Assessment of sexual behavior in rats: the potentials and pitfalls

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Abstract

In the field of behavioral neuroscience, it is essential to use the appropriate animal models for the topic of investigation. The danger of using the wrong model can result in false interpretation of the results. In this review we will discuss the animal models used to study sexual behavior, with a focus on rats. We will discuss the potentials and pitfalls of the different paradigms and try to make recommendations on how research in this field could be optimized. Both male and female sexual behavior are discussed, in addition to sexual motivation.

Key words: sexual behavior; incentive motivation; behavioral paradigm; rat; female; male

1 Introduction

Employing appropriate animal models for research in the field of behavioral neuroscience is essential. The use of the wrong animal model can result in misinterpretation of results and false assumptions about the neurobiological background of these results. In addition, it is possible that these misinterpretations and false assumptions set precedent for future research.

In this review we will explore sexual behavior in both male and female rats, discuss how this behavior should be analyzed and interpreted, and how it fits in behavioral paradigms. Furthermore, we will focus on behavioral paradigms for the investigation of sexual motivation in rats. For both the analyses of the behavioral observations and the paradigms, we will try to show their respective potentials and pitfalls, and argue for a careful approach to the operationalization of notions such as motivation and reward from the given sexual behavioral parameters.

It should be noted that this review is written in the context of the controlled environment of a laboratory. In their natural environment, rats copulate in groups consisting of one or several females and males [1, 2]. The sexual behaviors performed by the individuals is similar in nature

and in pair-tested tests, just as the complete sexual cycle. There are only some differences in the timing of behaviors, because rats in nature have more space to pursue conspecifics or might get distracted by the environment or fellow rats.

Before we discuss the sexual behavioral parameters, we deem it necessary to first describe the basic observations we can make during sexual encounters between a male and a female.

1.1 General behavioral aspects of the copulatory cycle in rats

The course of sexual interaction between a male and a female rat is to a large degree stereotypical (see Fig. 1) [3-5]. Broadly speaking, a copulation cycle can be divided into three parts, the precopulatory phase, copulatory phase and executive phase [6]. During the precopulatory phase, the male rat and the receptive female (i.e. being in hormonal or behavioral estrus) will engage in anogenital sniffing. The subsequent copulatory phase consists of the female drawing the male's attention with paracopulatory behavior: *hopping* (short jumps with all four legs off of the ground) and *darting* (short and sudden runaway movements, in which she presents her body to the male). In a reaction to these movements, the male rat will try to *mount* the female: he straddles the female from behind, and thrusts his hips in an attempt to locate the vagina with his penis. In the event of penile insertion into the vagina, the male rat continues his thrusting with a sudden deeper thrust. He then dismounts the female, visible as a short jump backwards, away from the female, sometimes raising his forepaws in the process. This behavior is recognized as an *intromission*. The physical stimulation caused by mounts and intromissions can cause the female to arch her back for easier vaginal entry, a receptive phenomenon known as *lordosis*. These behaviors tend to proceed in rapid succession, only to be intermitted by self-grooming, rest, and *pacing* by the female (runaway behavior). Finally, ejaculation constitutes the executive phase for

the male, which is followed by a period of male inactivity, usually lasting around 5 minutes. The beginning of a new cycle of sexual behavior marks the end of the *postejaculatory interval.* Auditory, olfactory and visual cues play an important role in sexual behavior. Interestingly, a cooperative function seems to exist for the different modalities in the induction of approach behavior of a potential mate [7].

2 Male rat sexual behavior

2.1 Parameters

The events described above (mounts, intromissions and ejaculations) are registered at the corresponding time points with a scoring device during sexual behavior assessment, either at the real time test or from video. When trained, an observer can easily recognize mounts, intromissions and ejaculations by looking at the associated behavior as described above. The act of intromission is for example very well correlated with the male rat behavior of a deep thrust and jumping backwards [8]. Analysis of the scoring output yields a set of parameters by which sexual behavior is assessed:

- Mount latency; time from introduction to the female until the first mount
- Intromission latency; time from introduction to the female until the first intromission
- Latency to first behavior; time from introduction to the female until the first behavior - i.e. mount or intromission
- Number of mounts
- Number of intromissions
- Number of ejaculations (if a test is used that allows for observation of multiple ejaculation series)
- Ejaculation latency; time from the first intromission to ejaculation

- Postejaculatory interval; time from ejaculation until next mount or intromission (often time to next intromission is used)

In addition, the following parameters are calculated:

- Intromission ratio; the number of intromissions divided by the sum of the number of intromissions and the number of mounts
- inter-intromission interval; the total test time divided by the number of intromissions, or the ejaculation latency divided by the number of intromissions
- Copulatory rate; the sum of the number of mounts and the number of intromissions divided by the time from first behavior to ejaculation

Sometimes, sexual behavior is expressed by means of a percentage of ejaculating rats or as a percentage of copulating rats (for example [9]). This makes sense when a treatment is so deteriorating on the sexual behavior of the rats, that there are too few events to score. Analyzing data from too few events can skew the data and augments the problem of how to deal with missing values. If possible, however, we recommend reporting sexual behavior testing results by reporting the abovementioned parameters.

2.2 Interpretation of results

In order to interpret an effect of a certain treatment on any of the mentioned parameters, we first have to more accurately define the key observed behaviors, i.e. mounts and intromissions, and elaborate on the role of those behaviors within the sexual behavior episode and its contribution to the copulatory and executive phase of copulation.

Penile stimulation through intromissions, with a minimum number of two, is essential for a male rat to reach ejaculation [10]. In addition, two or more intromissions are necessary for a female to get into progestational state, necessary to become pregnant [11]. Interestingly, rats that show an innate short ejaculation latency do not necessarily need less intromissions to achieve ejaculation [12]. Moreover, there is a low variability in the temporal pattern of male rat sexual behavior [12, 13], meaning that rapid ejaculators need less time to achieve the same amount of intromissions than normal and sluggish copulators. Indeed, normal and sluggish ejaculators show more mounts preceding ejaculation, essentially making rapid ejaculators more "efficient" than their sluggish and normal counterparts [12].

When we look at *mounts* in particular, it is difficult to establish what they really are. Are they failed intromissions? That is, is the "intention" of every mount to end in an intromission? Or, do they represent a behavior independently contributing to the copulation climax and/or do they serve a specific "purpose" within the sexual behavior? We have seen rats only intromitting and not mounting during an ejaculation series, which suggests that mounts are not necessary to reach ejaculation. It is clear, however, that mounts do contribute to the arousal state and facilitate ejaculation: when males mate with a female with a closed vagina for 40 minutes, less intromissions are necessary to achieve ejaculation during subsequent mating with an intact female. In addition, the ejaculation latency and number of mounts are decreased during this subsequent mating [14]. Mounting is also a self-maintaining behavior. Male rats continue to mount when they are prevented from intromitting through closure of the female vagina, or through local anesthesia of the penis [15, 16]. Intriguingly, although intromissions are the essential part of copulatory behavior leading to ejaculation, it is actually the mount bouts that determine the temporal pattern of copulation, independent of intromission behavior. This became evident from a study showing that the inter-mount-bout-interval (the time from the first mount of

one mount bout to the first mount of the next mount bout) was highly constant, independent of whether the preceding mount bout ended in a mount or an intromission. In addition, male rats do not keep mounting within a mount bout until they have achieved an intromission, suggesting that the mount bout is not "intromission driven" [17]. This proves that mounts are not just non-essential behaviors for reaching ejaculations, but central behaviors within the sexual behavior pattern of the male rat.

Consequently, interpretation of an effect on the number of mounts and/or intromissions preceding ejaculation is not particularly straightforward. A decrease in the *number of intromissions* preceding ejaculation could be interpreted as an increase of the "arousal state" of the rat, needing less stimulation to achieve ejaculation. It should be beared in mind though, that the lower need for stimulation in response to any treatment might also be the result of an increase in penile sensitivity. However, this does not mean that penile sensitivity changes are necessarily the mechanism through which rats can become more aroused. For example, male rats require less intromissions to reach ejaculation when the accessibility of the female is limited: single or multiple forced intercopulatory intervals (removing the female for a certain amount of time after intromissions) make the male need less intromissions to reach ejaculation [18, 19]. This could not be explained by an increase in penile sensitivity, but it does suggest that males can actually influence their efficiency and arousal state, depending on the circumstances. Another example of this phenomenon is seen in more "natural settings", in which female rats determine the pace of mating in a multiple choice arena. The non-preferred males in these tests are less often visited by the females, resulting in longer intercopulatory intervals, and become more efficient (more mounts result in intromissions), resulting in shorter ejaculation latencies than when they are tested in a situation where they can pace the mating themselves [20]. The efficiency of the rat is thus reflected in the *intromission ratio*. As mentioned before, the efficiency to reach ejaculation

is increased when the rat is more successful at achieving intromission when mounting. Because the occurrence of an intromission is dependent on the occurrence of an erection, effects on the intromission ratio may therefore reflect an effect on erectile function.

