BOOK CHAPTER

in: Animal Models of Reproductive Behavior, the Importance of Analyzing Sex Differences

SEXUAL INCENTIVE MOTIVATION

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Summary/Abstract

Motivation can be described as the processes that activate, direct and determine the persistence of goal-directed behavior. A certain level of sexual motivation is required to activate approach behavior which could potentially lead to copulation and reproduction. Here, we discuss the use of the sexual incentive motivation test to assess the magnitude of sexual motivation in rodents in response to different incentive stimuli. Our goal is to provide the reader with guidelines on how to set-up the test and what measures can be considered to study sexual incentive motivation.

1. Introduction

1.1 Sexual incentive motivation

Motivation can be described as the processes that activate, direct and determine the persistence of goal-directed behavior. A certain level of sexual motivation is required to activate approach behavior which could potentially lead to copulation and reproduction. As summarized in [1], different models of motivation have been discussed throughout history that mainly focused on the role of motivation for the continuation of the behavioral act. However, sexual motivation could be subdivided into two different components: the incentive motivation and the motivation to continue copulation. Incentive motivation could then be seen as the magnitude of motivation that is present *before* approach behavior has occurred, and the motivation to continue copulation as the processes needed to maintain the continuation of the behavior until satiety.

As proposed in a model from Ågmo and Bindra [1-3], sexual incentive motivation is based on three important elements: a central motive state, an incentive stimulus, and a certain level of general arousal. The central motive state was defined as "a hypothetical set of neural processes that promotes goal-directed actions in relation to particular classes of incentive stimuli" [2]. This internal state of the animal if brought to a certain level, can activate sexual incentive motivation and trigger the individual to take action. However, the internal state by itself will not induce approach behavior. For that, an incentive stimulus needs to be present, resulting in a positive effect on the central motive state. The incentive stimulus may be any stimulus with hedonic properties. With regard to sexual motivation, the incentive stimulus could for instance be the presence of an attractive partner. Last, but not least, a level of general arousal is also required to activate the central motive state. This means that the individual should be ready for the perception of an incentive stimulus. In a state of sleep, for instance, general arousal is absent, but even when awake, different factors can influence

general arousal, such as the hormonal status of the individual. When all elements are present and cause an enhancement of the central motive state, a level of sexual incentive motivation can be reached which may induce approach behavior towards the mate partner.

As already mentioned, gonadal hormones play an important role in the determination of sexual incentive motivation. Not only are hormones necessary for the appropriate internal state leading to sexual incentive motivation, and do they influence general arousal, potential partners will also only have an incentive value when they are in the appropriate hormonal state. A female rat, for instance, will approach an intact male more frequently when she is sexually receptive, i.e. on the night of the transition from proestrus to estrus, shortly after estrogen and progesterone levels have peaked [4, 5]. Similarly, only receptive females are normally sexually approached by intact males [6]. Castrated male rats and non-receptive females are not a sexual incentive to their conspecifics, and will also not sexually approach a potential mate [6]. Thus, the appropriate hormonal status that drives sexual motivation also causes animals to emit certain stimulatory signals making them sexually attractive. In rodents, the secretion of specific odors and pheromones is an example of such a signal that is important for the incentive value of males and females. Gonadally intact male rats approach the odor of sexually receptive females more than they approach non-receptive females [7-10], while anosmic males lose their capacity to distinguish between females in estrus or non-estrus [11]. Male odors also seem to have the same effect on female rats, i.e. receptive females prefer odors of intact males over odors of castrated males, and exposure to male urine can affect subsequent copulatory behavior [7, 12].

