Rapid changes in sociosexual behaviors around transition to and from behavioral estrus, in female rats housed in a seminatural environment

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ABSTRACT

Gonadally intact female rats display sexual behaviors only during a portion of the estrus cycle. In standard experimental setups, the on- and offset of sexual behavior is gradual. However, in naturalistic settings, it is almost instantaneous. We assessed the changes in sociosexual behaviors at the beginning and end of behavioral estrus in ovariectomized females treated with ovarian hormones. Rats were housed in a seminatural environment, in groups of three males and four females. We scored female and male behavior during the 8 min preceding and following the first and last lordosis of behavioral estrus. Immediately before the first lordosis, there was a sharp increase in female paracopulatory behaviors whereas the end of estrus was marked by a sudden decrease in these behaviors. There was no systematic change in other female behavior patterns. These data suggest that the display of female paracopulatory behaviors plays a key role. Both during transition into and out of behavioral estrus, most behavioral changes occurred within one minute. The rapid changes must be unrelated to ovarian hormone fluctuations in these ovariectomized females. Perhaps they can be explained in terms of hormone-induced, dynamic (chaotic) changes in the function of critical structures within the brain.

1. Introduction

Sexual behavior in female rats consists of a few stereotyped motor patterns. The basic behavior pattern, lordosis, is a concave arching of the back, raised hind legs and the tail moved to one side (see Kow and Pfaff, 1973). Females displaying lordosis are called receptive, and the state of being receptive is named receptivity. Additional female sexual behavior patterns are ear wiggling (fast lateral or vertical movements of the head giving the impression that the female is wiggling her ears), hopping and darting (rapid running away from the male with small hops and darting movements). These behaviors are often grouped together under the label "solicitation" or "proceptive" (Beach, 1976; Erskine, 1989). It has been suggested that the term "paracopulatory behavior" is more appropriate (Blaustein and Erskine, 2002), and we have followed that suggestion for the last couple of years.

Gonadally intact female rats show sexual behavior only during a short period of the estrus cycle (e.g. Long and Evans, 1922). This period is often called heat, behavioral estrus or just estrus. The transition from non-estrus to estrus, and conversely, have been described as a gradual increase/decrease in female sexual receptivity (e.g. Ball, 1937; Hardy, 1972; Madlafousek and Hliňák, 1977). At the beginning of estrus, only a

small fraction of the male's mounts produces a lordosis response. This fraction increases with time until most or all of the mounts lead to lordosis. At the end of the estrus period, the proportion of male mounts leading to lordosis is gradually reduced until no lordosis is displayed. These gradual changes in the sexual response have been described in experiments repeatedly subjecting females to discrete, short tests, during which females could not escape from a vigorously mounting male or from the experimenter's fingers when manual stimulation rather than a male was used (e.g. Blandau et al., 1941). Thus, the females were victims of forced sexual interaction. However, in the rat's natural context, or in seminatural environments, males rarely try to mate with non-receptive females (Chu and Ågmo, 2015b; Robitaille and Bouvet, 1976). In fact, males almost never pursue or mount females not displaying paracopulatory behaviors in such environments (Bergheim et al., 2015). It is, consequently, possible that the gradual change from non-estrus to estrus described in studies employing forced sexual interaction is an artifact caused by the observation procedure.

This supposition was confirmed in an experiment in which we observed the beginning and end of behavioral estrus in groups of naturally cycling rats living together with males in a seminatural environment. These females did not show the gradual changes in responsiveness to

the males as seen in a series of discrete, short tests. Instead, the females instantaneously changed from non-receptivity to full receptivity at the beginning of behavioral estrus, and then from full receptivity to non-receptivity at the end of estrus (Chu and Ågmo, 2014). A more detailed study revealed that the females suddenly started to display paracopulatory behaviors while the males started to pursue them less than a minute before the male initiated mounting. The first mount was invariably associated with female lordosis, and so were all subsequent mounts. At the end of estrus, an equally rapid change occurred. After the display of the last lordosis, no further paracopulatory behavior was shown and the males stopped pursuing and mounting the female (Chu and Ågmo, 2015a).

The results of the earlier study suggested that the sudden on- and offset of female sexual behavior was determined by rapid changes in female attractivity and behavior. In the gonadally intact females used in that study, short-term fluctuations in the release of ovarian hormones might underlie the rapid behavioral changes observed, including changes in attractivity. Pulsatile release of estradiol and progesterone has been described in several species (Bäckström et al., 1982; Baird, 1978; Schallenberger et al., 1985), and both steroids may have rapid actions in the central nervous system (reviewed in Gellersen et al., 2009; Tonn Eisinger et al., 2018). It has even been suggested that estradiol may act as a transmitter (Balthazart et al., 2018). In order to exclude the possibility of fluctuating steroid concentrations or other factors emanating from the ovaries as causes of the sudden behavioral changes, we now analyzed the transition from non-receptivity to receptivity and vice versa in ovariectomized females sequentially treated with estradiol + progesterone to induce estrus. In the present study, we made a more detailed description of female and male behavior in the seminatural environment around the transition into estrus than has previously been done. This would make it possible to determine the relationship between changes in female attractivity and behavior, male behavior and the females' responses to those behaviors in the absence of short-term variations in the concentration of circulating gonadal hormones. Specifically, we evaluated the hypothesis that the onset of copulatory behavior, i.e. the beginning of estrus, was a result of the appearance of female paracopulatory behavior, inciting the male to pursue and mount. The end of behavioral estrus would be caused by the reduction or disappearance of paracopulatory behavior. Confirmation of this hypothesis would reinforce the notion that sexual behavior in seminatural settings must be analyzed in terms of male – female interaction in which the behavior of one subject becomes the determinant of the behavior of the other. Furthermore, these data could inspire novel hypotheses concerning the dynamics of hormone action within the brain and the behavioral manifestations of these actions.

2. Material and methods

2.1. Subjects

Fifteen male and 20 female Wistar rats (300 g and 250 g upon arrival, respectively) were obtained from Charles River WIGA (Sulzfeld, Germany). Animals were housed in same sex pairs in Macrolon® IV cages in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %). The room was exposed to a 12:12 h light/dark cycle (lights on at 11:00). Food (RM1, Special Diets Services, Witham, UK) and tap water were available *ad libitum*. Females were ovariectomized through dorsal incision under isoflurane anesthesia, two weeks prior to the experiment. For a detailed description of the ovariectomy procedure, see Ågmo (1997).

