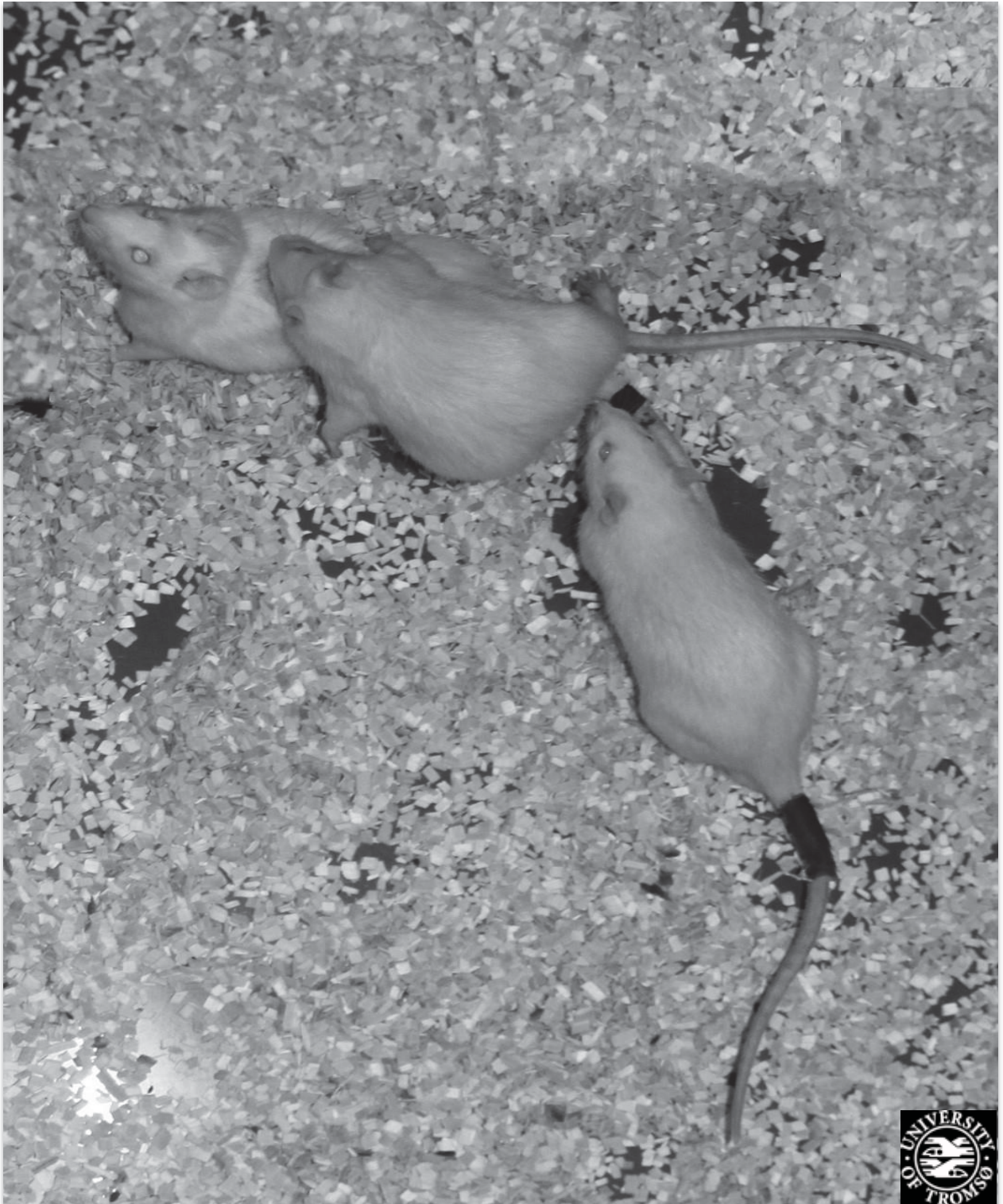


Department of Psychology

Sociosexual behaviors and reproductive success in a group of rats housed in seminatural environment

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A dissertation for the degree of Philosophiae Doctor – Jan 2015



Preface and acknowledgements

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Abstract

Wild rats employ a rather extensive area for their daily activities and share elaborate burrows with tunnel and chambers. When a female enters estrus, she will copulate with multiple males during the entire period of estrus. In a typical laboratory setting, rat sexual behavior is studied in pairs of opposite sex individuals. Apparently the experimental studies have been performed in this highly artificial setting is with low or no ecological validity. Thereby, we initiated systematic examinations of the behavioral effects of male and female in an ecologically valid context. Five groups of 4 intact cycling females and 3 intact males living in a seminatural environment (consisting of a big open area and complex burrow system) for a period of 8 days were used in this study. Sociosexual activities were recorded and analyzed from the beginning of the behavioral estrus until the end of it. During this period, female was sexually receptive to every male mount and showed no significant partner preference or avoidance to a particular male. Males and females copulate simultaneously in a rather random way. The sexual behavior of both sexes changes abruptly in the transition periods from nonreceptivity to receptivity and vice versa. Male did not mount on female before the first lordosis and after the last of her behavioral estrus. Females hardly displayed paracopulatory behavior (darting, running and ear wiggling), and male pursuit of females was very low outside of behavioral estrus. Instead of ejaculatory series, male behavior was observed in copulatory bout. The intensity of male sociosexual interaction is stable throughout the copulatory bout. Female sexual behavior was irrelevant of number of offspring, yet males with more intromissions and ejaculations achieved greater reproductive success. Both mount frequency and non-sexual interactions were unrelated to male fertility as well as the order of ejaculations did not determine reproductive advantage. Male and female preferences slightly influenced fertility in rats. Dominance in male showed no impact in reproductive success. Sexual events occurred mainly in the open area and social events in the burrow.

List of papers

Paper 1

Sociosexual behaviours in cycling, intact female rats (*Rattus norvegicus*) housed in a seminatural environment.

Paper 2

Sociosexual behaviors during the transition from non-receptivity to receptivity in rats housed in a seminatural environment.

Paper 3

Sociosexual behaviors of male rats (*Rattus norvegicus*) in a seminatural environment.

Paper 4

Sociosexual behaviors and reproductive success of rats (*Rattus norvegicus*) in a seminatural environment

1. Introduction

Sexual behavior in mammals, including humans, is an expression of reproductive physiology. To complete such a complex action requires the collaboration of the central nervous system (CNS), the endocrine system, and the reproductive system. Gonadal hormones regulating sexual behavior play a fundamental role in both males and females. There is no other behavior showing such strong hormone dependency. Therefore, sexual behavior has been one of the favorite areas of study for neurobiologists. Particularly, the behavioral studies in rodents have contributed valuable information on how hormones act in the brain when modifying behavior. To explore this, we first need to understand the basic behavior pattern of the subjects.

1.1 Male copulatory behaviors in rats

Rat sexual behavior is generally studied in a standard copulation cage. A small cage in which researchers can observe the interactions between pairs of opposite sex individuals is typically used. Shortly after a sexually active female is introduced to the single male in the cage he starts investigating the female by sniffing, grooming, and licking, particularly near the female's anogenital region. The head and ears of the female are reported as the secondary focus of the male's interests (Dewsbury, 1967). The majority of males start copulating within 1 min following the introduction of the female (Ågmo, 1997). A copulatory act, or "mount", is demonstrated as the male standing on his hind legs and placing his forepaws on the female's rump from behind and displaying pelvic thrusting. This mounting behavior is also observed in same sex pairings, such as male to male (e.g. Sodersten & Larsson, 1975) and female to female (e.g. Fang & Clemens, 1999). Intromission, defined as a penile insertion into the

female rat's vagina during a mount, is ended by a backward thrust and followed by genital grooming.

The first displayed copulatory event (mount or intromission) is the starting point of an ejaculatory series, which can last about 10-15 min in vigorous males. The actual duration of each copulatory act (mount or intromission) can be rather short, lasting about 400 ms (Moralí et al., 2003). The disappearance of penile erection often coincides with the male licking his penis after intromission (Carlsson & Larsson, 1962; Larsson, 1956). Intromission, unlike mount, is essential for successful mating. Without intromission and penile insertion the male would fail to achieve ejaculation (Beach & Jordan, 1956). After each mount or intromission the male rat takes a short rest before he resumes sniffing and pursuing the female again. Mounts and intromissions are performed alternately for 4 -10 min until an ejaculation occurs (Ågmo, 1997). During an ejaculation the penile insertion lasts longer than an intromission, approximately 1.5 s (Moralí et al., 2003) and is associated with rhythmic abdominal contractions. Dismount following ejaculation is rather slow and associated with an open arm posture (Lucio, Manzo, Martinez-Gomez, Sachs, & Pacheco, 1994).

An approximate 4 - 7 min of quiescent refractory period follows the first ejaculation. This interval has two phases, the absolute and relative refractory phase (Beach & Holtzucker, 1949). During the absolute phase the male rat takes a rest, alternating between genital grooming and self-grooming and does not respond to female solicitations (details explained later). The male rat resumes his interest in the female by increasing sniffing or pursuing of female during the relative refractory phase.

In humans male sexual activity usually terminates at the first ejaculation; multiple ejaculations can occur, but are infrequent (Kinsey, Pomeroy, & Martin, 1948). Rats can achieve 5-9 ejaculations before a state of sexual exhaustion or satiety is reached. After that no further copulatory acts are displayed until the males are fully recovered after resting for a

period lasting up to 15 days (Beach & Jordan, 1956; Rodríguez-Manzo & Fernández-Guasti, 1994; Tiefer, 1969; Ågmo, 1997). The number of mounts preceding the first ejaculation vary from 0-20 with the corresponding number of intromissions ranging from 5-10 (Tiefer, 1969; Ågmo, 1997). The exhibition of intromission is necessary for males to achieve ejaculation; males fail to ejaculate when they copulate with receptive females when their vaginas were surgically closed (Kaufman, 1953). A number of studies have reported the intromission number required to achieve an ejaculation declines following first ejaculation and increases postejaculatory intervals between subsequent ejaculations (e.g. Beach & Jordan, 1956; Carlsson & Larsson, 1962; Dewsbury, 1967; Larsson, 1956; Ågmo, 1997).

