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# Assessment of Sexual Behavior of Male Mice

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Professor Phillip Steindel,

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Thank you for the opportunity to resubmit our revised manuscript. We thank you and the reviewers for your constructive suggestions and comments. This time, I rewrite the introduction section thoroughly. I added more background information and references of mouse sexual behavior assessment. For the sake of reading and understanding conveniently, the definitions of key conception of sexual behavior were corrected to be consistent with past literature. Finally, our point-by-point answers to the reviewers' comments are below.

We hope that the revised manuscript is now acceptable for publication. We look forward to hearing from you.

Sincerely,

Ziwei Liu

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**TITLE:**

# Assessment of Sexual Behavior of Male Mice

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**KEYWORDS:**

ejaculation, intromission, male mice, mounting, ovariectomized estrus female, post-ejaculatory interval, sexual behavior.

**SUMMARY:**

This article describes how to perform sexual behavior tests in male mice.

**ABSTRACT:**

Sexual behavior is highly species-specific. Although rodents have slightly different sexual behaviors, mice and rats have a similar sexual behavioral pattern. The purpose of this article is to describe the hormone-induced estrus ovariectomized female model and the experimental procedure for the assessment of sexual behavior of male mice. The most important sexual behavioral elements are demonstrated in the video and illustrations. The critical steps, advantages, and limitations of the sexual behavior test are explained as well. Finally, the behavior parameters are presented, and mounting, intromission, and ejaculation processes in mating are distinguished. Behavioral parameters are assessed in terms of the occurred duration and counts during the test period.

## INTRODUCTION:

Sexual behavior in mature male mice results from the interaction of a series of related and interdependent hormonal systems and neural systems in different brain circuits[1]. It also requires developmental experiences, learning, context, and an appropriate partner. Behavioral analysis is an important reflection on neural or neurocrine function. Hence, sexual behavior study on animal models has been widely used in behavioral neuroscience and other related research[2]. The

ethogram of sexual behaviors in rodents has been explained in many articles and books[1,3,4]. For instance, Scahs and Barfield's description of sexual behavior in the rat[5] has helped understand a similar behavioral pattern in mice[5]. The mouse is one of the most commonly used subjects for behavioral studies. Hull et al.[6] gave a detailed introduction of male mouse sexual behaviors: When a male mouse encounters a female, it starts to investigate the anogenital region of the female. Then, the male presses his front paws against the female's flanks to mount the female from the rear. The female exhibits a characteristic sexually receptive posture, bending its spine down into a bow and moving its tail to one side of the body, exposing an opening introitus for the sexual penetration of the male (i.e., lordosis). Following the mounting, the male makes rapid, shallow pelvic thrusts, followed by slow and deep vaginal thrusts. After numerous intromissions, a long-lasting thrust results in the ejaculation of semen, during which the male mouse may freeze for about 25 s before dismounting or falling off from the female[6]. At ejaculation the male mouse accessory glands may produce a mixture containing semen that hardens to form the copulatory plug. Finally, following ejaculation, the male begins genital grooming and displays a lack of interest in the female. In brief, the basic sequence of male sexual behavior consists of sniffing, following, mounting, intromission, ejaculation, and post-ejaculation grooming. Mouse sexual behavior exhibits strain differences. For instance, ejaculation latencies range from 594 to 6943 s, and the numbers of intromissions range from 5 to more than 100. Post-ejaculation latencies range from 17 to 60 min. However, the introduction of a novel female can decrease this time interval. In some cases, the male ejaculates on the first intromission with the new female[7].