All experimental procedures employed in the present experiment were approved by the Norwegian Food Safety Authority and were in agreement with the European Union council directive 2010/63/EU.
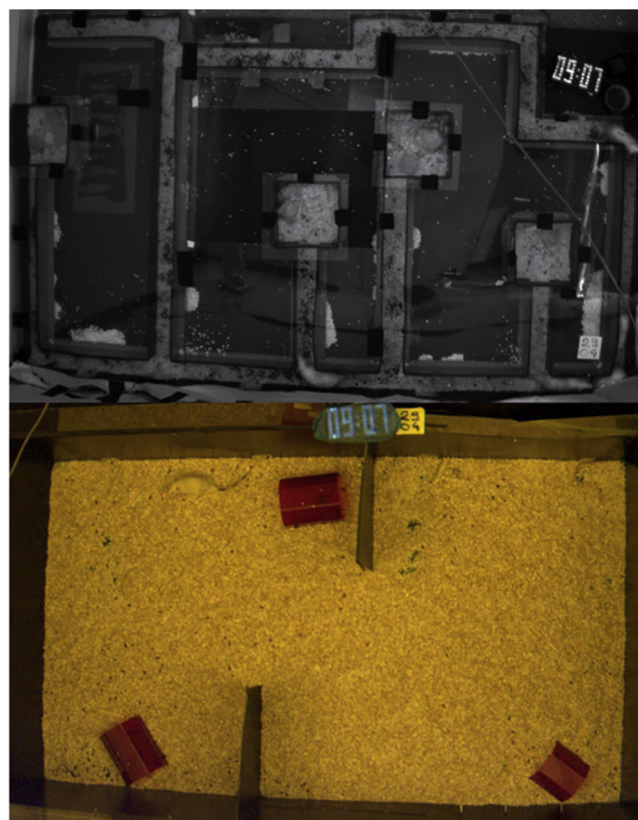

Fig. 1. Picture of the seminatural environment.

2.2. Apparatus

The seminatural environment used in this study has been described in detail elsewhere (Chu and Ågmo, 2015a, 2015b, 2014). Briefly, it consisted of a burrow system (120 × 210 cm) and an open area (120 × 210 cm) (Fig. 1). There were four small openings (8 × 8 cm) between the burrow and the open area. The burrow was maintained in complete darkness by a light-blocking wall of extruded polyethylene foam. Two infrared lamps (850 nm) provided light necessary for video recording. In the open area, the light/dark cycle was maintained, with the exception that a light of about 1 lx was maintained during the dark phase. Temperature and humidity were maintained as previously described. Two video cameras, one in the burrow and one in the open area, were fixed to the ceiling and recorded the entire experiment.

2.3. Hormones

Estradiol benzoate (EB) and progesterone (P) (both from Sigma Aldrich, St Louis, MO) were dissolved in peanut oil (Den norske eterfabrikk, Norway) and administered subcutaneously in a dose of 18 µg/kg and 1 mg/rat, respectively. Injection volume was 1 ml/kg for EB and 0.2 ml/rat for P.

2.4. Procedure

The floor of the seminatural environment was disinfected and covered with wood chips (Tapvei, Harjumaa, Estonia) prior to animal introduction. Twelve wood sticks and three plastic shelter huts were provided in the open area, and nest-building material was put in the nest boxes. About 3 kg of regular food pellets were provided in a corner of the open area, and 4 water bottles were also freely available in that corner. The subjects were marked with different shaving patterns and marks on the tail for identification purpose on video. The video recorders were started when the animals were introduced at 13:00 on day

0. Recording was then continuous for a period of 8 days. On day 5, females were injected with EB at 09:00. On day 7, they received P at the same hour. Additional procedural details can be found in Chu and Ågmo (2015a, 2014).

2.5. Design

Five groups were used in the seminatural environment. Each group consisted of 4 females and 3 males. All subjects were sexually naïve. The males were left gonadally intact, whereas all females received the hormonal treatment described above. Subjects in the same group came from different cages to ensure that they were unknown to each other at the beginning of observation.

2.6. Behavioral observations

First, we determined when each female displayed her first lordosis. This first lordosis was considered the beginning of behavioral estrus. The end of behavioral estrus was defined as the moment when a lordosis was displayed, not being followed by another one within 60 min. The time elapsed from the first to the last lordosis was the duration of behavioral estrus. This definition of estrus duration has been employed in earlier studies in the seminatural environment (Chu and Ågmo, 2015a, 2014). In the present study, estrus duration varied from 1.02–14.08 h, with a mean ± SEM of 6.39 ± 1.67 h.

We scored female and male behavior during the 8 min preceding and following the first lordosis, and the 8 min preceding and following the last lordosis (Fig. 2). Rat behavior was scored according to an ethogram previously established (Chu and Ågmo, 2015a, Table 1). Scoring was made with the Observer XT software, version 12.5 (Noldus, Wageningen, The Netherlands).

2.7. Data preparation and statistical analysis

2.7.1. Frequency and duration of behaviors

The number of occurrences and, whenever possible, the total duration of each behavior was recorded. We also calculated the mean duration of each episode of each behavior (total duration divided by the number of occurrences). Earlier studies (e.g. Bergheim et al., 2015) have shown that the mean duration of episodes of a behavior is more informative than the total duration. Therefore we only report the number of occurrences or frequency per min, and the mean duration of behavioral episodes. The data from the 8-min period preceding the first lordosis was compared to those from the 8-min period following that lordosis. Similar analyses were made for the 8 min period preceding and following the last lordosis. The *t*-test for paired observations, or the Wilcoxon test, when data deviated from the normal distribution according to the Shapiro-Wilk test, were used for these comparisons.

In order to more closely analyze the temporal aspects of behavioral changes, we divided the 8-min period preceding the first lordosis in 8 intervals of 60 s each. This was also done for the 8-min period following the first lordosis, and the periods before and after the last lordosis.

Separate one-way ANOVAs were used for analyzing the intervals within each of the 4 periods. Inspection of the data revealed that several behavioral changes occurred in the minute preceding or following the first and last lordosis. Therefore, post hoc tests were limited to comparing the interval closest to the lordosis with the seven intervals located further afar. To this end, we used Dunnett's test. When data did not satisfy criteria for parametric analysis, i.e. deviation from the normal distribution according to the Shapiro-Wilk test, we used Friedman's ANOVA. In case of significance, post hoc comparisons between the interval closest to the lordosis and all other intervals were made with Steel's test (Steel, 1959).

Statistical analyses were performed using R version 3.6.1 (core and PMCMRplus packages) and IBM SPSS Statistics, version 24. Two-tailed significance level was 0.050.

2.7.2. Co-occurrence analysis

Since the behavior patterns were recorded in chronological order, it was possible to determine how the structure of behavior was modified by transition from non-estrus to estrus and vice-versa. Thus, in order to fully exploit the data obtained, we subjected the behavioral record of all individuals to an analysis of co-occurrence. This procedure has been described in detail elsewhere (Le Moëne and Ågmo, 2019, 2018). Briefly, for each individual record of behavior, we used a moving window of four behavior patterns, and determined how often one behavior occurred together with another in the same window. This is defined as a co-occurrence. The window moved behavior by behavior, over the entire individual record. Descending hierarchical classification was used in order to find clusters of associated behaviors (Le Pape et al., 1997; Reinert, 1990, 1983). The descending hierarchical classification is based on the probability for a co-occurrence to be proportionally more present in a cluster than it is in the entire data set, as evaluated by χ^2 analysis. Each co-occurrence is permutated from one cluster to others in order to test the robustness of the classification, until statistically independent profiles of events appear (Marchand and Ratinaud, 2012). Clusters can therefore be interpreted as groups of behaviors significantly more co-occurring together than with behaviors of any other cluster. Co-occurrence networks were established and visualized using the Fruchterman-Reingold algorithm, a force-directed layout algorithm (Fruchterman and Reingold, 1991). Each behavior is regarded as a node, and the frequency of co-occurrence is the edge weight. Calculations were performed with the free software IRAMUTEQ (Interface de R pour les Analyses Multidimensionnelles de Textes et de Questionnaires; available at http://www.iramuteq.org/).

