Evaluation of sexual behavior in laboratory vs seminatural conditions

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Short title: Sex and seminatural environments

Abstract

Sexual behavior is, by necessity, sexually dimorphic. Males transfer sperm to females, whereas females receive sperm from males. Discussions of sex differences in copulatory behavior are consequently trivial. However, the behaviors associated with copulation, for example mate choice or postcopulatory reactions, may well be similar in males and females. Such differences, even subtle, are far easier to observe in seminatural environments than in the standard laboratory cage. We will present examples of the use of seminatural environments in insects and rodents. Even though most studies of insect sexual behavior are performed in relatively simple laboratory procedures, there are also some studies performed in natural or seminatural conditions. We briefly describe the most common procedures used, and mention the main results. It is noteworthy that insect studies focus on sexual approach behaviors, particularly the role of visual and olfactory stimuli in mate location. The actual copulatory behavior, i.e. how gametes are transferred from one individual to another, seems to be of less interest.

The sexual behavior of rats has traditionally been studied in heterosexual pairs, despite the fact that they often copulate in groups. Nevertheless, data obtained in the simplified environment have advanced knowledge of the endocrine and neurobiological control of sex behavior in a quite spectacular way. The understanding of the dynamics of the sexual interaction and the possible function of the many peculiarities of rat sexual behavior has not advanced to a similar degree. Studies in seminatural environments may provide valuable data concerning sociosexual interactions and how such interactions are modified by contextual events. Furthermore, observations made in an environment, which incorporates the basic features of rats' natural habitat, offer some external validity. This is of importance when we want to generalize our results to contexts outside the laboratory, and it becomes of paramount important when we want to make inferences about behavior in other species, for example the

human. We offer here a detailed description of an environment designed for studies of group-living rats, with notes on the observation procedure and the analysis of the large quantity of data generated in the environment.

Key words: Sexual behavior, Insects, Rodents, Representative design, Social interaction, Olfaction

1. Introduction

The vast majority of animals reproduce sexually. With the exception of self-fertilizing hermaphrodites, and perhaps animals with external fertilization, this obliges animals from the simplest to the most complex species to engage in some kind of sexual behavior. Consequently, sexual behavior has been studied in several classes of invertebrates [1] as well as in many chordates. Whereas many invertebrates have quite modest nervous systems, the insect brain processes sensory information, integrates different sensory modalities and controls motor systems in a way similar to the vertebrate brain [2]. In contrast to their complex behavior, the insect brain is small and simple compared to that of mammals. Therefore, insects may be used as model organisms for studying the neural bases of behavior. However, insects are often overlooked in reviews of sexual behavior. We find it important to draw attention to the valuable data obtained in these animals. Consequently, we devote the first part of this chapter to a synopsis of procedures employed in research on insect sexual behavior. The second part of this chapter is dedicated to a rather detailed presentation of a seminatural environment used in studies of rat sociosexual interactions.

In all species with internal fertilization, for example mammals, gametes need to be transferred from one individual to another. Males produce sperm and females are equipped with eggs. For some reason, nature has arranged that sperm must be deposited in the interior of the female reproductive organs before fertilization can occur. This is a necessity because of the dimorphic reproductive organs. The fact that the male expels gametes from his body whereas the females receive them in hers has made male and female sexual behavior entirely different. In fact, sexual behavior is inevitably dimorphic. At the same time, it must be remembered that males of most species are perfectly able to display typically female behavior patterns, and females can easily display male sexual behavior patterns. In rats, males now and then display lordosis and occasionally also paracopulatory behaviors. Female rats frequently

mount other rats, and sometimes they can show behavior most similar to the male behavioral pattern associated with vaginal penetration and even ejaculation. The difference is that the likelihood for a male to display a female pattern is far lower than the likelihood that he displays a male pattern. The inverse is true for females, i.e. the likelihood for a female to display male behavior patterns is far lower than the likelihood of displaying female patterns.

Even though male and female reproductive behaviors, i.e copulatory behavior and behaviors associated with parental care, are dimorphic by necessity, there is no *a priori* reason to believe that behaviors unrelated to reproduction are. Even though the frequency of many behaviors, for instance acts of aggression, may differ between males and females, such quantitative differences are not inevitable. To the contrary, only males can transfer sperm and only females can lactate. Seminatural environments offer excellent opportunity for observing a vast spectrum of non-sexual, non-dimorphic social behaviors as well as emotional reactions to aversive as well as attractive stimuli. We will briefly provide an example of this at the end of this chapter.

2. Examples of insect model organisms

Several insect species have been used in research studies for different purposes. Fruit flies (e.g., *Drosophila melanogaster*), with a lifespan well-suited to laboratory research, are small, abundant, and easy to manipulate. Currently studied by over 1800 labs around the world, they are the most powerful model insect in neuroscience for understanding multisensory signal processing in courtship (e.g., [3,4]), odor memory and synatic plasticity [reviewed by 5], as well as neurodegenerative diseases, like Parkinson's Alzheier's and Huntington's disease [reviewed by 6]. The majority of observations of sociosexual behaviors in fruit flies are performed in varied laboratory conditons, while only few studies systematically document fruit fly sexual behaviors in the natural habitat. Field observations

demonstrated that, like rats, the mating territory of each fly was rather small, and a number of flies could copulate in close proximity [7]. After orienting and approaching a potential mate, the male engaged in an approximate 2.5 minute species-specific courtship before attempting to mount and copulate [7,8]. During copulation, a third fruit fly might approach the mating pair, and according to the field work conducted by Dukas [9], a male fly was more likely to display approach to mating pairs than was a female fly. Multiple copulation with different sex partner is common in fruit flies. In addtion to fruit flies, lepidoperan species are also commonly used model insects in reproductive activities and pheromone tracing, e.g. silk moth (*Bombyx mori*) [10], as well as a group of sympatric heliothis moths that use the same sex pheromone components (air-borne chemicals) in different ratios for reproductive isolation (e.g., [11]). Species-specific sex pheromones plume released by the sexually mature female is first detected by olfactory sensory neurons located on the male antenna, and the male will demonstrate an upwind, zigzag flight [12] to trace the origin of these phermone signals. Multiple mating to different partners is common in heliothis moths, e.g. female tobacco budworm moths, *Heliothis virescens* [13], and female and male cotton bollworm moths, *Helicoverpa armigera* [14].

3. Multisensory processing in insects' mate choice and mate searching and its importance in experimental methods selection

Orientation to a proper mate is vastly important in reproductive success. Insects make good subjects for the study of discerning mechanisms and processes governing mate search. Many insects rely on simultaneous sensory signals in more than one modality to trace the potential mate partner. Within their small but complex nervous system, the insect first encodes the external information into electric signals via different sensory organs, and then computes this information into different strategical plans. Based on the tracing distance, we

find two types of reproductive searching, the short-distance and long-range mate tracing. Both short or long mate-tracing activities are multimodal navigation, which is one of the fundamental activities for insects to locate the source of an odor that may indicate food, a mating partner, proper oviposition site, or aggregation into swarms [15,16]. The short-distance exploration of the local environment for mates usually has a small traveling distance. For instance, male fruit flies orient to female at a distance of 1.5-2.5 mm [17]. Such tracing activity is based on the female mating signals combining olfactory, visual and wing-vibratory information [18]. Generally, male flies preferentially mate with larger, more productive females [19,8]. Impaired sensory perception can affect mate choice in fruit flies, particularly in females. It appeared that female mate choice persists in a rational, transitivity manner, only when one of the sensory cues, i.e. either visual or olfactory modality, is impaired, but not when both sensations are impaired [20].

The long-range tracing, on the other hand, occurs mainly in moth species, in which the males are extraordinarily good at finding the conspecific females from a distance of more than one kilometer [21]. With a supreme sensitivity, male moths can detect a single molecule of female-produced pheromone [22]. However, sensing attractive odors alone is not enough to result in approaching. Flying moths rely profoundly on the visual system when tracing an odor source [reviewed by 23]. Without visual feedback, moths find it very difficult to track airborne pheromone plumes successfully [24]. Contrary to our intuition, no odor-mediated behavior in moths is based on *chemotaxis,* i.e., there is no direct navigating response to odor concentration gradients. Moths rather steer against the odor source in correspondence with optomotor *anemotaxis* (*anemo*: wind, taxis: directed movement), which requires olfactory, visual and mechanosensory feedback [25].

Although insects may not necessarily utilize all components of a multisensory cue, a more ecologically relevant laboratory assays would greatly advance our understanding of the

mechanisms of sexual-behavior-related orientation. It is worth mentioning that selection of experimental method depends on the perspective of the individual research question. In the studies examining neurophysiological properties of individual sensation (e.g., olfaction, vision, audition, etc.), providing a single-component cue is highly desired. For instance, the experiments reporting the mapping of the pheromone pathways were conducted in such controlled manner, exposing the male moths to the female-produced pheromone puff and minimizing the interference of foreign emissions and other factors such as visual/auditory stimuli as much as possible (e.g., [26,27]). However, in complex behavioral studies, such as exploration of the mate-tracing flight pattern, to focus on only one sensory cue while neglecting the involvement of multisensory perception may lead to results with less external validity.

