well.

Therefore, in this study we aimed to develop a similar behavioral assessment tool for female rats by investigating the temporal copulatory patterns in more detail. We took advantage of the fact that the sexual activity of females is highly dependent on hormonal status (Snoeren, Chan et al., 2011; Brandling-Bennett, Blasberg, and Clark, 1999; Hliňák, 1986; Dominguez-Ordonez et al., 2015; Edwards and Pfeifle, 1983), which allowed us to compare the temporal patterns of normally receptive females with low receptive females. By using a within-subject design, we were able to follow the progress of each rat over multiple copulation tests and study the effects of sexual experience on their temporal copulatory patterns. Previous research has suggested that sexual experience facilitates sexual performance in female rats (Meerts, Park, and Sekhawat, 2016; Meerts et al., 2014; Meerts, Strnad, and Schairer, 2015; Blaustein et al., 2009), but the effects on their temporal copulatory patterns are still unknown. Additionally, as an extra proof of concept, we tested the rats once more after treatment with (R)-(+)-8-OH-DPAT, a 5-HT_{1A} receptor agonist. This pharmaceutical compound is known to inhibit paracopulatory behavior (Uphouse and Wolf, 2004; Kishitake and Yamanouchi, 2003; Mendelson and Gorzalka, 1986; Snoeren et al., 2010, 2014; Snoeren, Refsgaard et al., 2011), and could therefore provide information about how inhibition of female sexual behavior would be reflected in the temporal copulatory patterns, and thus help us assess the usefulness and interpretation of bout-based assessments. Finally, we added an experiment in which we investigated whether a paced mating condition would still be important in assessing female sexual behavior when using this new assessment tool for studying female sexual behavior.

2. Materials and methods

2.1. Animals

The experiment involved twenty-four sexually naïve adult female and twelve male Wistar rats, each weighing approximately 230 g, purchased from Janvier labs, France. Rats were housed in same-sex pairs in Macrolon IV ® homecages in a room with a reversed 12 h light/dark cycle (lights on between 23:00 and 11:00), controlled temperature (21 ± 1 °C), and humidity (55 ± 10 %). They were provided standard rodent food pellets (low phytoestrogen maintenance diet, #V1554, Ssniff, Germany) and tap water *ad libitum*.

After one week of acclimatization to the animal facility, female rats were ovariectomized under isoflurane anesthesia following the methods described by (Ågmo, 1997). A medial dorsal incision of about 1 cm long was made in the skin. Using blunt dissection, abdominal musculature was exposed just enough to allow a bilateral incision of about 0.5 cm in the dorsolateral abdominal musculature. The ovaries on each side were located and extirpated, while the fallopian tubes were ligated and gently placed back in the posterior abdomen. The incised muscle layer was closed with absorbable sutures (Vicryl 4–0, Ethicon), and the skin was closed using wound clips. The male rats, on the other hand, were sexually trained by allowing them to copulate for 30 minutes on three occasions with receptive females who are not further used or mentioned in this study. The sexually trained rats were then used as stimulus males in this study.

All animal care and experimental procedures employed in this study were conducted in agreement with the European Union Council Directive 2010/63/EU and in accordance with the Norwegian Food Safety Authority with the ethical approval number FOTS ID 30239.

2.2. Experimental groups (models)

The ovariectomized female rats were randomly assigned to a hormonally sub-primed (n = 12) and hormonally fully-primed group (n = 12). The hormonally sub-primed females were administered 5 μg of Estradiol benzoate (EB, Sigma-Aldrich, product Nr: E8875) alone, while hormonally fully-primed females were administered 5 μg of EB and 500 μg of progesterone (P, Sigma-Aldrich, product Nr: P-0130). Both EB and P were dissolved in peanut oil solution at 25 μg/mL and 2.5 mg/mL, respectively. EB and P were administered subcutaneously (0.2 mL) at different time intervals: EB was given 36 hours before the start of each copulation session, while P was administered 4 hours prior to each copulation session throughout all experiments in this study.

The administration of EB alone induces lower receptivity than the administration of the combination of EB and P, which induces full receptivity in female rats (Snoeren, Bovens et al., 2011). According to a previous study (Snoeren, Bovens et al., 2011), sub-primed (EB) females are intended to represent females with low receptivity, potentially modeling female sexual dysfunction, while the fully-primed group (EB+P) is used to model normally receptive females.

2.3. Apparatus

We used the paced and non-paced mating apparatus in this experiment. The paced mating apparatus consists of a rectangular steel box

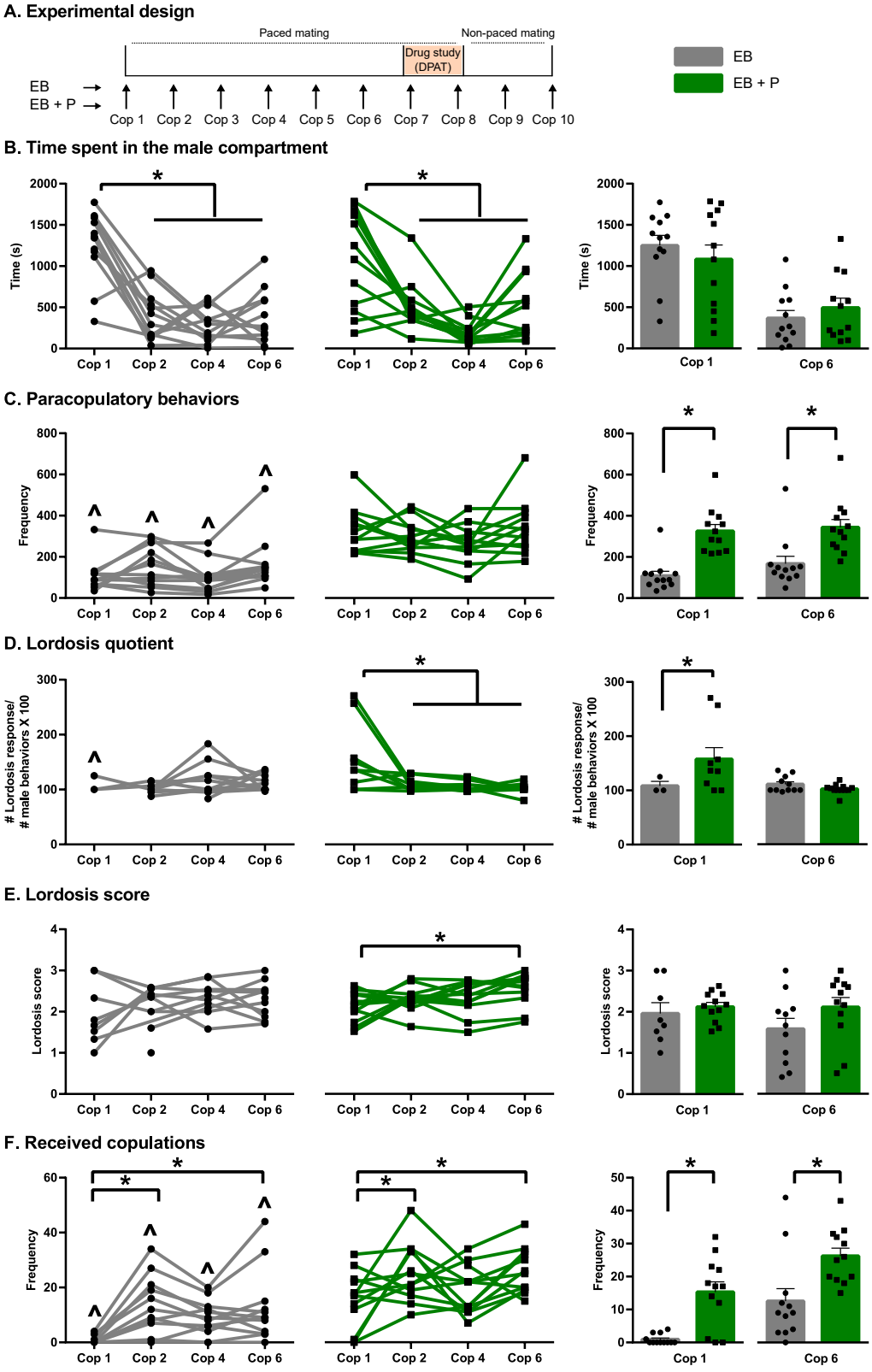

Fig. 1. Sexual experience and hormonal status and female rats copulatory parameters. (A) Schematic illustration of the experimental timeline, (B) Time spent in the male compartment (in seconds), (C) Total number of paracopulatory behaviors, (D) Lordosis quotient, (E) Lordosis score, (F) Total number of copulations (mounts, intromission and ejaculations) received by the females. **Panels to the left (B-F):** The data are shown with individual data points, with the lines connecting each rat across different Cop tests; panels **to the right (B-F)**: The data are shown with individual data points, with the bars representing the mean ± SEM, **All figures (B-F):** Data is shown for EB (n = 12) and EB+P (n = 12) female Wistar rats. Missing data points are caused by the lack of received copulations resulting in that no lordosis quotient could be calculated. *$p < 0.05$ significantly different between Cop tests, ^$p < 0.05$ is significantly different between groups (EB vs EB+P). Cop = Copulation test, EB = Estradiol benzoate, P = progesterone, DPAT = (R)-(+)-8-OH-DPAT.