The *inter-intromission interval* and *copulatory rate* are parameters that are often interpreted as a measure for temporal patterning of copulation. We question, however, whether these parameters do actually provide any useful information about the temporal pattern of copulation. Previously, we concluded that temporal patterning of copulation in the male rat is entirely determined by the mount bout. Consequently, the inter-intromission interval is actually a function of the intromission ratio and the inter-mount-bout-interval. This means that a decreased inter-intromission interval could be entirely due to a higher efficiency (increased intromission ratio), without any effect on the temporal copulatory pattern (defined by the inter-mount-bout-interval). The copulatory rate in its turn is also very dependent on the efficiency of the rat. For example, interpreting an increased copulatory rate as "increased copulation speed" would be a mistake if there were actually no effects on inter-mount-bout-intervals, but just an increase in the number of mounts within a mount bout, which means the rat is just less efficient – a completely different conclusion! To sum this up, we are inclined to ignore the inter-intromission interval and copulatory rate and instead look at the inter-mount-bout-interval as a measurement for copulation speed. Copulation speed is an interesting measurement in the light of a very basic theory of a "mount generator" within the brain, described by Ågmo [21]. Within this theory, mounts, intromissions and ejaculations all temporarily inhibit this mount generator, in which an intromission has a greater inhibitory effect than a mount. For example, 3-5 mounts (a mount bout) could be necessary to reach the inhibitory threshold already achieved by one intromission. Ejaculation results in the greatest inhibition, reflected by the post-ejaculatory interval (see below

for further discussion). In conclusion, measured effects on copulation speed could reflect an influence on the functioning of this mount generator

As for the practical side of scoring *inter-mount-bout-intervals*, it requires either a formula to calculate the parameter from the mount and intromission data points or it needs to be scored separately according to a clear recognizable behavioral definition. Sachs and Barfield defined the mount bout as "a sequence of mounts (one or more), with or without intromission, uninterrupted by any behavior (other than genital autogrooming) that is not oriented toward the female" [17]. This seems to be the only valid way to register mount bouts, since a definition cannot exist in terms of time between behaviors, because time is actually the parameter that is variable here.

Continuing with the interpretation of mounting parameters, *increased mounting* is often interpreted as a measure of motivation. However, a shorter ejaculation latency accompanied by less mounting and intromission behavior does not necessarily mean that the rat is less motivated. It might as well mean that the arousal state of the rat is increased. Another parameter that is usually considered to be a measure of motivation is the *latency to mount*. However, it should be considered that general activity, general arousal and sensory efficiency of the rat also affect this parameter. For example, a treatment that increases tactile sensitivity or sensitivity to smell can affect the ability of the male rat to localize the female and mount faster. Next to that, we cannot be sure in what way the female may affect the mounting latency of the male. Therefore, we need to be very careful when drawing any conclusions from effects on the latency to mount. Finally, there is no reason to believe that the rat has any active choice in starting copulation behavior with a mount or an intromission. Therefore, in contrast to what is common practice, we believe that no different interpretation should be given to whether the first behavior is a mount or an intromission. Consequently, we propose to only report the *latency to first behavior* as a measurement of latency to start copulation.

The interpretation of the *post-ejaculatory interval* is unclear [22]. It is sometimes interpreted as a measure of sexual motivation. However, the post-ejaculatory interval is in general not very variable, as is for example evident from the fact that innate rapid ejaculators do not have a shorter post-ejaculatory interval than other rats [12]. In addition, it is clear that the post-ejaculatory interval can be divided in an absolute and a relative refractory phase [23]. While the rat is absolutely unresponsive to any sexual stimuli, and copulation is completely inhibited during the absolute phase (the first 75% of the post-ejaculatory interval), the rat can be reactivated to start copulating again during the relative refractory phase, by arousing stimuli such as the introduction of a new receptive female, handling or electrical shock [24, 25]. Nevertheless, there are examples of treatments that do affect the post-ejaculatory interval, including the absolute refractory phase, sometimes in an extreme fashion (see for instance [23, 26]). Furthermore, it is known that the post-ejaculatory interval is not caused by a reduced excitability in the spinal cord control of penile reflexes [27]. Therefore, the post-ejaculatory interval is clearly an effect of some sort of inhibition within the brain. We remind the reader of the mount generator theory, which could explain the refractory period of the post-ejaculatory interval. Small treatment effects on the post-ejaculatory interval could well be effects on general arousal. More extreme effects may suggest an effect on the absolute refractory period. It would be an interesting study to research whether effects on inter-mount-bout-intervals are correlated with effects on the post-ejaculatory interval.

The current standard is to calculate the post-ejaculatory interval as the time from the ejaculation to the next first intromission. Since intromissions require penile erection and coordinated activity of the striated penile muscles, it was seen as a more important sexual behavior than mounts. However, as discussed before, we believe that mounts play an important

role in sexual interactions as well, and consider the latency to first behavior a more relevant parameter than the latency to first intromission. For the same reasons, we recommend to calculate the post-ejaculatory interval as the time from the ejaculation to the next first behavior. Only when we calculate the *latency to ejaculation*, the latency to first intromission might become relevant. In comparison to the other parameters, the latency to ejaculation could provide additional information about the efficiency from the first penile sensory stimulation to reach an ejaculation. Mounts do not involve penile insertion and are therefore not considered valid as penile sensory stimulation. Therefore, it could be useful to calculate the latency to ejaculation as the time from the first intromission to the ejaculation. However, with the previously mentioned arguments for that mounts play an important role in sexual interactions as well, it could just as well be interesting to calculate the latency to ejaculation from the first mount, or even the beginning of the test.

A very important point to be made with regard to explanation of results is definition of facilitation and inhibition of sexual behavior in the literature (see also [28-30]). A decreased ejaculation latency is frequently presented as a facilitation of sexual behavior, whilst it is often accompanied by a decrease in behaviors during the copulatory phase; the rat is more efficient (higher intromission ratio) or has a lower ejaculation threshold (less intromissions preceding ejaculation). On the other hand, decreased ejaculation latency could indeed be accompanied by an increase of behaviors during the copulatory phase, through an increase of the copulatory rate. The fact that the number of pre-ejaculatory intromissions positively influences the amount of sperm reaching the uterus of the female [31] and the chance of pregnancy [11], illustrates that inhibition of the copulatory phase combined with facilitation of the executive phase should not be considered as facilitation of sexual behavior in general, since it can actually have a negative

effect on fertility. This makes a case for clearly differentiating between facilitation of the copulatory phase on the one hand and facilitation of the executive phase on the other hand.

2.3 Behavioral paradigms

Excellent protocols have been written on testing paradigms for male rat copulatory behavior assessment [22, 32]. Therefore, we will briefly discuss the tests available and considerations that determine the choice of a test without going into too much detail.

Sexual behavior of the male rat is most often assessed by putting the male rat in a transparent test arena together with a receptive female rat. In this set-up, the male has continuous access to the female and can freely copulate at his own chosen pace. It is important to let the test subject pace the copulation, because copulation is only rewarding to the rat that is able to control the mating [33]. This is also illustrated by the fact that the structure of male copulation behavior in a seminatural environment, where females are capable of pacing the copulation, differs from that in a copulation test [5]. Often, the copulation test is conducted for one ejaculation series, ending after the first intromission after the post-ejaculatory interval. Alternatively, the test can be ended after a predefined time period (usually 30 minutes), independent of the amount of ejaculation series the rat has shown. Sometimes, rats are tested up until exhaustion.