4. Experimental methods used in laboratory

4.1 Small behavior chamber

The shape and size of the chamber used for studying insect sexual behavior are diverse, but they are generally small, suited for subjects like fruit flies for a short period of behavioral assay. The acoustic behavior chamber (Fig. 1A) used in studying auditory experience and sexual response in fruit fly was 50 mm long × 10 mm wide × 6 mm high [28]. The courtship chamber (Fig. 1B) used to examine the choice between feeding and sexual behaviors in fruit flies was a petri dish measuring 2 cm wide [29]. The number of available sexual partners in such behavioral tests is one in most of the cases. Thus, the observations of the behavioral patterns in a pair of flies or a few flies in this kind of chamber/apparatus usually are far less representative to the wild fruit flies living in their natural conditions.

A Y-tube or T-maze bioassay apparatus is also a commonly used behavior chamber in olfactometer experiments. As shown in 1C, the Y-tube used in Biasazin et al. [30] was

intended to test the detection of food volatiles in fruit flies. It was made of a borosilicate glass tube with an inner diameter of 3.1 cm. The length of the upstream arms was 16 cm each and the common arm was 14 cm long. The upstream end consisted of glass odor source chambers (8.5 cm and 2 cm id) containing a stimulus. Such apparatus with different size can be used in preference tests with other volatile organic compounds, e.g., sex pheromones, in fruit flies and moths [31,32].

4.2 Physiological recordings in individual insects

For studies focusing on understanding the physiological mechanism of sexual attractiveness, observing the response to sensory cues on a neuronal level is broadly used. The most relevant stimuli in these experiments are a group of olfactory cues functioning as sexual attractant or repellents. Sex pheromones, produced by a conspecific female serve as the main sexual attractant, whereas sex antagonists, produced by a sympatric heterospecific female, serve as repellents. Extracellular recording and intracellular recording/staining techniques allow to obtain the instant electrophysiological activity of individual neuron across different period around the stimulation window (e.g., [26,27,33]). Another method, the calcium-imaging experiment, is to monitor the change of cellular calcium signal in a group of neurons (e.g., [34,35]). In these assays exploring the odor perception, the subject is normally immobilized, and the stimuli are closely applied. However, mate searching is not based on olfaction exclusively. Flying or walking are commonly occurring simultaneously with olfaction perception. A modern experimental arena designed to allow the insect free flying in complex 3D virtual reality environments (MultiMoVR arena including airflow, odor plumes, skyglow, grass and trees, etc., see Fig. 2) was developed for studying insect long-range search behavior [36]. This experimental arena is most appropriate for behaving insects (particularly flying), yet it has not been used in studies of insect mate search strategy. This kind of methods are

highly inspiring and they have huge potential to impact behavioral neuroscience.

4.3 Observing apparatus used in a population of insects

In many species, the territorial area of each insect in the wild is rather small and multiple mating are common. Thus, the representative value of examining a pair's behavior is lower compared to analyzing a group of insects. A population observation cage was designed to observe sexual selection and sexual isolation in a such context [37-39]. In the sexual isolation studies [37,38], a total of 24 pairs (12 virgin females and males from one population and 12 virgin females and males from a second one) were used for random mating experiments for 90 min. Group copulation tests involving multiple pairs were also used in evaluating mating preference. In Korol et al. [39], 40 flies (10 pairs from one location and 10 pairs from a second location) were introduced simultaneously into a mating chamber and were observed for 60 min. As soon as a pair started copulating, the mated types were recorded before the pair was removed from the chamber [39]. A population cage has also been used in testing the tracing of sexual signals emitted by the opposite sex in the laboratory. Keys, Mills [32] investigated the attractiveness of female sex attractant (odor) in highly dense male angoumois grain moths (*Sitotroga cerealella*) population. The cage used in this study contained 600 individuals simultaneously, having a size of 63.5 cm long × 63.5 cm wide × 63.5 cm high, in which three traps with female-produced attractant were hang 20 cm from the top in a 30 cm triangular pattern in this cage and the number of trapped males was monitored.

4.4 Seminatural environment with host plants

In many of the experimental methods we mentioned above, the subject insects are either restrained or confined to a very small space with unnaturally strong experimental stimuli, such that the subject could not avoid perceiving them. Additional to these unwanted

scenarios, host plants were seldomly provided. The chemical, visual and tactile cues from the host plants are specifically important for some sexual behaviors, e.g., mate choice and female ovipositing activity. Only a few studies used ecological enriched laboratory assays, e.g., seminatural environment with host plants [40,41]. In a study by Arita and Kaneshiro [40], multiple male fruit flies were placed into a 23 cm long x 23 cm wide x 23 cm high glass cage with a potted Syngonium plant for a 24-hr period to acclimate to the cage conditions, before a virgin female was placed into the cage with the two males, and her choice of mate was recorded. A simplified version of seminatural environment was used in an experiment to test female egg-laying preference in gravid female hawk moth, *Manduca sexta* [41]. In that experiment, two species of nonflowering host plants of similar size were placed at the upwind end of a transparent wind tunnel (220 cm long × 90 cm wide × 90 cm high, see Fig. 3), and the oviposition deterrent effect of feces from conspecific larvae to the females were compared by the number of eggs between two plants.

5. Insect conclusion

This short overview of some of the procedures used for studying insect sexual behavior should illustrate that the main theme of research in this area concerns mate localization and mate choice. The stimuli, olfactory and visual, activating sexual approach behaviors have been studied in some detail, and the neurophysiological activities generated by these stimuli are starting to be understood. The sexual acts themselves have been far less studied. The motor patterns displayed during copulation and their sensorimotor control have not been as carefully described as the sexual approach behaviors. An important issue that has not been sufficiently addressed is whether the observations made in insects in any way contribute to the understanding of mammalian, including human, sexual behavior.

6. Why study rodent sexual behavior?

Among rodent behavior patterns, sexual behaviors are exceptional for several reasons. To justify this assertion, we can use the female rat as an example. Intact females usually reject mating attempts from males or other females with a series of rather stereotyped behaviors. For a short period every 4 or 5 days, depending on the length of the estrous cycle, the female's behavior is drastically changed. Instead of rejecting males' mounting attempts, the female actively incites males to mount with a few behavior patterns named paracopulatory (formerly proceptive) behaviors. When the male mounts, the female arches her back in a concave fashion, extends the hind legs and moves the tail to one side. Thereby the vaginal opening is made accessible for the male's erect penis, and vaginal penetration may occur. Neither this response to a mount, lordosis, nor the paracopulatory behavior are displayed outside this short period in the cycle. This short period, called proestrus, is preceded by increasing blood concentration of estradiol, and coincides with the LH surge, ovulation and a rise in progesterone. The female rat sexual behavior is, in fact, strictly dependent on ovarian hormones. No other known behavior shows this extremely rigid dependence on hormones. In male rodents, the sexual behavior is also dependent on gonadal hormones, but this dependence is not so immediate. Castration of a male rat will lead to a gradual decline in sexual behavior during a few weeks until the behavior disappears. It can be restored by the administration of testicular hormones, but restoration is gradual and needs one or two weeks. Nevertheless, it can be maintained that male sexual behavior is as dependent on gonadal hormones as female behavior is, although on a longer time scale.

Not only is rodent sexual behavior strictly hormone dependent, it is also highly stereotyped. In females, paracopulatory behavior and lordosis can be considered as simple reflexes, produced by stimuli from the male. The fundamental unit of male behavior, the mount accompanied by anteroposterior pelvic thrusting, is also highly stereotyped and can be

considered a reflex activated by tactile stimulation of the preputial and perineal areas by the female's back.

Sexual behaviors are biologically significant, yet they do not depend on organismic need as eating and drinking do. Abstinence from sex has no deleterious consequence for the individual. Furthermore, the behavior is unlearned. Most males and females will engage in sexual interaction when given the opportunity to do so, without any prior training.

Because of the reasons outlined above, sexual behavior is a perfect choice for those interested in studying the neurobiology of hormone-dependent modifications of behavior. There is no mammalian, hormone-dependent behavior pattern better described than lordosis in female rats. The sensory pathways from the skin on the flanks to the hypothalamus as well as the motor output to the muscles in the back responsible for the lordosis posture have been carefully mapped, and the molecular actions of estradiol in the crucial hypothalamic neurons are beginning to be elucidated [42-44]. Knowledge of the neural control of mounting and the ensuing penile insertion lags seriously behind, but progress has certainly been made ([45-47], see also [48] for a review).

7. The role of representative design in the studies of rat sexual behavior

The knowledge summarized above has been obtained in very simple behavioral procedures. One male and one female are put together in a cage, and the experimenter just observes what they are doing. This procedure is straightforward and efficient for obtaining data in a short time. It has proved invaluable in neurobiological studies of sexual behavior.

Now and then scientists are interested in making generalizations from their data to contexts outside of the laboratory. Some want to speculate about the adaptive value of some behavioral element, and others may want to generalize their rat data to the human, i.e. use rat sexual behavior as a model for human sexual behavior. Then the simple behavioral

procedures may not be appropriate. Enclosing two individuals of opposite sex in a barren arena dramatically limits the behavioral repertoire. Furthermore, since the subjects cannot escape from each other, and since few alternative behaviors are possible, they are almost forced to engage in sexual activities. The situation is entirely different in rats' natural habitat. According to observational data, a sexually receptive female attracts a pack of males, all trying to copulate with her. The female can control copulation by escaping into a burrow or elsewhere [49,50]. The natural environment also offers possibilities for other behavior patterns, like foraging, or non-sexual social interactions with other individuals appearing in the rat's home range. The behavioral repertoire is extremely rich in this environment. This means that motivational factors determine which of all possible behaviors the rat will choose to perform. The fact that rats copulate in groups rather than in couples changes the dynamic of the sexual interaction, and since alternative behaviors are available, motivation to have sex relative to all other motives becomes of paramount importance.