1.1.1 Measurements of male sexual behaviors

A number of behavioral elements are used to measure male copulatory patterns (Fig. 1). Both the number of mounts and number of intromissions preceding each ejaculation, as we described in the last section, are representative parameters of male sexual activity. The time from when the female was introduced to the moment the male displays the first mount and intromission are termed mount latency (ML) and intromission latency (IL) respectively. Both parameters characterize the male sexual reaction to the appearance of a potential mating partner. The intercopulatory interval (ICI) is calculated as the interval between two adjacent copulatory interactions regardless of whether they are mounts or intromissions. Similarly, the interintromission interval (III) is the interval between two adjacent intromissions. Furthermore, the intromission ratio (IR) represents the capacity of emerging intromission, calculated as the number of intromissions / (number of mounts + number of intromissions). The postejaculatory interval (PEI) refers to the rapidity of the male resuming copulation following ejaculations. PEI is measured as the duration from the previous ejaculation to when the male makes the next intromission.

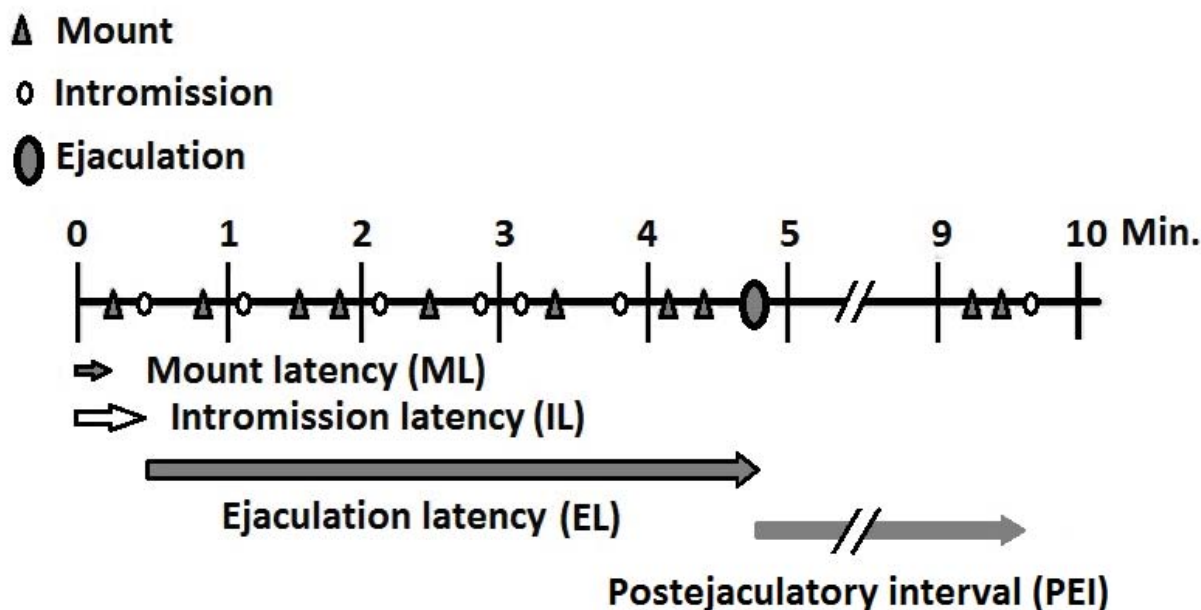


Figure 1. Typical indicators used to evaluate male sexual behaviors in an ejaculatory series.

1.1.2 Behaviors associated with copulation

A male rat usually sniffs and pursues a receptive female before he mates with her. Before a male rat displays a copulatory act, he frequently runs closely behind the potential mating partner. This pursuit of the female is considered to be connected with copulation; as the female is usually very active in running during mating. However, male pursuit of females does not appear very often in the traditional copulation cage due to space limitations.

1.2 Female copulatory behaviors in rats

1.2.1 Elements of female sexual behavior

A distinct spinal reflex is displayed by sexually active females or receptive females in response to male mounting during the copulation. This reflex, known as lordosis (Fig.2 A), lasts approximately 0.5 – 1.5 s, is a posture involving flexion of the back, extension of the neck, and elevation of the hindquarters and rump exposing female genitals and allowing penile intromission by the male. In rodents, lordosis is the ultimate criterion of female sexual

receptivity. Studies of female sexual behaviors undertaken in the 1970s concluded that female lordotic posture was triggered by male mounting behavior (e.g. Beach, 1976; Madlafousek & Hliňák, 1977; McClintock & Adler, 1978). However, it is now known that the appearance of lordosis is associated with hormone regulation and is triggered by tactile stimulations (Kow & Pfaff, 1973; Kow, Zemlan, & Pfaff, 1980).

To achieve this posture cutaneous stimulation via physical contact is essential. Specifically, lordosis is triggered by cutaneous stimulation female's flank when the male applies pressure to the posterior rump, tail base, and perineal region (Pfaff, 1999). Through this sexual interaction, tactile and pressure stimulation provided by the male activate female sensory neurons in the skin. These signals reach the spinal cord, where the contraction of axial muscles, including deep back muscles, are controlled by motor nerves. In some case, lordosis can be accomplished in response to non-copulatory stimulation (e.g. male sniffing or touching the female on any part of the body) and manual stimulations (Blandau, Boling, & Young, 1941).

In addition to tactile stimulation, the central nervous system plays a significant role in facilitating lordosis. According to a series of lesion studies, several brain sites and regions are involved in the mediation of lordosis. The detailed elucidation of neurobiological mechanisms of lordosis behavior is now well summarized in two of Pfaff's books (Pfaff, 1980; Pfaff, 1999). In the cytosol of neurons there is a class of special proteins referred to as hormone receptors that are responsible for detecting specific steroids. During estrus, circulating ovarian steroids first combine with the corresponding receptors (Fig. 2 B) localized in the ventromedial nucleus of the hypothalamus (VMN), the medial preoptic area (MPOA), and the medial anterior hypothalamus (MAH). Binding steroids causes a conformational change in these receptors. Steroid bound receptors activate transcription of variant genes via cofactors including coactivators and cointegrator families (Farach-Carson & Davis, 2003; Nilsson et al.,

2001). Consequently, steroid signaling triggers the production of specific proteins and peptides in neurons.

The newly produced compounds modulate various neuron functions, including transmitting nerve signals from VMN areas to the midbrain central gray region, midbrain reticular formation, and medial geniculated body (Fig. 2 C). The signals are projected via axons to the medullary reticular formation in the brain stem. From there signals reach the spinal cord through the descending fibers where, together with the information received from the cutaneous stimuli, the deep back muscles contract massively resulting in the lordosis posture.

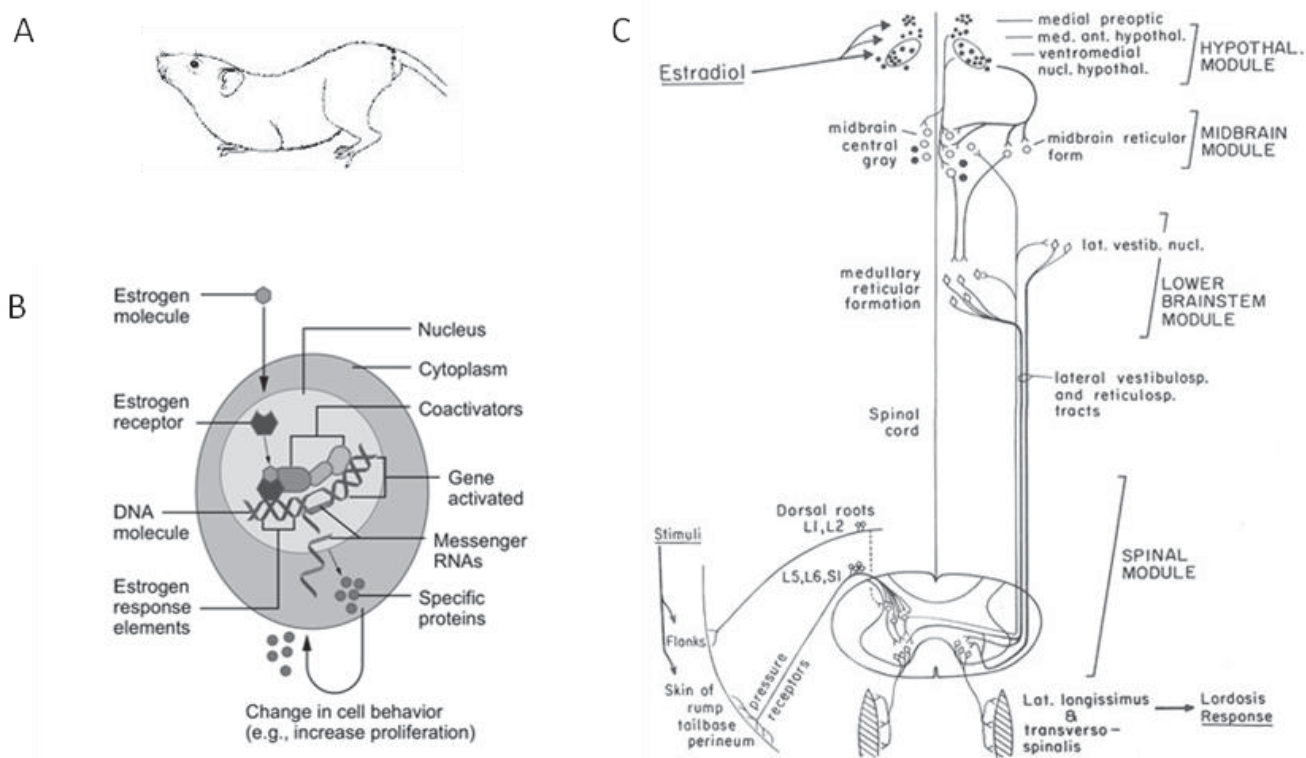


Figure 2. Mechanism of lordosis. (A) Lordotic posture in rats. (B) Diagram of hormone and hormone receptor (e.g. estrogen) shows receptor-mediated transcriptional activation. (C) Detailed diagram of neural circuitry mediating lordosis, the reflex is triggered by cutaneous stimuli, mainly facilitated by estrogenic action in ventromedial hypothalamic

neurons, and is manifest in massive contractions of the deep back muscles (bottom). Its neuroanatomic and neurophysiologic features indicated a modular construction (right), which turned out to match embryologic divisions of the neuraxis. Adapted from (Pfaff, 1999).