measuring 40 × 60 x 40 cm with a Plexiglas front and an arena covered with wood chips. The interior space is divided into two compartments by a transparent compartment divider. The divider has three 4 cm diameter holes at the bottom, which serve as an escape route for the females during copulation. As a result, one compartment (40 ×45 x 40 cm) is available for both the male and female, while a smaller compartment (40 ×15 x 40 cm) can only be accessed by the female. For the non-paced mating, we removed the transparent compartment divider in the paced mating apparatus, transforming the apparatus into one compartment (40 ×60 x 40 cm) where rats can copulate without an escape route for the females.

2.4. Behavioral testing

All copulation sessions started at noon and were conducted in a room with a 5 lux dim light. The experimenter was blinded to the experimental group of the subject animals. The female rats were placed in the female compartment and were allowed to habituate in the copulation apparatus for five minutes in which we assured they had crossed between chambers. Next, a stimulus male rat was placed in the male compartment, and they were allowed to copulate for 30 minutes. If during that time the stimulus male achieved an ejaculation, he was replaced with another stimulus male (the two males were circulated within the same copulation session). This was done to enable us to study the female rats' copulatory pattern independent of the male's post-ejaculatory interval. The entire copulation test was recorded to video, and behavioral assessment was carried out by annotating all female sexual behaviors with the use of observer XT version 17 software (Noldus, Wageningen, the Netherlands).

2.5. Experimental setup

2.5.1. Experiment 1

The goal of experiment 1 was to determine the temporal copulatory patterns of female rats and the effect of gaining sexual experience on these female temporal copulatory patterns. Two groups of sexually naïve female rats, EB alone (EB) and EB+P (EB+P) primed, were exposed every 5th day to a copulation test in the paced mating setup for 6 times (Cop 1 to Cop 6) (Fig. 1A). With this setup, we explored the temporal copulatory patterns of the female rats and the effects of sexual experience by comparing the data from naïve (Cop 1) versus sexually experienced (Cop 6) in EB and EB+P primed rats. To obtain the progress of gaining sexual experience, results of a selection of the test was sufficient, and therefore we scored Cop1, Cop2, Cop4 and Cop6. By testing (and hormonally priming) the rats every 5th day, we simulated their natural cycle and assured wash-out of the hormones from the previous test.

2.5.2. Experiment 2

In the second experiment, we tested the hypothesis that (R)-(+)-8-OH-DPAT (Sigma-Aldrich, product Nr: H140–5MG), a 5-HT_{1A} receptor agonist known to inhibit female sexual behavior (Snoeren et al., 2010), could disrupt the temporal copulatory patterns in females. For this purpose, we continued testing the female rats from experiment 1 in experiment 2 (Fig. 1A). The copulation test was performed twice in a within-subject Latin square design (with a 1-week interval, which served as a wash-out period). We administered 0.1 mg/kg of (R)-(+)-8-OH-DPAT or vehicle subcutaneously 10 minutes before the copulation test in a paced mating setup. Two EB rats were excluded from the data analysis, because they had accidently received a higher dose of (R)-(+)-8-OH-DPAT.

2.5.3. Experiment 3

Finally, the same female rats were tested in a non-paced mating setup for 30 minutes. The aim of the last experiment was to compare the temporal copulatory pattern of female rats in paced mating versus non-paced mating. With this, we can infer whether the female's temporal copulatory pattern is dependent on the setup in which she can withdraw from the male and thus actively pace her sexual encounters versus the setup in which she is not able to withdraw from the male during copulation. The same animals from experimental 1 and 2 were also used in experiment 3. Two non-paced mating tests were performed: the first non-paced mating test was done to familiarize the rats with the new concept, while the second test was used for data collection (Fig. 1A).

2.6. Behavioral analysis

We manually scored the following female behaviors: paracopulatory behaviors (hops and darts), female receptive behavior (lordosis behavior on a 4-point scale (Hardy and Debold, 1971)), sniffing (sniffing other body parts of the male), anogenital sniffing (sniffing the anogenital region of the male), genital self-grooming, other behaviors such as female running, head towards the male (head oriented in the direction of the male), and head away from the male (head oriented away from the male). The male behaviors that were annotated included mounts, intromissions, and ejaculations. In addition, we scored the occasions in which the female crossed over between compartments, so that we could calculate pacing behavior measures such as: the percentage of exits following copulations (mounts, intromissions, and ejaculations), contact return latencies following exits, and the total time spent in each compartment. Moreover, we calculated the lordosis quotient by dividing the total amount of lordosis responses by the number of received copulations times 100 %. Since female rats that are highly receptive can also display a lordosis upon tactile stimulation, this percentage can be higher than 100 % (reviewed in (Heijkoop, Huijgens, and Snoeren, 2018)).

Furthermore, using a similar approach as previously done for male rats (Sachs and Barfield, 1970; Huijgens et al., 2021a), we divided the female rats' temporal copulatory patterns into sexual bouts and time-outs. A female sexual bout was defined as a series of behaviors that begins and ends with either a paracopulatory behavior or lordosis response. All subsequent behaviors oriented towards the male (such as sniffing, anogenital snigging, genital self-grooming, head towards the male) were part of the same bout, until a behavior was displayed that was not oriented towards the male (such as attention away from the male, or rejection behaviors). Then the former last paracopulatory or lordosis behavior marked the end of a sexual bout, and a time-out was started and lasted until the next paracopulatory behavior or lordosis response (Fig. 2A). A self-written Python script was used to analyze all sexual behaviors and identify the female sexual bouts. These behavioral outcome measures are listed in Table 1.

2.7. Statistical analysis

Statistical analyses were conducted using SPSS software (version 29, IBM, Armonk, USA) with a level for statistically significant difference set at $p < 0.05$. A Shapiro-Wilk test confirmed that the data was not normally distributed, and therefore a linear mixed model was used that comprised of the factors (hormonal) treatment and experience for the sexual experience study (study 1), treatment and drug for the (R)-(+)-8-OH-DPAT study (study 2), and treatment and pacing for the paced and non-paced mating study (study 3). In the case of a significant interaction effect, a Bonferroni-corrected post hoc test was conducted. When a correlation test was used to analyze the relationship between the duration of sexual bouts and time-outs and other parameters, the Spearman correlation test was conducted.

Finally, in order to investigate what determines the duration of sexual bouts and time-outs, data points were z-scored within each rat using the following calculation: z-score = ((data point) - (mean of the data points for the rat)) / (standard deviation of the data points for the rat). Z-scores were then analyzed by means of the Kruskal-Wallis test, followed by Mann-Whitney U post hoc tests.