Whenever we wish to make assumptions about the function or adaptive value of a behavior we need to assure that we study the behavior in an experimental setup as similar to the natural habitat as possible. This was pointed out many years ago [51-53], and experimental designs incorporating these features are called representative. Only a representative design allows for generalizations from our results to the world outside of the laboratory. If we want to generalize our findings to another species in addition, it is likely that representative design, in the brunswikian sense, becomes still more important, as discussed elsewhere [54,55].

Here we need to mention that a variant of the standard observation setup allows the female to escape from the male, thereby acquiring control of the pace of sexual interactions [56,57]. The mating arena is simply divided in parts connected with small holes. The small female can pass through the holes, and is therefore free to move about the entire cage. The

larger male cannot penetrate the holes, and is, consequently, confined to one part. This variant, usually called paced mating, has been rather popular for many years. It approaches the natural situation in the way that the female can escape from the male, but the behavioral repertoire is still quite limited. Furthermore, a fundamental element of the natural context, group mating, is entirely absent.

In an effort to study rat sexual behavior with a representative design in the laboratory we have adapted a seminatural environment, originally described by McClintock [58-60]. Rats live in male – female groups in a large environment consisting of a burrow system similar to a real burrow, attached to a large open field with some objects available. Thus, the main features of rats' natural habitat (group living, a burrow, and a large open space) are included, making it possible to claim that this procedure satisfies the brunswikian criteria for a representative design. Detailed descriptions of sexual interactions in this context [61,55] have revealed several important differences from the standard small cage. The relationship between sexual behavior patterns and fertility has also been analyzed [62]. We believe that data obtained in this environment can be generalized to the world outside the laboratory. Whether it also can be generalized to other species remains an open question, although there are some indications that it actually can. However, the validity of such generalizations remains an open question.

In the following we will give a detailed description of the experimental setup as well as the procedure for performing experiments and analyzing the enormous amount of data obtained.

8. Materials

8.1 Apparatus

A drawing of the environment is provided in Fig. 4. The burrow is constructed on sheets of dark gray unplasticized polyvinylchloride. The distribution of the tunnels as well as of the nest boxes is based on descriptions of wild rat burrows [50]. The size of the tunnels and boxes, for example, are similar to those found in a real rat burrow. They are all built of sheet steel, covered with black plastic on the inside and fixed to the polyvinylchloride base. During experiments, Plexiglas fixed to the boxes and tunnels with building tape covers the entire area. The floor is covered with an approximately 2 cm thick layer of aspen wood shavings (Tapvei, Harjumaa, Estonia). Nesting material in the form of 6 pieces of square mats of non-woven hemp fibres (5 × 5 cm, 0.5 cm thick; Happi mat, Datesend) is put in each nest box. The burrow connects to an open area via 4 openings (8 x 8 cm). Twelve small wooden sticks (2 × 2 cm, 10 cm long, Tapvei) are randomly put on the floor of the open area. Three plastic shelters of red polycarbonate (15 × 16.5 cm, height 8.5 cm; Datesend, Manchester, UK) are also provided. The walls surrounding the open area are 75 cm high, which is enough to ensure that the rats are unable to escape from the environment even though no cover is used. Like in the burrow, the floor is covered by about 2 cm of aspen wood shavings. Four 500 ml drinking bottles hang on the exterior wall, with the spout extending 5 cm through a hole about 10 cm above the floor on the inside.

The burrow and the open area are separated by a wall of heavy dark cloth extending from floor to ceiling and from wall to wall. This makes it possible to maintain the burrow in complete darkness even though the open area has a 12:12 h light/dark cycle. The light needed for video recording comes from two infrared lamps (850 nm; model Sal-60, New Surway Digital Technology (Shenzhen), Guangdong, P.R. China) located on opposing walls of the room. In the open area, the transition from light to darkness and vice versa is made gradually during 30 min at the beginning and end of the light phase. Light intensity is 180 lx at floor level during daytime and 1 lx during nighttime.

One digital video camera (Basler acA2000-50gc, Ahrensburg, Germany) is located about 2 m above the burrow and another camera is fixed above the open area. Both are connected to a computer equipped with Noldus Media Recorder 2.5 (Noldus, Wageningen, the Netherlands).

The animal quarters' ventilation system produces a constant background noise of about 40 dB.

8.2 Animals

We have constantly used Wistar Han IGS rats from Charles River (Sulzfeld, Germany). Other strains and other providers would probably work equally well. Rats are ordered as adults (250 g for females, 300 g for males) and kept in the animal quarters for at least two weeks before experiments. They are housed under the light/dark schedule to be used during experiments in a room with constant temperature (21 ± 1 °C) and controlled relative humidity (55 ± 10 %). Same-sex pairs are cohabitating in Macrolon® IV cages, with food (RM1, Special Diets Services, Witham, UK) and tap water constantly available.

In some experiments, the rats are gonadectomised or given intracerebral injections, or whatever treatment may be required. This is always done during the two-week period between arrival and start of experiments, allowing the animals sufficient time for complete recovery.

8.1 Procedure

8.1.1 General considerations

The groups used in the environment consist always of 3 males and 4 females. This sex ratio (57 % females) is similar to what has been reported for adult rats observed in the wild [61]. The population density in the environment is 1.4 rat per square meter. This should be considered a very high density in the wild. Practical considerations do not allow for

maintaining a population density more similar to the wild condition. In experiments requiring treatment of some kind, e.g. drug administration or manipulations of gene expression, a reasonable number of subjects in each treatment is necessary. This means that several groups must be run in the environment before a reasonable number of rats has received each experimental treatment. The smaller the number of subjects in each group, the larger the number of replications needed. The number of replications becomes important because of the large size of the environment and the consequent need for substantial lab space combined with the length of the procedure.

8.1.2 Preparation of the animals

When seven animals are to be observed simultaneously in a large and rather complex environment, easy identification of each of them is fundamental. Therefore, we shave different areas of the back of the animals A rectangle, about 2 × 4 cm, is carefully shaved on the back of the rats about 3 h before introduction into the environment. On one female, the rectangle is close to the tail, in another it is in the middle of the back, and in a third it is close to the neck. The fourth female is not marked on the back. Males are marked exactly as the females. Because of their bigger size there is no danger of confusion between males and females. The tail is marked with one, two, three or four transversal, thick black lines. Any permanent marker pen can be used, e.g. Edding 500 (Edding international, Ahrensburg, Germany). The double marking makes it possible to identify the rats on the video record with a certain ease.

8.1.3 Preparing the environment

The floor needs to be covered with fresh wood shavings, a Plexiglas cover needs to be fixed over the burrow, food (about 2 kg of standard rat pellets) must be put on the floor in one

corner of the open area, and water bottles must be filled. The video recording system should be checked and started.

8.1.4 Using the environment

The rats are introduced in rapid succession over a period shorter than 5 min. Only one animal per cage is used, assuring that all animals are unknown to each other at the beginning of the experiment. When all 7 rats are in the environment, humans leave the room. The rats remain there for 8 days. The only disturbance during that time is a daily, human visit to check the proper functioning of the equipment and that water remains in the bottles. This visit is always made shortly before the end of the light phase of the cycle.

Pilot studies indicated that rats showed elevated locomotor activity when introduced into the environment. Activity was gradually reduced over the first 48 h, and remained stable for the rest of the observation period. It also appeared that social interactions became stable after 48 – 72 h. Nevertheless, we do not start experimental manipulations until day 5 in the environment, and whenever possible we wait until day 7. This might be an unnecessary precaution, but we want to have the social group solidly established at the moment experiments are performed. In purely observational studies, we use data from any moment of the period, as appropriate. For example, when we wanted to see how devocalization affected the initial social interaction, we observed behavior during the hour following introduction of the animals into the environment [64].

In some experiments, all or some of the animals need to be manipulated (e.g. [65]). Drugs or hormones can be injected whenever appropriate. This requires capture of the animals, which is easily accomplished. During capture, we usually block the entrances to the open area. All animals that are to receive treatment are first captured, then treated and thereafter reintroduced into the environment at the same time. Injections or other treatments

are performed in another room in order to avoid disturbing animals that shall not be treated and reduce the likelihood that possible aversive experiences during treatment become associated with the environment. As far as we have observed, removal of the animals, injection and subsequent return to the environment have only a short-lasting effect on behavior. Within 15 min of return, the rats have resumed their regular activity.

All kinds of events may be introduced into the environment, something useful when we want to study emotional reactions. Pleasant and aversive odors may be distributed in the burrow or in the open area or both. We use an olfactory stimulation equipment (Olfactory Stimulus Package, Medical Associates, Georgia, VT) connected to nozzles placed on the walls of the tunnels in the burrow and in the open area. Assuring an efficient flow of scented air, the rats can be exposed to any odor. We have used the aversive odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Contech, Delta, BC, Canada), smelling of fox, and the pleasant odor of lavender oil (AromaBio, Lyon, France). Tasty foods, e.g. chocolate tasting pellets (Supreme Mini-Treats 1 g; F05472; Bio Serv, Frenchtown, NJ) can be provided in limited amounts, allowing observation of who has first access and how much each individual consume, among other things. Fear-inducing, sudden events, like intense white noise, can be introduced. This is easily produced by a noise generator (e.g. from Lafayette instruments, Lafayette, IN) and loudspeakers placed in the ceiling above the burrow and open area. A sound intensity of 90 dB is sufficient for producing an immediate fear reaction.