3. Results

Two females never displayed behavioral estrus, i.e. neither lordosis nor paracopulatory behaviors. Thus, eighteen females were included in the analyses. The first and last lordosis of behavioral estrus in each individual rat was used as anchoring points (time 0) for behavioral observation. The lordoses fixing the anchoring points were not included in the analyses since they were a constant. Likewise, the mounts or
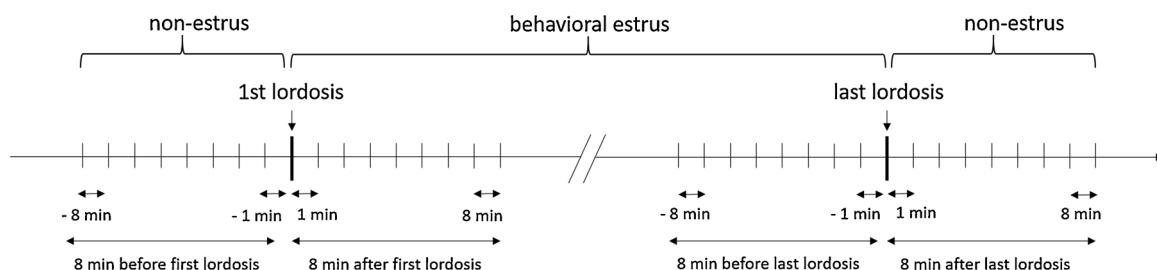

Fig. 2. Illustration of the temporal division between estrus and non-estrus used in the observations and analyses. Before and after the first and the last lordosis, comparisons were made between (1) non-estrus and estrus; (2) the eight 60 s segments before and after the first lordosis, as well as the segments before and after the last lordosis.

Table 1
Description of scored behaviors. For each behavior, different parameters were registered.

Behavior category	Behavior	Behavior description
Female sexual behavior	Paracopulatory behaviors**	Approach to a male followed by runaway, often associated with hops, darts, ear wiggling.
Female attractivity	Male pursuit**	Male pursues and runs closely behind a female.
	Male anogenital sniffing**	Male sniffs, occasionally grooms and licks, a female's anogenital region.
	Male copulatory behavior*	Male mount, intromission or ejaculation: male standing on its hind legs and places its forepaws on a female rump from behind.
Male attractivity	Rejection*	Female kicks, bites or turns around against its suitor.
	Sniffing a male**	Female places its snout close to any body part, except the anogenital region, of a male.
Female prosocial behavior	Sniffing another female**	Female places its snout close to any body part, except the anogenital region, of another female, while its whiskers move briskly.
Female antisocial behavior	Nose off**	Female faces another rat, male or female, either standing on four legs or while rearing; includes boxing and teeth showing.
	Flee*	Female runs away from another rat, male or female, after an agonistic interaction.
Female non-social behavior	Self-grooming**	Female grooms any part of its body, including the anogenital region.
	Drinking**	Self-explanatory.
	Eating**	Self-explanatory.
	Resting**	Female rests in a corner or nest box.
	Rearing**	Female sniffs the air while standing on hind legs.

* frequency.
** frequency and duration.

intromissions associated with these first and last lordoses were excluded from analysis.

Five different groups were observed in this experiment. Even though the rats were randomly assigned to these groups, it is possible that there were some unintended differences.

In order to check for this, we analyzed the effect of group on the frequency of one social (nose-off) and one exploratory (rearing) behavior with the Kruskal-Wallis test. Both males and females were included in these analyses. There was no significant group difference during any of the intervals analyzed (before and after the first lordosis, before and after the last lordosis; all $p > 0.066$; data not shown).

3.1. Female behavior

3.1.1. Female behavior during the 8-min period preceding and following the first and last lordosis

The number of paracopulatory behaviors was lower before than after the first lordosis ($t_{17} = 2.153$, $p = 0.046$). It was higher before the last lordosis than after it ($t_{17} = 2.720$, $p = 0.015$) (Fig. 3A). The mean duration of paracopulatory behaviors seemed to follow a similar pattern but the difference did not reach significance around the first lordosis ($t_{17} = 1.996$, $p = 0.062$). It was shorter after the last lordosis than before it ($t_{17} = 2.597$, $p = 0.019$) (Fig. 3B). The number of female flights from males was not different between the periods preceding and following the first lordosis (Wilcoxon test, $z = 1.484$, $p = 0.138$), however, females fled from the males significantly more often after the last lordosis than before it ($t_{17} = 3.651$, $p = 0.002$) (Fig. 3C). No other difference was found in female behavior when comparing the periods before and after the first and last lordosis (all $ps > 0.079$, data not shown).

3.1.2. Detailed temporal analysis of female behavior during the 8-min period preceding and following the first and last lordosis