The major events for the evaluation of sexual behavior are mounting, intromission, and ejaculation. Behavioral scientists have recommended the measurement of not only the frequency of each action, but also its latency and time interval[5,8]. Some major measurement indicators in past studies include: number of mounts, number of intromissions, mount latency, intromission latency, ejaculation latency, post-ejaculatory mount latency (or post-ejaculatory intervals), post-ejaculatory intromission latency, number of copulatory series, and duration of copulatory series. Park et al.[8] and Sachs et al.[5] described how to identify each action of mounting, intromission, and ejaculation of rodents. Mounting is defined as the male mounting the female from the rear, palpating her flanks with his forelegs, and thrusting his penis rapidly and repeatedly without penile insertion. Intromission, also known as penile insertion, is identified by one or more of the following acts: a long, deep thrust after rapid shallow thrusts, a rapid kick with one hindleg, and a marked lateral withdrawal of the male from the female. Ejaculation is identified by a terminal pelvic thrust that is slower and deeper than that of an intromission and a reduction in the elevation of the hindleg. A copulatory series is identified by each sequence from mounting to ejaculation. The definitions of behavioral parameters used in the present study are listed as follows: 1) *Mounting latency:* the time from the introduction of the female to the first mounting of the male; 2) *Intromission latency:* the time from the introduction of the female to first intromission; 3) *Ejaculation latency:* the time from the first intromission to first ejaculation (generally following the last pelvic thrust); 4) *Post-ejaculatory mount latency:* the time from ejaculation to the next mounting; 5) *Post-ejaculatory intromission latency:* the time from ejaculation and the next intromission; 6) *Number of mounts:* the number of mounting times before first ejaculation; 7) *Number of intromissions:* the number of intromissions before the first ejaculation; 8) *Number of copulatory series:* the number of copulatory series during the

observation period; 9) *Duration of copulatory series:* the time of all copulatory series during the observation period.

Sexual behavior and related behavior can be conducted in either the male's home cage or in an enclosed arena, among which an apparatus called Rissman's "No Secrets" mirrored box is introduced to observe the mating behavior[3]. A video camera is placed in front of the box to simultaneously record the action of the mice from a lateral view and through an inclined mirror from a ventral view. However, this method requires bright lights, which inevitably leads to longer habituation in order to eliminate environmental stress in mice. As for the measuring method, video-based behavioral analysis is recommended to record and quantify behavior[4]. A video recorder that has a frame-by-frame video advance option with recommended shutter speeds greater than 1/1000 s can be used to record rapid mouse movements. The high resolution infrared camera is necessary when recording in a dark environment. To analyze the film, a computer with a frame grabber to allow the individual frames of behavior to be captured for computer manipulation is needed. Mice are extremely versatile and can display compensatory behavior after almost any treatment. Ambiguity can exist about every moving body part[4]. Hence, the analysis of some behaviors may require still greater resolution and higher speed cameras.

Male sexual behaviors in mice are affected by many factors, including strain differences, hormone changes, and gene mutantions[1,3,9,10]. McGill and Blight[11] illustrated the strain differences in mouse mating behaviors. For example, C57BL/6 males typically gain intromission quickly and ejaculate in about 20 min[11]. DBA/2 males are slow to gain intromission but ejaculate rapidly. BALB/c males are slow to achieve ejaculation (average latency of 1 h) due to a long period of courtship[11]. Testosterone facilitates and maintains male sexual behavior[2], and changes in testosterone levels can alter sexual behavior performance[12]. Both surgical castration and antiandrogen treatment can reduce the level of testosterone and result in a rapid decline of sexual behaviors and even sexual motivation and sexual arousal[13]. Administered testosterone can restore precopulatory and copulatory behaviors in castrated mice. Lastly, knockout and knockdown mice display differences in facets of sexual behaviors compared to wild type mice. For example, male mice with targeted mutations of Adcy3, Cnga2, and Gnao exhibit a reduced ability to detect pheromones, whereas Trpc2 knockout mice show altered partner preference[14-16]. Other effects of transgenics and knockouts on the sexual behavior of mice are explained by Crawley[3].

Here, one of the most common procedures to assess sexual behavior in the pairing of a male mouse with an ovariectomized female that has been hormonally primed to be receptive is described. An experimental protocol is presented for conducting sexual behavior experiments in mice. In addition, an example of changing sexual behavior patterns resulting from social isolation in CD-1 mice is shown.