In summary, ovariectomy, lesion in the certain brain regions, and reduction of estrogen receptors can result in a lordosis deficit (e.g. Moreines & Powers, 1977; Pfaff & Sakuma, 1979; Spiteri, Ogawa, Musatov, Pfaff, & Ågmo, 2012). Thus, the occurrence of lordosis requires 3 conditions: (i) an adequate amount of circulating ovarian hormones, (ii) sufficient quantity of steroid receptors in the functional VMN, and (iii) cutaneous stimulation of the flanks of the female. Hormonal regulation of female reproductive behavior is addressed below. The level and frequency of lordosis can be used as an index to measure the status of female sexual receptivity. Moreover, the lordosis quotient (LQ), calculated by dividing the number of lordoses displayed by the number of copulatory acts received multiplied by 100, is also used to quantify female response to male mounting.

1.2.2 Behaviors associated with female sexual activities

For the period of copulation, the female displays a series of stereotypical motor activities including ear wiggling, running, and darting. This copulatory facilitated pattern in females has been described as proceptive (Beach, 1976), precopulatory (Madlafousek & Hlišák, 1977) and/or female solicitation behaviors (McClintock & Adler, 1978). Since the complexity of this behavior has been studied in detail previously, these activities are further subdivided into various behavior patterns. Some of these are reviewed by Erskine (1989). Nevertheless, despite different interpretations on the purpose of ear wiggling, running, and darting are all considered paracopulatory activities exhibited by females during mating (e.g. Blaustein, 2009). The function of such behavior can be summarized as (i) female initiation of

sex, (ii) female encouragement of a male to mate, and (iii) female response to the male's approaching. Parallel to lordosis, paracopulatory behaviors are highly hormone dependent, which are enumerated through frequency and duration depending on the test's purpose.

As stated above, lordosis is a tactile reflex which indicates that in order to demonstrate of lordosis, the distance between the female and male should be reasonably small. The female approaching a male or staying in close proximity with a male increases the possibility of sexual interactions. Therefore, displays of paracopulatory behavior seldom occur when a male is a large distance from the female. As with lordosis, paracopulatory behavior is also intensely activated by tactile stimulation provided by the male (Ågmo, 2007; Ågmo, Turi, Ellingsen, & Kaspersen, 2004). However, unlike lordosis tactile stimulation is not necessary for the manifestation of paracopulatory behavior.

Paracopulatory behavior, as an indicator of facilitation of female sexual activity, only appears while the female is enthusiastic for copulation. In contrast, when the female is unwilling to mate she exhibits a copulatory inhibiting action known as rejection; the female performs a kicking or boxing action towards the suitor (male or female), or assumes a belly up posture. This interaction can occur before, during, or after a mating period. The amount of rejection can be used as description of anti-receptive behavior since a fully sexual active female rarely shows rejection.

1.3 Estrous cycle and hormonal regulation

1.3.1 Estrous cycle

Estrous or heat cycle, is the reproductive cycle in most mammals. Estrus duration and interestus intervals vary from species to species. One commonality is that across species females are more sexually receptive during the estrus period than at other times. In most species, estrus is the only period a female is receptive to sexual interactions. Rats, a species

with non-seasonal estrus, usually have a cycle period of 4 – 5 days. In intact female rats, paracopulatory behavior and lordosis are often observed only during estrus, meaning females will mate only during this period. In rats, estrus and ovulation are highly associated. Since the fertilizing life of sperm is about 14 h, mating in estrus will increase the possibility of egg fertilization (Soderwall & Blandau, 1941). If no mating occurs during estrus, female rats do not undergo a functional luteal phase, resulting in a rapid reappearance of the estrous cycle.

The estrous cycle consists of 3 stages: diestrus, proestrus, and estrus (Fig. 3A). Each stage has different physiological and histological features in the ovaries, uterus, vagina, and vaginal smear. Of these the smear is the most common technique to distinguish estrus stage in the laboratory. Estrous stage could be determined by fluctuation in hormone levels during the cycle resulting in changes in the number and types of cells present in the vagina. This is mostly due to cyclical hypertrophy and subsequent sloughing of the uterine epithelium and the concomitant invasion of white blood cells scavenging dead and dying cells. For instance, there is a growing population of cornified squamous epithelial cells with pyknotic nuclei when a female approaches estrus, whereas leukocytes gradually infiltrate during diestrus. Ovulation and sexual receptivity in rats normally take place nocturnally, between proestrus and estrus.

In some studies, despite estrus phase being determined by cytological change, the period with the appearance of sexual receptivity is termed as behavioral estrus (Long & Evans, 1922). Expressed during actual period of sexual interaction, this term is more appropriate in the aspect of behavioral evaluation.

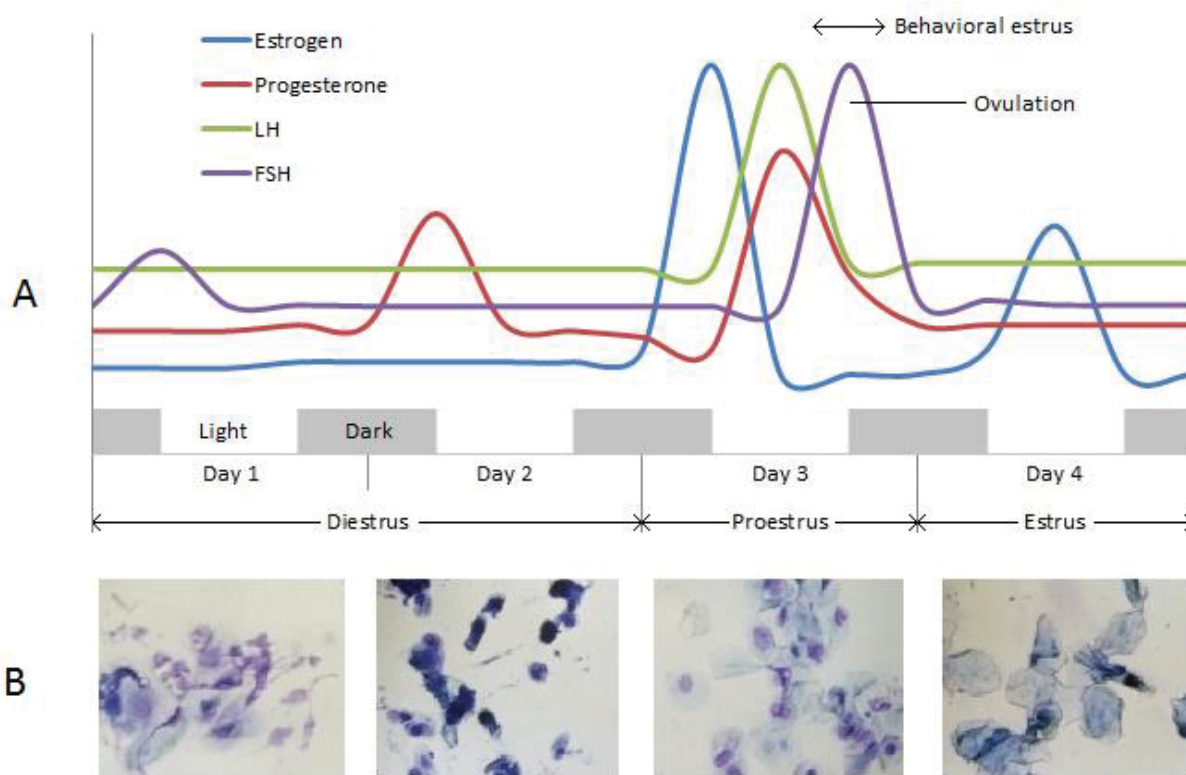


Figure 3. Estrous cycle in an intact female rat. (A) Hormone concentrations altering during 4 days cycle; (B) Cytological change of vaginal cells in estrous cycle.

1.3.2 Hormonal regulation of sociosexual behaviors

As shown in Fig. 3 A, the hormone concentrations exhibit cyclical fluctuations. Modulation of sociosexual behaviors of females during estrous is very much attendant on endocrine levels. Steroids regulate existing neural circuits or initiate a cellular response via receptors that trigger signal transduction pathways. All these may result in behavioral modification. Ovarian steroids, estrogen and progesterone, play a significant role in modulating female sexual and related behaviors during estrus.

An intact female responds to male approach differently depending on estrous cycle stage. She will display rejections towards males while in diestrus and reacts with lordoses during the late proestrus and early estrous stages. It is established that the cyclicity of occurrence of receptive behavior coincides with hormone changes. Using ovariectomized

(OVX) females, studies concluded that sexual receptivity disappears in OVX female rodents but can be restored with estrogen treatment (e.g. Boling & Blandau, 1939; Meyerson, 1964). In young females estrogen injection induces the display of lordosis at an earlier age (21-25 days) (Beach, 1942b). This has provided evidence that estrogen is one of the essential aspects of the appearance of sexual receptivity.

Estrogen signals mainly function through receptors widely distributed in the rat brain (Pfaff, 1968). There are two different coding receptors, ER α and ER β . It has been reported that only ER α is necessary for female reproductive behavior based on studies using knock-out mice (Ogawa et al., 1999; Ogawa et al., 1998). The density of ER α in the VMN was found to enhance female sexual receptivity and paracopulatory behavior and reduce the amount of rejections in rats (Spiteri et al., 2010), and similar results were reported in mice (Musatov, Chen, Pfaff, Kaplitt, & Ogawa, 2006).