In general, all significant differences among groups can be identified by only looking at the data for the first ejaculation series, except for the number of ejaculations within a defined period of time. Still, the effect of an increase in the number of ejaculations will logically be accompanied by a decreased ejaculation latency and/or a shortened postejaculatory interval, and would therefore automatically be reflected in the data from the first ejaculation series. However, although it might not be expected, treatment effects could also only become evident in later ejaculatory series. For example, the ejaculation latency in the first series may remain normal,

while it is affected in the following series. Therefore, we recommend to always conduct a 30 min test, if only to rule out this possibility. While the focus of data analysis will lie with the first series, we might come across something unexpected in any of the following series. Additionally, Chan et al. (2010) discussed an interesting argument in favor of the 30-minute test: when testing pharmacologically active substances, a 30 minute time period will control for individual difference in pharmacokinetics better than a single ejaculation series test [32].

A problem that presents itself when analyzing data from a 30-minute test is whether to compare results from the total test time or only from corresponding ejaculation series. In wildtype rat sexual behavior, the number of mounts and intromissions decline during the second to the fourth series, after which the numbers increase again for the series following. Also, the post-ejaculatory interval increases for each ejaculation series after the first [22]. This makes it very difficult to determine how to compare and interpret total test data (except for total ejaculations). Consider the complication in comparing a rat that only ejaculates once, right before the end of the test, with a rat that ejaculated four times. The fast ejaculator will have had four post-ejaculatory intervals, so about 15 min out of 30 min without activity, while the slow ejaculator has been active during the whole duration of the test. Total test number of mounts and intromissions are in this case incomparable between the two situations. The previous example only emphasizes the complexity of drawing conclusions from the data. Therefore, we believe it is most preferable to report raw data as they are, total test and per series, instead of just the interpretations of results. This practice will maintain objectivity in the results as much as possible.

In the end, choosing a suitable test is very dependent on the effect that one is looking for. If the only interest is, for example, an increased or decreased ejaculation latency, a test with one ejaculatory series is obviously sufficient. This is especially applicable in translational research, because humans achieve most often only one ejaculation. For example, in order to assess whether

a drug could function as treatment for premature ejaculation, it is sufficient to investigate the effects on the delay in the latency to first ejaculation. However, in case the research is quite fundamental and focuses on mechanisms in rat sexual behavior, it is recommended to assess all effects on behavior which is then tested in a 30-minute test. As an example, a treatment might affect the post-ejaculatory interval in such a way that instead of increasing over ejaculatory series in time, it remains the same within each ejaculation series. This effect would not be found in a single ejaculation series test, but will be reflected in data from a 30-minute test.

With the use of the 30-minute test, it was also discovered that sexual behavior of the male rat is highly variable between rats. A typical population of wild type Wistar rats will show that 10-20% of the animals are so called 'sluggish copulators' and 10-20% of the animals are 'rapid copulators'. Rapid copulators reach double the amount of ejaculations than normal copulators in the same time span, while sluggish copulators will reach less than half of that of normal copulators [12]. Similar endophenotypes can also be found in females, in which about 37%, the male-avoiders, spent significantly less time in the male compartment and showed lower levels of paracopulatory behaviors than the male-approachers. This behavior is also constant over multiple paced-mating tests [34].

3 Female sexual behavior

3.1 Parameters

Just as with testing male sexual behavior, the events can be registered by a trained observer at the corresponding time points with a scoring device during sexual behavior assessment. Analysis of the scoring output yields a set of parameters by which sexual behavior is assessed or calculated:

- Number of lordosis responses assessed on a 4-point scale (0-3 with zero as no lordosis and 3 as a full lordosis with a hollow back and lifted head of 45 degrees or more [35]), from which can be calculated:
    - Lordosis score (the mean of all lordosis intensities)
    - Lordosis quotient (the number of lordosis responses divided by the number of received sexual stimulation times 100%)
- Number of paracopulatory behaviors (darts and hops)
- Number of received sexual stimulations (mounts, intromissions and ejaculations)
- Time spent with the male
- Percentage of exits after sexual stimulations (total number of exits after the stimulation within a certain time-frame divided by the total number of the stimulation times 100%). This parameter should be given separately for mounts, intromissions and ejaculations.
- Contact-return-latency (the average time the female needs to enter the male compartment again after an exit). This parameter should be given separately for mounts, intromissions and ejaculations.

Ear wiggling is sometimes also calculated and added to the number of paracopulatory behaviors. Ear wiggling is a rather fast lateral shaking of the head that is visible as a quiver of the ears, a behavior that is very difficult to score, because it happens very regularly and fast. Therefore, many researchers leave this behavior out of their analysis. In fully receptive females, ear wiggling almost always accompanies the darts and hops, and could therefore (out of practicality) also be considered part of this paracopulatory act of behavior as one event.

346 *3.2 Interpretation of results*

347 Lordosis is the most studied component of female sexual behavior. The *lordosis quotient*
348 (LQ) is considered a measure of sexual receptivity, whereas the *lordosis score* (LS) represents the
349 magnitude of the lordosis response. Lordosis is a reflexive behavior that is very much depending
350 on the hormonal state of the female. The presence of estrogen alone is sufficient to induce
351 receptivity, but progesterone facilitates the estrogen-induced lordosis response [36]. Older studies
352 concluded that lordosis was triggered by sexual stimulations from the male [3, 37], but more
353 recent studies have shown that this hormonally regulated response can also be triggered by other
354 forms of tactile stimulations (e.g. upon male sniffing or touching the female or manual
355 stimulations) [38, 39]. Surprisingly, researchers keep scoring only the lordosis responses upon
356 mounts, intromissions and ejaculation resulting in a lordosis quotient of maximal 100%. So far,
357 the extra lordosis responses have been measured and reported in only a few publications (e.g. [4,
358 40, 41]), which is a missed opportunity. There is a variation between rat strains, but as showed in
359 Snoeren et al. (2011), Wistar rats almost always show an LQ of 100% when the appropriate
360 hormonal treatment is given to ovariectomized rats [40]. Only when females were treated with a
361 low dose of 2 µg of estradiol benzoate alone, an LQ of 40% was (sometimes) found, but the LQ
362 reached 100% in all cases as soon as progesterone was added. Consequently, if the researchers
363 would not have scored the extra lordosis responses to other tactile stimulations, they would not
364 have discovered the positive drug effects on lordosis [40]. The drug-induced increase in LQ is an
365 important finding, because it indicates that the females were extra sensitive to tactile stimulation,
366 which probably is a result of an increased receptivity. This conclusion could never have been
367 drawn if the extra lordosis responses were not measured, and the drug would have been evaluated
368 as having “no results on receptivity”. We therefore suggest that the extra lordosis responses
369 should always be reported in future studies in order to prevent from misinterpretation of results.

370 It is generally accepted that LQ and LS are the ultimate criterion for female sexual
371 receptivity, but there are some reasons to be careful with the interpretation of the resulting data.
372 For example, sexual behavior tests performed under paced and non-paced mating conditions have
373 resulted in different outcomes on lordosis behavior. POA lesions, for instance, cause an increase
374 in lordosis quotient compared to sham-operated females in a non-paced mating test, while the
375 same lesions disrupt lordosis when the females were allowed to pace their sexual stimulations
376 [42]. Similar conflicting findings were observed on the role of estrogen α receptors in the VMN
377 on lordosis; in a non-paced mating test, females without estrogen α receptors showed impaired
378 lordosis responses [43], while sexual behavior tests performed in a seminatural environment (in
379 which females can escape from the male) indicated normal lordosis capacity in these females
380 [44]. Together, this suggests that the lordosis response might not solely reflect the receptive state
381 of the female, but could also be influenced by her motivational state. In a paced mating set-up, a
382 female can escape from the male when she is not motivated for copulation, while in a non-paced
383 mating paradigm she either overrides her motivation and participates with lordosis responses (in
384 case of the increase in LQ) or she prevents the male from mounting by fighting and/or
385 suppressing the lordosis response (in case of the decrease in LQ). Interestingly, this actually
386 shows that also the reflexive response can be actively suppressed. Therefore, carefulness is
387 needed when analyzing lordosis behavior in a non-paced mating set-up. It actually makes us
388 recommend to always study female sexual behavior in paced mating conditions.
389
390 Another measurement for female sexual behavior is the number of paracopulatory
391 behaviors. *Paracopulatory behavior,* also called solicitation or proceptive behavior, is usually
392 described as the species-specific behaviors displayed by an estrus female during sexual
393 interaction in which she encourages the male to mate and regulates the pattern of copulation (also