We have used a sequence of aversive and attractive events administered during part of the dark period, trying to mimic the events a rat is likely to encounter during a nightly walk in the home range [66-68]. Only the scientist's creativity imposes limits on the events that can be used.

8.2 Behavioral observations

All observations of behavior are made from the video records. The records from the burrow and the open area are synchronized and shown on the same screen. Before beginning observation, it is indispensable to establish an ethogram, such as the one shown in Table 1, including all the behavior patterns of interest. In addition to record the frequency and duration of the behavior patterns defined in the ethogram, it can be extremely useful to determine to whom the behavior was directed (in case of social or sexual behavior) and where in the environment it occurred. If social interaction is of interest, it is fundamental to know not only the emitter of the behavior but also the receptor. Behavior patterns are not uniformly distributed in the environment. Therefore, the spatial distribution of behavior can offer important information. In addition to the obvious distinction between burrow and the open area, it can be useful to divide the open area in sectors. Thus, besides recording emitter and receptor, we systematically record location.

Efficient software, fixing behavior patterns in time besides recording the data mentioned in the preceding paragraph, is essential for the recording of behavior. We use the Observer XT, version 12.5 (Noldus, Wageningen, The Netherlands) to that end. The normal procedure for making observation is to observe one rat at a time, during a prefixed time interval. Since there are 7 rats in the environment, this interval must be multiplied by 7 when estimating the time needed for behavioral observation. The amount of time required for data collection is probably the main drawback of this procedure. Furthermore, observers must be trained, and interobserver reliability must de determined if more than person is in charge of the observations.

Although not feasible at present, it is most likely that highly efficient algorithms incorporating deep learning may be able to replace human observers in the near future. Specific body parts and their position relative to each other as well as their movement can already be determined and linked to specific behaviors (e.g. [69]). It should be possible to

identify behaviors like grooming, mount and lordosis, etc. with relative ease. Moreover, it appears that the identity of similar looking animals, like rats or mice, can be preserved even when the animals are interacting closely [70]. When the entire ethogram can be computerized, an enormous amount of information can be extracted with minimal investment of human labor.

8.3 Data analysis

Classic statistical procedures may be used both for describing and analyzing the data. Depending on the number of behavior patterns and the complexity of social interactions, such analyses may be cumbersome and time consuming. Nevertheless, since they produce a lot of easily understandable information, they are certainly most useful. Often, the number of behavior patterns that are of interest calls for a multivariate analysis, particularly if some treatment has been given to the animals. Multivariate analysis can be performed using standard statistical packages such as SPSS (IBM Corp., Armonk, NY, USA), or with the free software R. The latter has the advantage of including non-parametric, multivariate analyses [71], a feature not found in SPSS. It is likely that a few multivariate analyses rather than a large number of univariate tests appear more attractive to statisticians concerned with multiple hypothesis testing.

The Observer software records the behaviors in chronological order from the start of observation. The record is, then, a description of the continuous flow of behavior, in which one behavior pattern always is followed by another. Instead of just counting the frequencies and duration of these behavior patterns, the sequence of behavior can be analyzed. One way to do so is to determine how often one specific behavior pattern is followed by another specific behavior. The most obvious example would be how often an intromission is followed by genital grooming. Another example would be how often nose-off is followed by flight.

However, limiting the analysis to adjacent behaviors gives little new information in addition to the obvious. To the contrary, it is most informative to establish a window of 4 or 8 behaviors, and determine how often two behaviors occur in the same window. This is a procedure known as co-occurrence analysis. The window moves, by steps of one behavior pattern, over the entire individual record. The frequency of co-occurrence is subjected to a descending hierarchical classification with the purpose of identifying clusters of temporally related behaviors. This classification is based on the probability for an item to be proportionally more present in a cluster than it is in the entire data set, as evaluated by chi-square analysis. Each item is permutated from one cluster to another to test the robustness of the classification, until statistically independent profiles of items appear. The criterion for including elements in their respective cluster is a higher frequency of co-occurrence compared to the average occurrence, as well as an association with the cluster determined by chi-squared values equal to or higher than 3.84. This gives an error margin of 0.05 when df = 1.61 These rather complex statistical procedures have been described elsewhere [72,73]. Clusters can be interpreted as groups of behaviors significantly more co-occurring together than with items of another cluster. The clusters and their relationship are visualised using the Fruchterman-Reingold algorithm [74].These calculations are performed with the software IRAMUTEQ (Interface de R pour les Analyses Multidimensionnelles de Textes et de Questionnaires; available for free at http://www.iramuteq.org).

The co-occurrence analysis can be applied to each of the treatments included in an experiment. Even when classical analyses fail to detect significant treatment differences, a co-occurrence analysis can reveal that the behavior patterns are grouped differently under different treatments. An example is shown in Fig. 5. In fact, this analysis makes it possible to detect subtle and unpredicted changes in the structure of behavior, something particularly important when the animals are able to express a substantial part of their behavioral

repertoire. Statistics designed for hypothesis testing does exactly what they are intended for, but does not allow for the discovery of unexpected behavioral changes produced by the experimental manipulations or the dynamics of complex social interactions in unmanipulated animals.

9. Seminatural environments and sex differences in non-sexual behaviors

We already pointed out that copulatory behavior by necessity is dimorphic. Search for sex differences in that behavior is, therefore, a triviality. There is no such obligatory sexual dimorphism in non-reproductive behaviors. Although not the theme of the present chapter, we want to briefly mention that seminatural environments can offer an externally valid procedure for analyzing sex differences in all kinds of behaviors and in all kinds of situations. For example, when the mixed-sex groups in the seminatural environment were exposed to a highly aversive stimulus, sudden, intense white noise, the behavioral responses of males and females were surprisingly similar [67]. However, when a co-occurrence analysis of the behavioral record was made, males and females were assigned to different clusters. Interestingly, the sex difference was more apparent before presentation of the aversive stimulus than during exposure to it (see Fig. 6). This is a modest example of potential uses of seminatural environments in the study of sex differences. At least it shows that representative designs are possible also in this field of research.

10. A note on human sexual behavior and seminatural conditions

In this context, it may be interesting to make a brief comparison between rat and human sexual behavior. Outside the laboratory, human sexual interactions are similar to those of rats in the seminatural environment. Sex mostly occur in a place which the participants have chosen and which they are free to leave at any moment. Furthermore, the participants

have always the possibility to engage in a considerable number of alternative behaviors, meaning that sexual activity is only one possibility among many others. There is, however, one important difference: Humans normally copulate in couples, whereas rats engage in group sex. Even though humans may do the same, it does not seem to be the most common form of sexual interaction. For reasons that are evident, laboratory studies of human sexual interactions are extremely rare. In fact, only the ground-breaking work of Masters and Johnson [75] employed observations of people copulating in the laboratory. Studies before and after have either employed questionnaires or recordings of sexual responses to solitary sex (masturbation). Exceptionally, a partner has been asked to stimulate the genitals of the experimental subject (e.g. [76,77]). This is probably the closest we have come to a natural sexual interaction in the laboratory. Thus, it can be maintained that most laboratory studies of human sexual responses do not satisfy the criteria for a representative design. The consequences of this are probably far less important than the consequences of absent representative design in rat studies. In the case of rodents, observations must not only be generalized to contexts outside the specific setup used, but also to another species. In humans, generalizations are only made from one context to others within one species.

In one exceptional study women were asked to record their behavior or genital responses to sexually relevant stimuli at home as well as in the laboratory (e.g. [78]). The former is very close to a representative design. Interestingly, there were important differences between the data obtained in the lab and those obtained at home, showing that even intra-species generalizations from one context to another are not perfect. We know of only one additional study in which genital responses were recorded in a home setting [79]. Unfortunately, no laboratory data were obtained, making comparisons between the two contexts impossible. We feel it is important to draw attention to the fact that excellent

representative designs could easily be used in humans. In insect and rodent studies, we are only able to approximate such designs through the use of seminatural environments.

11. Conclusion

Experimental setups incorporating basic characteristics of the experimental subjects' social and physical environment, and permitting the expression of as large as possible part of the natural behavioral repertoire, can be expected to be externally valid. This means that results from such setups can be generalized to conditions outside of the laboratory setting. Whenever the purpose of a study is to elucidate the biological function, adaptive value or evolution of particular behavior patterns, the use of an externally valid procedure is mandatory. The preceding presentation of this kind of procedures in insects and rodents will hopefully contribute to an enhanced interest in and use of seminatural environments. The development of powerful image analyzing tools may make data acquisition far more time-efficient than at present, and novel statistical approaches may provide the possibility to fully explore the millions of data points generated in complex environments. Perhaps this will contribute to a break-through in our understanding of brain – behavior relations.