Olfactory stimuli are not the only factors determining the incentive value of a stimulus. As approach behavior to a potential mate can be induced by distant stimuli, other potential sensory modalities involved in mate choice include audition (e.g., via ultrasonic vocalizations) or vision (the sight of a potential mate). In rats, visual stimuli are not as

important, since males approach females even in complete darkness [13, 14]. The role of auditory stimuli is potentially more complex. Although rats emit 50 kHz ultrasonic vocalizations (USVs) in the presence of a sexual partner and during copulation [15-17], we have shown that these USVs do not have any incentive value for male and female rats, i.e. playback of USVs alone do not induce any preference, and devocalized potential mates have the same incentive value as intact mates [9, 18]. Interestingly, a study in which the different modalities and the combination of these modalities in inducing sexual incentive value was studied, showed that there is a synergistic function for the different modalities in increasing the incentive value of an individual and thus the induction of approach behavior towards a potential mate [19]. Specifically, the presence of a single-sensory sexual stimulus with one modality (olfaction, vision, or 'others', but not audition) was sufficient to induce approach, but not above approach towards a social stimulus animal. A multisensory stimulus of multiple modalities was necessary to induce incentive sexual motivation (i.e. a preference over a social stimulus) [19].

Another important fact is that sexual incentive motivation, at least in rats, is unconditioned, meaning that sexual experience is not necessary for sexual motivation to be induced by sexual incentives. In fact, sexual experience does not even modify sexual incentive motivation in either male or female rats [6, 20]. This is remarkable, because gain of sexual experience reduces mount/intromission latency and ejaculation latency in a copulation test in males [21], and influences paced mating behavior in females (e.g. return latencies after intromission and ejaculation) [22]. Urine of a receptive female alone can elicit approach behavior in gonadally intact male rats, even when still sexually naïve. However, when bedding of a receptive female is used as olfactory stimulus (meaning a mix of urine with other odors), sexual motivation is induced in sexually experienced, but not in sexually naïve animals [6]. This illustrates an important point; whereas sexual motivation itself is innate and

unconditioned, it seems that a learning component through rewarding sexual experience leads to a greater capability of perceiving sexual incentives during future encounters under complex conditions with several (non-sexual) stimuli present.

After the induction of sexual motivation, approach behavior could follow leading to the start of copulation. Sexual behavior as a whole can thus be divided into different phases: the introductory phase, copulatory phase and executive phase (ejaculation or orgasm) [23, 24]. It is important to note that these different phases may be driven by different neurobiological processes, at least in rats [25]. Different subregions of brain areas are activated during the different phases [26], suggesting that different mechanisms are involved in the different parts of sexual behavior. The dissociation between motivation, copulation and ejaculation is also evident in lesion studies showing e.g. that motivation or copulation can be abolished while ejaculation is stimulated [27], or chemogenetic studies in which incentive motivation remained unaffected while ejaculation was disrupted [28]. Furthermore, the relative magnitude of sexual incentive motivation (measured as relative preference for a sexual vs. a social incentive) does not correlate with several outcome parameters of copulation assessment in either male or female rats. For example, the latency to mount and number of paracopulatory behaviors, which are often taken as measures of sexual motivation in copulation tests, as well as number of mounts and intromissions, ejaculation latency, and duration of post-ejaculatory interval in males, and percentage of exits and return latency after mounts and intromissions in females, do not correlate with sexual incentive motivation measured in a separate test[24, 29]. These lack of relationship between these measures can then be attributed to the different elements of sexual motivation, with incentive motivation on the one side and motivation to continue copulation on the other. Together, the lack of a correlation between motivation and copulatory measures as well as the ability of physiological manipulations to affect motivation but not copulation stress the relevance for why it is important to study the different phases of

sexual behavior (incentive motivation and copulation) separately with the appropriate tests (reviewed in [24]).

1.2 Assessing sexual incentive motivation

Several paradigms have been used to study sexual motivation (reviewed in [24]). Importantly, not all paradigms measure the same aspects of sexual motivation. Briefly, it is important to distinguish between paradigms that measure incentive sexual motivation versus paradigms that actually measure 1) motivation to continue copulation, 2) the willingness to work or overcome adverse events in order to access a mate, or 3) reward anticipation. In contrast to sexual incentive motivation, these measures are dependent on previous sexual experience and heavily influenced by the rewarding properties of copulation.

In our lab, we assess sexual motivation in rats with the sexual incentive motivation test (SIM test). In the SIM test, a subject animal is tracked for 10 minutes during exploration of a large arena to which two stimulus boxes are attached that house either a sexual incentive or a social incentive. The time spent in the vicinity of the sexual incentive, relative to the time spent in the vicinity of the social incentive, is a measure for the sexual incentive motivation of the subject rat. Measuring sexual motivation above and beyond motivation for social interaction in the same test is a clear advantage of the SIM test set-up over other tests in which only one incentive is used. As rats are highly social animals, any sexual incentive animal is inherently also a social incentive. Since tests of sexual motivation often neglect this aspect, this needs to be taken into account when assessing the motivation to approach a sexual incentive.