Lordosis frequency remained stable during the 8 min following the first lordosis ($\chi^2_{(7)} = 8.723$, $p = 0.273$). This was also the case for the 8 min preceding the last lordosis ($\chi^2_{(7)} = 5.951$, $p = 0.546$) (Fig. 4A). To the contrary, the frequency of paracopulatory behaviors increased during the intervals preceding the first lordosis ($\chi^2_{(7)} = 28.103$ $p < 0.001$). The first interval before the first lordosis showed higher frequency of these behaviors than previous intervals (all $ps < 0.05$). The frequency of paracopulatory behavior was stable after the first lordosis ($\chi^2_{(7)} = 7.481$, $p = 0.381$). Before the last lordosis, paracopulatory behaviors frequency varied between the 8 intervals ($\chi^2_{(7)} = 14.492$, $p = 0.043$), but post hoc tests did not reach significance (all $ps > 0.05$). The frequency of paracopulatory behaviors also decreased during the 8 min following the last lordosis ($\chi^2_{(7)} = 14.654$, $p = 0.041$), but the post hoc test for this period did not reach significance either (all $ps > 0.05$) (Fig. 4B). The mean duration of paracopulatory behaviors increased before the first lordosis ($\chi^2_{(7)} = 27.078$, $p < 0.001$). The first 60-s interval preceding the first lordosis showed longer mean duration of these behaviors than earlier intervals (all $ps < 0.05$). The mean duration of paracopulatory behavior was stable during the 8 min following the first lordosis ($\chi^2_{(7)} = 4.800$, $p = 0.684$). This was also the case for the 8 min preceding the last lordosis ($\chi^2_{(7)} = 8.381$, $p = 0.300$). After the last lordosis, the mean duration of paracopulatory behaviors decreased ($\chi^2_{(7)} = 14.217$, $p = 0.047$), although post hoc tests did not reach significance (all $ps > 0.05$) (Fig. 4C). The rejection frequency slightly increased before the first lordosis ($\chi^2_{(7)} = 16.022$, $p = 0.025$) but none of the post hoc tests reached significance (all $ps > 0.05$). The rejection frequency was stable after the first lordosis ($\chi^2_{(7)} = 9.762$, $p = 0.202$) and before the last lordosis ($\chi^2_{(7)} = 7.628$, $p = 0.367$). However, rejection frequency
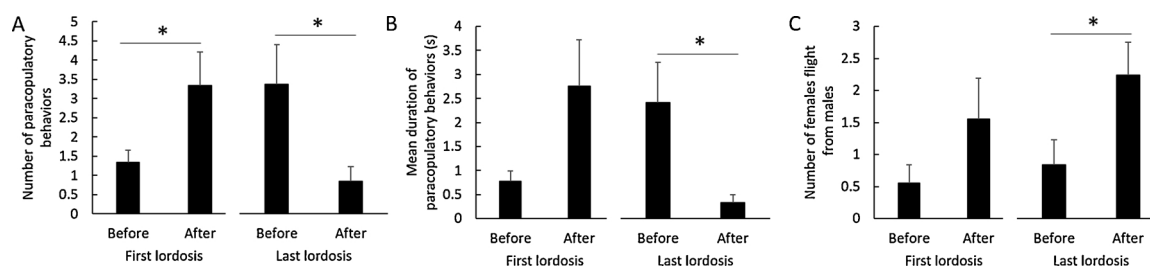

Fig. 3. Number and mean duration of female behaviors performed during the 8-min period preceding and following the first and the last lordosis. A. Number of paracopulatory behaviors. B. Mean duration of paracopulatory behaviors. C. Number of female flights from males. Data are mean + SEM. *, $p < 0.05$.
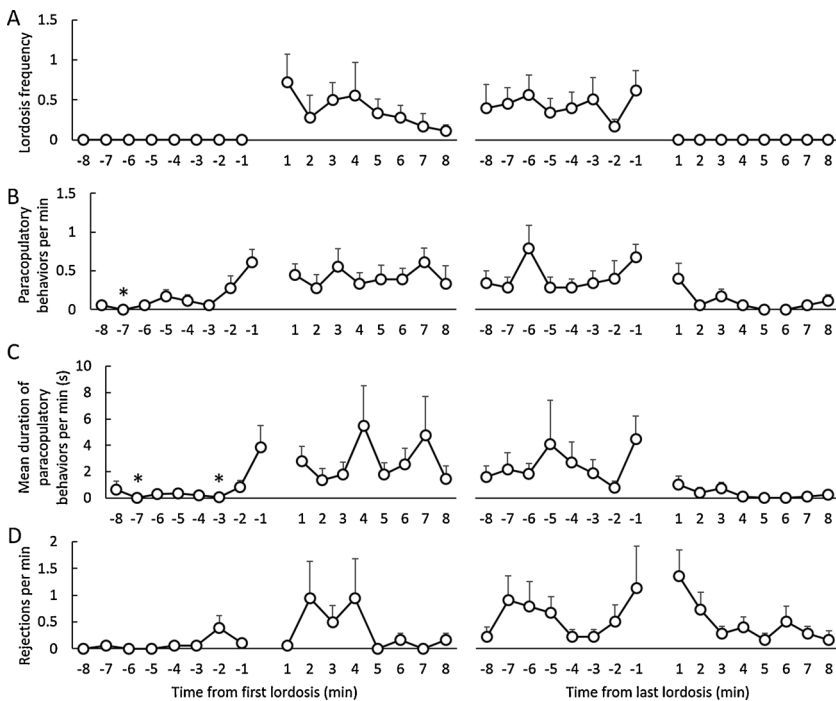
Fig. 4. Female behavior during the eight 1-min segments preceding and following the first and the last lordosis. A. Lordosis frequency. B. Paracopulatory behaviors frequency. C. Mean duration of paracopulatory behaviors. D. Rejection frequency. Data are mean + SEM. Different from the interval closest to the lordosis: *, p < 0.05.

decreased after the last lordosis ($\chi^2_{(7)}$ = 15.562, p = 0.010). Again, post hoc tests did not reach significance (all ps > 0.05) (Fig. 4D).

The frequency of females sniffing conspecifics showed the same pattern as the mean duration of this behavior. We only present data for mean duration. Mean duration of sniffing a male conspecific did not vary between intervals before the first lordosis ($F_{7,119}$ = 1.344, p = 0.236), but increased after the first lordosis ($F_{7,119}$ = 3.583, p = 0.002). The mean duration of this behavior was lower during the first interval following the first lordosis than the latest interval following it (Dunnett's test, p = 0.003) (Fig. 5A). There was no effect of time interval on females sniffing males around the last lordosis (all ps > 0.111). We did not find any effect of time interval on sniffing directed to other females, independently from the observation period regarded (all ps > 0.053).

The frequency of female nose-off episodes to male conspecifics showed the same pattern as the mean duration of this behavior. We only present data for mean duration. The mean duration of nose-off episodes directed to males was stable before the first lordosis

($\chi^2_{(7)}$ = 9.814, p = 0.199). After the first lordosis, nose-off directed to males increased over intervals ($\chi^2_{(7)}$ = 16.422, p = 0.022), but post hoc tests did not reach significance (all ps > 0.05) (Fig. 5B). Nose-off behavior directed towards males was not modified around the last lordosis (all ps > 0.365). Female nose-off behavior directed to other females was not affected by the time intervals, regardless of the observation period regarded (all ps > 0.429) (Fig. 5B). Finally, the frequency of female fleeing from the males was heterogeneous before the first lordosis ($\chi^2_{(7)}$ = 18.868, p = 0.009), but post hoc tests did not reach significance (all ps > 0.05). The fleeing frequency was stable after the first lordosis ($\chi^2_{(7)}$ = 11.866, p = 0.105) and before the last lordosis ($\chi^2_{(7)}$ = 4.555, p = 0.714). However, after the last lordosis, the frequency of female fleeing from males decreased ($\chi^2_{(7)}$ = 23.452, p = 0.001). The first interval following the last lordosis showed a higher flight frequency than following intervals (all ps < 0.05) (Fig. 5C). Fleeing from female conspecifics was never impacted by time intervals, regardless of the period regarded (all ps > 0.429) (Fig. 5C). We did not find any other modification of female behavior during the
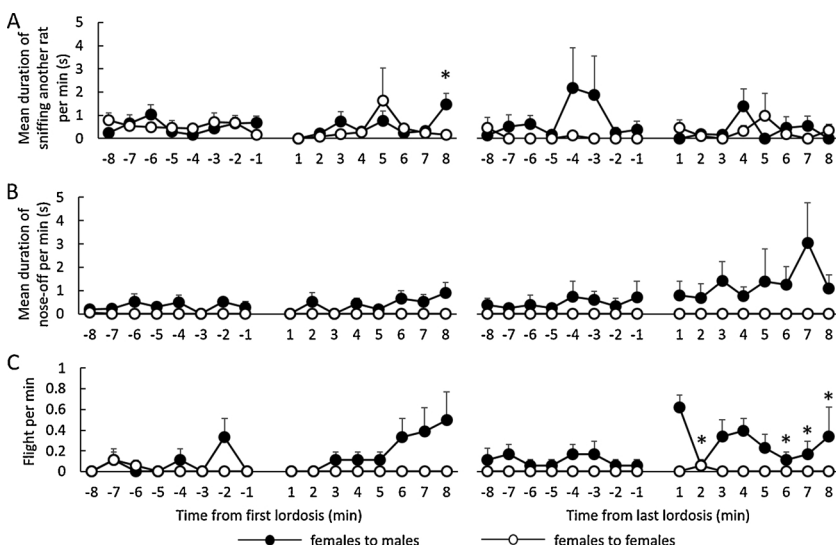