References

1. Hodgson AN (2011) Reproduction and sex in invertebrates. Encyclopedia of Life Support Systems. UNESCO, Paris

2. Kinoshita M, Homberg U (2017) Insect brains: Minute structures controlling complex behaviors. In: Shigeno S, Murakami Y, Nomura T (eds) Brain Evolution by Design: From Neural Origin to Cognitive Architecture. Springer Japan, Tokyo, pp 123-151. doi:10.1007/978-4-431-56469-0_6

3. Oh SM, Jeong K, Seo JT, Moon SJ (2021) Multisensory interactions regulate feeding behavior in *Drosophila*. Proc Natl Acad Sci USA 118(7):e2004523118. doi:10.1073/pnas.2004523118

4. Griffith LC, Ejima A (2009) Multimodal sensory integration of courtship stimulating cues in Drosophila melanogaster. Ann N Y Acad Sci 1170:394-398. doi:10.1111/j.1749-6632.2009.04367.x

5. Keene AC, Waddell S (2007) Drosophila olfactory memory: single genes to complex neural circuits. Nature Rev Neurosci 8(5):341-354. doi:10.1038/nrn2098

6. Moloney A, Sattelle DB, Lomas DA, Crowther DC (2010) Alzheimer's disease: insights from Drosophila melanogaster models. Trends Biochem Sci 35(4):228-235. doi:10.1016/j.tibs.2009.11.004

7. Spieth HT (1974) Courtship behavior in *Drosophila*. Annu Rev Entomol 19(1):385-405

8. Ewing AW (1961) Body size and courtship behaviour in *Drosophila melanogaster*. Anim Behav 9(1):93-99. doi:https://doi.org/10.1016/0003-3472(61)90055-0

9. Dukas R (2020) Natural history of social and sexual behavior in fruit flies. Sci Rep 10(1):21932. doi:10.1038/s41598-020-79075-7

10. Takasaki T, Namiki S, Kanzaki R (2012) Use of bilateral information to determine the walking direction during orientation to a pheromone source in the silkmoth *Bombyx mori*. J Comp Physiol A 198(4):295-307

11. Lelito JP, Myrick AJ, Baker TC (2008) Interspecific pheromone plume interference among sympatric heliothine moths: a wind tunnel test using live, calling females. J Chem Ecol 34(6):725-733. doi:10.1007/s10886-008-9475-6

12. Cardé RT, Hagaman TE (1979) Behavioral responses of the gypsy moth 1 in a wind tunnel to air-borne enantiomers of disparlure 2. Environ Entomol 8(3):475-484. doi:10.1093/ee/8.3.475

13. Lamunyon CW (2000) Sperm storage by females of the polyandrous noctuid moth Heliothis virescens. Anim Behav 59 (2):395-402. doi:https://doi.org/10.1006/anbe.1999.1294

14. Hou M, Sheng C (1999) Fecundity and longevity of *Helicoverpa armigera* (Lepidoptera: Noctuidae): Effects of multiple mating. J Econ Entomol 92(3):569-573. doi:10.1093/jee/92.3.569

15. Shepherd GM (1974) The synaptic organization of the brain: an introduction. Oxford University Press,

16. Guo X, Yu Q, Chen D, Wei J, Yang P, Yu J, Wang X, Kang L (2020) 4-Vinylanisole is an aggregation pheromone in locusts. Nature 584(7822):584-588. doi:10.1038/s41586-020-2610-4

17. Connolly K, Cobb M, Burnet B (1985) Courtship behaviour in the melanogaster species sub-group of Drosophila. Behaviour 95(3-4):203-230. doi:https://doi.org/10.1163/156853985X00136

18. Halfwerk W, Varkevisser J, Simon R, Mendoza E, Scharff C, Riebel K (2019) Toward testing for multimodal perception of mating signals. Front Ecol Evol 7(124). doi:10.3389/fevo.2019.00124

19. Byrne PG, Rice WR (2006) Evidence for adaptive male mate choice in the fruit fly Drosophila melanogaster. Proc Royal Soc B: Biol Sci 273(1589):917-922

20. Arbuthnott D, Fedina TY, Pletcher SD, Promislow DEL (2017) Mate choice in fruit flies is rational and adaptive. Nature Commun 8(1):13953. doi:10.1038/ncomms13953

21. Priesner E, Witzgall P, Voerman S (1986) Field attraction response of raspberry clearwing moths, *Pennisetia hylaeiformis* Lasp.(Lepidoptera: Sesiidae), to candidate pheromone chemicals. J Appl Entomol 102(1-5):195-210

22. Kaissling K-E, Priesner E (1970) Die Riechschwelle des Seidenspinners. Naturwissenschaften 57(1):23-28. doi:10.1007/bf00593550

23. Baker TC, Hansson BS (2016) Moth sex pheromone olfaction. In: Allison JD, Cardz RT (eds) Pheromone Communication in Moths: Evolution, Behavior, and Application. University of California Press, Oakland,

24. Willis MA, Avondet JL, Zheng E (2011) The role of vision in odor-plume tracking by walking and flying insects. J Exp Biol 214 (24):4121-4132. doi:10.1242/jeb.036954

25. Kennedy JS, Marsh D (1974) Pheromone-regulated anemotaxis in flying moths. Science 184(4140):999-1001

26. Chu X, Heinze S, Ian E, Berg BG (2020) A Novel Major Output Target for Pheromone-Sensitive Projection Neurons in Male Moths. Front Cell Neurosci 14:147. doi:10.3389/fncel.2020.00147

27. Kymre JH, Liu X, Ian E, Berge CN, Wang G, Berg BG, Zhao X, Chu X (2021) Distinct protocerebral neuropils associated with attractive and aversive female-produced odorants in the male moth brain. eLife 10:e65683. doi:10.7554/eLife.65683

28. Li X, Ishimoto H, Kamikouchi A (2018) Auditory experience controls the maturation of song discrimination and sexual response in Drosophila. eLife 7:e34348. doi:10.7554/eLife.34348

29. Cheriyamkunnel SJ, Rose S, Jacob PF, Blackburn LA, Glasgow S, Moorse J, Winstanley M, Moynihan PJ, Waddell S, Rezaval C (2021) A neuronal mechanism controlling the choice between feeding and sexual behaviors in Drosophila. Curr Biol 31(19):4231-4245. doi:10.1016/j.cub.2021.07.029

30. Biasazin TD, Chernet HT, Herrera SL, Bengtsson M, Karlsson MF, Lemmen-Lechelt JK, Dekker T (2018) Detection of volatile constituents from food lures by tephritid fruit flies. Insects 9(3):119 doi:0.3390/insects9030119

31. Ren L, Ma Y, Xie M, Lu Y, Cheng D (2021) Rectal bacteria produce sex pheromones in the male oriental fruit fly. Curr Biol 31(10):2220-2226.e2224. doi:https://doi.org/10.1016/j.cub.2021.02.046

32. Keys RE, Mills RB (1968) Demonstration and extraction of a sex attractant from female angoumois grain moths1. J Econ Entomol 61(1):46-49. doi:10.1093/jee/61.1.46

33. Namiki S, Kanzaki R (2019) Morphology and physiology of olfactory neurons in the lateral protocerebrum of the silkmoth Bombyx mori. Sci Rep 9(1):16604. doi:10.1038/s41598-019-53318-8

34. Ian E, Kirkerud NH, Galizia CG, Berg BG (2017) Coincidence of pheromone and plant odor leads to sensory plasticity in the heliothine olfactory system. PLoS One 12(5):e0175513

35. Wu H, Xu M, Hou C, Huang L-Q, Dong J-F, Wang C-Z (2015) Specific olfactory neurons and glomeruli are associated to differences in behavioral responses to pheromone components between two Helicoverpa species. Front Behav Neurosci 9(206). doi:10.3389/fnbeh.2015.00206

36. Kaushik PK, Renz M, Olsson SB (2020) Characterizing long-range search behavior in Diptera using complex 3D virtual environments. Proc Natl Acad Sci USA 117(22):12201-12207. doi:10.1073/pnas.1912124117

37. Koukou K, Pavlikaki H, Kilias G, Werren JH, Bourtzis K, Alahiotis SN (2006) Influence of antibiotic treatment and Wolbachia curing on sexual isolation among *Drosophila melanogaster* cage populations. Evolution 60(1):87-96

38. Ehrman L (1965) Direct observation of sexual isolation between allopatric and between sympatric strains of the different *Drosophila paulistorum* races. Evolution:459-464

39. Korol A, Rashkovetsky E, Iliadi K, Michalak P, Ronin Y, Nevo E (2000) Nonrandom mating in *Drosophila melanogaster* laboratory populations derived from closely adjacent ecologically contrasting slopes at "Evolution Canyon". Proc Natl Acad Sci USA 97(23):12637-12642. doi:10.1073/pnas.220041397

40. Arita LH, Kaneshiro KY (1988) Body size and differential mating success between males of two populations of the Mediterranean fruit fly. Pac Sci 42(3-4): 173-177.