394 reviewed in [45]). Beach suggested that the darts and hops constitute the female's assumption of
395 initiative in establishing or maintaining sexual interaction [3], which is then translated in a
396 measurement for female sexual motivation. McClintock and Adler (1978) showed that 90% of
397 intromissions were preceded by female approach, while only 3% of intromissions occurred upon
398 approach of a male towards a female [37]. It was, therefore, believed that copulation occurred
399 upon initiation of the female rats. However, a recent study by Bergheim et al. (2015) performed
400 in a seminatural environment showed that the copulatory acts were a consequence of a subtle
401 interaction between the male and female. This indicates that the behavior of both rats are equally
402 important in the initiation of copulation, and thus not controlled solely by the female [46]. Still,
403 there is a linear relationship between the amount of paracopulatory behavior and the amount of
404 copulation: females who dart less, receive less sexual stimulations, while actively darting females
405 receive more sexual stimulations [46]. There is thus an equal proportion of paracopulatory
406 behavior leading to a sexual interaction. Based on the definition that the intensity of execution of
407 a behavior is strictly dependent on the level of motivation (as discussed in [47]), this indicates
408 that paracopulatory behaviors are indeed a parameter for sexual motivation. This idea is
409 strengthened by the observation that the rate of paracopulatory behaviors decreases over time
410 after having received multiple sexual stimulations [48], which attenuates the levels of sexual
411 motivations.

412 However, some scientists believe that paracopulatory behaviors are not adequate as
413 measure of sexual motivation. They argue that paracopulatory behaviors are very stereotyped,
414 and can be considered entirely reflexive, because hormonally primed females can also show
415 paracopulatory behaviors (just as lordosis responses) upon manually stroking the hind flanks, and
416 thus in a non-sexual context [49]. However, as mentioned before, lordosis is a clear reflexive
417 behavior, that might also be influenced by the motivational state of the female, since lordosis can

be actively suppressed when required. In case paracopulatory behaviors are indeed reflexive, it does not prove that this behavior is not a measurement of motivation. Although they can occur upon manually stroking of hind flanks in a non-sexual context, darts and hops performed during copulation can still reflect sexual motivation. An alternative explanation we would like to introduce is that the paracopulatory behaviors might represent the motivational level of keeping participating in the sexual intercourse rather than of the female's intrinsic sexual motivation. In order to measure the level of intrinsic motivation, a sexual incentive motivation test (as mentioned later in this review) is a better method to use.

Overall, it is important to report the scientific findings as objectively as possible. We could argue that the number of paracopulatory behaviors could be an indicator of the level of sexual motivation, but clear empirical evidence is not available at this moment. Besides, alternative options should not be neglected. We, therefore, strongly support Blaustein and Erskine (2002) in using the term *paracopulatory behavior* instead of the older terms (proceptive, solicitation, precopulatory), simply because it obviates the assumptions about the female's sexual motivation to initiate mating [50].

When a paced mating paradigm is used (as described later), the *time spent with the male* can also be measured. This parameter is thought to reflect the female's motivation to continue participation in copulation. However, caution should be taken when analyzing this behavior, because this parameter is also affected by a component of social behavior. Male rats do normally not attempt copulating with non-receptive females, defined as females who are not in behavioral estrus. Non-receptive females, therefore, can safely spend time with the male without the risk of being mounted. The parameter of time spent with the male is probably only a reliable measurement in hormonally primed females who have signs of receptivity. For example, the

442 smell of a receptive female stimulates the male to attempt to mount the female. Now the not-
443 willing female can only reject or escape from the male to be left alone, which is then indicated in
444 less amount of time spent with the male compared to the willing females.

445 To continue with other components of pacing behavior, it has been shown in the past that
446 the *percentage of exits* increases with the intensity of the received sexual stimulus [51]. In the
447 same line, the *contact-return latency* (CRL) of the female to return to (or to press a lever for)
448 sexually males also changes with the intensity of the previously received sexual stimulus [51-53];
449 after a mount females return to the male quicker than after an intromission or ejaculation. These
450 parameters are therefore always given per type of stimulation; e.g. percentage of exits after
451 mount or CRL after intromissions. Interestingly, this pacing behavior seems to be a very stable
452 behavior that is innately present in females upon their first sexual contact [54].

453 Several studies have shown that certain conditions or treatments can have a different
454 effect on the percentage of exits and the CRL [34, 40, 55, 56], suggesting that these
455 measurements of pacing behavior have different read-outs that might be regulated through
456 different brain mechanisms. For example, no differences in percentage of exits were found in
457 ovariectomized females treated with only estradiol or a combination of estradiol and
458 progesterone, while the presence of progesterone decreases the CRL [40]. Furthermore, no
459 change in percentage of exits, but an increase in CRL's after intromissions was found in females
460 receiving more than 15 intromissions [48]. The percentage of exits could, therefore, reflect the
461 female's short-term response to the intensity of the copulatory stimulus (sensory component),
462 while CRL is more a direct measure of the female's motivation to reinitiate mating [57].

463 However, it is essential to be cautious with the interpretation of the data for a few reasons.
464 First of all, females are more likely to delay their return upon intromissions after they have
465 received multiple intromissions along with ejaculations than after receiving only a few

466 intromissions [45], suggesting that the pacing behavior of the female seen in a copulation test (as
467 described below) is highly dependent on the copulatory activity of the male rat. Since the activity
468 of the male is uncontrollable when studying the sexual behavior of the female, this makes the
469 parameters of pacing behavior very unreliable as indicator of sexual desire or arousal of solely
470 the female. Second, a CRL can only be measured when a female does escape from the male with
471 an exit. As a result, the CRL parameter is biased for the moments that the female escapes from
472 the male and neglects the moments in which the female continues in copulation. At the same
473 time, no clear definition of an exit exists, or an exit is measured with a certain cut-off time,
474 meaning that an escape is scored as exit only if the female runs away from the male within for
475 example 10 or 20 seconds (but also 120 seconds has been used). But what does this cut-off point
476 mean and what is it based on? Female rats regularly start running around the cage after a
477 stimulation, in which she might "accidentally" run through her own female compartment before
478 immediately re-entering the male compartment. This would then count as an exit and
479 immediately as a very short CRL, but she might not participate in the sexual interaction
480 straightaway (which is the reason why missing data points for the CRL due to no escape cannot
481 be filled with a zero second count). This kind of situations influence the outcome without
482 explaining the female's short-term response to the stimulation or her motivation to reinitiate
483 mating. One might suggest it is better to calculate a CRL with the time to the next first
484 paracopulatory behavior instead, but since the female often darts in her own compartment, this
485 measurement would also have no significance. In addition, Ellingsen and Ågmo (2004) have once
486 calculated the relationship between ambulatory activity and the propensity to escape from the
487 male. By calculating the probability that the female would randomly enter her own compartment,
488 and then compare this to the proportion of escapes after mounts, they discovered that an increase
489 in percentage of escapes (e.g. upon amphetamine treatment) can rather be an effect on

ambulatory activity than an increase in sensory responsiveness [58]. Altogether, this supports the idea that the percentages of exits and CRL are useless as indicators for the female's sensory and motivational state. We therefore suggest that if the percentage of exits and CRL are estimated, they should always be evaluated in combination with other parameters of female sexual behavior and never as a measurement of its own.

3.3 Behavioral paradigms

When studying female sexual behavior, different kinds of tests can be used. In many studies, researchers focused solely on investigating lordosis. This was commonly done by allowing females to receive 10 mounts or intromissions and measuring the number of lordosis responses. The lordosis quotient, which is the number of lordosis responses divided by the 10 copulatory stimulations times 100%, was considered a measure of sexual receptivity. This method could be very convenient for the researcher, because it does not take much time to observe 10 mounts, but a disadvantage of this method is that it is always performed in a non-paced mating set-up. As discussed before, female rats seem to be able to suppress the lordosis response to sexual stimulation when no escape possibility is available, which could lead to misinterpretation of the results. But a more important argument for the uselessness of this paradigm is that one only investigates one aspect of the female's sexual behavioral repertoire. Even though, the LQ might provide the information of the receptivity of the female, it does not reflect the willingness of the female to participate in sexual interactions.