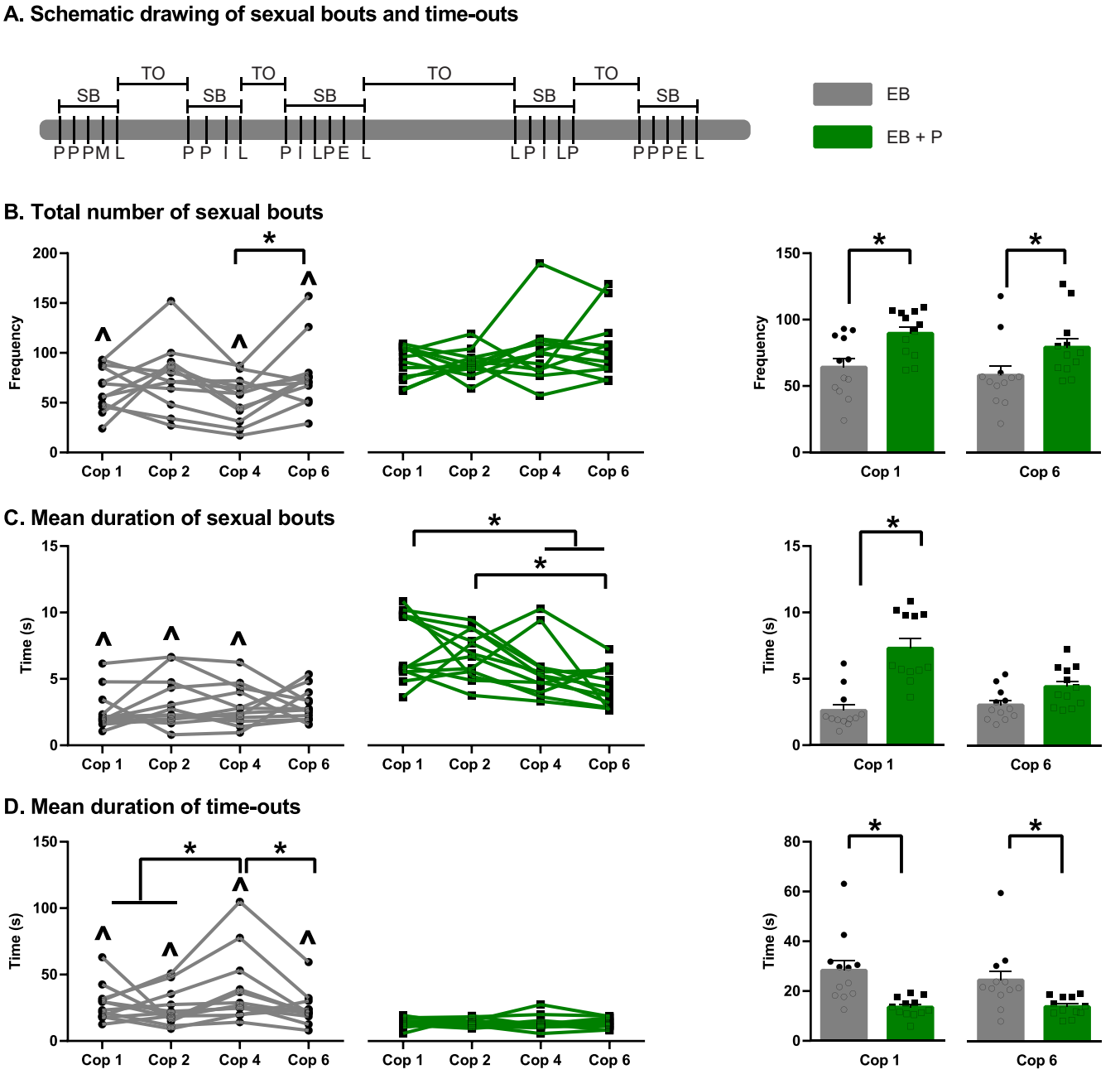

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Fig. 2. Female temporal copulatory patterns are organized into sexual bouts and time-outs. (A) Schematic representation of a hypothetical female rat temporal copulatory patterns divided into sexual bouts (SB) and time-outs (TO) with paracopulatory behavior (P) and lordosis (L) marking the start and the end of sexual bouts. M = mount, I = intromission, E = ejaculation, (B) Total number of sexual bouts. (C) Mean duration of sexual bouts (in seconds). (D) Mean duration of time-outs (in seconds). (E) Boxplot of z-scores of individual sexual bouts duration divided into groups based on the numbers of paracopulatory behaviors within the bout, (F) Boxplot of z-scores of individual sexual bouts duration divided into groups based on the received copulations within the bout. (G) Boxplot of z-scores of individual time-out duration divided into groups based on the numbers of paracopulatory behaviors within the preceding bout, (H) Boxplot of z-scores of individual time-out duration divided into groups based on the received copulations within the preceding bout. **Panels to the left (B-D):** The data are shown with individual data points, with the lines connecting each rat across different Cop tests; panels **to the right (B-D):** The data are shown with individual data points, with the bars representing the mean ± SEM, **Figures B-D:** Data is shown for EB (n = 12) and EB+P (n = 12) female Wistar rats. $*p < 0.05$ significantly different between tests, $\hat{}p < 0.05$ is significantly different between groups (EB vs EB+P). **Figures E-H:** $*p < 0.05$ significantly different between sexual bout groups. Cop = Copulation test. P = paracopulatory behavior, M=mount, I=intromission, E = ejaculation.

Table 1
Definitions of behavioral parameters used in the experiment.

Behavioral outcome	Definition
Paracopulatory behavior	Darts and hops in both male and female compartments (note: ear wiggles were not included).
Paracopulatory behaviors in the female compartment	Darts and hops in the female compartment only.
Lordosis responses	Postural reflex of the female with dorsiflexion of the vertebral column.
Lordosis quotient	The number of lordosis responses divided by the number of received copulations (mounts, intromissions, ejaculations) multiplied by 100 %.
Lordosis score	Lordosis responses were assessed on a 4-point scale (0 – 3), with 0 as no lordosis and 3 as a full lordosis with a hollow back and lifted head of 45° or more. From this, we calculate the lordosis score.as the number of lordosis responses with score 1 times 1 + number of lordosis responses with score 2 times 2 + number of lordosis responses with score 3 times 3, divided by total number of received copulations. (Hardy and Debold, 1971).
Mounts	Received mounts.
Intromissions	Received intromissions.
Ejaculations	Received ejaculations.
Received copulations	Sum of received mounts, intromissions, and ejaculations.
Time spent in the male compartment	Total time spent in the male compartment.
Percentage of exits after mount, intromission, and ejaculations	The total number of withdrawals to the female compartment after received mount, intromission or ejaculation within 5 seconds, divided by the total number of received mounts, intromissions or ejaculations, multiplied by 100 %.
Contact return latency (CRL) after mount, intromission, and ejaculation	The mean duration for the females to re-enter the male compartment after she withdraw upon a mount, intromission, or ejaculation.
Sexual bouts	The total number of sexual bouts defined as a series of behaviors that begins with either a paracopulatory behavior or lordosis response and continues until a behavior was displayed that was not oriented towards the male. The last paracopulatory or lordosis behavior marked the end of a sexual bout, and the start of a time-out consisting of behaviors that are not oriented at the male.
Mean duration sexual bouts	Mean duration of all sexual bouts, calculated from the first paracopulatory or lordosis behavior in a sexual bout until the last behavior within the sexual bout.
Mean duration time-outs	Mean duration of all time-outs, defining a time-out as the time from the end of a sexual bout to the start of the next sexual bout.
Paracopulatory behavior in a sexual bout	Mean number of paracopulatory behaviors within a sexual bout that contains at least more than 2 paracopulatory or lordosis behaviors.
Lordosis behavior in a sexual bout	Mean number of lordosis behaviors within a sexual bout that contains at least more than 2 paracopulatory or lordosis behaviors.
Copulations in a sexual bout	Mean number of mounts and intromissions within a sexual bout that contains at least more than 2 paracopulatory behaviors or lordoses.

3. Experiment 1

In experiment 1, we attempted to develop an improved behavioral assessment tool by studying the female rats' temporal copulatory patterns in detail. In addition, we investigated the effects of sexual experience on these temporal copulatory patterns. By using EB and EB+P primed females, the effect of low versus normal receptivity, respectively, could be assessed.

To make this result section more readable, we moved all statistical outcomes on the hormonal treatment, experience, and/or interaction effects into a separate table (Table 1) and report only on the significant relevant results in this paper.