41. Zhang J, Bisch-Knaden S, Fandino RA, Yan S, Obiero GF, Grosse-Wilde E, Hansson BS, Knaden M (2019) The olfactory coreceptor IR8a governs larval feces-mediated competition avoidance in a hawkmoth. Proc Natl Acad Sci USA 116(43):21828-21833. doi:10.1073/pnas.1913485116

42. Pfaff D (2017) How the vertebrate brain regulates behavior. Direct from the lab. Harvard University Press, Cambridge, MA

43. Pfaff DW (1999) Drive. Neurobiological and molecular mechanisms of sexual motivation. MIT Press, Cambridge, MA

44. Pfaff DW (1980) Estrogens and brain function: neural analysis of a hormone-controlled mammalian reproductive behavior. Springer, New York

45. Holmes GM, Chapple WD, Leipheimer RE, Sachs BD (1991) Electromyographic analysis of male rat perineal muscles during copulation and reflexive erections. Physiol Behav 49(6):1235-1246

46. Sachs BD (1982) Role of striatal penile muscles in penile reflexes, copulation, and induction of pregnancy in the rat. J Reprod Fertil 66(2):433-443

47. Moralí G, Soto MAP, Contreras JL, Arteaga M, González-Vidal MD, Beyer C (2003) Detailed analysis of the male copulatory motor pattern in mammals: Hormonal bases. Scand J Psychol 44(3):279-288

48. Hull EM, Rodríguez-Manzo G (2017) Male sexual behavior. In: Pfaff DW, Joëls M (eds) Hormones, Brain and Behavior (Third Edition). Academic Press, Oxford, pp 1-57. doi:https://doi.org/10.1016/B978-0-12-803592-4.00001-8

49. Robitaille JA, Bovet J (1976) Field observations on the social behaviour of the Norway rat, Rattus norvegicus (Berkenhout). Biol Behav 1:289-308

50. Calhoun JB (1962) The ecology and sociology of the Norway rat. USPHS Publication No. 1008. US Governnment Printing Office, Washington,D.C.

51. Brunswik E (1955) Representative design and probabilistic theory in a functional psychology. Psychol Rev 62(3):193-217

52. Petrinovich L (1980) Brunswikian behavioral biology. In: Hammond KR, Wascoe NE (eds) Realizations of Brunswik's representative design. New directions for methodology of social and behavioral science, 3. Jossey-Bass, San Francisco, CA, pp 85-93

53. Petrinovich L (1989) Representative design and the quality of generalization. In: Poon LW, Rubin DC, Wilson BA (eds) Everyday cognition in adulthood and late life. Cambridge University Press, New York, NY, US, pp 11-24

54. Chu X, Ågmo A (2016) Sociosexual interactions in rats: Are they relevant for understanding human sexual behavior? Int J Psychol Res 9(2):76-95

55. Chu X, Ågmo A (2015) Sociosexual behaviors of male rats (Rattus norvegicus) in a seminatural environment. J Comp Psychol 129(2):132-144

56. Peirce JT, Nuttall RL (1961) Self-paced sexual behavior in the female rat. J Comp Physiol Psychol 54(3):310-313

57. Erskine MS, Kornberg E, Cherry JA (1989) Paced copulation in rats - effects of intromission frequency and duration on luteal activation and estrus length. Physiol Behav 45(1):33-39

58. McClintock MK, Adler NT (1978) The role of the female during copulation in wild and domestic Norway rats (*Rattus norvegicus*). Behaviour 67:67-96

59. McClintock MK, Anisko JJ (1982) Group mating among Norway rats I. Sex differences in the pattern and neuroendocrine consequences of copulation. Anim Behav 30(2):398-409.

60. McClintock MK, Anisko JJ, Adler NT (1982) Group mating among Norway rats II. The social dynamics of copulation: Competition, cooperation, and mate choice. Anim Behav 30(2):410-425.

61. Chu X, Ågmo A (2014) Sociosexual behaviours in cycling, intact female rats (*Rattus norvegicus*) housed in a seminatural environment. Behaviour 151(8):1143-1184

62. Chu X, Guarraci FA, Ågmo A (2015) Sociosexual behaviors and reproductive success of rats *(Rattus norvegicus*) in a seminatural environment. Physiol Behav 151:46-54

63. Schein MW (1950) The relation of sex ratio to physiological age in the wild brown rat. Am Nat 84(819):489-496

64. Heinla I, Chu X, Ågmo A, Snoeren E (2021) Rat ultrasonic vocalizations and novelty-induced social and non-social investigation behavior in a seminatural environment. Physiol Behav 237:Article number 113450. doi:https://doi.org/10.1016/j.physbeh.2021.113450

64. Le Moëne O, Hernández-Arteaga E, Chu X, Ågmo A (2020) Rapid changes in sociosexual behaviors around transition to and from behavioral estrus, in female rats housed in a seminatural environment. Behav Processes 174:104101. doi:https://doi.org/10.1016/j.beproc.2020.104101

66. Le Moëne O, Ågmo A (2018) Behavioral responses to emotional challenges in female rats living in a seminatural environment: The role of estrogen receptors. Horm Behav 106:162-177

67. Le Moëne O, Ramírez-Rentería ML, Ågmo A (2020) Male and female immediate fear reaction to white noise in a semi-natural environment: A detailed behavioural analysis of the role of sex and oestrogen receptors. J Neuroendocrinol 32(10):Article number e12902. doi:10.1111/jne.12902

68. Le Moëne O, Stavarache M, Ogawa S, Musatov S, Ågmo A (2019) Estrogen receptors a and β in the central amygdala and the ventromedial nucleus of the hypothalamus: Sociosexual behaviors, fear and arousal in female rats during emotionally challenging events. Behav Brain Res 367:128-142. doi:10.1016/j.bbr.2019.03.045

69. Mathis A, Schneider S, Lauer J, Mathis MW (2020) A primer on motion capture with deep learning: Principles, pitfalls, and perspectives. Neuron 108(1):44-65. doi:https://doi.org/10.1016/j.neuron.2020.09.017

70. Lauer J, Zhou M, Ye S, Menegas W, Nath T, Rahman MM, Di Santo V, Soberanes D, Feng G, Murthy VN, Lauder G, Dulac C, Mathis MW, Mathis A (2021) Multi-animal pose estimation and tracking with DeepLabCut. bioRxiv Preprint. doi:https://doi.org/10.1101/2021.04.30.442096

71. Ellis AR, Burchett WW, Harrar SW, Bathke AC (2017) Nonparametric inference for multivariate data: The R package npmv. J Stat Softw 76(4):1-18. doi:10.18637/jss.v076.i04

72. Reinert A (1983) Une méthode de classification descendante hiérarchique: Application à l'analyse lexicale par contexte. Les Cahiers de l'analyse des Données 8(2):187-198

73. Valax MF, Marine C, Reinert M (1990) Traitement de données structurées par un ordre temporel ou hiérarchique : Utilisation dans l'analyse de l'activité. Le Travail Humain 53(1):79-89

74. Fruchterman TMJ, Reingold EM (1991) Graph drawing by force-directed placement. Softw-Pract Exp 21(11):1129-1164. doi:10.1002/spe.4380211102

75. Masters WH, Johnson VE (1966) Human sexual response. Little, Brown & Co., Boston

76. Georgiadis JR, Kortekaas R, Kuipers R, Nieuwenburg A, Pruim J, Reinders AATS, Holstege G (2006) Regional cerebral blood flow changes associated with clitorally induced orgasm in healthy women. Eur J Neurosci 24 (11):3305-3316. doi:https://doi.org/10.1111/j.1460-9568.2006.05206.x

77. Georgiadis JR, Holstege G (2005) Human brain activation during sexual stimulation of the penis. J Comp Neurol 493 (1):33-38

78. Bloemers J, Gerritsen J, Bults R, Koppeschaar H, Everaerd W, Olivier B, Tuiten A (2010) Induction of sexual arousal in women under conditions of institutional and ambulatory laboratory circumstances: A comparative study. J Sex Med 7(3):1160-1176. doi:10.1111/j.1743-6109.2009.01660.x

79. Poels S, Bloemers J, van Rooij K, Goldstein I, Gerritsen J, van Ham D, van Mameren F, Chivers M, Everaerd W, Koppeschaar H, Olivier B, Tuiten A (2013) Toward personalized sexual medicine (part 2): Testosterone combined with a PDE5 inhibitor increases sexual satisfaction in women with HSDD and FSAD, and a low sensitive system for sexual cues. J Sex Med 10 (3):810-823. doi:10.1111/j.1743-6109.2012.02983.x

Table 1. Typical ethogram used in the rat seminatural environment. Both the number of occurrences (frequency) and the duration of each occurrence were registered for most behavior patterns. For some, like lordosis, duration is not always possible to record accurately from the video record. For other behaviors, duration does not offer any useful information beyond that contained in frequency. f = frequency; d = duration

Category	Behavior pattern	Definition
Female sexual behaviors	Lordosis; f	Posture of the female arching her back, exposing her vagina.
	Paracopulatory behaviors; f, d	Approach to a male followed by runaway, often associated with hops, darts, and ear wiggling.
	Rejection; f	Female kicks, boxes or assumes a belly up posture.
Prosocial behaviors	Resting with other females; f, d	Rests immobilized in relaxed position at a distance shorter than one rat from one or several females.
	Resting with males; f, d	Rests immobilized in relaxed position at a distance shorter than one rat from one or several males.
	Sniffing other females; f, d	Snout close to a female, sniffing the fur.
	Sniffing males; f, d	Snout close to a male, sniffing the fur.

	Hiding with another rat; f, d	Immobilized in a corner or in a nest box within one body length of the other rat.
Antisocial behaviors	Nose-off male; f, d	The female faces a male, nose to nose, heads up, with or without boxing.
	Nose-off female; f, d	The female faces another female, nose to nose, heads up, with or without boxing.
	Flee from male; f	Escapes from agonistic interaction by running away or simply turning head away from a male.
	Flee from another female; f	Escapes from agonistic interaction by running away or simply turning head away from a female.
Non-social behaviors	Resting alone; f, d	Rests immobilized in relaxed position at a distance longer than one rat to a conspecific.
	Drinking; f, d	Self-explanatory.
	Self-grooming and scratching; f, d	Self-explanatory.