A better method to study the full aspects of female sexual behavior would be a complete copulation test in which the female shows its repertoire of copulatory behaviors: ear wiggling, darts, and hops, besides lordosis. A standard copulation test as used for male sexual behavior would be an option. However, this paradigm is also not ideal, because females are not able to

pace their sexual interaction. Research has shown that coital stimulations are more effective in inducing pregnancy in a paced mating situation than under non-paced mating conditions [59], suggesting that intromissions become more effective in changing neuroendocrine changes in the female. Besides, copulation only has rewarding properties for a female, when pacing opportunities are available [60]. Thus, a test set-up in which paced mating can be investigated, reflects the *voluntary* participation in sexual behavior better in female rats.

Two standard paced mating set-ups are used for studying female sexual behavior: a bilevel chamber and a two-compartment paced mating set-up in which the chambers are connected with holes (of 4 cm in diameter) through which the female fits, but the male does not (because of his larger size). The bilevel chamber is designed in a such a way that the female can run around and avoid the male by changing levels that are connected by a set of ramps on either side in a narrow cage. This makes it more difficult for the male to mount her during a chase. The disadvantage of this paradigm, however, is the fact that the female needs to keep escaping instead of having a location away from the male to rest. In that perspective, the two-compartment paradigm seems a better way to investigate female sexual behavior. The female can now decide when and for how long she visits the male and receives sexual stimulations, which results in a more direct translational approach.

In the two-compartment paradigm, it is important to mention that the accessibility of multiple holes is essential. If only one hole is available for the female to enter the male compartment, the male can block the hole in his eagerness to get to the female. Practically, this results in less time she spends with the male and less received sexual stimulations, which is then not a measurement of her receptivity, but rather a lack of possibility to visit the male. By making multiple holes accessible, she always has the option to enter the male compartment.

Previously, in the review under male sexual behavior, we discussed the potentials and pitfalls of the 30-minute test versus the first ejaculatory series. When studying female sexual behavior in paced mating paradigms, 30-minute tests are the standard, although shorter and longer tests have also been used. Just as the lordosis test based on only 10 mounts, a study during only 1 ejaculatory series would not be an appropriate measurement of female sexual behavior. Even though the performance of the male is probably dependent on the accessibility of the female (and thus her sexual motivation and receptivity), it is still better to evaluate the female behavior as independently as possible from the male's performance. A complete 30-minute test would minimize the influences from the male, because it would include enough time for a combination of mounts, intromission and ejaculations, whether or not she copulates with a fast or sluggish male. In fact, females spend equal amounts of time and show the same amount of paracopulatory behaviors in the vicinity of a sluggish and a fast male [34], when a sufficient amount of test time is provided. Therefore, we recommend to study the sexual behavior of females in a 30-minute paced mating set-up in which all behaviors of the female (lordosis, paracopulatory and pacing behaviors) are evaluated. A two-compartment paradigm seems to be the best option.

4 Behavioral paradigms for sexual motivation

Whereas the paradigms mentioned above describe sexual behavior, they do not investigate sexual incentive motivation. As mentioned before, sexual behavior is divided into three phases, where sexual incentive motivation is part of the first, precopulatory phase. Some of the aforementioned measures of copulation are described (by others) to express motivation. Given the weight motoric responses have in the execution of this behavior, however, we think sexual incentive motivation, as described by the interaction between internal motivational state and incentive stimulus is not a factor in these phases of copulation. If these measures of

561 copulation indicate a kind of motivation, they rather reflect the propensity to continue to
562 participate in copulation.
563 To investigate sexual incentive motivation, the earlier phase of identification of sexual
564 incentives, and initiation of the efforts to gain physical contact with that incentive, some
565 paradigms have been proposed.
566
567 *4.1 Runway paradigm*
568 The *straight-arm runway*, as described by Lopez et al. [61], consists of a startbox (25 x 25
569 x 20 cm), a runway (160 x 10 x 20 cm), and a Plexiglas goalbox (45 cm diameter, 40 cm height;
570 see Fig. 2). A removable, transparent barrier within the goalbox prevents physical contact
571 between subject and stimulus, while retaining access to visual, auditory and olfactory cues. Both
572 the startbox and the goalbox are separated from the runway by removable doors, allowing the
573 entry of the subject to the runway to be controlled. Entry to the runway and subsequent entry to
574 the goalbox are automatically timed by infrared light sensors, which provides a measurement of
575 time needed for the subject to cross the runway and reach the goalbox. Before the subject rat can
576 take a run, they are placed in the goalbox with the target animal first, with the transparent barrier
577 in place. The subject is subsequently placed in the startbox, and the door is opened to start the test
578 and allow the subject to run for the known target stimulus. The runway test has successfully been
579 used with other incentives than sex, e.g. food [62], water [63] and drugs [64].
580 As shown by Lopez et al. (1999), male rats run faster towards a receptive female than to a
581 non-receptive female or male rat. The previously obtained sexual experience in the goal box did
582 not affect running times. Only after the experience of an ejaculation, the males seem to run faster
583 towards the goal box, but this effect was found for both a receptive female and a non-receptive
584 female as stimulus. Therefore, this confirms previous findings that copulatory experience is not

585 required in order for the male to prefer receptive females over non-receptive females [65-67], or
586 males [68-70]. This indicates that the runway paradigm is indeed suitable to study sexual
587 incentive motivation, and is usable for both sexually naive and experienced rats.

588 The key benefit of this test for motivation is that it (literally) is straightforward, as its
589 main measurement is the latency to reach the stimulus. If one expresses male sexual motivation
590 as the preparations and actions intended to gain physical contact with a female, the most direct
591 measurement of this approach behavior is the time needed to travel the distance between location
592 A and location B, where the female is. The directness of this test, however, also limits the
593 strength of the measurement: with a relatively short runway, the latency to reach the target is
594 short (in Lopez et al. (1999) a male reaches a receptive female within 25 seconds), which may
595 limit the possibility to discriminate between subject groups or stimuli. In addition, the short travel
596 time may allow internal states, such as anxiety or stress, and (distracting) extraneous stimuli, such
597 as sound, light, or movement, to possibly prolong or shorten the travel time, and thereby affect
598 the outcome. These effects can be filtered out easier in tests with a longer duration, and indeed,
599 this runway test has been used with runways up to 3 meters in length [71]. In any runway
600 paradigm, to reduce this vulnerability to extraneous effects, rats should be habituated to the test
601 set-up in order to reduce exploring and other novelty-associated behavior, and the startbox and
602 runway should be thoroughly cleaned between tests to reduce unwanted olfactory cues.

603 Compared to procedures where stimulus preference is measured (as in the sexual
604 incentive motivation test, see below), i.e. the subject has the choice between two or more targets
605 with different incentive properties (e.g. receptive female, non-receptive female, male), only one
606 target is present in the runway set-up. Whereas some stimulus preference procedures allow
607 distinction between sexual and social components of the incentive stimuli within one test, the
608 runway test only measures the total incentive value of the stimulus in the goalbox. However, this

609 is a relatively minor objection, since different incentive targets can still be tested with a within-
610 subject design by conducting multiple tests with the different stimuli. In that case, similar
611 conditions should be applied.

612
613
614 *4.2 Sexual incentive motivation test*
615
616 The *sexual incentive motivation (SIM) test* consists of a rectangular arena (100 x 50 cm)
617 of which the short sides are oval shaped (See Fig. 3, based on [72]). On both long sides, but
618 diagonally opposed to each other, a small box (25 x 10 x 25 cm) containing a stimulus can be
619 attached [72]. The arena and stimulus boxes are separated by steel mesh, physically separating
620 the subject from the stimuli, but allowing visual, auditory, and olfactory cues to be perceived by
621 both. Five minutes prior to testing, the stimulus rats are introduced into their respective stimulus
622 boxes. The subject, which is habituated to the arena on three consecutive days before the test, is
623 subsequently placed in the middle of the arena and allowed to move freely during a fixed period
624 of 10 or 20 minutes, after which the subject is taken out of the arena. Stimulus box A and B can
625 be interchanged to prevent influences of spatial memory. The room in which the SIM test is
626 located is dimly lit, so that a video camera, positioned above the arena, can take recordings,
627 which can be analyzed with tracking software. Using this software, two areas measuring 20 x 30
628 cm in front of the stimulus boxes are defined, and are called incentive zones. Thus, a host of
629 variables can be measured: time spent in incentive zones, number of visits to the zones, distance
630 moved during the test, and average movement speed. From these variables, the preference score
631 (time spent in incentive zone A/(time spent in incentive zone A + time spent in incentive zone B))
632 can be calculated. In addition, a number of basic behavioral observations, such as general
633 mobility, self-grooming, freezing, and rearing can be made using the video files.