Fig. 5. Female non-sexual behavior during the eight 1-min segments preceding and following the first and the last lordosis. A. Mean duration of female sniffing other male or female conspecifics. B. Mean duration of nose-off directed to males or females. C. Frequency of female flight from male or female conspecifics. Data are mean + SEM. Different from the interval closest to the lordosis: *, p < 0.05.
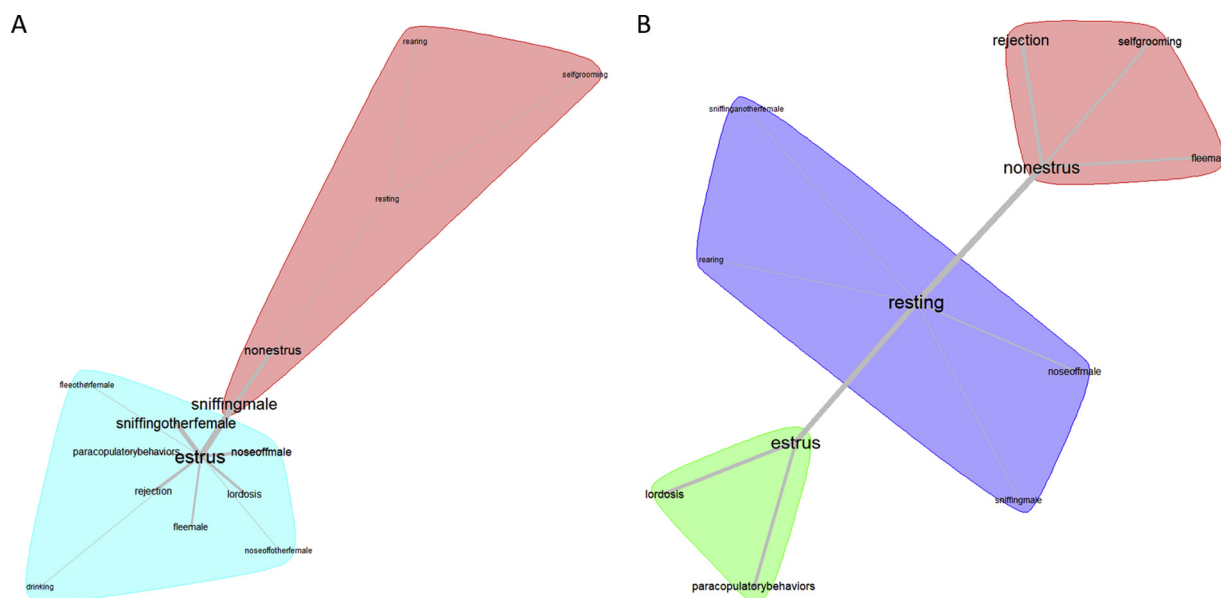
A

B

Fig. 6. A. Co-occurrence analysis showing main behavioral associations typical of the transition from non-estrus to estrus for females. B. Co-occurrence analysis showing main behavioral associations typical of the transition from estrus to non-estrus for females. Clusters of behavioral association are represented in halos of different colors. The size of the words is proportional to their occurrence frequency. The thickness of the branches is proportional to the frequency of association of the two items linked. Cluster shape and color are arbitrary.

intervals around the first and the last lordosis (all ps > 0.096).

3.1.3. Co-occurrence analysis

When looking at the structure of female behavior transitioning from non-estrus to estrus, two distinct clusters appeared. Non-estrus was associated with non-social and exploratory behaviors, as well as sniffing the males. Estrus was associated with the sexual behaviors (lordosis, paracopulatory behaviors), rejection, anti-social behaviors to males and females, as well as drinking. The behavioral repertoire seemed to be far more extensive during estrus than before estrus (Fig. 6A). The co-occurrence analysis of female transitioning from estrus to non-estrus revealed three clusters of behavioral patterns. Estrus was associated with lordosis and paracopulatory behaviors, whereas non-estrus was associated with fleeing from males and rejection, as well as with self-grooming. An intermediate cluster was characterized by the behaviors resting, rearing and sniffing other males and females. Nose-off to other males also appeared in this cluster (Fig. 6B).

3.2. Male behavior

3.2.1. Male behavior during the 8-min period preceding and following the first and last lordosis

Male copulatory behavior was strongly affected by female estrus. The number of male mounts was higher after than before the first lordosis (Wilcoxon test, $z = 3.086$, $p = 0.002$). Interestingly, 4 out of the 18 (22 %) first lordoses were elicited without any mount. This can happen when female display lordosis to non-copulatory tactile stimulation, as observed before (e.g. Chu and Ågmo, 2014). The number of intromissions was not significantly increased during the 8-min period following the first lordosis (Wilcoxon test, $z = 1.732$, $p = 0.083$). The period preceding the last lordosis showed more mounts (Wilcoxon test, $z = 2.829$, $p = 0.005$) and intromissions (Wilcoxon test, $z = 2.251$, $p = 0.024$) than the period following it (Fig. 7A). Eight out of the 18 last lordoses (44 %) were elicited without any mount.

The number of episodes of male pursuit of the female increased from before to after the first lordosis ($t_{17} = 2.820$, $p = 0.012$) whereas this behavior decreased after the last lordosis compared to before it ($t_{17} = 2.812$, $p = 0.012$) (Fig. 7B). The same pattern was observed for the mean duration of male pursuit of the female (first lordosis: $t_{17} = 2.366$,

$p = 0.030$; last lordosis: $t_{17} = 2.527$, $p = 0.022$) (Fig.7C). We did not find any difference in the frequency and duration of females receiving anogenital sniffing from the males before and after the first and the last lordosis (all ps > 0.275, data not shown).