Other advantages of the SIM test include the fact that no physical interaction between subject and stimulus animals is necessary, and that the outcome measure for magnitude of sexual motivation is not dependent on operant responses, nor on sexual experience, and less

sensitive to variations in motor function than other measures (e.g. operant responses). Even if ambulatory activity is affected by certain treatments, it is first and foremost easy to assess this by looking at distance traveled, velocity, and immobility during the test, but it would also most likely not affect the outcome of the test because the subject animal will still spend relatively more time in the vicinity of the sexual incentive compared to the social incentive. This relative preference has been observed in the face of both enhanced ambulatory activity (e.g. upon amphetamine administration) as well as decreased ambulatory activity (e.g. upon cis(Z)-flupenthixol administration) [30].

1.3 Sex differences

Sexual behavior in general is very different in males and females. Male rats, for example, copulate in mount bouts in which they show patterns of mounts, intromissions and ejaculations [24, 31], while females show paracopulatory behaviors and lordosis [24]. The set-up of a copulation test is often also different for male and female rats, as the sex that is under investigation should be allowed to control the pace of copulation [24]. The different types of behaviors and different testing set-up make it difficult to compare results between the different sexes. Still, by comparing the behaviors in the right phases, it remains possible to draw parallels [23].

In contrast to the copulation test, the SIM test is conducted in exactly the same way for both sexes, and does not need to be modified for sex differences. Both males and females show similar levels of sexual incentive motivation, despite the fact that different gonadal hormones are involved. As long as the rats are sexually mature and in the appropriate hormonal state, male and female rats spend similar amounts of time with a sexual incentive and a social incentive in the SIM test, resulting in equal levels of sexual incentive motivation (ratio typically around 0.7) [20]. It is then also expected that certain manipulations will have similar effects in males and females on sexual incentive motivation. For example, both male

and female rats lose sexual incentive motivation when they have engaged in copulation with extensive stimulation (4 hours of copulation up to exhaustion in males, 3 ejaculations received in females) [20]. These similarities between males and females, as well as the aforementioned overlapping importance of hormonal status, the reliance on the sensory modality of olfaction, the role of sexual experience, and the lack of a correlation between incentive motivation and copulation parameters, make the SIM test a very suitable test to study sex differences.

## 2. Materials

2.1 Equipment

The setup for the sexual incentive motivation test consists of the following parts:

- SIM apparatus
- Dim, non-directional light source
- Digital, light-sensitive camera, connected to computer
- Computer with tracking software

2.2 Apparatus and set-up

The SIM apparatus consists of a rectangular arena (50 x 100 cm) covered with dark gray polyvinyl chloride, with short sides that are rounded (Fig. 1). The walls are 60 cm high and made of 0.6 mm thick coated sheet steel. They are coated on the inside with a black polymer, which minimizes light reflections and makes the walls easy to clean. In either of the long walls of the arena, a 23 x 23 cm square opening is routed. On the outside of the openings two vertical guide rails allow stimulus boxes to slide in place. These boxes (25 x 15 x 25 cm,

and closed with a lid on hinges) are made from the same material as the arena and connect with the arena with a wire mesh.
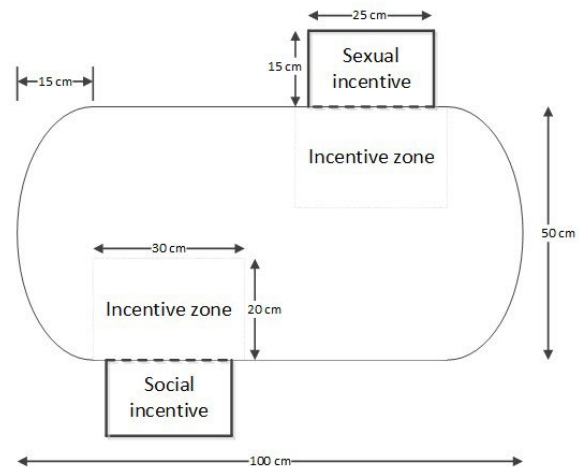
Figure 1: *Photograph (left) and schematic drawing (right) of the sexual incentive motivation test set-up*