**PROTOCOL:**

All experiments were performed in compliance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication No. 80-23, revised 1996) and under the approval and supervision

of the Academy of Experimental Animal Centre of the Institute of Medicinal Plant Development (China).

**1. Animal husbandry**

1.1. House female and male mice at 25 °C for 12 h light/12 h dark cycles.

1.2. Provide free access to water and a standard pelleted diet.

1.3. Allow mice to acclimate to their environment for 7 days prior to the operation if transported from a different facility.

**2. Ovariectomy in female mice**

2.1. Anesthetize the female (8 weeks postnatal, not less than 6-weeks-old) with isoflurane (~4−5% for induction, ~1−2% for maintenance) in 100% oxygen via a face cone mask.

2.2. Check that the appropriate depth of aesthesia has been achieved by making sure that there are no voluntary movements for over 30 s, in combination with an appropriate respiratory rate (e.g., 1 breath per 2 s or longer). Alternatively, test the mouse's response to gentle pressure on the toes of the hind paws.

NOTE: The normal respiratory rate is ~180/min. A rate drop of 50% is acceptable during anesthesia[17].

2.2.1. Use ophthalmic ointment to prevent corneal drying and eye trauma while under anesthesia.

2.2.2. Keep the body temperature of the mouse at or above 36 °C. Provide supplemental heat support during the period of anesthesia when necessary.

2.3. Sterilize and disinfect all surgical instruments and hard surfaces of the operating table with 75% ethanol prior to use.

2.4. Place animal on a sterile drape.

2.5. Shave fur bilaterally over the lumbar spine on the back of the mouse to expose the skin.

2.6. Sterilize the exposed skin with 75% ethanol.

2.7. Make a single midline incision (about 0.5 cm in length) on the back from the center of the two thigh roots toward the head of ~1 cm distance (position is shown in **Figure 1**).

2.8. Use small scissors to penetrate the skin to gently free subcutaneous tissue from the

underlying muscle in order to expose the muscle layer.

2.9. Locate the ovary under the thin muscle layer and make a small incision (about 5 mm in length) to gain entry to the peritoneal cavity.

1.1. Use small tweezers to pull the tissue slightly on the left side of the abdominal cavity to show the left-hand ovary wrapping around the white adipose tissue (a translucent, irregular mass as seen by the naked eye, see **Figure 1**).

2.10. Retract the ovarian fat pad surrounding the ovary with blunt forceps to expose the oviduct.

2.11. Perform a single ligature around the oviduct to prevent bleeding.

2.12. Use small scissors to sever the oviduct gently and remove the ovary.

2.13. Check the oviduct carefully to confirm that all ovarian tissues are removed. The ovary is about 5 mm × 4 mm × 3 mm with irregular nodules on the surface.

2.14. Place the remaining part of the oviduct back into the abdominal cavity.

2.15. Suture the muscle layer with absorbable sutures.

2.16. Pull the skin to the right side to expose the muscle layer on the right-hand side and remove the right ovary by repeating steps 2.9–2.16.

2.17. Close the skin incision by using absorbable sutures.

2.17.1. Inject each mouse intraperitoneally with penicillin sodium (10,000 units/10 g per mouse) to prevent infection.

2.17.2. Inject lidocaine (4 mg/kg, 0.4 mL/kg of a 1% solution) beneath the skin along the site of the incision. Also provide ibuprofen (50–60 mg/kg/day; 10 mL of Children's Motrin in 500 mL of water) continuously in the drinking water for 3 days for pain treatment.

2.18. Place each mouse into a sterilized cage individually.

2.19. Keep under close observation for approximately 1–2 h until fully recovered from anesthesia.

2.19.1. Recover animals on paper towels in a clean cage without bedding. This step minimizes the risk of tracheal obstruction or pneumonia. Provide supplemental heat support during anesthetic recovery. Monitor the surgical site to prevent the rupture of the wound.