3.2.2. Detail temporal analysis of male behavior during the 8-min period preceding and following the first and last lordosis

We did not find any effect of time interval on the mount and intromission frequencies, independently from the observation period regarded (all ps > 0.051) (Fig. 8A). Neither the frequency nor the mean duration of episodes of male pursuit of the females was affected by time intervals around the first and the last lordosis (all ps > 0.067) (Fig.8B). Similarly, no effect of time interval was observed on the frequency and mean duration of male anogenital sniffing of the females (all ps > 0.076) (Fig.8C).

3.2.3. Co-occurrence analysis

The co-occurrence analysis of male behavior associated with female transition from non-estrus to estrus revealed two clusters. One shows that pre-estrus period was associated with pursuit of the females, while a second cluster associated estrus with male copulatory acts (mount, intromission) and anogenital sniffing of the females (Fig. 9A). Two clusters of male behaviors toward females transitioning from estrus to non-estrus appeared. One cluster associated the estrus period with male copulatory acts and pursuit of the females. Another cluster associated post-estrus period with anogenital sniffing (Fig. 9B).

4. Discussion

Transition from pre-estrus to estrus occurred rapidly. Most changes appeared within the minute preceding the first lordosis, including the sharp increase in both the frequency and episode duration of para-copulatory behaviors. Noteworthy, in a seminatural environment, about 97–98 % of male mounts are preceded by a paracopulatory behavior (Bergheim et al., 2015; McClintock and Adler, 1978). However, most paracopulatory behaviors are not followed by any response from the male. In fact, it has been reported that the duration of each episode of paracopulatory behavior is an important determinant of whether it will incite a male to mount or not (Bergheim et al., 2015). Episodes of
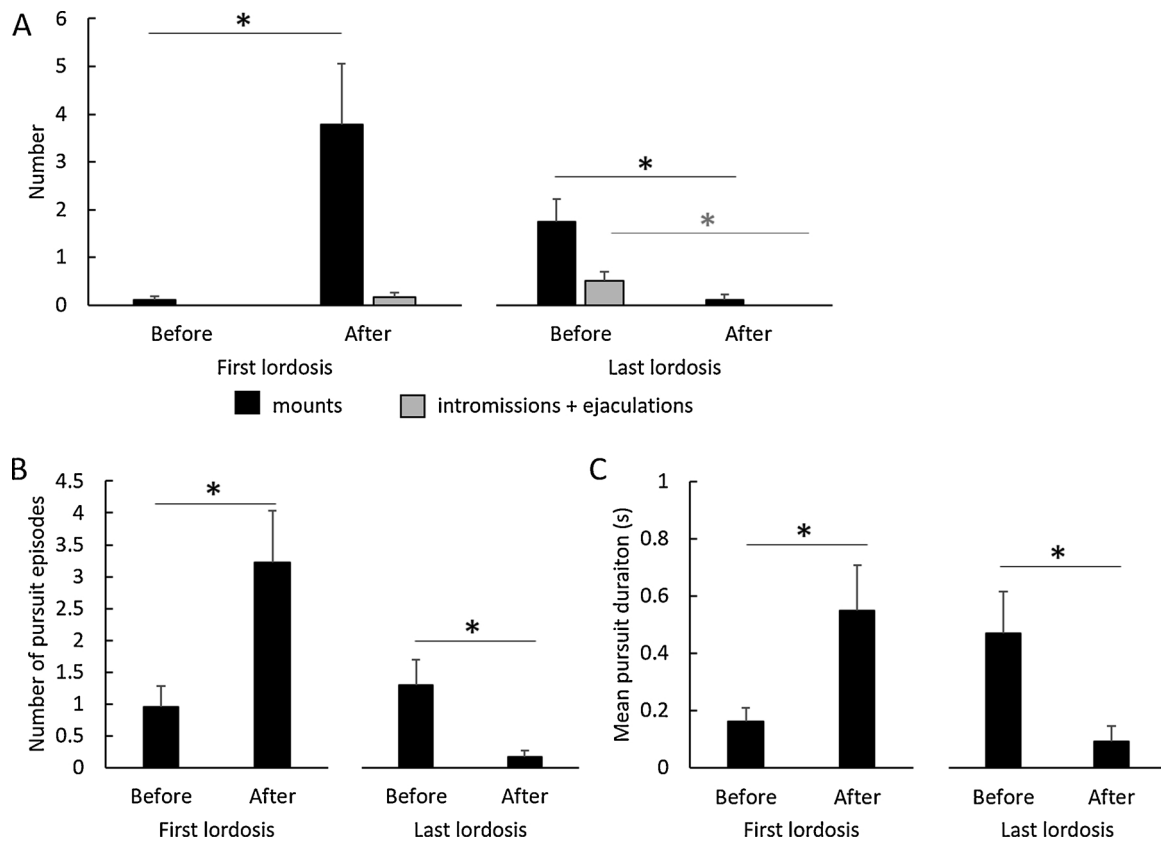
Fig. 7. Number and mean duration of male behaviors received by the females, during the 8-min period preceding and following the first and the last lordosis. A. Male copulatory behaviors: Number of mounts, number of intromissions. B. Number of episodes of male pursuit of the females. C. Mean duration of male pursuit of the females. Data are mean + SEM. *, p < 0.05.

paracopulatory behavior followed by a mount are about twice as long as those not followed by mounting. We therefore propose that the lengthening of the episodes of paracopulatory behavior immediately preceding the first lordosis is the main factor causing the male to mount, which in turn leads the female to display lordosis. This nicely illustrates that copulatory behavior is a result of female - male interactions, and that the behavior of both needs to be studied if the intricacies of the behavior are to be understood.
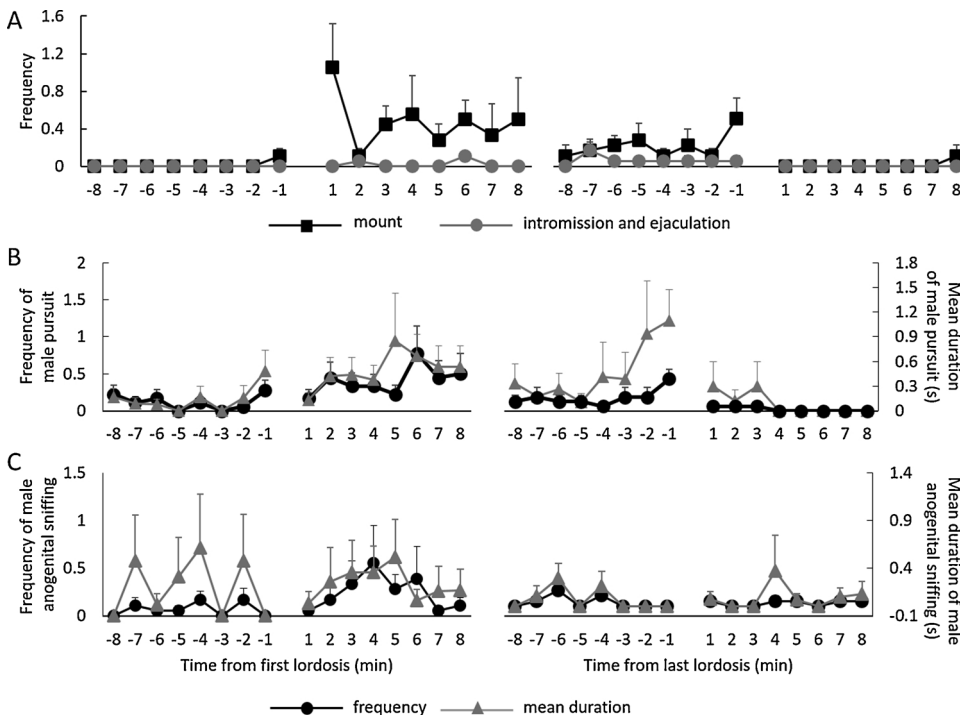