A light-sensitive, digital camera is positioned roughly 200 cm above the SIM apparatus so that the whole apparatus is in view. A 15 Watt (85 lumen), incandescent light source, mounted just above the camera, provides about 5 lux of light in the arena, roughly comparable to light of a full moon. This is just bright enough for proper functioning of the camera and tracking software. To track the subject animal in the SIM arena, we use Ethovision XT16 (Noldus, Wageningen, The Netherlands), but any behavior tracking software that enables predefining areas within the test arena will work.

Beforehand, we design an experiment in the Ethovision software. For details on how to do this, we refer to the manual provided by Noldus (Noldus, Wageningen, The Netherlands). It is important to classify the arenas of your test set-up, and define two virtual incentive zones (20 x 30 cm; A4 paper size for convenience) in front of the stimulus box openings in each arena. The subject's position is tracked with a frequency of at least 5 Hz.

2.3 Animals

We have always used adult Wistar rats, but we see no reason why other rat strains, or even other animal species, could not be used in this same test (see Note 7). Animals are typically kept on a reversed light-dark cycle (11 am-11 pm) and testing is performed during the dark period (typically between 12 and 5 pm). Subject and stimulus animals can undergo a wide variety of treatments for testing purposes before the SIM test. In case the animals undergo invasive treatment (e.g. stereotaxic brain surgery), a recovery period of at least one week is used.

Most often we use conspecifics as an incentive stimulus (see Note 4), even though other types of stimuli can be used as well (see Note 6). If the subject animal is a male, one stimulus box contains an intact male as a social incentive and the other a sexually receptive female as sexual incentive. In order to induce sexual receptivity at the moment of testing, these stimulus females undergo a surgical procedure at least one week prior to the SIM test for ovariectomy and implantation of a Silastic capsule containing 10% 17β-estradiol in cholesterol (see for a protocol: [32], and see Note 4). Four hours before testing, the females are hormonally primed by subcutaneous injection with progesterone (dissolved in peanut oil, 5 mg/mL, 0.2 mL). In our experience, these females can be used as stimulus at least twice a week for at least 3 months. An ovariectomized female without hormonal treatment (non-receptive female) could also be used as social incentive instead of a male rat for a male subject.

If the subject animal is a female, one can decide whether to study naturally cycling females or ovariectomized and hormonally primed females (also see Note 5). In case naturally cycling females are used as subjects, we have good experience using an estrous cycle monitor (Muromachi Kikai Co., Tokyo, Japan) to track the estrous cycle of female rats. For a female

subject, the sexual incentive is an intact male, and the social incentive is either a castrated male or another female. All stimulus animals are usually sexually experienced.

3. Methods

3.1 Habituation

It is essential that subject animals are familiarized to the arena to avoid potential influences of novelty on the behavioral outcomes. During habituation, subject animals are allowed to freely explore the SIM apparatus for 10 minutes, with the stimulus boxes attached, but without stimulus animals present. The habituation takes place under the same light conditions and is repeated on three different days separated by at least 24 hours, preferably one week prior to the first SIM test. In case subject females are used, there is no need to hormonally prime them before the habituation session.

3.2 Testing

After habituation, the test sessions can start. The SIM test can be repeated as many times as the experiment calls for, with a minimum of 24 hours between test sessions. The same type of incentives or a variety in incentives could be used for the different SIM tests, depending on the purpose of the experiment (see also Note 6). Both, within- and between-group comparisons are possible. When conducting the SIM test in combination with a copulation test, see Notes 1-3 for some considerations.

Before each test session the incentive animals are placed in their respective stimulus boxes. About 5 min later, the first experimental subject can be introduced into the middle of the arena and the 10 min of observation is started. The experimenter can then leave the room, or stay in the room quietly. At the end of the test, the subject animal and any feces and urine

on the floor are removed from the arena. Then the following subject animal can immediately be introduced.