2.20. Following the recovery period (approximately 24 h after surgery), place mice back to their home cage.

2.21. Do not perform the experiment for at least 2 weeks after surgery.

**3. Hormone-induced estrus in females**

3.1. Determine the estrous stage of the females by performing a vaginal smear as described in McLean et al.[18]. No estrus cycle change indicates that the ovariectomy of the female was successful.

3.2. Inject estradiol benzoate (20 µg per mouse, dissolved in 0.1 mL of sterilized olive oil, intraperitoneally) 48 h prior to the sexual behavior test.

3.3. Inject progesterone (500 µg per mouse, dissolved in 0.1 mL of oil, intraperitoneally) 4 h prior to the sexual behavior test.

NOTE: The eligibility of an estrus female is determined by the female accepting genital insertion of a male mouse 3 or more times, when they cohabit with a sexually active and experienced male in one cage.

**4. Preparation for the sexual behavior test**

4.1. Conduct the sexual behavior test of male mice in a rectangular and open field box (40 cm x 40 cm x 40 cm) with black Plexiglass walls, except for a transparent front wall that allows for the observation of mouse movement.

4.2. Set the general room lighting to 650 lux.

NOTE: The mice should not be illuminated directly to avoid abnormal behavior patterns.

4.3. Use a digital camera linked to a computer to videotape the movement and behavior of the mice.

4.4. Perform the behavioral test on the male mice during the first hours of the dark cycle.

**5. Habituation**

5.1. Keep the experiment room quiet.

5.2. Place the mice to be tested in the center of the open field box, allowing them to explore the environment freely for 30 min.

5.3. Habituate mice for 2 consecutive days prior to the testing day in the apparatus to prevent stress from the new environment.

NOTE: Mice should move and explore freely without feeling stress. Both male and female mice need to be habituated to the testing environment.

**6. Behavioral assays**

6.1. Turn on the camera prior to the beginning of the test.

6.2. Place the mouse to be tested in the center of the open field in the test box, allowing free exploration for 5 min to acclimate to the environment.

6.3. Place a female in estrus into the test box.

6.4. Record the social and mating behaviors and the interactions between the male and female mice for 30 min.

6.5. Turn off the camera and confirm that the video is saved.

6.6. Take the female out of the test box and record the formation of a vaginal plug.

NOTE: A female mouse that accepted mating cannot be employed in another sexual behavior test in one day.

6.7. Place the female back to its home cage.

6.8. Return the male to its home cage.

6.9. Clean the urine, feces, and padding within the apparatus.

6.10. Remove the smell of the tested mice with 75% ethanol.

6.11. Start the test of the next male mouse by repeating steps 6.1–6.10.

**7. Behavioral data extraction**

7.1. Play back the video recording and extract the behavioral parameters (see **Figure 2**).

7.1.1. Record the number of mounts in 30 min.

7.1.2. Record the number of intromissions in 30 min. Count a pelvic thrust as an intromission.

7.1.3. Record the time from the introduction of the female to the first mounting as mounting latency.

7.1.4. Record the time from the introduction of the female to the first intromission as

intromission latency.