Fig. 8. Male behavior received by the females during the eight 1-min segments preceding and following the first and the last lordosis. A. Number of male mounts and intromissions. B. Mean duration and number of episodes of male pursuit of the females. C. Mean duration and number of episodes of male anogenital sniffing of the females. Data are mean + SEM.
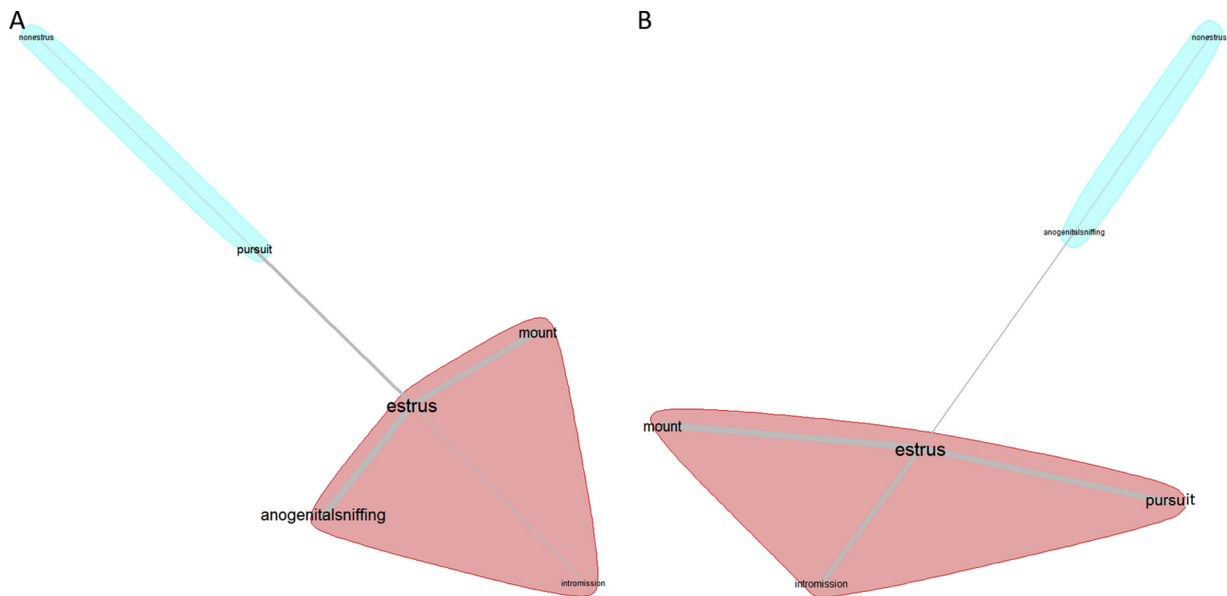
Fig. 9. A. Co-occurrence analysis showing main behavioral associations typical of the male behavior around female transition from non-estrus to estrus. B. Co-occurrence analysis showing main behavioral associations typical of the male behavior around female transition from estrus to non-estrus. Clusters of behavioral association are represented in halos of different colors. The size of the words is proportional to their occurrence frequency. The thickness of the branches is proportional to the frequency of association of the two items linked. Cluster shape and color are arbitrary.

It is well known that the display of paracopulatory behavior requires a more intense and longer exposure to gonadal hormones than the display of lordosis (e.g. Fadem et al., 1979; Tennent et al., 1980). Thus, when the female starts to display paracopulatory behavior, like we observed right before the onset of behavioral estrus, she is invariably fully receptive. This fact can explain why there is no gradual increase in receptivity in the seminatural environment. In a "forced" sexual interaction procedure, the male will mount the female regardless of whether she shows paracopulatory behavior or not. In tests performed before the appearance of these behaviors, females may be only partly receptive, giving the impression of a gradual increase in receptivity at the beginning of estrus. Since males in the seminatural environment do not mount in the absence of paracopulatory behavior, this graduality cannot be observed.

Males did not mount the females prior to the beginning of behavioral estrus, but consistently mounted them after their first lordosis. Most males used in this experiment (14/15) did not attain intromission at their first mount. Whenever a mount does not end in intromission, it is frequently followed by another mount within a few seconds (Sachs and Barfield, 1970). Even though male copulatory acts associated with the elicitation of the first and last lordosis were not included in the analyses, we did observe a higher number of mounts in the first minute following the first lordosis than in the following minutes. During the 8 min period following the first lordosis, the males mounted regularly. This is most likely related to the sustained high frequency and long duration of episodes of paracopulatory behavior during this period.

None of the other behavior patterns recorded seemed to be related to the onset of copulatory behavior. This impression is confirmed by the co-occurrence analysis. Before the beginning of estrus (see non-estrus in Fig. 6), the typical behaviors were non-social, viz. resting, rearing and self-grooming. After the first lordosis (estrus in Fig. 6), there were obviously all the sexual behaviors, but also prosocial and antisocial behaviors. It appears that the increase in all kinds of social interactions was a consequence of the display of sexual behavior rather than a cause for the initiation of that behavior.

Transition from estrus to non-estrus also occurred rapidly. Females drastically decreased the number and duration of paracopulatory behaviors, and consequently the males immediately stopped mounting and pursuing them. The females showed a high amount of rejections

immediately after the last lordosis, but the number was rapidly reduced because of the waning male interest in the females. The end of estrus was also accompanied by increased female flight from the males. Please note that fleeing was defined as running away from another rat regardless of whether that rat was approaching or not. It would appear that the post-estrus females actively avoided the males. This supposition is confirmed by the co-occurrence analysis in which post-estrus was associated with antisocial behaviors directed against the males and self-maintenance (grooming).

It might be thought that the end of behavioral estrus depended on male exhaustion, and that no lordosis was displayed because the males had stopped mounting. However, our data did not confirm such a hypothesis, since the number of mounts displayed during the 8 min preceding the last lordosis was similar to the number displayed during the 8 min following the first lordosis (mean difference: 1.67 ± 1.25; *t*-test : $t_{17} = 1.33$, p = 0.200). Thus, there is no sign of male sexual exhaustion. It seems far more likely that male copulatory behavior disappeared because of a lack of sufficient stimulation from the female in the form of paracopulatory behavior, as already suggested.