The position of the incentive animals is semi-randomly changed throughout the experimental session to rule out location bias for the sexual vs. social incentive. This is done by switching the detachable stimulus boxes on the arena while stimulus animals can remain in the boxes. Stimulus animals are often changed between tests to prevent effects of dehydration, food deprivation, and stress of small space confinement. We typically change the stimulus animals after every third SIM test during a testing day. It is essential to place the same type of stimulus animal (social or sexual incentive) in the previously used stimulus box, so that odor contamination is avoided. If a different type of incentive stimulus animal must be placed in a previously used box, careful cleaning is highly recommended between changing the animals.

After the last test of a day, the SIM arena is cleaned with 0.1% acetic acid solution, and the stimulus boxes are washed with washing machine of the animal facility.

3.3 Data analysis

The tracking software program can determine the following main outcome parameters:

- The time spent in each of the virtual incentive zones (i.e., time spent near the sexual incentive and time spent near the social incentive)
- The latency to enter each of the incentive zones
- The frequency of entering each of the incentive zones
- Mean duration of visits to each of the incentive zones
- The distance moved during the test
- The mean velocity of movement
- The time spent moving

In addition, a preference score can be calculated which is the time spent in the sexual incentive zone divided by the sum of time spent in the sexual and social incentive zones. The time spent in the rest of the arena is thus not included in this calculation. The preference score has a value between 0 and 1 and with .5 as no preference for either the social or sexual incentive and above 0.5 to indicate the sexual incentive was efficient to induce sexual motivation. For comparison between incentives, the preference score as well as the time spent in the vicinity of the incentives should differ in order to consider one incentive as different from another. A double criterion is needed in order to avoid false positive effects. A high preference score could be caused by a very short time spent in the social incentive zone just as well as by a very long time spent in the sexual incentive zone. However, a short time spent in the vicinity of the social incentive zone does not necessarily indicate a superior sexual incentive stimulus. Consequently, the chance of false positive differences between incentives is reduced by using a combination of both criteria.

Furthermore, the number of entries into each incentive zone as well as the latency to visit and mean duration of each visit can be determined. As indicators of ambulatory activity, we use the total distance moved during the test, the mean velocity of movement while moving, and the time spent moving.

The newest version of Ethovision XT 16 allows for detection of head and tail leading to the exploration of more behavioral outcomes. This gives the possibility to add tracking parameters such as head directions, and distance between nose and predetermined points of interest in the arena (i.e. the wire mesh). In addition, other behavioral observations, such as general mobility, self-grooming, freezing, and rearing can be made using the video files.

4. Conclusion

Sexual incentive motivation is of the same magnitude in gonadally intact male and receptive female rats and is similarly affected by different types of manipulations. The SIM test is highly suitable to assess sexual incentive motivation and is conducted in the same way for males and females. The many advantages and potentials of the SIM test advocate for this test to be used much more in the study of sexual behavior, for example with different types of incentives (Note 6), other animal species (Note 7), in translational research (Note 8), and in studies with timed interventions or when needing measures to be relatively unpolluted by motor reflexes (Note 9).

5. Notes

1. The SIM test can be conducted on the same day as a copulation test. In that case, it is important to perform the SIM test before the copulation test, as prior sexual stimulation can negatively affect sexual incentive motivation [20], as also noted in the introduction. Pre-exposure to sexual odors in the SIM test may have some influence on copulation in the copulation test, but these conditions will be the same for all subject animals (unlike amount of sexual stimulation in the copulation test).

2. It is recommended to not use stimulus animals in the SIM test that have just been used before as stimulus animals in a copulation test. After copulation, they can carry odors from the mate partner, which can mask the odors of the stimulus animal itself.

3. It is possible to modify the stimulus boxes so that the front wire mesh can be removed and the stimulus box opens towards the arena. Now the stimulus animal can enter the arena and studying the transition from motivation to copulation in one single test becomes possible. It

should be noted though that if the subject animal is a female, she cannot pace the copulation in this set-up. Importantly, this modification has not been tested or taken in use yet.