7.1.5. Record the time from first intromission to the first ejaculation as ejaculation latency.

7.1.6. Record the time from ejaculation to the next mounting as post-ejaculatory mount latency.

7.1.7. Record number of copulatory series in 30 min. A copulatory series is each sequence from mounting to ejaculation.

7.1.8. Record the time of all copulatory series in 30 min as the duration of the copulatory series.

**REPRESENTATIVE RESULTS:**

A comparison of sexual behavior between CD-1 mice reared in isolation and group-housed CD-1 mice is shown. Male CD-1 mice were randomly assigned into an isolation-reared group (IS, one mouse per cage, n = 30) and a group-housed group (GH, five mice per cage, n = 15). The mice underwent isolation rearing from postnatal day 23 to day 93. Then, both groups of mice were assessed for sexual behavior. Our study found that the success rate of copulation tended to be lower in the IS group than in the GH group (IS: 80.0%, GH: 86.7%), although no statistically significant difference between groups was observed ($p = 0.458$). Mounting latency was longer in the IS group than in the GH group ($p = 0.002$, **Figure 3A**), indicating that the former required more time to initiate sexual behavior. Intromission latency was longer in the IS group than in the GH group ($p = 0.015$, **Figure 3B**), indicating that the former required a longer time to perform the insertion of the penis into the female's vagina. No statistically significant difference between the two groups was observed in terms of ejaculation latency and post-ejaculatory mount latency. Duration of the copulatory series was shorter in the IS group than in the GH group ($p = 0.002$, **Figure 3C**). No statistically significant differences between the two groups were observed in terms of the number of mounts, number of intromissions, and number of copulatory series[19] (see **Table 1**).

**FIGURE AND TABLE LEGENDS:**

**Table 1: Sexual behavioral parameters of isolation-reared and group-housed mice.**

**Figure 1: Ovariectomy of female mice.** The position of vertical incision and the right-hand ovary are shown.

**Figure 2: Process of sexual behavior of mice.** The red arrow indicates the female and the yellow arrow indicates the male. (**A**) The male's sniffing of anal-genital areas at the beginning of sexual behavior. (**B**) The male mounting the female. (**C**) The intromission posture of the male. (**D**) The ejaculation of the male. (**E**) The male grooming the genital areas after ejaculation.

**Figure 3: Results of sexual behavior test.** (**A**) Box plot of the mounting latency of IS and GH mice. (**B**) Box plot of the intromission latency of IS and GH mice. (**C**) The total mating duration of IS and GH mice. $*p < 0.05$, $**p < 0.01$. This figure has been modified from Liu et al.[19].

**DISCUSSION:**

There are a few critical steps in the presented protocol. Regarding the ovariectomy of females, the surgery incision opening from the back is less detrimental than that from the abdomen. Given that the position of the ovary is deep, pulling other organs when the incision is cut open from the abdomen often leads to bleeding and results in unclear surgical vision[20]. We performed the incision on the back to reach the ovary easily and shorten the surgical time, as well as to ensure the safety of the surgery.

The maintenance of sterile conditions during surgery is important for survival. Four main variables are considered during a surgical procedure: the surgical space, the instruments, the surgeon, and the animal. For the surgical space, including workspaces and surgical rooms, all necessary surfaces are cleaned and disinfected with appropriate disinfectants (e.g., diluted bleach, hydrogen peroxide products) prior to the surgery. Additionally, pressurized steam with an autoclave is recommended. During a surgical procedure, traffic flow is limited in the surgical room. In a surgical procedure, newly sterilized instruments and materials should be used for every animal. When instruments fall outside the sterile field or become contaminated, they should be immediately replaced. Meanwhile, after washing/scrubbing hands and arms thoroughly, surgeons need to don all sterile attire, including a sterile gown and sterile surgical gloves. If any material is contaminated, the affected article needs to be changed immediately prior to surgery (e.g., new gown or surgical glove). Finally, hair from the surgical site and surrounding area should be removed for the prevention of contamination. The skin must be scrubbed with 75% ethanol after hair removal. Electric clippers or depilatory cream can be used to remove the hair. Prior to surgery, a sterile drape is placed over the animal allowing access to the surgical site for the prevention of contamination.