3.1. Results traditional parameters

When looking at the more traditional parameters first, our data revealed that EB-primed females spent a similar amount of time in the male compartment as EB+P-primed females, and that this time decreases after a single sexual experience (Fig. 1B). Additionally, EB females exhibited fewer paracopulatory behaviors compared to EB+P females across all copulation (Cop) tests (Fig. 1C), including when only paracopulatory behaviors performed in the female compartment were considered (Suppl. Figure S1). Furthermore, EB females demonstrated a longer latency to their first paracopulatory behavior (measured from the male's introduction) than EB+P females, particularly in the first copulation (Cop 1) test (Table 2). Although sexual experience did not alter the overall number of paracopulatory behaviors, it did increase the number of darts displayed in the female compartment for both EB and EB+P females. Additionally, sexual experience reduced the latency to the first paracopulatory behavior in EB females, bringing it in line with EB+P females.

Regarding lordosis behavior, we found that sexually naïve (Cop 1) EB+P females had a significantly higher lordosis quotient (LQ, calculated as number of lordosis responses divided by number of received copulations times 100 %) than EB females (Fig. 1D). This difference, however, disappeared after the females gained sexual experience. The lordosis score (LS, intensity of the lordosis response), on the other hand, seemed to increase in Cop 6 compared to Cop 1 in EB+P females, but no differences were found between EB and fully primed females (Fig. 1E). Similar results would also be found when the lordosis responses upon

Table 2
Effects of hormonal priming and sexual experience on female sexual behavior.

Parameters		Cop 1	Cop 2	Cop 4	Cop 6
Latency to 1st paracopulatory	EB	67.98 ± 6.26 *[a]	18.01 ± 6.26[b]	14.57 ± 6.26[b]	4.34 ± 6.26[b]
	EB+P	20.52 ± 6.26	6.07 ± 6.26	5.90 ± 6.26	2.12 ± 6.26
Mounts	EB	0.67 ± 1.17 *	2.75 ± 1.17 *	1.92 ± 1.17	2.75 ± 1.17 *
	EB+P	5.42 ± 1.17[ab]	6.08 ± 1.17[ab]	3.50 ± 1.17[a]	7.92 ± 1.17[b]
Intromissions	EB	0.25 ± 1.85 *[a]	8.75 ± 1.85 *[b]	6.00 ± 1.85 *[b]	8.42 ± 1.85 *[b]
	EB+P	9.33 ± 1.85[a]	16.92 ± 1.85[b]	12.83 ± 1.85[ab]	16.08 ± 1.85[b]
Ejaculation	EB	0.00 ± 0.00[a]	1.33 ± 0.35[b]	1.00 ± 0.35 *[ab]	1.42 ± 0.35[b]
	EB+P	0.58 ± 0.35[a]	2.17 ± 0.35[b]	2.50 ± 0.53[b]	2.25 ± 0.35[b]
Latency to 1st ejaculation	EB	1800 ± 0.00 *[a]	1023.90 ± 162.90 *[b]	1232.65 ± 162.90 *[b]	1093.53 ± 162.90 *[b]
	EB+P	1326.78 ± 162.90[a]	495.23 ± 162.90[b]	380.55 ± 162.90[b]	506.00 ± 162.90[b]
Percentage of exits after mounts	EB	18.57 ± 15.19	36.86 ± 10.04	24.14 ± 10.49	22.33 ± 10.04
	EB+P	1.59 ± 9.47	15.61 ± 8.95	18.06 ± 8.95	11.67 ± 8.95
Percentage of exits after intromissions	EB	12.94 ± 25.98[ab]	17.26 ± 9.93[a]	60.48 ± 9.96[b]	61.23 ± 9.62[b]
	EB+P	2.07 ± 10.20[a]	24.51 ± 9.32[c]	49.31 ± 9.32[bd]	44.22 ± 9.32[b]
Percentage of exits after ejaculations	EB	-	47.13 ± 17.11	66.67 ± 18.48	83.59 ± 17.11
	EB+P	33.36 ± 18.48	26.39 ± 13.08	48.61 ± 13.07	53.47 ± 13.07
Contact return latency after mounts	EB	8.93 ± 92.59	27.39 ± 46.93	114.05 ± 54.12	83.14 ± 53.81
	EB+P	33.56 ± 64.77	113.93 ± 41.81	81.97 ± 46.63	85.72 ± 46.56
Contact return latency after intromissions	EB	-	56.74 ± 21.64	106.62 ± 16.79	79.35 ± 17.69
	EB+P	70.44 ± 37.34	86.76 ± 21.64	108.27 ± 17.69	56.12 ± 16.79
Contact return latency after ejaculations	EB	-	66.33 ± 54.28 *[a]	323.65 ± 54.28[b]	173.97 ± 48.57[ab]
	EB+P	111.63 ± 76.74	234.99 ± 54.26	269.98 ± 41.04	209.94 ± 41.04
Paracopulatory per sexual bout	EB	2.66 ± 0.33 *	3.15 ± 0.34 *	2.75 ± 0.34 *	3.29 ± 0.33 *
	EB+P	5.29 ± 0.33[a]	4.70 ± 0.33[ab]	3.92 ± 0.33[b]	5.22 ± 0.33[ac]
Lordoses per sexual bout	EB	0.03 ± 0.05 *[a]	0.20 ± 0.05[ab]	0.25 ± 0.05[b]	0.22 ± 0.05 *[b]
	EB+P	0.25 ± 0.05	0.32 ± 0.05	0.28 ± 0.05	0.40 ± 0.05
Copulations per sexual bout	EB	0.03 ± 0.05 *[a]	0.22 ± 0.06[ab]	0.27 ± 0.06[b]	0.25 ± 0.05 *[b]
	EB+P	0.26 ± 0.05	0.35 ± 0.05	0.31 ± 0.05	0.44 ± 0.05

Note: Data shown as mean ± SEM.
*$p < 0.05$ different with EB+P females rats of the same Cop test.
[ab] values within a row with unlike letters significantly differed between Cop tests. $p < 0.05$. Cop = copulation test.

only mounts, intromissions and ejaculations, were calculated (data not shown). It should be mentioned, though, that two rats (1 from the EB and 1 from the EB+P group) were removed from the data analysis regarding (only) lordosis behavior. This was done because they displayed an extraordinary number of lordoses in Cop 1 with receiving only 1 copulation and were therefore considered statistical outliers. The removal did not change the final conclusions of the study.

When exploring the number of received copulations, we found that EB+P rats received significantly more copulations compared to EB rats in all Cop tests (Fig. 1F). Both EB and EB+P rats showed an increase in received copulations in the 2nd and 6th Cop tests compared to Cop 1, driven primarily by the number of received intromissions, as the number of received mounts did not vary with experience (Table 2). Interestingly, although the stimulus male rats were sexually experienced from the start of the experiment, both EB and EB+P females received significantly fewer ejaculations in the sexually naïve versus experienced state. This suggests that female behavior may play a role in regulating male behavior. Furthermore, when comparing the latency to 1st ejaculation calculated from the 1st paracopulatory behavior, we observed that EB+P- primed females reached their 1st ejaculation more quickly than EB rats in all Cop tests (Table 2). Additionally, sexual experience reduced the latency to 1st ejaculation in both EB and EB+P females, with sexually experienced rats achieving ejaculation faster than their sexually naïve counterparts. Still, no differences in number of received ejaculations were found between EB and EB+P females (Table 2).

Additionally, we investigated the effects of hormonal status and sexual experience on traditional paced-mating behaviors such as the percentage of exits and contact-return latencies. No differences were found between EB and EB+P females with regard to percentages of exits after mounts, intromissions, or ejaculations (Table 2), nor was there an effect of sexual experience on the percentage of exits after mounts or ejaculations. The level of sexual experience did increase the percentage of exits after intromissions, but post-hoc analysis revealed that this effect was only found in EB+P rats. With the generally low percentages of exits, the number of data points to determine the contact-return latencies (CRL) became rather small, and no effect of hormonal status or experience was found on CRL after mounts, intromissions, or ejaculations (Table 2).