As with many gonadal steroid actions, estrogen mediates behavior through a slow transcriptional process (Nilsson et al., 2001). This classical genomic pathway may take hours to several days after the concentration peak of estrogen to affect changes in behavior (Farach-Carson & Davis, 2003; Marino, Galluzzo, & Ascenzi, 2006). For instance, female sexual receptivity (displaying lordotic response to tactile stimulation) appears at least 16 hours after estrogen administration in the OVX female (Green, Luttge, & Whalen, 1970). Most of this latency period is spent facilitating the structural plasticity of neurons, particularly estrogen sensitive neurons including the VMN. The morphology of dendrites, patterns of synaptic connectivity, neuronal soma size, and the overall regional volume can be modulated by the circulating estrogen level (Cooke & Woolley, 2005). These structural modifications appear along in the presence of estrogen and reverse when hormone levels decline (Arnold & Breedlove, 1985). It is described that there is a threshold level of circulating estrogen which is sufficient to induce receptivity. The level in intact females appears to be variable, as

individual females show different estrogen thresholds to achieve sexual receptivity (Powers & Valenste, 1972). Once the threshold is met, the presence of estrogen is no longer required to initiate and maintain a female's receptivity (Södersten & Eneroth, 1981). However, in OVX female rats, the reaction to estrogen treatment is dose dependent (e.g. Spiteri & Ågmo, 2006). At low doses the female displays lordosis in response to a small proportion of mounts and rejection behavior could be repeatedly demonstrated to the mounting male. With increasing dosage the proportion of mounts with corresponding lordosis increases and the frequency of rejections is reduced. Eventually the female will display lordosis to every mount.

In intact females, the modulation of receptivity is mainly regulated by estrogen. The level of estrogen is relatively low during diestrus and no lordosis is observed during this period. Proestrus is the most highly receptive period. The onset of sexual receptivity is gradual, in the sense that only a fraction of a male's mounts activate lordosis (e.g. Madlafousek & Hliňák, 1977). This is also the case at the end of the period of sexual receptivity. For only a few hours in the middle of this period does the female responds with lordosis to all male mounts (e.g. Hardy, 1972). When manual stimulation is used to activate lordosis, it appears that the stimulation needs to be more intense at the beginning and the end of the period of sexual receptivity than in the middle of this period (Blandau et al., 1941).

Besides lordosis, estrogen level is also significant in a female's displays of paracopulatory behavior. Estrogen enhances the production of sexual stimuli such as odors or pheromones, both of which make the female sexually attractive to the male. As mentioned, physical contact provided by a male induces female paracopulatory behavior. Specifically, the male's approach increases the amount of tactile stimulation causing an increase in paracopulatory behavior. Estrogen also increases a female's willingness to approach a male. Approaching a male is an action often preceding the occurrence of paracopulatory behavior. There is data supporting that females in estrus (with high level of estrogen) prefer to spend a

longer time with intact males than with castrated males or a females; this preference disappears when the estrogen level reduces, for example in diestrus (Eliasson & Meyerson, 1975). Giving OVX females a low dose of estrogen or reducing ER α expression in the VMN alter the quantity of paracopulatory behaviors, suggesting these are estrogen and ER α dependent (e.g. Spiteri et al., 2010; Tennent, Smith, & Davidson, 1980; Ågmo et al., 2004)). It should be reiterated that while estrogen action upon ER α effects sexual behavior directly, it also primes the expression of receptors for another ovarian hormone, progesterone.

Progesterone receptors (PRs) can be found throughout the rat brain. It is that this increase of expression of PRs mostly occurs within the estrogen receptor containing neurons (Blaustein & Turcotte, 1989). Therefore, estrogen primed PRs appear generally in the brain regions associated with sexual behavior, such as MPOA and VMN, and arcuate nucleus (ARC) (Blaustein, King, Toft, & Turcotte, 1988). In OVX females, progesterone administration increases the receptive behavior in estrogen primed females (Beach, Etkin, & Rasquin, 1942; Boling & Blandau, 1939; Boling, Blandau, Rundlett, & Young, 1941). It also promotes female sexual related behaviors such as paracopulatory behaviors and inhibition of rejections (e.g. Fadem, Barfield, & Whalen, 1979; Frye, Bayon, & Vongher, 2000; Frye, Bayon, Pursnani, & Purdy, 1998). However, administering progesterone alone in OVX female does not induce sexual receptivity and paracopulatory behavior.

In intact females, increasing receptivity and paracopulatory behavior during estrus are facilitated by the interaction between raised progesterone levels and estrogen induced PRs. There is a gradual increase in progesterone, which precedes the appearance of sexual behavior by a few hours. The gradual onset of lordosis response to male mounting coincides not only with changes in serum concentrations of estrogen, but progesterone as well (Södersten & Eneroth, 1981). Nevertheless, unlike the estrogen, progesterone is not required for sexual

receptivity and does not effect the duration of receptivity (Powers, 1970; Södersten & Eneroth, 1981).

1.3.3 Hormones and female social activity

In addition to the established effects on sexual and sexual related behaviors, estrogen influences non-reproductive actions. Both estrogen receptors were reported to be strongly expressed in hypothalamic neuronal groups, in addition to the brainstem, forebrain, and dorsal raphe (Shughrue, Lane, & Merchenthaler, 1997; Shughrue & Merchenthaler, 2001). In these brain regions estrogens play an important role in behavior and emotionality. Studies in rodents show that estrogen level associate with motor activity, stimulus response, fear, and anxiety. In running wheel tests, estrogen treated OVX female mice performed more activities than control (e.g. Morgan & Pfaff, 2001, 2002). When intact females are in a proestrus state they are also more active than ovariectomized ones, as estrogen was at its peak level (Deelvira, Persaud, & Coen, 1992; Gerall, Napoli, & Cooper, 1973). In the elevated plus maze or the open field test, estrogen treated animals are less active than control animals due to the enhanced anxiety and fear (Frohlich, Morgan, Ogawa, Burton, & Pfaff, 2002; Mora, Dussaubat, & Diaz-Veliz, 1996; Morgan & Pfaff, 2001; Nomikos & Spyraiki, 1988).

1.3.4 Male behaviors affected by female estrous cycle

Male sexual response to receptive females is a rapid process, even in sexually naive males. Therefore, the modification of male sexual behaviors can be induced by female's performances in different states of the estrous cycle. Durations of male pursuit of a female, the time spent with a female, and approaching frequencies, are often used to quantify male sexual motivation.

Several sensory stimuli expressed by females, including olfactory, tactile, and visual stimulations, function in conjunction with hormones and central nervous system (CNS) signal the male to begin mating with the female (Beach, 1942a). Olfactory stimuli play the greatest role in male sexual motivation. The production of these stimuli is hormone dependent since odors from OVX females are far less attractive than odors from intact estrous females or from estrogen treated OVX females (Carr, Loeb, & Dissinge, 1965). Some researchers revealed male rats prefer to spend time with an estrous female, even if direct physical contact with the female is prevented (Landauer, Wiese, & Carr, 1977; Merks, 1983). The attractiveness of these odors, produced by female preputial glands, varies across states of the estrous cycle. Odors from female rats in proestrus were the most attractive to male rats; female rats during diestrus the least attractive. It appears that estrogen plays an escalating role of the odors attractiveness in OVX females, alternatively, a single dose of progesterone results in an opposite outcome (Lucas, Donohoe, & Thody, 1982).

Generally an intact female is not sexually receptive outside of estrus. She displays a series of intense rejections if a male attempts pursue and mount her. However, a vigorous male may sometimes mount a nonestrous female, particularly when he has been previously sexually excited by a receptive female. Since the non-receptive female does not display the back arching posture, ejaculation is not achieved as lordosis is essential for male intromission. Intromission is required for an ejaculation. This indicates that male rats can only achieve a complete copulation with estrous female.

Paracopulatory behavior is also highly hormone dependent as it appears exclusively when the female is in estrus. This behavior is reported to be a critical determinant of sexual motivation in nonresponsive, sexually inexperienced, and recently castrated males, as well as males in long-term castration given low doses of testosterone (Hlinak & Madlafousek, 1977;

Hlinak, Madlafousek, & Mohapelova, 1979; Madlafousek & Hlinák, 1983; Madlafousek, Hlinak, & Beran, 1976; Wishaw & Kolb, 1985).

1.4 Partner preference in sexual interaction

Both male and female rats are sexually promiscuous. The choice of sexual partner can be important in reproductive success since the preferred animal usually has an advantage in siring litters. There is a specific standard for heterosexual mating partner preferences. In order for a rat to demonstrate preference for a rat of the opposite-sex it needs to have the possibility of choice between at least two rats of the opposite sex simultaneously. The determination of male or female preferences can be evaluated on various sociosexual interactions. To choose which interaction as the most appropriate behavioral indicator depends on the purpose of the investigation. For instance, Winland et al. (2012) defined a preferred male rat as an individual rat with whom the female rat spends the longer period of time in a short test when there are two males available. In addition to a behavioral indicator, preference could also be expressed by the choice the testing animal makes, for instance, when the rat was allowed to press a bar for access to one of two obtainable rats of the opposite sex (French, Fitzpatrick, & Law, 1972).

The attractiveness to the opposite-sex conspecific is one potential determinant of mating preference. Attractive incentives can be visual, olfactory, or auditory. Olfactory stimuli are the most powerful of these three. Male sex odors act as a key stimulation to attract the female, likewise female sex odors are very attractive to males. For example, the urinary odors of estrous females are highly attractive to intact male rats (Brown, 1977; Sachs, 1997). Males prefer the urine of estrous females to that of non-estrous females (Carr & Caul, 1962; Carr, Loeb, & Wylie, 1966). On another hand, an anosmic male rat does not immediately

distinguish such differences and shows a constant decrease in social interaction (Thor & Flannelly, 1977).

Most mammals, including rats, have two distinct parts of their olfactory system: a main olfactory system and an accessory olfactory system. The main olfactory system detects volatile and airborne substances, whereas the accessory olfactory system senses mostly pheromones. As reviewed by Keller, Baum, Brock, Brennan, & Bakker (2009), both the main and accessory olfactory systems are able to process sexual chemosignals and both support mating recognition and sexual behavior. Destroying the main olfactory epithelium (MOE) but not the vomeronasal function of female mice renders them no longer able to reliably distinguish odors between an intact and a castrated male (Keller, Douhard, Baum, & Bakker, 2006). This illustrates that the MOE is primarily involved in the detection and processing of odors that are used to localize and identify the sex and endocrine status of conspecifics. Pheromones of conspecifics, detected by the accessory olfactory systems, evolve through the vomeronasal sensory neurons (VSNs) in the vomeronasal organ (VNO), could also modify various reproduction related behaviors (Johnston, 1983).