Three aspects of the post-surgical treatment of the animals require attention: anesthetic recovery, analgesia and surgical site monitoring, and suture removal. Upon the completion of the surgical procedure, animals must be monitored during recovery from the anesthesia. The animal should not be left unattended until it has regained sufficient consciousness to maintain sternal recumbency and should not be returned to the company of other animals until it has fully recovered. In addition, appropriate recovery conditions must be provided, including a warm environment free from objects that could cause harm. For example, paper towels instead of corn cob bedding are used in the recovery of the mice and large toys or water bowls are removed from large animal pens. According to the guidelines of the University of Minnesota, the ovariectomy of female causes moderate to severe pain. Thus, analgesia must be administered directly post-operatively by parenteral injection or oral gavage[21]. In this study, a lidocaine injection beneath the skin was performed after surgery and water containing ibuprofen was administered for at least 1–2 days for pain treatment. However, a veterinarian must be consulted in the development of the analgesic plan. Finally, the animal's post-surgical health and the surgical site must be observed and recorded for a minimum of 3 days. An operating line or wound clips are used for suturing the incision, which should be removed from the skin 7–14 days after surgery[21]. In this study, an absorbable line to suture the incision to avoid suture removal was used.

The estrus of the female was artificially controlled with ovariectomy and hormone usage, instead

of using a female with natural estrus. This step was taken to ensure the consistency of the sexual receptivity of the female in the test and guarantee the reliability of measurements when monitoring male mating behavior. Furthermore, the hormone-induced estrus female can be reused in a set of experiments, and the influence of pregnancy is prevented. Estrus in the female is induced by injecting estradiol benzoate and progesterone before the experiment. This method is easy to manage, has a high success rate, and multiple estrus females can be obtained at the same time, thus greatly improving the efficiency of the test.

Sexually naïve and experienced mice show different behavior patterns. Attention needs to be paid to the test-retest reliability of the experiment in various stages of mice development. The dynamic change in sexual behavior needs to be considered before conducting the test and in the experimental design stage. In this study, sexually naïve males were used for the sexual behavior test and only the first occurrence of sexual behavior was measured without any training prior to the test. Copulation is the final outcome of a series of pheromone detection, mounting, intromission, and ejaculation. There is a limitation to the present experimental protocol when applied to mutant mice. For example, male mice with targeted mutations of Adcy3, Cnga2, and Gnao exhibit reduced ability to detect pheromones[14-16], whereas the Trpc2 knockout mice show altered partner preference[22]. The present protocol may not be able to exhibit the sexual behavior of mutant mice because of its reduced ability to detect pheromones.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure1