4. As mentioned in the method section, different kind of social stimuli can be used when male or female subjects are tested. In case of a male subject, one can choose between another male rat or a non-receptive female as social stimulus that is paired with a receptive female as sexual stimulus [6]. Ovariectomized females without hormonal priming are usually used as non-receptive female. As receptive female, either an intact female in the appropriate phase of the estrous cycle or an ovariectomized females primed with hormones is used. The primed ovariectomized female can either be implanted with a 5 mm long Silastic capsule (medical grade Silastictubing, 0.0625 in. inner diameter, 0.125 in. outer diameter, DeganiaSilicone, Degania Bet, Israel) containing 10% 17β-estradiol (Sigma,St. Louis, MO, USA) in cholesterol (Sigma, St. Louis, MO, USA) with medical grade adhesive silicone (Nusil Silicone Technology, Carpinteria, CA USA)-sealed ends (leading to average estradiol serum levels of 75 pg/mL [33]) , or primed with a estradiol benzoate 25 µg/rat (in peanut oil) subcutaneous injection 52 hours before the test. In both cases, the females should be primed with progesterone 1 mg/rat (in peanut oil) 4 hours before testing. Hormone priming protocols may vary in the literature. It is most important that the result is that the female stimulus is sexually receptive, as would be reflected in a lordosis quotient of 100 or more.

When testing female subjects, either a castrated male or another female rat can also be used as social incentive stimulus (e.g. [34]), whereas an intact male rat is used as sexual incentive.

5. When studying female subjects, a consideration is whether to study naturally cycling females or hormonally primed ovariectomized females. The advantage of the latter is simplicity, as all females can be tested whenever they are hormonally primed. It has however been shown that the dose of estradiol (in addition to progesterone dose kept constant) affects the preference for an intact male over a castrated male [35] and affects paracopulatory and

lordosis behaviors [36]. Therefore, naturally cycling subject females might be the most natural option to not mask possible effects of hormonal manipulations on the assessed sexual incentive motivation. When choosing to study ovariectomized female subjects, it should be considered to lower the dose of estradiol in order to better mimic natural hormone levels (e.g. 18 µg/kg estradiol benzoate [35, 37]). Finally, in female rat copulation studies, intact females are sometimes primed with estradiol only [38]. We have never employed this in the SIM test, but it could be a consideration if surgery is to be avoided. 6. The SIM test is highly suitable to test approach towards any kind of incentive, besides towards behaving animals. We have for example assessed approach behavior towards anesthetized animals, devocalized animals, auditory stimuli (playback of ultrasonic vocalizations), and odors [9, 18, 19]. Odors can for example be introduced to a stimulus box by leaving an animal into the stimulus box for 6 hours prior to the test in order to collect urine and feces in the box [9, 19]. Auditory stimuli, on the other hand, can be introduced by placing a loudspeaker in one of the stimulus boxes [9, 18, 19].

7. An advantage of the SIM test is that it should be suitable to be used to test any kind of small animal species. The SIM test has been successfully used in mice [39], and similar set-ups have been used for hamsters [40], and prairie voles (although direct contact between animals was also possible in this test) [41].

8. The SIM test can be used as a valid behavioral paradigm to assess sexual motivation in the translational setting. Whereas copulation is quite different in humans compared to rodents, sexual approach is rather similar between these species (for a discussion, see [29]). The SIM test is not often used for characterization of effects of pharmacological or other interventions in animals for translational purposes. However, using the SIM test or a very similar set-up, it has for example been demonstrated that SSRI's, which can reduce sexual motivation in both human sexes, also reduce sexual incentive motivation in male (fluoxetine; [42]) and female

rats (paroxetine; [43]). We advocate for the SIM test to be used in this translational context more often.

9. The SIM test is highly suitable for the study of any kind of intervention, from pharmacology/chemogenetics [28, 44, 45], to optogenetics and photometry. For example, in optogenetic experiments, the set-up can easily be exploited for timed intervention when the animal is in any of the zones, even in a "closed loop" with the help of the right tracking software that can be linked to TTL signals. For photometry, the SIM test is especially beneficial because signals will be mostly unpolluted by motor reflexes, in contrast to for example operant response paradigms in which specific motor patterns are required for the performance of the behavior.

6. References

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