1.4.1 Female sexual preference

Female preference could also be evaluated by male independent, non-sexual behaviors, e.g. such as the time she spent with males as well as the duration and/or the frequency of approaching, sniffing, or anogenital sniffing of the males. The frequency of lordosis reflex and paracopulatory behaviors could be used as indicators as well. However, these two behaviors are not accurate indicators as both are mostly male dependent.

A female is more active and will spend a significantly longer time with urine samples from an intact male than those of castrated male (Carr et al., 1965; Scott & Pfaff, 1970).

When alternative partners are all intact males the female usually shows preference (staying for

a longer time) for one of them; sometimes the preferred male is consistent among different female rats (Ferreira-Nuno et al., 2010; Ferreira-Nuño, Morales-Otal, Paredes, & Velázquez-Moctezuma, 2005; Lovell et al., 2007; Winland et al., 2012).

Preferred males have shorter intromission and ejaculation latencies than non-preferred males (Taylor & Weiss, 1987). A male with higher a gonadal hormone level is more likely to be preferred; female rats choose to stay with the male with a high concentration of testosterone in their urine (Taylor, Haller, & Regan, 1982). In addition, a study reports that females prefer the male who recently engaged in sexual activity (with females) rather than a male had not (Galef Jr, Lim, & Gilbert, 2008). Studies in other rodent species show similar results. For instance, female mole rats choose to spend a longer period of time next to males with higher testosterone levels in the blood and urine and high blood androstenedione levels than next to those with lower levels of these hormones (Gottreich, Zuri, Barel, Hammer, & Terkel, 2000).

1.4.2 Male sexual preference

When a male rat mates simultaneously with two or more females the preferred female can be defined as the one receiving more copulatory acts from the male (mount, intromission, and ejaculation) than the other females (e.g. Bartos & Trojan, 1982; Tiefer, 1969)). Other criteria for determining male preference have been employed; for example, female preference may be determined by spending more time with a certain female or more non-sexual contact displayed to one of the females, such as pursuit of or sniffing females. Male rats prefer receptive females over non-receptive female or male rats (Carr et al., 1965; Carr et al., 1966; Krames & Shaw, 1973; Ågmo, 2003).

Studies in male mating preference are not as extensive as those conducted with females. Only a few related studies are available in rats. In multiple chamber tests, males

spend a longer time with the first encountered female, and display more mounts and intromissions to her compared with females they meet subsequently (Snoeren, Helander, Iversen, & Agmo, 2014). Results of this study are contradicted by the impression that female's sexual attractiveness is based on the individual differences of the odors, since there is no difference between the duration of male sniffing of individual females. It may be that male mate choice is random.

1.4.3 Dominance in male rats

As female dominance is rarely observed in a laboratory setting (Adams & Boice, 1989; Blanchard, Fukunaga-Stinson, Takahashi, Flannelly, & Blanchard, 1984), it will not be further discussed here. Dominance among males is believed associated with copulation advantage. The pattern of dominance behavior is very well described by Blanchard et al. (1984). The criteria for dominance vary from study to study; the detailed methods of determining dominance rank within a group of subjects are described by Lehner (1998). Dominant males may have more access for limited resources such as food, water, shelter, and even receptive females (Calhoun, 1962; Flannelly & Lore, 1977). A dominant male rat is more attractive to females than a subordinate (Carr, Kimmel, Anthony, & Schlocker, 1982); the latter's mating behavior may be inhibited by the former (Blanchard et al., 1984; Thor & Carr, 1979).

1.5 Fertility and reproductive capacity in rats

Female rats are polyestrous. Male rats are able to copulate at any time after puberty. Female rats ovulate 10-20 eggs during each estrus and the usual litter size ranges from 6-18 pups. After copulation, the pregnant females make nests. The average gestation time of rats is 21 to 23 days.

1.5.1 Female fertility

Regardless of health and the age, the determination of reproductive capacity for young adult female rats relies more on physiological than behavioral features. Several complex mediators such as gonadal hormone level, ovulation process, and vaginal-cervical stimulation, may be involved in female reproductive success. Rats have spontaneous ovulation, meaning that the egg-releasing process is not dependent on whether females copulated previously. Ovulation is triggered by the surge of luteinizing hormone (LH) (Fig. 3). Females usually ovulate at night, about 8 -10 h after the beginning of behavioral estrus (John L. Boling, Blandau, Soderwall, & Young, 1941; Melmed & Conn, 2005; Zarrow, 1964). The timing an LH surge might positively associate with ovulation and female receptivity. This means the early appearance of an LH surge could incite early ovulation and result in greater rates of lordosis during copulation (Gans & McClintock, 1993). The litter size a female produces depends on the ovulation rate (quantity of releasing eggs). Ovulation rate may be manipulated by modifying several different hormone levels or drugs; there are almost no studies reported that ovulation rate may be mediated by females' sociosexual behavior.

Generally a female rat who received little or no copulatory stimulation during estrus demonstrates only a short lasting, non-functional corpus luteum. As a result, progesterone level decreases soon after estrus ends. Although her eggs can be fertilized by a single ejaculation, under this low concentration of progesterone, the fertilized eggs are not able to implant into the uterus or if they have implanted, they will be spontaneously aborted. However, with appropriate stimulation, female rats can maintain a prolonged luteal phase. A rise in serum concentration of progesterone occurs when the female receives consecutive vaginal-cervical stimulations provided by the male's intromission during mating (Feder, 1984; Uchida, Kadowaki, & Miyake, 1969). Thus multiple intromissions (4 or more) preceding the ejaculation are crucial for female's reproductive success (Wilson, Adler, & Le Boeuf, 1965).

1.5.2 Male fertility

In a standard paired test conducted in a small cage, about 7 – 9 ejaculations are achieved by the males before they fail to display sexual activity for 30 - 60 min (e.g. Beach & Jordan, 1956; Larsson, 1956; Rodríguez-Manzo & Fernández-Guasti, 1994). The number of pups a male fathered does not vary among the first 6 ejaculations, but declines sharply after the 6th ejaculation (Austin & Dewsbury, 1986; Tlachi-López, Eguibar, Fernández-Guasti, & Lucio, 2012; Toner & Adler, 1985).

When there is more than one sexually active male available, an estrous female receives mounts, intromissions, and ejaculations from all males as long as she is receptive. It takes 15 - 30 min for sperm to arrive in the fallopian tube (Austin & Short, 1982) and the fertilizing life of rat sperm is about 14 h (Soderwall & Blandau, 1941). After copulation, a majority of ova are fertilized by sperm cells during the third hour following ovulation (Maeda, Ohkura, & Tsukamura, 2000). It is very likely the female is carrying offspring from several males in the same litter, as multiple paternity has been reported (Dewsbury & Hartung, 1980). Although the factors determining a males' fertility success are poorly understood, several hypotheses have been proposed.

Due to female sexual promiscuity and the long fertilizing life of sperm, the postcopulatory competition between the sperm of different males actually takes place in the female reproductive tract. The male who deposited more sperm would have an increased chance of fertilizing the female's eggs and siring offspring. Therefore, the ejaculation order or the number of ejaculations achieved with a particular female are associated with fertility, as the first copulator (male rat) with 5 complete ejaculations displays a significant reproductive advantage over the second copulator (Lanier, Estep, & Dewsbury, 1979). However, it has been reported that a second copulator sires more offspring than the initial male (Moore &

Wong, 1992). This result was later shown occur only when copulation with the second copulator was initiated immediately following the first one's ejaculation (Coria-Avila, Pfaus, Hernandez, Manzo, & Pacheco, 2004). If there is an interval ranging from 5 - 10 min between the two males, the first male sired more offspring. Additional hypotheses for determinants of male fertility include the sperm quality of individual males (Birkhead & Pizzari, 2002) and the position of the seminal plug (Matthews & Adler, 1978).

Behaviorally, dominance has been considered to be associated with reproductive advantage. Again, a dominant male rat is more attractive to females, and has better performance in both sexual behavior and siring offspring than a subordinate males (Dewsbury, 1982). Female preference, another potential determinant of male reproductive success, has been described in many species: females prefer to copulate with highly attractive males rather than with less attractive males (Andersson & Simmons, 2006). Male fertility may also relate to male sexual preference, since male mice showed higher reproductive capacity when they mated with preferred females than with non-preferred female (Gowaty, Drickamer, & Schmid-Holmes, 2003).

1.6 Studies of wild rats

Wild rats live in groups, and occupy a rather extensive area for their daily activities and share elaborate burrows with tunnels and chambers (Calhoun, 1962). A typical organization of a group of rats consists of several females, a small number of males, and offspring (McClintock, 1987). It was observed by Calhoun (1962) that approximately one day prior to a wild female rat entering behavioral estrus, she dragged her anogenital region over the soil around trees, bushes, and both sides of a burrow to mark her scent. Shortly after male rats noticed the female scent they marked their own scent as well. The female rat wandered beyond the limits her normal home range and actively sought males. No copulatory behavior

was observed before she was fully receptive; she copulated with multiple males during the entire period of estrus. As a consequence, many of the males involved in this group sex successfully copulate with the estrous female (Robitaille & Bouvet, 1976), multiple paternity appears to be common in wild rat populations (Miller, Russell, MacInnes, Abdelkrim, and Fewster, 2010). Wild male rat dominance occurs when a male dominant individual is among a group of mixed-sex rats; the dominant male may engage in more sex with receptive females (Calhoun, 1962; Flannelly & Lore, 1977).

Test crosses of wild and laboratory rats revealed that naïve wild rats had some difficulty at first intromission while sexually experienced wild rats required fewer mounts and intromissions to achieve ejaculation (Price, 1980). McClintock & Adler (1978) established that wild pairs had a 17% longer interintromission interval than laboratory males. Apart from these, the differences in behavioral interaction and copulation pattern were relatively scant between the two strains (Boice, 1981).