The present data show that ovariectomized females, sequentially treated with estradiol and progesterone, show an onset of behavioral estrus just as fast as previously reported for gonadally intact, cycling rats (Chu and Ågmo, 2015a). Likewise, the end of estrus is equally sudden. Within a few minutes, the female changes from complete non-receptivity to full receptivity at estrus onset, and the opposite change occurs within the same time frame at estrus offset. Since there can be no fast variations in the availability of the ovarian hormones in the ovariectomized females, it can be concluded that fast changes in the serum concentration of these hormones are unrelated to the beginning and end of estrus. In fact, the peak concentration of estradiol occurs about 1 h after subcutaneous administration of estradiol benzoate (Cheng and Johnson, 1974), whereas that of progesterone occurs within a few min (O'Brien et al., 1980). Rising hormone concentrations are, therefore, not responsible for the initiation of sexual receptivity and the display of paracopulatory behaviors.

It has been reported that the onset of receptivity in gonadally intact females occurs during the rise of progesterone concentration, whereas the end of estrus is unrelated to any decline in estradiol or progesterone (Södersen and Eneroth, 1981). This means that the ovariectomized

females used here are different from intact females, since progesterone concentration was declining in the former when sexual receptivity started. Yet the behavioral manifestations of transitions from non-receptivity to receptivity were most similar. We suggest that the hormone-initiated, intracellular processes in both kinds of females are very similar despite the difference with regard to the timing of receptivity in relation to raising or maximum progesterone concentrations.

In the present experiment, the transition from non-estrus to estrus occurred about 2 h after the beginning of the dark phase. In naturally cycling females observed in the seminatural environment, estrus started during the late afternoon, about two h before the end of the light phase (Chu and Ågmo, 2014). This discrepancy is probably due to the timing of the P injection in the present experiment. Despite the different timing, the behavioral changes during the transition from non-estrus to estrus were similar in intact and ovariectomized, hormone-treated females This observation suggests that intrinsic properties of the hormone actions are more important than extrinsic factors like phase of the light/dark cycle.

Estradiol initiates a series of intracellular processes developing over some time, eventually leading to behavioral estrus (Cohen and Pfaff, 1992; Micevych et al., 2017). These processes are reinforced by progesterone (reviewed in Camacho-Arroyo et al., 2017). Rapid, nongenomic actions of estrogens (see Introduction) could, at least in principle, be superimposed on the slow actions, and cause the fast behavioral changes observed in the present study. This presupposes equally rapid changes in local estradiol concentration at appropriate brain sites. In these ovariectomized females, such changes must come from de novo synthesis of estradiol. Such synthesis has been reported to occur at hypothalamic sites important for sexual behavior (reviewed in Rossetti et al., 2016). The environmental factors triggering de novo brain synthesis of the ovarian hormones in rats are mostly unknown. However, it is not impossible that such synthesis underlies the rapid behavioral changes observed in the present study. This hypothesis could easily be tested by administering an aromatase inhibitor to the females.

In addition to speculations about possible de novo synthesis of steroids, the issue of how the rather slow hormone actions manifest themselves in fast behavioral changes can, at least theoretically, be resolved by analyzing the cellular actions of hormones in terms of dynamic systems, often called chaotic systems. Within such systems, a phenomenon known as bifurcation regularly occurs (Zhou, 2013, see also Fasoli et al., 2018). Bifurcation is defined as a sudden qualitative change in a dynamic system caused by a small change in parameter values, such as hormone-initiated intracellular modifications. This qualitative change may be a phase shift, as when water turns into ice. Fast and slow intracellular changes brought about by the ovarian hormones may bring the system to a bifurcation point, hence to a qualitative change in the system's function and the ensuing drastic modifications of behavior. Thus, the abrupt change during the transition from non-receptivity to receptivity may be caused by a phase shift in the function of critical structures within the brain.

The kind of reasoning outlined in the preceding paragraph has been used to explain the transition from sleep to wakefulness and other sudden changes in arousal (Pfaff and Banavar, 2007; Quinkert et al., 2011). It might also be mentioned that within the field of dynamic neuroscience the notion of criticality has become central (Brochini et al., 2016). When one or several system parameters are close to the point of phase shift (a bifurcation point), the system is in a critical state, or at criticality. Some scientists maintain that information processing is particularly efficient at criticality, and that the brain therefore maintains itself in that state (reviewed in Cocchi et al., 2017; Hesse and Gross, 2014). Consequently, phase shifts are likely to be frequent in nervous function, for example when a female becomes sexually receptive and somewhat later starts to display paracopulatory behaviors. This account is, at the moment, purely theoretical but it could eventually be transformed into testable, experimental hypotheses.

It could be argued that a more parsimonious explanation for the

rapid behavioral changes observed is that fast estradiol actions on the membrane receptor (GPR30, also called GPER1) underlie these changes. This, however, is extremely unlikely, since the GPER30 has been exposed to estradiol for as long time as the intracellular receptors. Furthermore, in the studies in which the GPR30 was shown to facilitate lordosis, agonists for this receptor were injected 48 h before progesterone and behavior was observed 4–6 h later (Anchan et al., 2014) or the females were primed with estradiol benzoate 48 h before a GPR30 agonist was administered (Long et al., 2014). In fact, no immediate effect of this receptor on sexual behavior in non-primed females has been reported. For these two reasons, it is unlikely that estradiol actions on the GPR30 are responsible for the fast changes between estrus and non-estrus, and even less for the transition from estrus to non-estrus. Obviously, it is possible that other membrane-bound, fast-acting estradiol receptors are involved (e.g. Vail and Roepke, 2019). However, such explanations need to be based on the unlikely supposition that de novo estradiol synthesis suddenly is initiated.

5. Conclusions

In a procedure avoiding forced sexual interactions and employing continuous behavioral observation for an extended period of time, it turns out that the female transition from non-estrus to estrus as well as from estrus to non-estrus occurs rapidly and without any gradual increase in receptivity. This is the case both in gonadally intact, cycling females and in ovariectomized, hormone-treated females. Furthermore, it appears that the sudden display of paracopulatory behavior incites the males to pursue and mount the females, thereby providing the stimulus necessary for the display of lordosis. Whereas male and female behavior is closely interrelated and likely equally important during the period of behavioral estrus (Chu and Ågmo, 2015a, 2014), it is likely that sudden changes in female behavior play a key role in the onset of sexual interaction at the beginning of the period of behavioral estrus. Likewise, changes in female behavior seems to signal the end of estrus.

CRediT authorship contribution statement

Olivia Le Moëne: Formal analysis, Writing - original draft, Writing - review & editing. **Enrique Hernández-Arteaga:** Investigation, Formal analysis, Writing - original draft. **Xi Chu:** Conceptualization, Methodology, Writing - original draft. **Anders Ågmo:** Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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