Several studies performed in this paradigm showed that male rats have a significant preference for a receptive female, when given the choice between this female and a male or non-receptive female [72, 73], expressed by a preference score >0.5. Sexual experience does not affect this outcome. Castration of the male, on the other hand, does lower the preference score by spending more time in the neutral zone instead of in the incentive zones [72]. These effects are reversible with suppletion of testosterone propionate.

Similar results have been found with female rats, which spend significantly more time in the incentive zone of an intact male rat than with a castrated male or female rat [58, 74]. Interestingly, the sexual incentive motivation test investigates not only the interaction between internal motivational state and a stimulus, but also the relative strength (incentive valence) of specific properties of a stimulus: e.g. a non-castrated male is preferred over a castrated male, while a devocalized male has the same incentive valence as a sham male. The test can also be used to study the incentive value of isolated properties. For example, when only the odor of a receptive and non-receptive females was used in the stimulus boxes, both experienced and in-experienced males prefer the odor of the receptive female. Interestingly, the inexperienced males do not show a preference when the odor of the receptive female was mixed with another odor, e.g. when the bedding was used instead of urine, or when combined with almond odor [72].

Central to the validity of this paradigm of relative choice is the question whether the propensity for a subject to prefer one incentive zone over the other not only depends on the attractiveness (positive incentive value) of the preferred stimulus, but also on the repulsiveness (negative incentive value) of the non-preferred stimulus. This is especially important in a situation where a male stimulus serves as a control for a female stimulus. In a series of tests, Ågmo showed that a male control stimulus does not have a negative incentive value in the SIM test [72]. First of all, male subjects did not show a preference for non-receptive females over

male stimuli: no significant differences were found in the preference score, the number of visits, duration of visits, and time spent in incentive zone. Both inexperienced and experienced males showed these results. Second, in a comparison between the first five minutes of the third habituation (empty stimulus boxes) and the first five minutes of the test with either a male stimulus or a non-receptive female stimulus, the experimental rat spent significantly more time in incentive zones when an animal was present. Together, these results rule out the existence of a negative incentive value of either male or non-receptive female stimuli in this sexual incentive motivation test.

Because of the longer and fixed test duration, it seems plausible that the SIM test has a higher discriminative power than the runway test: random, short distractions will have less impact on a ten-minute test than on a 30-60 second test. In addition, because two stimuli are present at the same time, and it is even possible for the subject to withdraw from contact with either of them, it is possible to separate social motivation from sexual motivation. The preference score reflects a measure of stimulus preference relative to the other stimulus (A/(A+B)), thereby taking the potential social motivation out of the equation.

Again, familiarization of the experimental rat to the environment seems to be of specific importance. In a test with male subjects unfamiliar to the environment, the subjects showed no preference for the receptive female compared to a male stimulus. However, when the test was repeated 7 days later, the subject did show a significant preference for the receptive female, suggesting that a previous experience in the test set-up is sufficient to induce the required conditions for the test [72]. Ågmo suggested that one 20-minute session in the presence of incentive animals offers sufficient familiarization, but others have confirmed that habituation to the environment without stimuli present for 3 times 10 minutes offers the same result [73].

682 *4.3 Level searching paradigm*

683 Level searching as a measurement for sexual motivation is a phenomenon first described
684 by Mendelson and Pfaus [75]. It occurs when a sexually experienced rat moves through a familiar
685 behavioral test set-up with different levels, in an apparent search for a sexual partner.

686 The testing chamber was previously described by Mendelson and Gorzalka (see Fig. 4),
687 who developed the apparatus for easier evaluation of sexual behavior [76]. It consists of a
688 Plexiglas box, with dimensions of approximately 60 x 25 x 15 cm. 28 cm above the floor, a
689 platform with the length of 40 cm is mounted. Ramps on either side connect this platform to the
690 floor, enabling the rats to move freely. In a typical experiment, a sexually experienced male rat is
691 allowed to explore the chamber for 5 minutes, after which a female is introduced. A trial lasts
692 until the male rat reached ejaculation or for 15 minutes, depending on the receptive state of the
693 female.

694 In a series of experiments, Mendelson and Pfaus showed that male rats that were paired
695 with receptive females had, in the 5-minute period before the introduction of the female,
696 increasing level-to-level movements with successive trials, whereas rats that were paired with
697 non-receptive females showed no increase in level changes. Only after these rats had
698 subsequently been paired with receptive females did their level changing rate increase too.
699 Additionally, male rats that had achieved a stable number of level changes (during the 5 minutes
700 before introduction of the stimulus) were then either paired with a non-receptive female or left
701 alone in the chamber for 15 minutes. Rats that were left alone showed a decreased number of
702 level changes in trial 4-7 compared to the first trial. Rats that were paired with a non-receptive
703 female did not show a decrease in level changes, a finding that Mendelson and Pfaus explained as
704 a response to a conditional reinforcer, where presence of the non-receptive female was assumed
705 to have an association with previous sexual activity in the chamber.

When the bilevel chamber is used to observe copulation behavior, an obvious advantage of this set-up is the relatively natural aspect of it: all behaviors leading to, and including copulation are possible. In addition, the combination of specific components that make up the total incentive value of both female and male is intact. Visual, olfactory, tactile, and auditory cues can be perceived, and free movement enables female pacing and male pursuit. It is doubtful, however, that this matters when this chamber is used in experiments aimed at *incentive motivation*. After all, the measurement of level changes takes place in the absence of a receptive female, and thus the absence of the sexual incentive. It can therefore be argued that the resulting behavior, in the form of level changes, is not as much attributable to an intrinsic response to a stimulus with a certain positive incentive value, but could rather be explained as a kind of reward anticipation. In the level searching set-up, rats have to be sexually trained in the bilevel chamber in order to obtain a stable number of level changes as measure for 'sexual motivation': they need to know what will happen in this box before they start showing this kind of behavior. As a result, the rewarding aspects of the copulation will get linked to the environment, turning the environment into a conditioned stimulus. Thus, the number of level changes seen by Mendelson and Pfaus could reflect this reward anticipation, which is elicited by the total emotional valence connected to the test environment by previous experience, instead of solely reflecting sexual incentive motivation.

These phenomena of sexual motivation and reward anticipation might have different neuroanatomical substrates. This seems to be supported by the juxtaposition of two papers that investigated the role of the μ-opioid receptor antagonist naloxone on sexual motivation. Using the bilevel chamber, Van Furth and Van Ree found that systemic administration of naloxone to experienced and inexperienced male rats decreases the number of level changes during both the anticipation and the interaction period [77, 78]. Ågmo, however, using the SIM test, found no

difference between rats that had been injected naloxone, and control rats that had been injected saline: both had an equal preference for a receptive female over a male [79]. This suggests that different neural substrates are activated in different tests, and thus that level changes measure something else than pure sexual incentive motivation (see also Holloway [80]). The level searching paradigm would therefore be unsuitable to study this type of sexual motivation.

The elucidation of these distinct mechanisms is further complicated because naive rats cannot be tested in the level searching paradigm. Sexual experience is a *conditio sine qua non* when level searching and extinction are measured. Sexual experience has been proven to be a modulator for both responses to olfactory stimuli in, and for copulation itself [61, 72]. In fact, olfactory cues appear to be the most salient for incentive motivation in experienced males [7], and inexperienced males only seem to react to unambiguous odors [72]. In the bilevel chamber, Van Furth and Van Ree also found odor to be of particular relevance. Rats with a surgically impaired olfactory capacity did not show increased level changes during either the anticipation or the interaction phase, while their copulation behavior was comparable to control animals [77]. These results made them suggest that previously found level changes might have been induced by odors that were still present in the set-up from previous trials. These findings further stress the necessity to remove all odor of receptive females from the chamber in between trials.