Exploratory behaviors and ambulatory activity	Hide alone; f, d	Immobilized in a corner or nest box at a distance longer than one body length to another rat.
	Grabbing; f	Grabbing food with paws or mouth.
	Eating; f, d	Chews on food pellet.
	Freezing; f, d	Immobilized in rigid position without any movement, including those of vibrissae.
	Startle; f	Sudden reflex contractions of the major muscles of the body, leading to a little jump on the spot.
	Sniffing the floor, f, d	Sniffs the floor material with all four paws on the floor.
	Rearing; f, d	Sniffs the air while standing on the hind legs.
	Transitions; f	Displays a behavior in a zone different from the one in which the previous behavior was displayed.

Figure legends

Fig. 1. Small behavior chambers. (A) Acoustic behavior chamber , reproduced with permission (CC BY 4.0) from Fig.2 in [28]. (B) Example of courtship chamber, in which a male fruit fly is allowed to choose between feeding source (blue circle) and courting a receptive female. (C) Y-tube bi-choice chamber, reproduced with permission (CC BY 4.0) from Fig.1 in [30].

Fig. 2. MultiMoVR arena. (A) The MultiMoVR arena is a prism-shaped arena composed of three monitors, measured 32 cm wide and 60 cm high. The behaving apply fly is placed in the center of the arena. (B) The insect is surrounded by a dynamic seminatural scenery according to its flying activity. Directional wind and odor are provided as well. The figure is reproduced with permission (CC BY-NC-ND 4.0) from Movie S1 in [36].

Fig. 3. Example of seminatural environment with host plant. (A) Two host plants of *M. sexta* coyote tobacco (*N. attenuata*, Left) and jimson weed (*D. wrightii*, Right). (B) Schematic drawing of the behavioral assay to test the oviposition deterring effect of larval feces. The figure is reproduced with permission (CC BY-NC-ND 4.0) from Fig.1 in [41].

Fig. 4. The seminatural environment. The thick black lines extending from the walls in the open area are 40 cm high, black dividers about 40 cm long. The purpose with them is to enhance complexity in the open area.

Fig. 5. The graphical representation of the result of a co-occurrence analysis. In this experiment, female rats had been treated with either estradiol benzoate (EB), the estrogen receptor α agonist propylpyrazoletriol (PPT), the estrogen receptor β agonist diarylpropionitrile (DPN) or oil. All subjects received progesterone (1 mg/rat) 48 h after the priming injection and 4 h before the start of observation. Each treatment appeared in a different cluster. The sexual behaviors, lordosis and paracopulatory behavior, were associated

with EB treatment. The ERα agonist was related to the antisocial behaviors of nose-off and fleeing, and to a minor degree with anogenital sniffing. The females treated with the ERβ agonist were characterized by the antisocial behavior of rejection and the solitary activity of drinking. Oil treatment was mainly related to the self-centered behavior of grooming, resting, and social and nonsocial sniffing. Rearing was also typical for these females. Many of the associations between treatment and particular behavior patterns were not detected in the analyses of frequency or duration, revealing the superior power of the co-occurrence analysis. For further details of this experiment, see [66]. Reproduced with permission.

Fig. 6. Co-occurrence analysis of behavior in male and female rats housed in a seminatural environment before (A) and during (B) exposure to a 90 dB white noise. Reproduced with permission (CC BY 4.0) from [67].