3.2. Results sexual bouts and time-outs

With the original scope of this paper of determining whether females (just like males) copulate in so-called sexual bouts, we next examined the female rat's copulatory patterns into sexual bouts and time-outs. As a reminder, paracopulatory or lordosis behaviors marked the start of a sexual bout, and all subsequent behaviors oriented towards the male are part of the same bout. As soon as a behavior was displayed that was not oriented towards the male, the former last paracopulatory or lordosis behavior marked the end of a sexual bout and the start of the time-out (Fig. 2A).

We found that EB+P females copulated with more sexual bouts than EB females in all Cop test (except Cop 2, Fig. 2B), a phenomenon that was seen both with and without counting single, isolated paracopulatory behaviors as a sexual bout. Additionally, when the mean duration of these sexual bouts was calculated, we found that in all Cop tests (except Cop 6), EB+P rats also spent on average more time in a sexual bout than EB rats (Fig. 2C). While the number of sexual bouts was also found to remain stable from sexually naïve to experienced states, the mean duration of a sexual bout declined upon gaining sexual experience in EB+P rats. No effect of experience, however, was found in EB rats. Similar differences were found in the mean duration of the time-outs between the sexual bouts. EB+P females had shorter time-outs in all Cop tests compared to EB rats (Fig. 2D). In addition, the mean duration of time-outs did not change upon gaining sexual experience and remained stable over the course of the copulation tests in EB and EB+P rats (except for an unexplainable increase in time-out duration in Cop 4 in EB rats).

Analysis of the sexual bouts in a more detailed manner revealed that sexual bouts of EB+P females consisted of more paracopulatory behaviors (considering only the sexual bouts that contained two or more

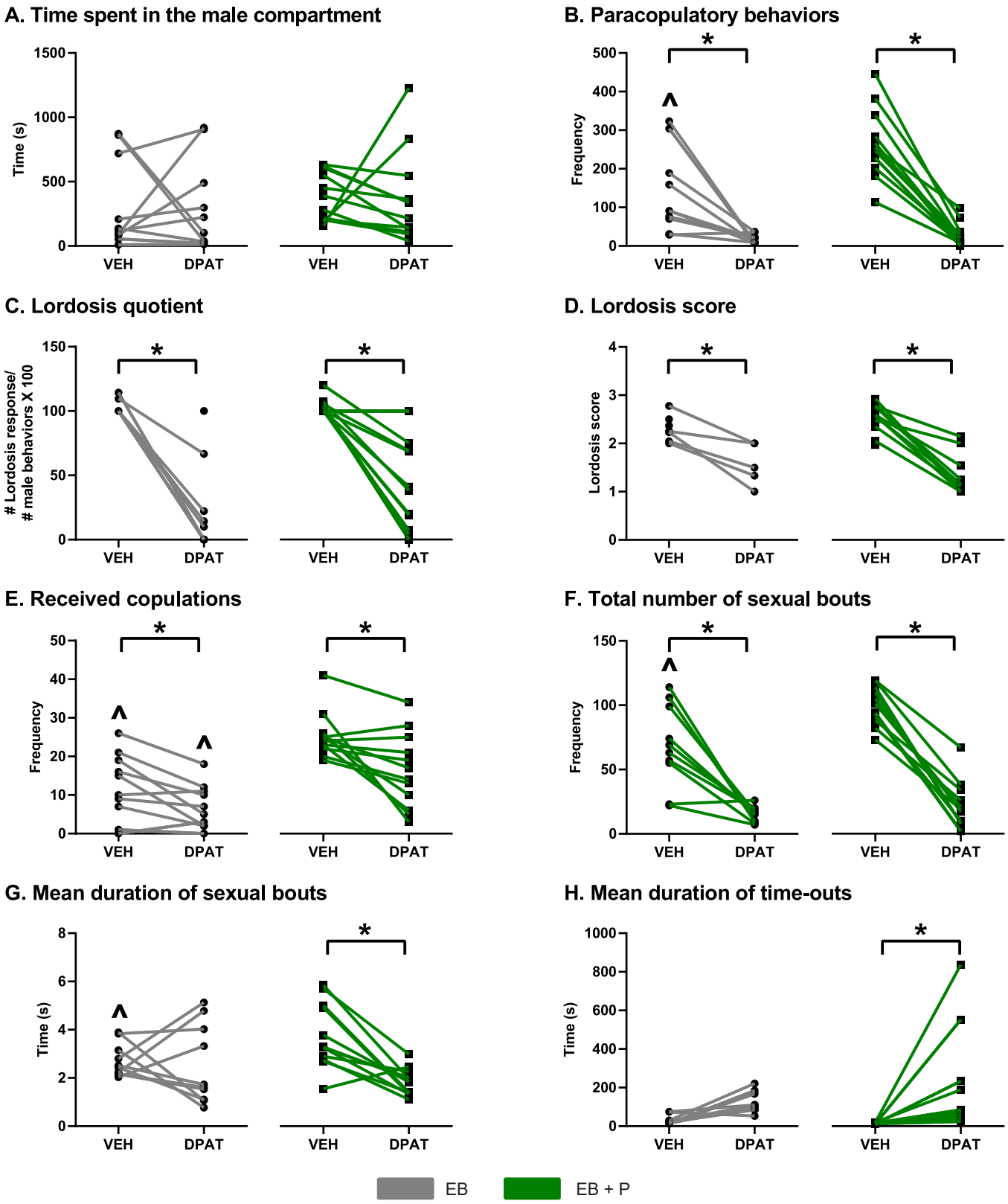

Fig. 3. Effects of 0.1 mg/kg of (R)-(+)-8-OH-DPAT on female sexual behavior. (A) Time spent in the male compartment (in seconds), (B) Total number of paracopulatory behaviors, (C) Lordosis quotient, (D) Lordosis score, (E) Total number of received copulations, (F) Total number of sexual bouts, (G) Mean duration of sexual bouts (in seconds), (H) Mean duration of time outs (in seconds). **All panels:** Data is shown for EB (n = 10) and EB+P (n = 12) female Wistar rats with individual data points, with the lines connecting each rat for both treatments. Missing data points are caused by the lack of received copulations resulting in that no lordosis quotient could be calculated. *$p < 0.05$ significantly different between tests, ^$p < 0.05$ is significantly different between groups (EB vs EB+P). VEH = vehicle, DPAT = (R)-(+)-8-OH-DPAT.