1.7 Seminatural environment and its external, ecological validity

Behavioral experiments in rats are mainly conducted in a laboratory setting, in which a pair of opposite-sex domestic rats are observed in a small copulation cage for a short period. This setting is convenient and efficient for researchers because each of the research trials only consist of one ejaculatory series, for example, studies of the molecular actions of hormones required for activation of sex behavior or the mechanism of generating copulatory acts. However, as soon as the laboratory observations need to be generalized to the natural world this structure no longer satisfies the demands of a representative design. One of the key concepts of a representative design is that it requires the researcher to perform the experiment in a random sample under an ecologically and externally valid circumstance in order to remove laboratory restrictions; these results would be more effective for real world application (Brunswik, 1955; Petrinovich, 1979). Compared with wild rats' living conditions,

observing the behavioral patterns of a pair of domestic rats in a standard copulation cage has neither ecological nor external validity. Consequently, the results from such tests cannot reasonably be generalized to situations outside of the laboratory setting. Thus in the study of sociosexual behavior, the importance of representative design becomes more significant, particularly when the experimental findings are applied to answer questions involving behavioral patterns and their adaptive value or “biological function”.

The standard copulation cage is a rather small device in which normally only a pair of rats are observed. This means that while the partner can be switched between trials, the number of available sexual partner in such behavioral test is always one. This is completely contrary to the intention of representative design. Rats in the wild live together as a group, and group interactions are far more complex than the interaction of two individuals. Under this paired context, both male and female subjects are prohibited from following the promiscuous nature of rats. Furthermore, the one male and one female setting eliminates the possible implications of group dominance and sexual preference.

A vast majority of experimental studies of rodent social and sexual behavior have employed domestic rather than wild subjects. Nevertheless, to use a domestic strain is not a problem, as behavior differences between wild and domestic rats are limited. It appears that the physical environment is a more important determinant of rat behavior than genetic background (Adams & Boice, 1989; Lore & Flannelly, 1981).

Studying behaviors of wild rats in their native pen satisfies all the demands of a representative design. However, meeting all the requirements of representative design might be not suitable to the present research, since domestic strains have been used in numerous comparable studies and it may potentially impose further limits on the possibility of generalizations to wild rats. An alternative option of representative design is to assure that the laboratory setting shares as many elements as possible with a natural setting (Petrinovich,

1980). To generalize observations upon a group of mixed-sex domestic rats in a lab-based seminatural setting is more applicable and controllable either a copulation cage or native pen.

A seminatural environment is a large device having a large open area and burrow system with complex tunnels and rest boxes. Studies undertaken in a seminatural environment have revealed that the female and male mating pattern in a group is rather different from that observed in a pair (McClintock & Anisko, 1982; McClintock, Anisko, & Adler, 1982). For instance, males and females took turns mating with the opposite-sex; the number of estrous females was associated with the number of intromissions and ejaculations. Furthermore, the dominant male had more intromissions and more ejaculations than the subordinate males. Females participated in same-sex competition for receiving the dominant male's ejaculation. McClintock and colleagues also described that between males and females, it was the female who initiated the majority of sexual interactions; the females were observed darting, hopping, or running, to the attention of the male rats prior to a copulatory event (McClintock & Adler, 1978). These are just a few examples among many valuable results established using the seminatural environment.

1.8 Purpose of the study

Systematic observation of copulatory behavior in a group of rats in a seminatural environment has only been reported in two papers (McClintock & Anisko, 1982; McClintock et al., 1982). A total of 3 groups of rats, consisting of 2 males and 5 females each, were observed. Although sociosexual behaviors were carefully described, many relevant questions were not addressed; such as how the female changes her sociosexual behavior including response to males during estrus, how female behaviors modify when estrus starts and ends, and whether male behavior modifies female receptivity, how male's activities in response to estrus in females, and whether there is a fertility advantage associated with male and female

behaviors. The purpose of the present study is to address these questions by exploring the validity of a seminatural environment.

Additionally, low subject number in the McClintock's studies means the results may have been influenced by individual behaviors. With the development of more advanced software (Observer XT) for recording and analyzing a large number of behavioral items, it is feasible to obtain much more detailed descriptions of behavior within a group than was possible previously. In light of this, a replication and extension of her studies is needed. It is hoped that this example of an approximate representative design will offer new insights into some of the intricacies of rat sociosexual behaviors.

2. General Method

In the present studies we observed 5 groups of intact rats housed in a seminatural environment (Fig. 4) for 8 days. Each group consists of 3 males and 4 cycling females. The seminatural environment (Fig. 1) was based on descriptions of wild rat burrows (Calhoun, 1962) and was similar to the environment used by McClintock and Adler in a series of studies (e.g. McClintock & Adler, 1978; McClintock, et al., 1982). Briefly, the 2.8 x 2.4 m device consisted of a complex burrow system and an open area. There were 4 small openings (8 x 8 cm) between the burrow and the open area. The floor was made of dark grey unplasticized polyvinylchloride. A light-blocking wall of extruded polyethylene foam was used to divide the room into two parts, thereby providing the possibility to vary the light intensity in the open area while maintaining the burrow in complete darkness. Infrared lamps provided light for the video camera centered above the burrow. Another camera was centered above the open area.

The Observer XT 10 (Noldus, Wageningen, The Netherlands) was used to determine the frequency and/or the duration of the behaviors defined in Table 1. The emitter and recipient of the behavior were recorded whenever relevant. The location of the subjects interacting within the environment was also registered.

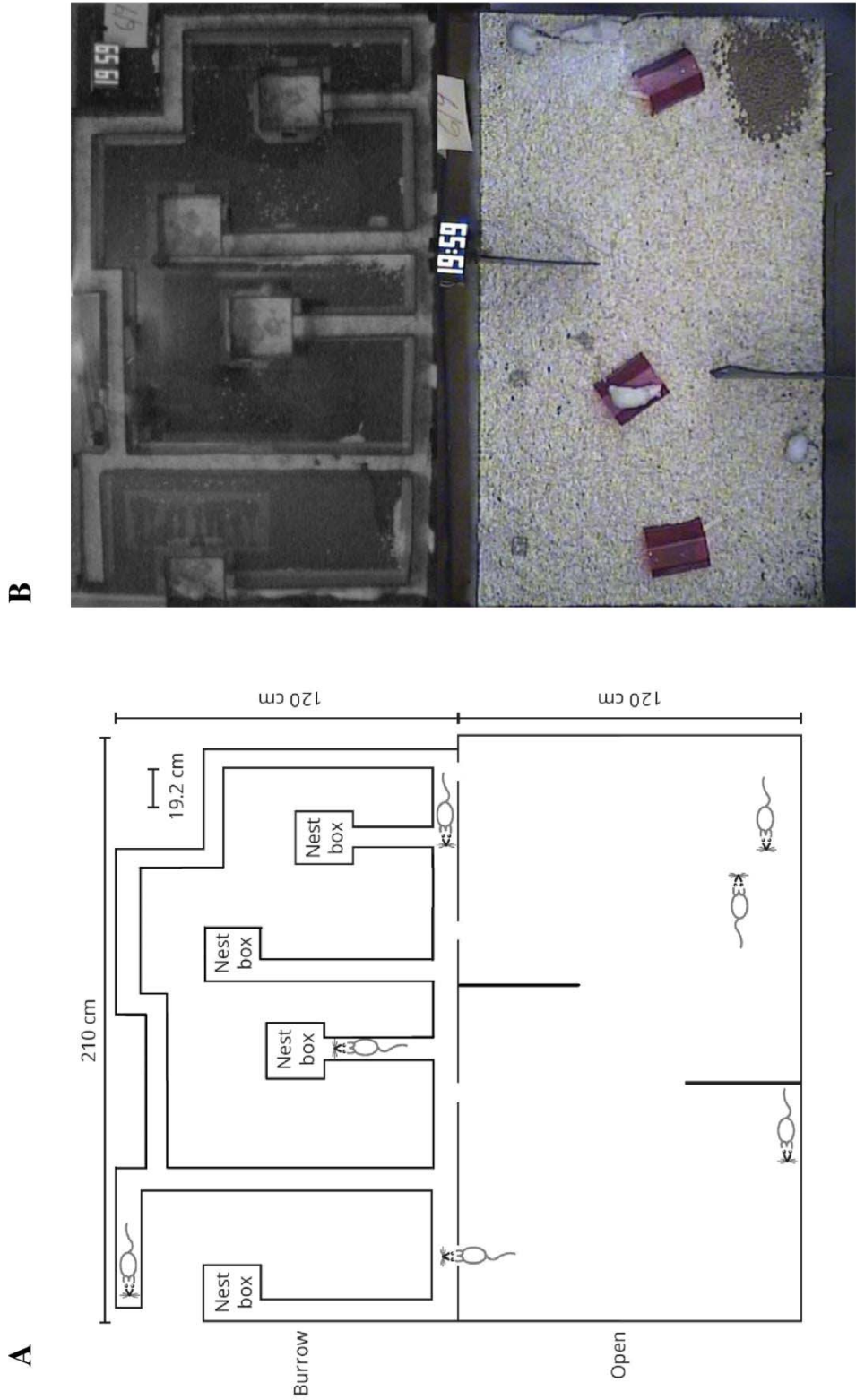


Figure 4. Schematic diagram (A) and photograph (B) of the seminatural environment used in this study. For further details, see Paper

1.

Male and female behavior	Data collected as	Behavior description
Sniffing	Duration	The rat places its snout close to any body part, except the anogenital region, of another rat while its whiskers move briskly.
Anogenital sniffing	Duration	The rat sniffs, occasionally grooms and licks, another rat's anogenital region.
Pursuit	Duration	The rat runs closely behind another rat.
Fight	Duration	Two rats form a tight ball, rolling around while biting.
Nose-off	Duration	Facing another rat either standing on 4 legs or while rearing; it includes boxing and teeth showing.
Allogrooming	Duration	The rat grooms another rat.
Flee	Number	The rat runs away from another rat after an agonistic interaction.
Male copulatory behavior		
Mount	Number	The rat stands on its hind legs and places its forepaws on another rat's rump from behind and displays pelvic thrusting.
Intromission	Number	Mount associated with penile insertion. The mount is ended by a backward thrust and is followed by genital grooming.
Ejaculation	Number	Penile insertion lasts longer than at intromission and is associated with rhythmic abdominal contractions. Dismount is slow and associated with an open arm posture.
Female behavior		
Paracopulatory behavior	Duration	Approach to a male followed by runaway, often associated with hops, darts, ear wiggling.
Lordosis	Number	Female stands immobile with the back arched downward and the rump pushed upward while the tail is deflected to the side.
Rejection	Number	The rat kicks, bites or turns around against its suitor.