Figure 1

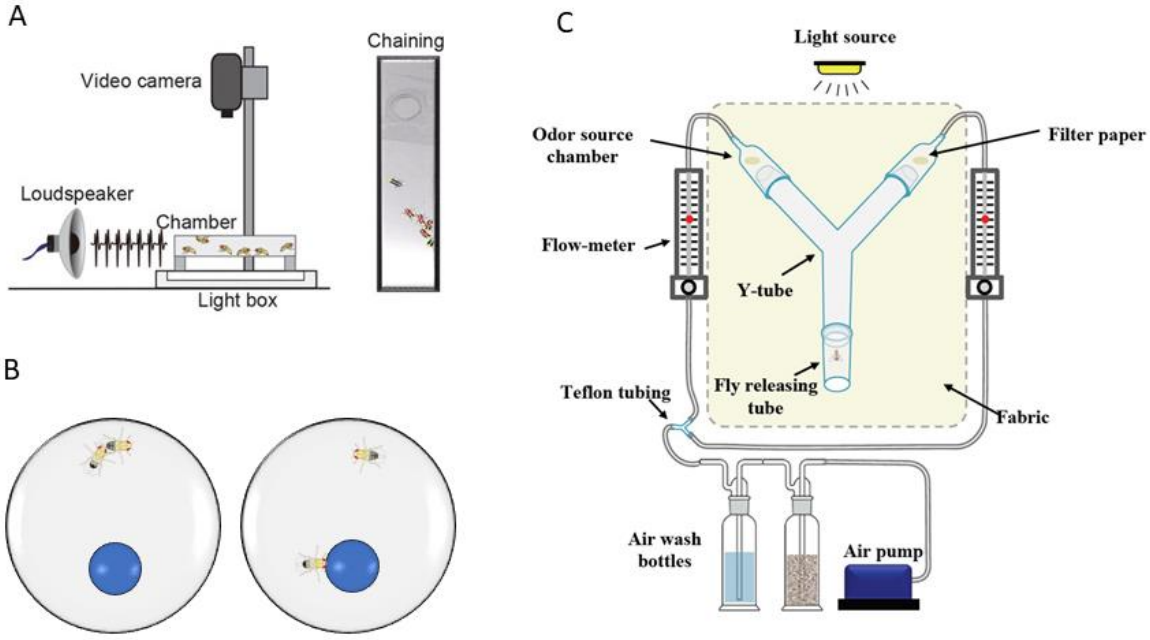


Figure 2

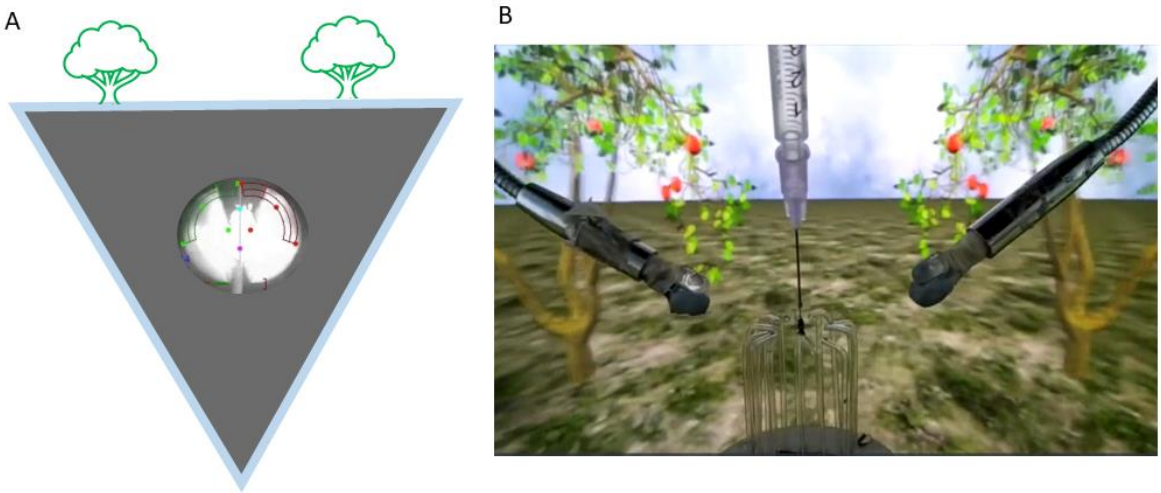


Figure 3

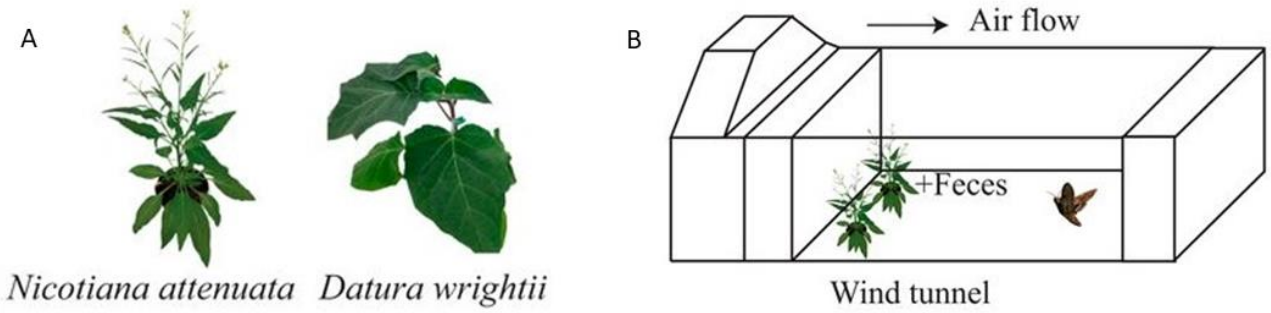


Figure 4

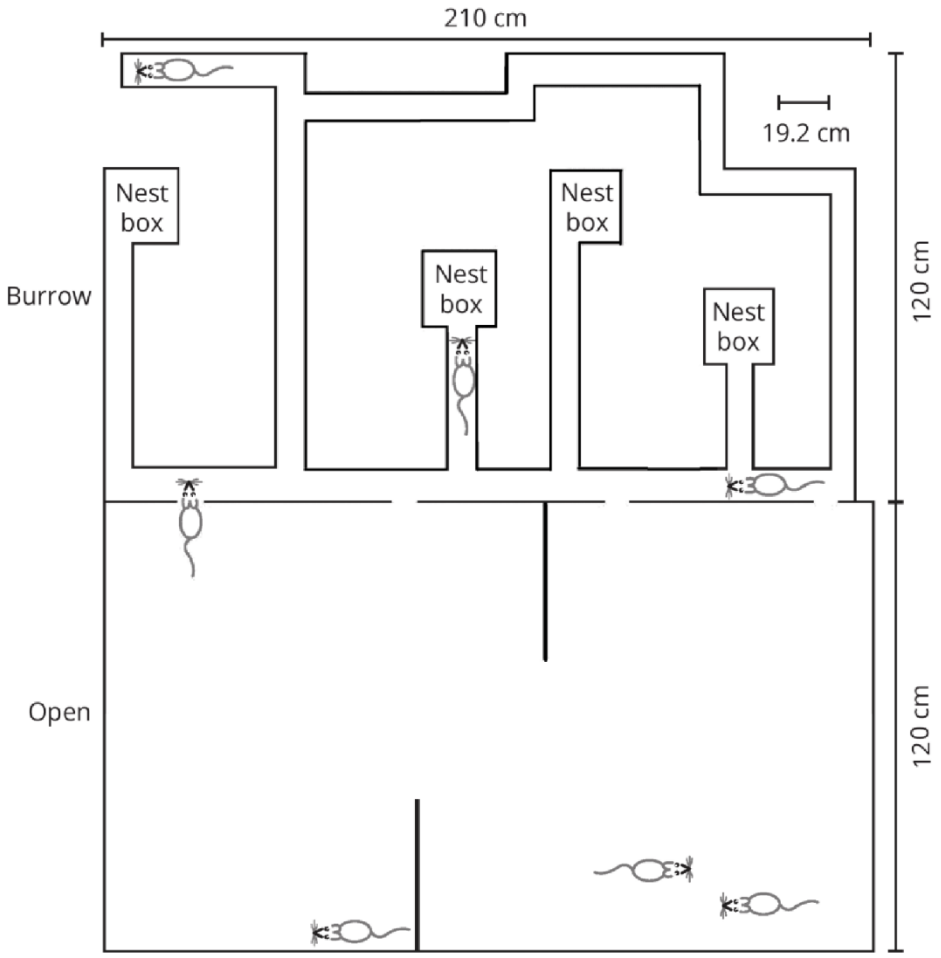


Figure 5

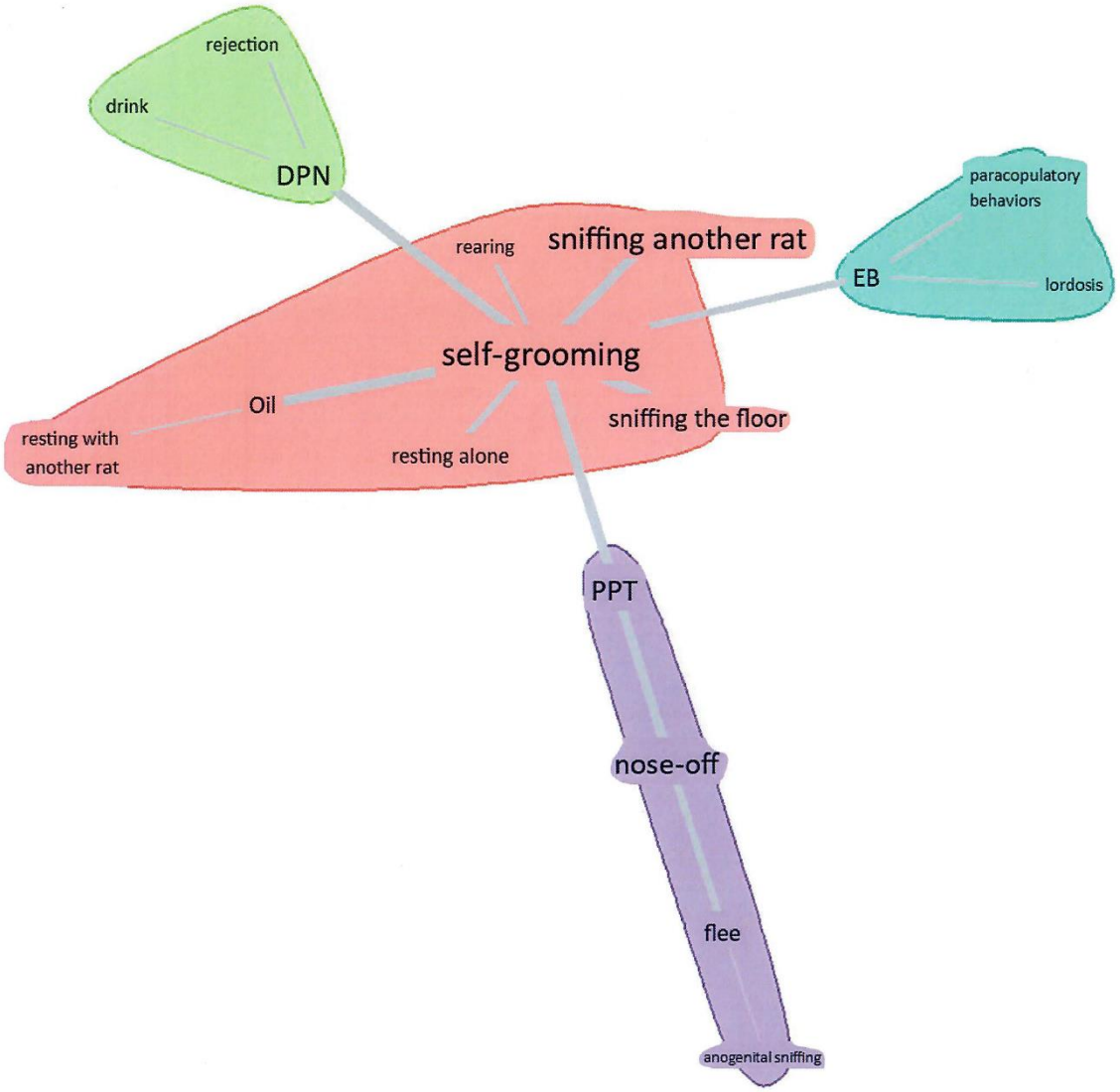
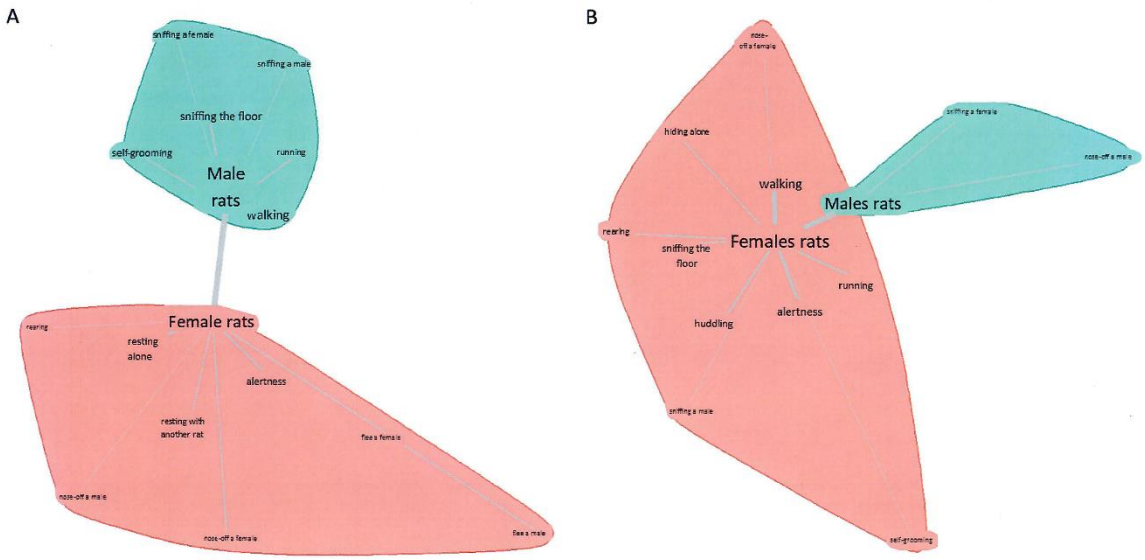

rejection
drink
DPN
paracopulatory
behaviors
EB
lordosis
rearing
sniffing another rat
self-grooming
Oil
resting with
another rat
resting alone
sniffing the floor
PPT
nose-off
flee
anogenital sniffing

Figure 6


A
sniffing a female
sniffing a male
sniffing the floor
self-grooming
running
Male rats
walking
Female rats
rearing
resting alone
alertness
resting with another rat
flees female
nose-off a male
nose-off a female
flees a male
B
nose-off a female
hiding alone
sniffing a female
nose-off a male
walking
Males rats
rearing
Females rats
sniffing the floor
running
huddling
alertness
sniffing a male
self-grooming