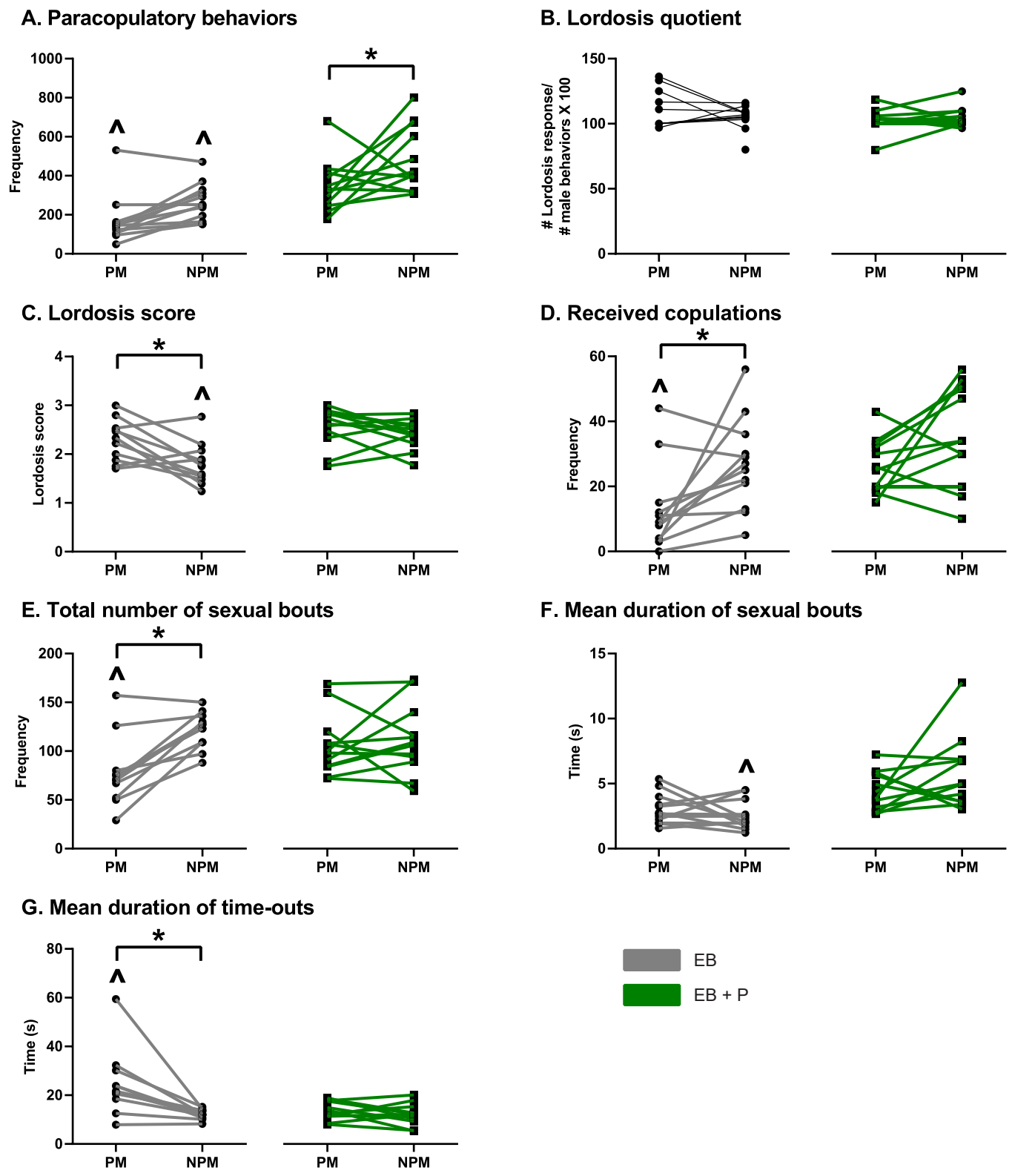

Fig. 4. Female sexual behavior in a paced and non-paced mating set-up. (A) Total number of paracopulatory behavior, (B) Lordosis quotient, (C) Lordosis score, (D) Total number of received copulations, (E) Total number of sexual bouts, (F) Mean duration of sexual bouts (in seconds), (G) Mean duration of time outs (in seconds). **All panels:** Data is shown for EB (n = 12) and EB+P (n = 12) female Wistar rats with individual data points, with the lines connecting each rat for both Cop tests in different set-up. Missing data points are caused by the lack of received copulations resulting in that no lordosis quotient could be calculated. *$p < 0.05$ is significantly different between tests, ^$p < 0.05$ is significantly different between groups (EB vs EB+P). PM = paced mating, NPM = non-paced mating.

paracopulatory or lordosis behaviors) than EB rats in all Cop tests, and more lordosis responses in Cop 1 and Cop 6 (Table 2). While the number of paracopulatory behaviors per sexual bout generally remained stable over the course of the copulation tests, a slight increase in the mean number of lordoses responses per sexual bout was found after obtaining sexual experience in EB, but not EB+P rats (Table 2). Since lordosis is most often a stereotypical response upon a received copulatory stimulation from the male, it is then not surprising that the mean number of copulation behaviors within these sexual bouts follows a similar pattern as the lordosis responses (Table 2).

To understand the newly proposed behavioral analysis of sexual bouts and time-outs better, we investigated what determines the length of these bouts and time-outs. Therefore, we z-scored the durations of each bout and time-out per rat and divided the z-scored bouts and time-outs into groups based on the behaviors that were included in the related sexual bout. As such, to determine the influence of the number of paracopulatory behaviors on the duration of the sexual bouts (without taking into account the copulatory behaviors), the data was divided into four groups: bouts containing 1–5, 6–10, 11–20, and more than 20 paracopulatory behaviors. We found that increasing numbers of paracopulatory behaviors per bout did increase the sexual bouts duration (Fig. 2E). Interestingly, when we evaluated the role of different kinds of received copulations during the sexual bouts, we found that the sexual bout durations increased when more intromissions and ejaculations were received, in comparison to only mounts or just 1 intromission or ejaculation (Fig. 2F). It is thus the increased number of copulation within the sexual bout, rather than the type of stimulation that lengthens the sexual bout. Since these copulations often occur with more paracopulatory behaviors, this suggests that the length of a sexual bout is most likely determined by the number of paracopulatory behaviors.

Regarding what determines the length of the time-outs, we found that the duration of time-outs is negatively correlated, but only weakly, with the duration of sexual bouts (data not shown), meaning that longer sexual bouts result in shorter time-outs. Since longer sexual bouts generally contain more paracopulatory behaviors, we next determined the influence of the number of paracopulatory behaviors on the duration of the time-outs (not taking into account the copulatory behaviors). We found that increased numbers of paracopulatory behaviors per bout did not affect the length of the time-outs (Fig. 2G). Interestingly, when we evaluated the role of different kinds of received copulations during the sexual bouts, we found that already 1 intromission in the sexual bout lengthens the time-out that follows compared to when the bout consisted of only mounts (Fig. 2H). When an intromission was accompanied by mounts or more intromissions, even longer time-outs were seen compared to bouts with only 1 mount, but these time-outs were shorter than time-outs following sexual bouts with 1 intromission. The longest time-outs were found when an ejaculation was received (independent of the co-occurrence of more mounts and/or intromissions within the same sexual bout). Overall, this suggests that while the length of the sexual bout is most likely determined by the number of paracopulatory behaviors, the time-outs are mostly determined by the type of received stimulation and lengthened as soon as an ejaculation was included in the sexual bout.

3.3. Discussion Experiment 1

In summary, our findings support our hypothesis that female rats copulate in temporal copulatory patterns organized into sexual bouts and time-outs. The copulatory patterns are dependent on the hormonal status of the rats, as EB+P rats copulated with more and longer sexual bouts and shorter time-outs than lower receptive EB females. Sexual experience, on the other hand, did affect the temporal copulatory patterns by shortening the mean duration of the sexual bouts in EB+P experienced females (to the level of EB females), without changing the total number of sexual bouts and time-out durations. As expected, EB+P females performed more paracopulatory behaviors and received more male copulations than EB rats. While EB+P females had a higher LQ than EB rats in Cop 1, but not in other copulation tests, no differences were found on LS or pacing behaviors such as exits and CRL after received copulations. Besides a decrease in the lordosis quotient and a small increased the lordosis score in EB+P rats, sexual experience did not affect other female sexual performance parameters in EB and EB+P rats. Sexual experience did only result in receiving more copulations from the second copulation test compared to the 1st in both EB and EB+P rats.

In our study, EB rats were hormonally primed with a low dose of estradiol benzoate (EB) alone, while EB+P females were primed with estradiol benzoate and progesterone (EB + P). Although EB alone is known to induce hormonal receptivity in female rats, P has been shown to facilitate the effect of EB on e.g. paracopulatory, approach, and lordosis behaviors in female rats (Snoeren, Chan et al., 2011; Brandling-Bennett, Blasberg, and Clark, 1999; Hliňák, 1986; Dominguez-Ordonez et al., 2015; Edwards and Pfeifle, 1983). Our findings concerning the difference between EB and EB+P females on the traditional parameters of paracopulatory behaviors and lordosis quotient and score are therefore in line with these previous studies. Interestingly, though, the difference in LQ between EB and EB+P females disappeared after gaining experience and the LQ of EB females remained around 100 % despite the lack of progesterone. As discussed before (Heijkoop, Huijgens, and Snoeren, 2018), this suggests that lordosis is not solely dependent on progesterone and that the lordosis behavior is maybe more than just a reflex. This is also why we have chosen to include the lordosis responses performed upon any stimulation, not solely upon received stimulations. It should be noted, though, that a LQ (or LS) can only be calculated when at least 1copulation was received. As shown in Fig. 1F, not all EB females received copulations and are thus not represented in the LQ (or LS) analysis, which would not have been different when we reported the LQ or LS upon received stimulations only.