Table 1 Description of registered behaviors.

3. Summary of the papers

Paper 1. Sociosexual behaviours in cycling, intact female rats (Rattus norvegicus) housed in a seminatural environment

Behavioral estrus was defined as starts at the first lordosis response observed in the environment and ends at the moment of a lordosis that was not followed by another one within 60 min. Sociosexual behaviors of the males and the females were recorded whenever one or several females were in behavioral estrus. In order to describe female behavior changes during behavioral estrus, we equally divided this period into 20 equal units, and each unit consisted of 5% of the length of estrus. The duration of behavioral estrus varied between 4.05 and 10.87 h, with a mean of 7.41 ± 0.49 h. In order to analyze changes in behavior during estrus, the entire period was divided in 5 % segments for each female. The frequency and/or duration of some behaviors changed during the period of estrus, while others remained stable. Most remarkable among the latter are the frequency of lordosis and the lordosis quotient. From the start of estrus until the end, the females responded with lordosis to every male mount. Rejections were most frequent at the beginning of the period of estrus. Then there was a decline, and thereafter rejections remained at a low level, even when the end of estrus was approached. Paracopulatory behaviors reached a maximum towards the end of the first quarter of the estrus period. There was no evident decline at the end of estrus. Females having a high frequency of lordosis showed more paracopulatory behaviors and were pursued more by the males than females with low lordosis frequency. When the behavior of females with a large number of offspring was compared to that of females with a low number of offspring, no difference in sexual behaviors was found. Comparisons between the females' behavior in response to preferred (either defined as the male producing the largest number of lordosis

responses or as the male that was most sniffed by the female) and non-preferred males revealed a few minor differences. Location of behavior was also analyzed. Some important differences were found in the comparisons made between the environment's open area and burrow and between different sectors in these parts.

Paper 2. Sociosexual behaviors during the transition from non-receptivity to receptivity in rats housed in a seminatural environment

In a seminatural environment with several males and females, an estrous female consistently displays lordosis in response to every male mount from the start of behavioral estrus until the end of it. This means that the female suddenly changes from a state of complete non-receptivity to a state of full receptivity and then abruptly changes back to non-receptivity. The mechanisms behind this swift receptivity change remain unclear. Here we present the results of a detailed study of sociosexual behaviors during the transition from non-receptivity to receptivity and vice versa. A preestrus phase was defined as a period before the initial lordosis and the postestrus phase was the period following the final lordosis. The duration of the preestrus and postestrus phases analyzed here was 5% of the length of estrus. Behaviors during these phases were compared to those observed during the first and last 5 % of behavioral estrus. The frequency of male mounting of the female was close to 0 both before and after estrus. It remained at a constant, high level throughout the period of estrus. The duration of the female's paracopulatory behaviors and of the males' pursuit of the female changed drastically from a very low level before estrus to a high level in estrus. It was strongly reduced in the postestrus phase. The female sniffed, anogenitally sniffed and pursued males equally before, during and after estrus. Avoiding behaviors such as fleeing from the male, rejection and nose-off did not change when the female entered into or went out of

estrus. By comparing the behaviors in short segments immediately before and after estrus, it turns out that most of the transition-associated changes occurred within 30 s preceding or following the first and last lordosis. These data show that the main changes occurring when the female enters into behavioral estrus do not occur in female but in male behavior. The males pursue the female a lot more, and they start to mount her. Likewise, at the end of estrus they do no longer pursue the female and they don't mount her. Since the paracopulatory behaviors mainly are responses to the males' approaches, they increase and decrease in parallel with the males' behavior. It appears that the female becomes attractive to the male only when she is in a state of full receptivity. In fact, there are data showing that receptivity requires less estrogen than attractiveness. The important role of the male in the determination of the duration of the female's behavioral estrus could not have been detected in a short mating test in a small cage housing a pair of rats. This is another example of the utility of seminatural environments for understanding the details of sexual behaviors.

Paper 3. Sociosexual behaviors of male rats (Rattus norvegicus) in a seminatural environment

Male behaviors were examined in copulatory bout and ejaculatory series. A copulatory bout was defined as the time between the initial mount or intromission and the beginning of a period of sexual inactivity lasting for more than 60 min. The results showed that the traditional way of conceiving male sexual behavior as a series of ejaculations in which the last ejaculation is followed by a long period of sexual inactivity turned out to be inadequate. Instead, male sociosexual behaviors occurred in bouts, which could be ended either by mount (mean \pm 95 % confidence interval was 38 ± 13 % of all bouts), intromission (21 ± 15 %), or ejaculation (40 ± 18 %). The males performed a median \pm semiinterquartile range of 101 ± 67

mounts, 59 ± 16 intromissions, 6 ± 2 ejaculations and 4 ± 1 copulatory bouts during the 8 days experiment. Copulatory bouts had median duration of 2.8 ± 0.9 h. During a bout, 77 ± 4 % of the time was spent resting and grooming whereas 8 ± 2 % of the time was used pursuing the receptive female. The copulatory acts themselves took a very small fraction of the time (less than 0.3 %). Within a bout, the intensity of sexual behavior remained quite stable. Interestingly, there was no decline as the end of the bout approached. The interbout interval was 98 ± 22 min. There was no relationship between the last event in the preceding bout and the interbout interval. Regardless of whether there was single or multiple females in estrus, the males copulated with all available females in an apparently random way. When more than one female was in estrus, the males switched partner more frequently after intromission or ejaculation than after mount. Nevertheless, it appears that male rats are completely promiscuous and change partner several times within a bout. Concerning the use of space, it was found that social behaviors like sniffing and anogenital sniffing were more frequent in the burrow whereas sexual interactions (copulatory acts and pursuit of the receptive female) were more frequent in the open area.

*Paper 4. Sociosexual behaviors and reproductive success of rats (*Rattus norvegicus*) in a seminatural environment*

When rats mate in groups, females copulate with multiple males during estrus, which often results in litters with multiple paternities. A promiscuous pattern of copulation has been reported in both wild and domestic rats. The purpose of the present study was to determine whether male sociosexual behaviors are associated with reproductive success or not. Paternity of the offspring sired was determined by analyses of single nucleotide polymorphisms (SNPs). Fertility was quantified either as total number of offspring or the mean proportion of

offspring from all females sired by the male. Although the total numbers of male social and sexual behaviors during the 8 days period was unrelated to reproductive success, male fertility was correlated with the number of intromissions and ejaculations performed with a particular female. In addition intromission and ejaculation ratios (the proportion of mounts ending in intromission and the proportion of the total number of ejaculations received by a female and the number contributed by a particular male, respectively) were also correlated with male fertility. Fewer mounts as well as fewer male pursuits of female were demonstrated by males siring entire litters than by males siring multipaternity litters. Ejaculation order was unrelated to fertility. Male or female preferences (based on various sociosexual interactions) were not strongly related to fertility. Female preference only showed a limited effect. Specifically, preferred males (males who were sniffed most) sired fewer pups. Male dominance status did not affect female preferences, copulatory behavior or fertility. It appears that only behaviors directly related to sperm transfer are important for fertility. Social behaviors both in males and females play a very limited role in determining rats' fertility.

4. Discussion

Both female and male rats copulated with the opposite sex randomly. Females received mountings, intromissions, and ejaculations from any available male. The males displayed copulatory acts to any available female. Aggressive activities, such as fighting, were rarely observed. Subjects of either sex did not show a significant preference or avoiding pattern to a certain sexual partner.

4.1 Female behaviors and female role in fertility

4.1.1 The modification of female receptivity in estrus

Female rats are normally considered to be in behavioral estrus whenever they demonstrate lordosis, the duration of behavioral estrus varied among individuals. In order to describe behavioral changes during behavioral estrus, the estrus period was divided into short units. Each unit consisted of 1/20 of the length of estrus. Preestrus and postestrus intervals were the same length as that unit. Change in female receptivity gave the impression of a sudden and increasing onset, then stayed stable throughout the entire period, and ended abruptly.

Displaying lordosis is a sensory reflex rather than an initial activity. Therefore, the significance of the frequency of lordosis was minor; the LQ value (lordosis quotient = number of lordosis / number of received copulatory act) is more descriptive of female response to males. During estrus the LQ was always slightly over 100% (Paper 1), meaning the female responded to all the male's mountings until the male stopped mounting her. Except for male mounting, in Paper 1 and 3, female sexual receptivity highly correlated with paracopulatory behavior and male pursuit of the female, but no correlation exists between receptivity and intromission or ejaculation. Therefore, it was not surprising to learn that female receptivity showed no relationship with female or male fertility capacity.

According to our understanding of behavioral estrus, it was expected that females would show no lordosis outside of behavioral estrus, even in the preestrus and postestrus intervals. Lordosis started when any male mounted a female for the first time. From then the lordosis level was constant during the entire estrus. This result was contradicted Madlafousek & Hlišák (1977), who found that the onset of female receptivity is gradual and that at the beginning of estrus females only responded to some of the male mountings. Although lordosis is strongly affected by estrogen, we hypothesized the onset of this rapid appearance of receptivity was unlikely to be estrogen dependent. Sensory stimulations could induce female lordosis display. Along with tactile stimulation mentioned above, olfactory stimuli played a crucial role in the onset of receptivity. Data supports that hormone primed OVX females showed a slightly increased number of cells expressing ER α after 2 h exposure to male odors (Bennett, Greco, Blasberg, & Blaustein, 2002). The possible causes of this abrupt behavioral modification might involve female-male communications, male-triggered interactions, and the mediations of several neuron transmitters, the details of which were discussed in Paper 2.

4.1.2 The modification of paracopulatory behavior

As reported in Paper 1, females displayed a high degree of paracopulatory behavior (hopping, darting, and ear wiggling) during estrus. Within several minutes before a female turns to be sexual receptive, there is almost no paracopulatory behavior displayed. It appeared that paracopulatory behavior increased suddenly from almost zero to the full level within a short duration of 30 s prior to the first lordosis displayed.