4.4 Lever press paradigm

A well-known paradigm to research motivated behavior is the second-order schedule of reinforcement, in which the subject learns to perform work in order to receive a conditioned stimulus (CS), and ultimately the unconditioned stimulus (US). In an elaborate sequence of

752 experiments, Everitt et al. operationalized this paradigm for use in the exploration of male sexual
753 motivation [81].
754 A Plexiglas box measuring 28 x 26 x 28 cm is fitted with two retractable levers. Between
755 these levers a magazine for the delivery of food pellets is placed. A small light source that
756 functions as the CS is placed on the same wall as the levers. White noise (also CS) can be
757 produced in the chamber. On top of this operant chamber, immediately above a trap door, a
758 second, smaller box is placed, which contains a receptive female (US). Upon reaching of the
759 necessary responses on the lever, the trap door opens and the female enters the center of the
760 operant chamber, making her available for copulation. Prior to testing, rats are allowed to gain
761 sexual experience. The full subsequent second-order schedule can be found in Everitt et al. 1987
762 and Everitt and Stacey 1987 [81, 82]. In short, the main measurement for sexual motivation is
763 expressed as the number of responses in a fixed, 15-minute interval.
764 During the development of this paradigm, Everitt et al. reported some interesting findings,
765 which we will summarize briefly, after which we will discuss the role a second-order paradigm
766 can play in the investigation of sexual behavior: 1) On average, male rats took around 30-36
767 sessions to reach stable levels of performance. 2) Conditioning with both CS+ and CS- yielded
768 the same results as conditioning with only CS+. 3) Omission of the CS+ during a single session
769 resulted in a significant decrease in responses. 4) Rats that did not have a restricted diet (i.e. food
770 *ad libitum* the night prior to testing), did not respond to food, if the food was used as the US.
771 Rats' responses to gain access to the female, however did not decrease. 5) During a
772 postejaculatory interval (PEI), the willingness to work for a sexual reward was reduced, but the
773 willingness to work for food remained intact. 6) ejaculation latency is negatively correlated with
774 number of earned CS+'s (i.e. rats that were more willing to work, or more successful to perform
775 the task, had a shorter ejaculation latency). In addition, rats that were more successful with the

776 lever presses, showed less intromissions before ejaculation at the moment they had access to the
777 mate.

778 An obvious advantage of this paradigm is that both a form of motivation and copulatory
779 behavior can be registered in one test, just as in the level searching paradigm, but not in the
780 runway or SIM test. This way, as shown above, the willingness to work (which serves as a
781 measure for motivation) can be directly linked to the subsequent copulatory parameters. This is a
782 property which makes the test suitable for pharmacological interventions. However, a clear
783 downside of this test paradigm, is that the susceptibility to motor, memory and attentional side
784 effects is high. The paradigm employs learned operant responses as bar pressing for access to a
785 mate. In case pharmacological interventions induce an increase in the number of responses, this
786 could be mistaken for effects of learning, or memory of the procedure. Even more significant,
787 however, is that the rate or speed of responding is an important factor in this operant procedure.
788 A change in the motoric capacity of the subject could, therefore, severely affect the motivational
789 read-out. The SIM test, on the other hand, employs permanence in a particular area as an index of
790 motivation, minimizing the requirement of motor capacities. The SIM test can, at the same time
791 as investigating sexual motivation, measure the indices of ambulatory behavior (e.g. distance
792 moved and speed of movement) in order to exclude potential effects on motor functions and to
793 diminish the risk of false interpretations. To the contrary, although more relevant in this
794 paradigm, this lever press paradigm alone cannot control for ambulatory behavior. A separate test
795 of motor function can however be added.

796 More disadvantages can be described to the lever press paradigm, like the lack of
797 relevance for the incentive value of the female as soon as the male had paired the effort to the
798 reward. This lack of relevance is even more present here than in the bilevel chamber, because the
799 male rat will be motivated to work based on previous experiences and the expectation of that

800 happening again, but not because of the inherent attractiveness of the female. This was also
801 evident when the receptive female was substituted by a non-receptive female. Even though it is
802 likely that the male rat had a possibility, however limited, to smell, hear and see the female, it
803 would continue to show the lever press levels as before. Only in session 6 and 7 there were signs
804 of extinction, with the lever press activity decreasing by more than 50%. One explanation of this
805 phenomenon is that the lever press action is decoupled from the incentive properties of the
806 rewarding activity, and that the levers themselves gain reinforcing properties.
807 Regarding the ease of use, this second-order paradigm would demand involvement of a
808 highly skilled and experienced researcher: planning and execution are intricate and time-
809 consuming, while proper analysis of the data is complex.
810
811 *4.5 Interpretation of results*
812 In conclusion, the different test paradigms for sexual motivation actually measure
813 different components of motivation or reward anticipation. It is clear that the interpretation of
814 results is complicated and need extra attention. Based on our review, we believe that the level
815 searching and lever press paradigms are not suitable to test sexual incentive motivation. They
816 instead seem to measure reward anticipation more than the interaction between internal
817 motivation state and incentive stimulus. Motivation can be split up in a component of innate
818 sexual incentive motivation, that is activated by a perceived sexual stimulus, and a sexual
819 motivation obtained by previous experiences. The second motivation could, thus, be seen as a
820 strengthened incentive motivational response to the sexual stimuli by an increase in arousal
821 caused by previous rewarding experiences. This complete incentive motivation, however, is still
822 different from reward anticipation, because it is always a response to the presence of a sexual
823 stimulus (which could be a receptive female or just the smell of a receptive female), rather than a

reaction towards an associated situation like an environment without the stimulus. In this perspective, only the SIM test and the runway test are suitable to study sexual incentive motivation.

5 Concluding remarks

In summary, after describing all potentials and pitfalls of the different behavioral paradigms to study sexual behavior in rats, a few important lessons can be learned. First, it is absolutely crucial to use the appropriate model for the research. Whereas an incentive sexual motivation test is used to study sexual motivation, a copulation test until the 1st ejaculation can be useful to study e.g. the drug efficiency to treat premature ejaculation. On the other hand, when studying female sexual behavior, the use of a paced mating test allowing the female to control her sexual interactions is important. Second, in all cases, it is essential to be critical of the interpretation of results. We have given some examples in which a parameter was interpreted one way in the past, but where new knowledge has changed the perspective of interpretation. Third, some studies have not always investigated all aspects of the sexual behavioral pattern. Especially in female rat research, a shortcut was often taken by only measuring lordosis behavior and neglecting the paracopulatory behaviors. Therefore, we propose that the measured parameters should always be described in the most complete and neutral sense as possible. When all behaviors are described as they are, it allows for 1) changes in interpretations and 2) comparisons with other studies in the future.

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847

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Fig. 1: Sexual behavior cycle

Schematic overview of typical sexual behavior. M = mount, I = intromission, E = ejaculation, L = lordosis, ● = dart/hop, PEI = postejaculatory interval.

Fig. 2: Runway test

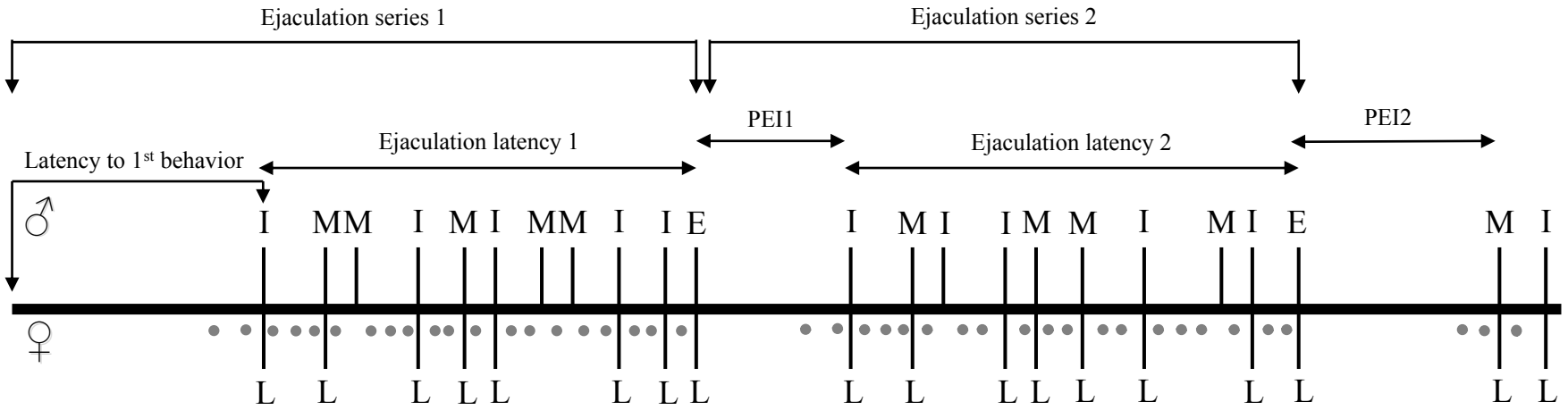
Mechanically removable doors separate the runway from the start and goalbox. Infrared photocell emitter–detector pairs situated at the beginning of the runway and just inside the goalbox allow measurement of the time the rat spends inside the runway.