Furthermore, we did not find any effect of hormonal priming on different traditional pacing parameters such as percentages of exits and contact-return latencies (CRL). These findings are not consistent with previous studies on paced mating behavior in female rats that showed that progesterone priming improves CRL compared to EB priming alone (Coopersmith, Candurra, and Erskine, 1996; Erskine, 1985, 1992; Brandling-Bennett, Blasberg, and Clark, 1999; Chan et al., 2011). The reason for the discrepancy between our study and previous studies could be the difference in test strategy. While others allow the rats to acclimatize in the test cages several times before the first test, we only habituated the rats for 5 minutes before the start of the test. Though, as we observed many crossing throughout COP 1 in both EB and EB+P females (EB: 18.8 ± 5.1; EB+P: 14.9 ± 5.1), we doubt this underlies the different findings. Another reason, however, could also be the cut-off point of 5 seconds that we used to mark an exit, in contrast to an open ending in other studies. This means an escape is considered as an exit only if the female runs from the male to the female compartment within 5 seconds of receiving a stimulation. We choose this cut-off to assure the escape was causal response to the received stimulation. In most cases in our study, the females did not exit after receiving stimulations, but rather continued copulating or took a pause in the male compartment, and this resulted in fewer exits and, thus, fewer data points for contact-return latency calculations. Because females do not always exit immediately after copulatory stimulations, and therefore a causal relationship between stimulation and escape can't be proven, we have argued that percentage exits and contact return latencies do not fully reflect females' motivation to continue copulating (reviewed in Heijkoop et al., 2018).

With this study, we hoped to develop a complementary improved assessment tool to study sexual motivation and copulatory rate, and therefore introduced the sexual bouts and time-outs. The observation that female rats copulate in temporal copulatory patterns, structured into sexual bouts and time-outs, is consistent with the copulatory

patterns found in male rats (Huijgens et al., 2021a). As can be expected, rats with higher levels of receptivity, such as our EB+P females, consistently copulated with more sexual bouts and shorter time-outs compared to low receptive females. The question that then arises is what do these bouts and time-outs exactly represent? The time-outs are probably the easiest to explain, as they could be considered a measure of motivation to continue copulation. Low receptive female rats are thought to have lower levels of motivation, which is also reflected in our data by having longer time-out durations. Similarly, the fact that the duration of time-outs is related to the type of stimulation the female received in the preceding bout, with an ejaculation lengthening the time-out duration, reflects the concept that higher intensities of stimulations require longer pauses before continuing copulation.

Sexual bouts, on the other hand, are more dependent on the females' paracopulatory behaviors, and could rather reflect the females' copulatory speed. A traditional and comparable parameter in male rats, copulatory rate, is often interpreted as copulatory speed. We previously argued how mount bouts are a better method to describe the copulatory speed in males than copulatory rate (reviewed in (Heijkoop, Huijgens, and Snoeren, 2018)), strengthening the concept of sexual bouts as reflection of the females' copulatory speed. Our data shows that sexually experienced females with low receptivity (EB) copulate with fewer, instead of shorter bouts, compared to normal receptive females in a sexual experienced state. This suggests that the number of sexual bouts, rather than the duration of sexual bouts, reflect this copulatory speed. Although it is hard to define what sexual 'efficiency' means for a female rat, the fact that EB+P females, in contrast to EB females, copulate with more sexual bouts and shorter time-outs, led us to conclude that these copulatory patterns should be considered optimal pacing strategy of female rats. More research would be needed to determine if it is an improvement (e.g. how this aligns with previous findings showing that pacing behavior is important for reproductive success (Coopersmith and Erskine, 1994; Erskine, 1989)).

Finally, our data revealed that sexual experience does not strongly affect female sexual behavior, but rather increases the received copulations. Previous research has suggested that sexual experience facilitates sexual performance in female rats (Meerts, Park, and Sekhawat, 2016; Meerts et al., 2014; Meerts, Strnad, and Schairer, 2015; Blaustein et al., 2009). The reported effects are mainly visible in measures like increased number of received copulations and faster contact-return latencies, but also increased paracopulatory behaviors. Based on this, we hypothesized that sexual experience would modify female rats' temporal copulatory patterning by reducing time-out duration. Interestingly, although we did not confirm the effects of experience on contact-return latencies or number of paracopulatory behaviors, we observed that sexual experience, in normally functioning EB+P rats, did reduce the sexual bout durations without affecting the number of sexual bouts or time-out durations. In combination with the observation that the number of paracopulatory behaviors remained stable, this implies that the paracopulatory behaviors are displayed in a shorter period when performed. As such, we found that EB+P females exhibited significantly more sexual bouts consisting of a single paracopulatory behavior in Cop 6 compared to Cop 1 and Cop 2 (data not shown). Our findings suggest that sexual experience does modify the temporal copulatory patterning of female rats, but the effects are rather small.

4. Experiment 2

In the second experiment, we used (R)-(+)-8-OH-DPAT (DPAT), a serotonin-1A receptor agonist known to inhibit female sexual behavior (Uphouse and Wolf, 2004; Kishitake and Yamanouchi, 2003; Mendelson and Gorzalka, 1986; Snoeren et al., 2010, 2014; Snoeren, Refsgaard et al., 2011), to study the effects on female temporal copulatory patterns in both EB and EB+P rats. This allowed us to assess the usefulness of the new behavioral assessment tool and help us interpret the sexual bouts and time-outs.

4.1. Results

First, we confirmed our previously reported finding regarding the differences between EB and EB+P females in the vehicle (VEH) treated rats. The details of these results can be found in Suppl. Table 2 . Here we will continue focusing on the differences in effects of (R)-(+)-8-OH-DPAT (DPAT) treatment.

Our data showed that (R)-(+)-8-OH-DPAT did not affect the time spent in the male compartment of EB and EB+P females (Fig. 3A). However, (R)-(+)-8-OH-DPAT significantly reduced the number of paracopulatory behaviors in both hormone groups compared to vehicle treatment (Fig. 3B). Additionally, (R)-(+)-8-OH-DPAT administration led to a decrease in both the lordosis quotient (LQ) and lordosis score (LS) in EB and EB+P females, relative to vehicle treatment (Fig. 3C/D). Moreover, (R)-(+)-8-OH-DPAT treatment resulted in a reduction in the number of copulatory events, including mounts, intromissions, and ejaculations (Fig. 3E, Suppl. Table 2).

When looking at the traditional pacing parameters, we found that (R)-(+)-8-OH-DPAT increased the percentage of exits after mounts in EB, but not EB+P females (Suppl. Table 3). No differences in percentage of exits after intromissions or ejaculations were found between vehicle and (R)-(+)-8-OH-DPAT in either EB or EB+P females (Suppl. Table 3). (R)-(+)-8-OH-DPAT did not affect contact return latencies after intromissions and ejaculations, but did increase the CRL after mount in fully-, but not EB rats (Suppl. Table 2).

Next, we investigated whether (R)-(+)-8-OH-DPAT treatment affects the temporal copulatory patterns divided in sexual bouts and time-outs. As expected, our data revealed that (R)-(+)-8-OH-DPAT administration caused a reduction in number of sexual bouts in both EB and EB+P females compared to vehicle (Fig. 3F). Additionally, (R)-(+)-8-OH-DPAT shortened the duration of sexual bouts in EB+P females, but not in EB rats (Fig. 3G). Simultaneously, (R)-(+)-8-OH-DPAT treatment led to a significant increase in the mean duration of time-outs in EB+P, but not EB, rats (Fig. 3H).

4.2. Discussion experiment 2

In summary, the results of this 2nd study showed that the temporal copulatory patterns can indeed be manipulated by a sexual inhibiting drug treatment. While (R)-(+)8-OH-DPAT reduced the number of sexual bouts in both EB and EB+P females, it only shortened the duration of these bouts and increased the subsequent time-out durations in only EB+P rats. Furthermore, (R)-(+)-8-OH-DPAT had an inhibiting effect on the number of paracopulatory behaviors, LQ, and LS in both EB and EB+P females without clearly affecting traditional pacing parameters.