The sudden change of paracopulatory behavior immediately prior to the first lordosis is unlikely to be related with ovarian hormones. As mentioned in Paper 1, paracopulatory behavior was highly correlated with male pursuit of the female and male pursuit normally preceded a mount (Ågmo, 1999). During preestrus the appearance of the first lordosis

triggered by male mount was always preceded by an episode of paracopulatory behavior of a relatively long duration. Male rats could distinguish the female by their sexual status, since males only pursued the female already in estrus but not the female in preestrus or postestrus state. Length of paracopulatory behavior episode could be an indicator of female attractiveness.

Paracopulatory behavior exhibited an extremely close relationship with female receptivity (Paper 1). Albert et al. (1991) suggested that female attractiveness requires more ovarian hormones than the activation of receptivity. This might provide the explanation of declined paracopulatory behavior after the last lordosis displayed in estrus (Paper 2). Paracopulatory behavior is also highly correlated with males displaying copulatory acts, especially the quantity of mounts (Paper 3), but made no contribution towards reproductive success (Paper 4).

4.1.3 Female social activity

The amount of affiliated behaviors, such female anogenital sniffing and pursuit of male, were consistently low in both transitions and behavioral estrus. Of these, female pursuit of a male was representative a negative factor in female fertility; the more a female pursued the fewer pups she produced (Paper 1). Female sniffing of males occurred more often than the two activities listed above. Sniffing showed a strong association for male copulatory behavior, as the female sniffing a male correlated with the number of mounts but not to intromission or ejaculation (Paper 3). Furthermore, female sniffing of male may have a limited impact on male fertility since females who sniffed males on average sired fewer pups (Paper 4). During estrus females rarely ran away from males, but some displayed a nose-off posture to males. The duration of female initiated nose-off did not change during the time from nonreceptive to

receptive or vice versa (Paper 2), and female nose-off to male showed a negative correlation with male ejaculation (Paper 3).

This result coincided with several studies which reported that a preferred male does not produce more offspring than the less preferred one (Taylor & Weiss, 1987; Winland et al., 2012; Zewail-Foote, Diehl, Benson, Lee, & Guarraci, 2009). Although female preference (e.g. sniff more or nose-off less) for a particular male means the preferred male might have the advantage in sex, it has no significance for that male's reproductive success.

Rejections, as another nonaffiliative activity, did not change from preestrus to estrus, but declined from the beginning to about a third of the way through estrus, after that it remained consistently low even in the postestrus transition. During both transitions (non-receptive to receptive and receptive to non-receptive), the number of rejections did not change by alteration of receptivity status. However, ovarian hormone level is important to displays of rejecting behavior. As already explained, progesterone increased along with the appearance of intromission. The decline which took place shortly after estrus started could be induced by the increased progesterone level, as progesterone inhibited the female rejection during copulation (Fadem et al., 1979; Frye et al., 2000; Frye et al., 1998). Thus rejection did not seem to be associated with the appearance and disappearance of sexual receptivity (Paper 2). Female rejection demonstrated no influence in either male sexual behavior (Paper 3) or fertility capacity of either the female or the male (Paper 1 and 4).

One of the interesting discoveries proposed in Paper 4 was that female behavior and/or attractiveness might play a greater role in cases of multiple paternity. It was found that females with offspring from multiple fathers were more pursued and mounted by the males; these females displayed more paracopulatory behaviors than females in which there was a single father. It appears that these females were more attractive to the males and/or responded more to the male's approaches.

4.1.4 Female promiscuity and sexual preference

Females in behavioral estrus responded with lordosis to any copulatory act displayed by any male, and the sequence of male partners appears as a disordered pattern. A female might switch partners from one lordosis to another, and it was found the possibility of partner change was time dependent (Paper 3). It appeared that if a male could perform two conjunctive copulatory acts with an interval of less than 2-4 min, the female would continue copulating with him. There was no stable sexual preference to a certain male that could be established upon lordosis or paracopulatory behavior. This result coincided nicely with a recent study in which females copulated with multiple sexual partners in random order (Snoeren et al., 2014; Snoeren & Ågmo, 2014)

4.2 Male behavior and male fertility

Male rats showed less sexual interest in nonestrous female when their sexual experience increased. Actually the male only copulated with female(s) when they were fully receptive, therefore they reduced pursuit of female rapidly after her estrus approaching to the end (Paper 2). Male sexual events occurred in copulatory bouts; there could be one or several copulatory bouts during female behavioral estrus. The length of a bout is unrelated to the intensity of male sexual behaviors (e.g. mount and intromission) but is associated with the total amount of those behaviors. Male's copulatory behaviors only took a very small proportion of the total time (less than 0.3 %) compared with other social interactions, and they were quite stable throughout the bout. Male rats were not always sexually active even when there was female available since there was at least one hour quiescent between bouts. After the first bout, there appeared a decline in intromission and ejaculation in the second bout. The

reason for the existence of the long pause between copulation did not involve sexual exhaustion. All of these findings are discussed in Paper 3.

4.2.1 Male copulatory behavior

Since a male approaching a female increased the opportunity of sexual interaction, there was no spurious association between the quantity of mount, male pursuit of female, lordotic response and paracopulatory behavior (Paper 1 and 3). Apart from this, mount was found to have no relation to male reproductive success (Paper 4). This finding was not unexpected as mounts did not necessarily equate to sperm transfer. The number of intromissions did not effect male fertility capacity directly; however, the indirect effects including eliciting ejaculation were essential. In contrast, ejaculation was required for reproductive success, meaning this copulatory behavior was more significant. In Paper 4, we discussed two possible determining factors of male fertility with regards to ejaculation: total number of ejaculations and ejaculation order. The proportion of the variance in fertility that can be attributed to the number of ejaculations is only 15%. Other factors, for example the position of the seminal plug (Matthews & Adler, 1978) or the quality of sperm (Birkhead & Pizzari, 2002; Dewsbury, 1982) might be far more important in determining male fertility than copulatory behavior.

4.2.2 Male social behavior

In a copulatory bout male rats used 77 % of their time resting, wandering, or other activities without interaction with another subject. This “self-entertaining” behavior accounted for greatest proportion of time. Males only used about 12% of this time to pursue (8%) and sniff (4%) receptive females (Paper 3). Male social behavior, including affiliative

and non-affiliative, activities, turned out to be irrelevant to intromission or ejaculation and had no importance to male fertility (Paper 3 and 4).

Dominance in the present study seemed to be only a vague pattern. One reason was males hardly provoked any fights; the most common aggressive behavior observed was nose-off. There was indeed an avoidance action between males that were described as a male taking flight or running away from another male. In some groups, even flight was very uncommon, and dominance based upon flight did not confer any advantage in sex or fertility. This coincides with the observation in wild rats that inter-competition for sex between males is unusual in the wild (Barnett, 1958, 1975) and wild rats usually live together peacefully in large packs (Eibl-Eibesfeldt, 1961) with attacks only occurring towards unfamiliar introducers.

4.2.3 Male promiscuity and sexual preference

Males preferred receptive females over nonreceptive females, except for this, they did not display any preference towards a certain female when having copulated with multiple females. Compared with McClintock's studies, our studies illustrated male mated promiscuously which contradicted their description of male copulating in turn. McClintock et al. (1982) described when one male copulated with a female, another male waited for the former one to enter a postejaculatory interval, during which the second male copulated until ejaculation. The first male then resumed copulation and continued to copulate until he again ejaculated, upon which the second male started to copulate, and so on. However this turn-taking was not observed in our males, they copulated in a random pattern with female(s) (Paper 3).

4.2.4 Male role in sexual interaction

In Paper 2, we observed that before a male triggered a female to display her first lordotic reflex, the male barely sniffed or pursued the female. Similar behaviors were exhibited by the male after the female displayed her last lordosis. Male pursuit of the female increased from nearly zero within the last 30 s before the male's first mounting. Whether a male or a female initiated the leading action preceding a copulatory act was studied by McClintock & Adler (1978). The authors mentioned females initiated the majority of sex interactions and females regulated the pace of copulation by displaying paracopulatory behavior. As discussed in Papers 1 and 3, in seminatural environment the proportion of female induced male pursuit was not higher than male self-initiated pursuit. McClintock's results were based on only 100 encounters of 6 males, and were likely influenced by the small sample size. Thus the male's role in sexual interactions was underestimated while the female's was likely exaggerated.

4.3 Spatial distribution of sociosexual activity in a seminatural environment

In accordance with the results presented in Papers 1 and 3, during the time when no female was in behavioral estrus, both males and females generally stayed inside the burrow and only occasionally visited the open for a short period. When there was a receptive female, the receptive female spent more time in the open area than the nonreceptive females thereby inciting the males to leave the burrow. The location of the female displaying the first lordosis was a preferred spot for a female to display subsequent lordosis. It is quite likely that female spatial preferences determined the location of sexual interaction. Sex and sex-related interactions occurred mainly in the open area, while the burrow seemed to be used as a place of retreat to avoid approaches of other group members.

General conclusion

When several males and females copulate simultaneously, they show a promiscuously and random copulatory pattern. Sexual behaviors are quite independent from non-sexual social behaviors including affiliative and non-affiliative activities, in both sexes. Sexual events occurred mainly in the open area whereas social events in the burrow, which is more likely determined by female spatial preference. Female rat remains completely receptive from the first lordosis displayed in the period of behavioral estrus until the last. There is no reduction in paracopulatory behaviors and no increase in rejections towards the end of estrus. It appears that, in prolonged observation of groups of rats in a seminatural environment, female paracopulatory behavior and receptivity as well as male pursuit change in a most abrupt way at both initiation and termination of estrus. The amount of ovarian hormones needed for activating lordosis is lower than that needed for supporting female attractiveness, which might express as relatively long episodes of paracopulatory behaviors, since there is almost no such paracopulatory behavior appears outside of behavioral estrus. It seems like male would not pursue the female unless she is attractive, and the duration of behavioral estrus in a seminatural environment might be determined by the males' responses to the female as much as the female's responses to the male. The intensity of male sociosexual interaction is stable throughout the copulatory bout. Unlike mating in small cage, male rats do not copulate beyond their maximal reproductive capacity when a representative design is used. Non-sexual, social behavior including dominance and preferences among rats is rather unrelated to reproductive success. Although males with greater reproductive success achieve more intromissions and ejaculations, however the order of ejaculations shows no reproductive advantage. Actually the position of the seminal plug or the quality of sperm might be far more important determining factor of fertility than ejaculations and intromissions.

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