Fig. 3: Sexual incentive motivation test

Design of the sexual incentive motivation test setup.

Fig. 4: Bi-level chamber

Schematic impression of the bilevel chamber used in the level searching paradigm (not on scale)


Ejaculation series 1
Ejaculation series 2
PEI1
PEI2
Ejaculation latency 1
Ejaculation latency 2
Latency to 1st behavior
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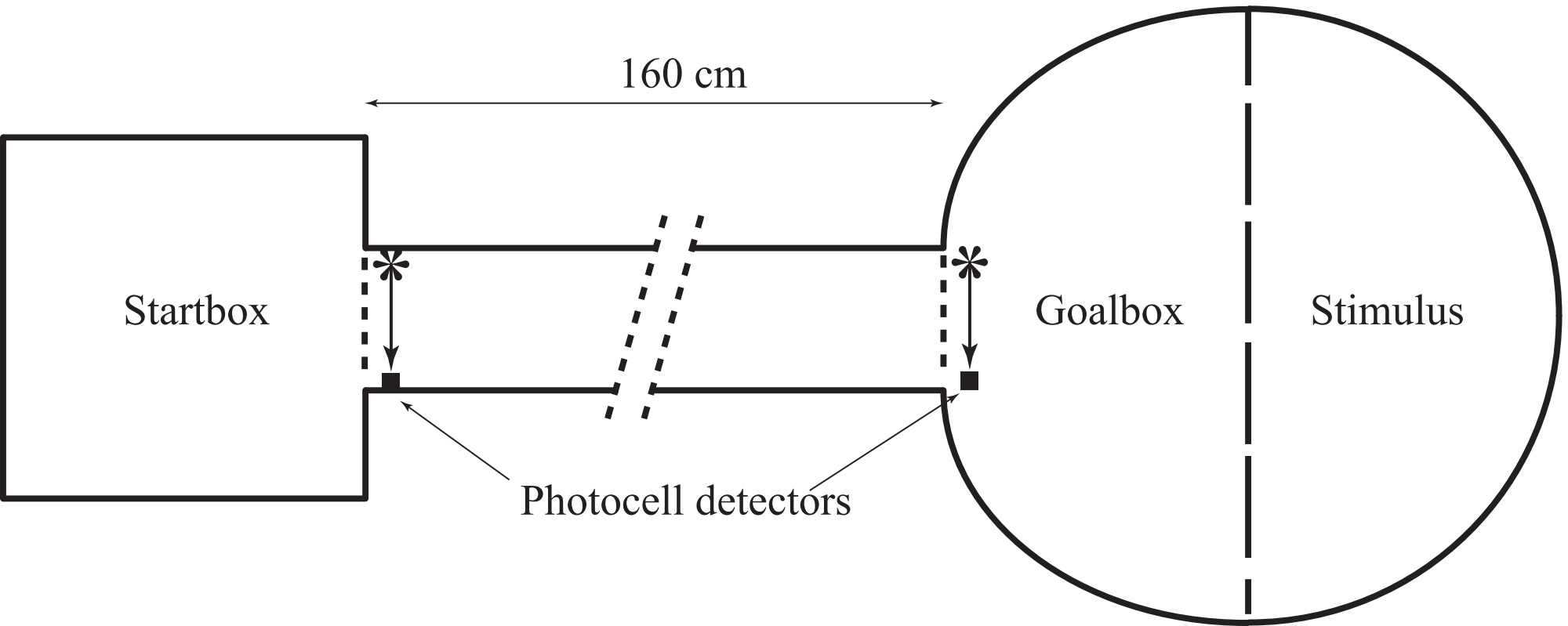
160 cm
Startbox
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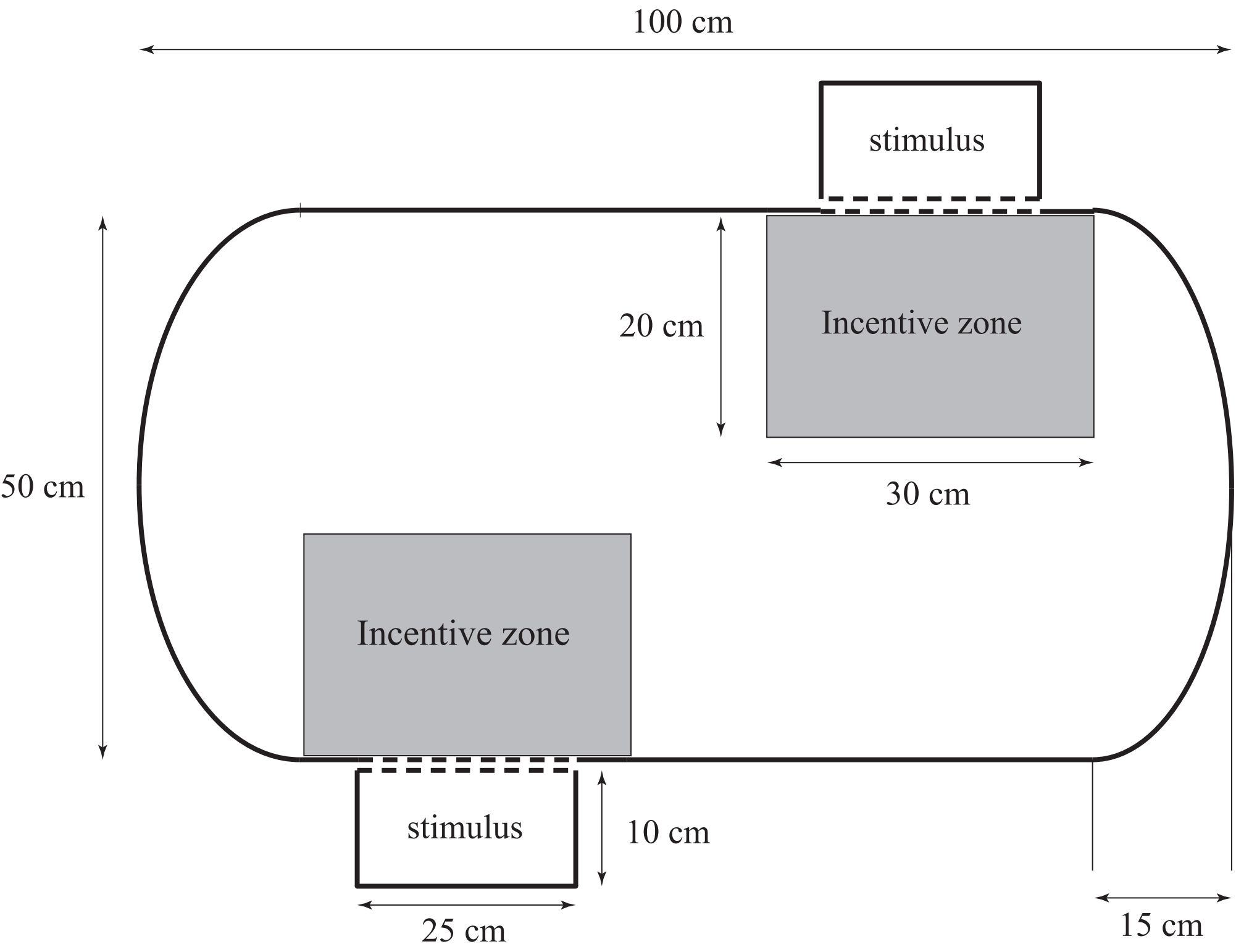
Photocell detectors


100 cm
stimulus
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