These results suggest that the serotonin 1 A receptor agonist inhibits female sexual behavior by impacting both sexual motivation (as reflected in the duration of time-outs) and copulatory speed (as reflected in the number and duration of sexual bouts). Our findings are consistent with previous studies, which have also shown that (R)-(+)-8-OH-DPAT inhibits paracopulatory and lordosis behaviors in EB and EB+P rats (Snoeren et al., 2010; Snoeren, Refsgaard et al., 2011; Uphouse, Caldarola-Pastuszka, and Montanez, 1992; Ahlenius, Larsson, and Fernandez-Guasti, 1989; Kishitake and Yamanouchi, 2003; Olivier et al., 2011). The lack of significant changes in traditional pacing parameters, such as exits and contact-return latency, is unsurprising given the limited number of data points caused by the use of a 5 second cut-off. Although some effects on pacing were observed, such as an increase in exits after mounts in EB females and a change in contact-return latency in EB+P females, these findings were not consistent or corroborated by other metrics.

Overall, this reinforces our view that traditional pacing parameters, such as percentage of exits and contact-return latencies, may be less useful for assessing pacing behavior in female rats if a cut-off is used to assure a causal relationship between received stimulation and exit (and thus CRL). In contrast, our new behavioral paradigm, which divides

copulatory behavior into sexual bouts and time-outs, offers a more functional explanation of female sexual performance. Pacing is the principle of timing sexual interactions. In a traditional small testing environment, this becomes difficult for a female and therefore the presence of a second chamber helps her to pace her sexual interactions by withdrawal to the other compartment where the male can't chase her. Percentages of exits and contact-return latencies are therefore in theory an excellent measure to study the female's pacing behavior in such test conditions. However, as we have more clearly outlined in our introduction, these parameters come with constraints as they might depend on whether or not the female immediately withdraws after a sexual stimulation. Our proposed improved behavioral analysis, on the other hand, studies the same principle of pacing behavior, but is now independent on whether or not the female makes use of the second compartment. No decision needs to be made on whether to use a cut-off for qualifying an exit (leading to a CRL), and simply the behavior (her focus on something else than the male) determines a start of a time-out. A time-out now includes both occasions: the situations in which the female escapes to another chamber and the situations in which she is fine with taking a break near the male. Both situations are considered pacing, as long as she can determine the start of a new sexual bout.

Notably, EB+P rats were more affected by (R)-(+)-8-OH-DPAT administration than EB rats, which may be due to differences in underlying mechanisms or a ceiling effects, given the already low performance of EB females. While research is needed to explore the potential biological underpinnings of these differences, the ability to manipulate new behavioral parameters such as sexual bouts and time-outs, and also obtain subtle differences between treatment groups, underscores the usefulness of this paradigm for advancing our understanding of female rat sexual behavior.

5. Experiment 3

In the final experiment, we aimed to investigate whether a paced mating condition would still have added value over a non-paced condition if this new assessment tool is used for studying female sexual behavior. We therefore compared the temporal copulatory patterns of female rats in a paced mating (PM) vs. non-paced mating (NPM) set-up (Fig. 1A).

5.1. Results experiment 3

Again, we confirmed that EB females show lower levels of sexual activity than EB+P females. This effect was often found in both non-paced and paced mating settings. The details on this data can be found in Suppl. Table 4, and we only report the relevant findings comparing non-paced to paced mating in this section.

Our data revealed that non-paced mating females displayed more paracopulatory behaviors than paced mating females when EB+P primed (Fig. 4A). The latency to start performing paracopulatory behaviors, on the other hand, was not different (Suppl. Table 5). While no differences were found of pacing conditions on lordosis quotient (Fig. 4B), EB females showed a lower lordosis score in the non-paced setting compared to the paced mating set-up (Fig. 4C). EB+P females, however showed a similar lordosis score in non-paced and paced mating cages. Furthermore, females received more male copulations in the non-paced mating setting than paced mating set-up (Fig. 4D), especially the EB females.

Since the parameters exits and contact return latencies are not available in non-paced mating -tested rats, we were not able to assess these, but a detailed analysis of sexual bouts and time-outs revealed an increase in the number of sexual bouts in EB females tested in non-paced vs paced mating rats (Fig. 4E). EB+P rats, on the other hand, performed the same amount of sexual bouts under paced and non-paced mating conditions. The pacing conditions did not have any effect on the mean duration of the sexual bouts (Fig. 4F), but EB (but not EB+P) females did have shorter time-outs in the non-paced vs paced mating setting (Fig. 4G).

5.2. Discussion experiment 3

In summary, our findings show that the mating conditions have a small effect on female sexual performance. In EB+P females, non-paced mating conditions resulted solely in more paracopulatory behaviors compared to paced mating. EB females, on the other hand, showed lower LS, but received more copulations in the non-paced mating setting. While no differences were found in EB+P females on the number and duration of sexual bouts and time-outs, EB females mated with more sexual bouts and shorter time-outs in the non-paced mating vs paced mating set-up.

Our findings are opposite to Coopersmith et al., who showed that female rats tested in a paced mated set-up displayed more paracopulatory behaviors than in a non-paced mating set-up (Coopersmith, Candurra, and Erskine, 1996). Another study reported no difference between the total number of paracopulatory behaviors between female rats tested in a paced and non-paced mating set-up (Hernández-Munive et al., 2018).

Overall, this suggests that the conditions in which female rats copulate might not be so relevant when they are normally receptive (EB+P rats) but could matter with regard to copulatory speed and motivation to continue copulation when females are in a low status of receptivity (EB rats). This could indicate that the presence of, and potentially the extra pressure from, the male could stimulate the female in increasing her copulatory speed. It should be mentioned, though, that while they can escape in a paced mating setting, female rats in a non-paced mating set-up instead show sexual rejection behaviors. Just as other studies have found (Coopersmith, Candurra, and Erskine, 1996; Arzate et al., 2011; Hernández-Munive et al., 2018; Nyuyki et al., 2011), we observed significantly longer sexual rejection behavior episodes (such as fighting, boxing, and kicking) in female rats tested in the non-paced mated set-up (non-paced mating: 35.40 ± 7.30 vs paced mating: 16.05 ± 7.30). This confirms that non-paced mating may not be more engaging but rather more aversive and less rewarding compared to paced mating. The increase in paracopulatory behaviors in EB+P rats, and the increase in sexual bouts in the EB rats might instead reflect an induced response to the male behavior rather than a voluntary behavior. Altogether, this strengthens the recommendation that paced mating paradigms should be used to study female sexual behavior, as it will lead to a more comprehensive understanding of female sexual behavior and motivation from the female's willingness to participate in copulatory activity. Moreover, by observing females in a more naturalistic setting, in which they can pace their sexual encounters, we may uncover behavioral consequences, and potentially underlying neural mechanisms, that were previously overlooked or inconclusive in non-paced paradigms.

6. Conclusion

Overall, the goal of the study was to develop an improved behavioral assessment tool to study temporal copulatory patterns in female rats in more detail. We found that female rats copulate in patterns of sexual bouts and time-outs. While the duration of the sexual bouts solely depends on the female paracopulatory behaviors, the time-out duration is also related to the male's copulatory stimulation received in the preceding bout. By using low (EB) and normal (EB+P) receptive females, we determined that higher levels of receptivity result in more sexual bouts and shorter time-outs. This indicated that sexual bouts can be interpreted as measures of copulatory speed and time-outs as a measure of motivation to continue copulation.

Furthermore, our study showed that sexual experience did not improve the sexual performance of female rats themselves by a large extend, but do result in receiving more mounts, intromissions and

ejaculations from the (experienced) male. Finally, we found that the conditions in which female rats copulate (non-paced vs paced mating) might not be so relevant in normal functioning rats, but could matter for females in a low receptive state. Still, sexual rejection is the main focus, paced mating conditions are recommended to study female sexual behavior.

CRediT authorship contribution statement

Eelke M.S. Snoeren: Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **John C. Oyem:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Roy Heijkoop:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used sometimes ChatGPT in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.beproc.2025.105148.

Data availability

Data will be made available on request.

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