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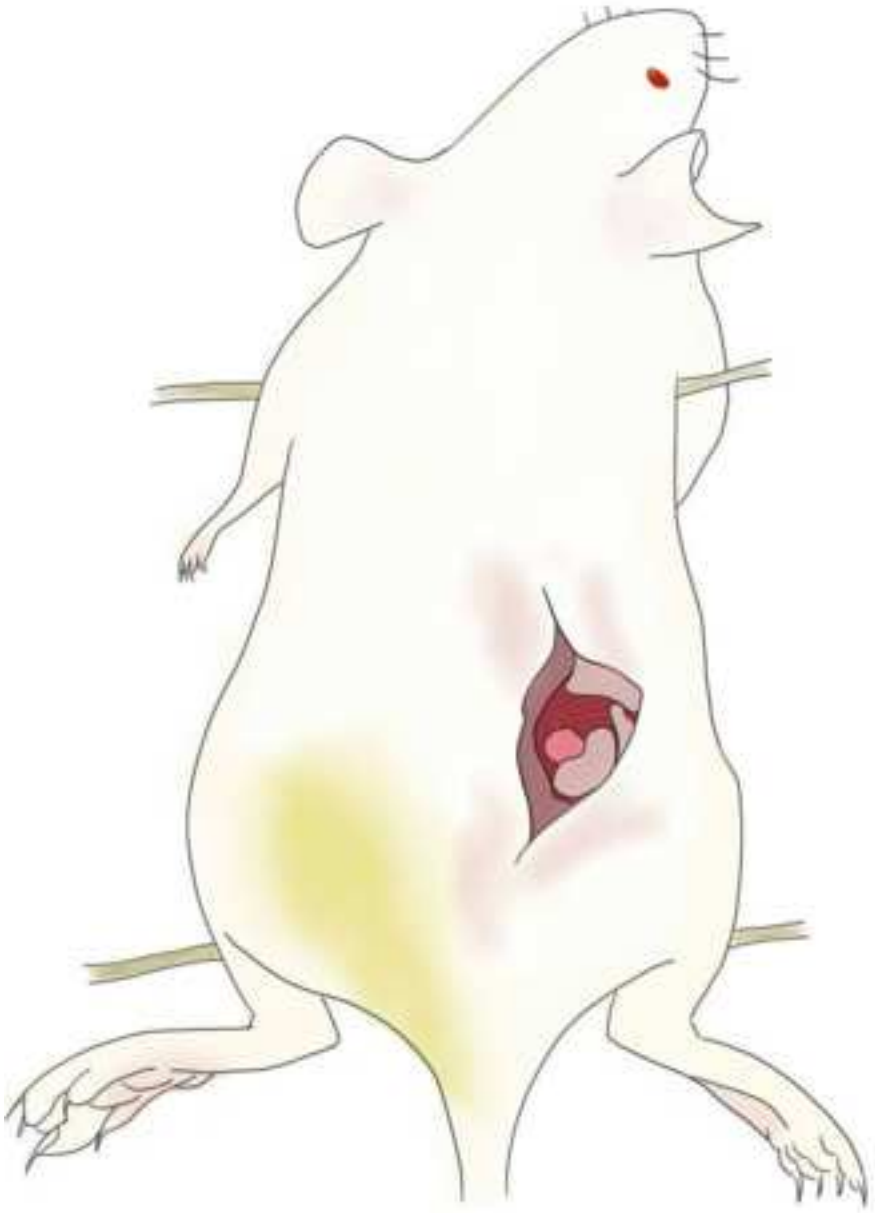

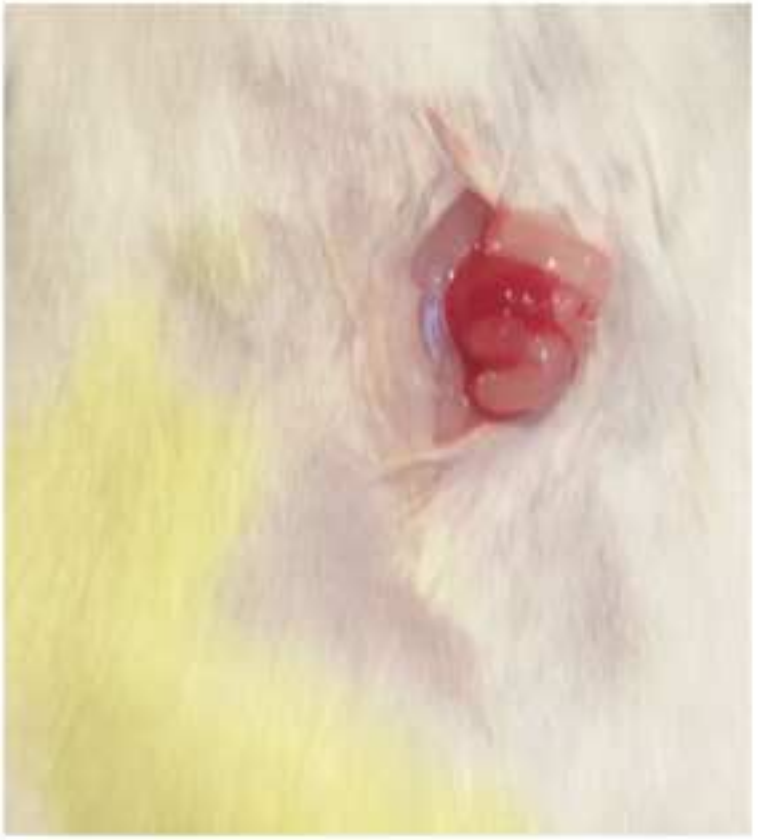

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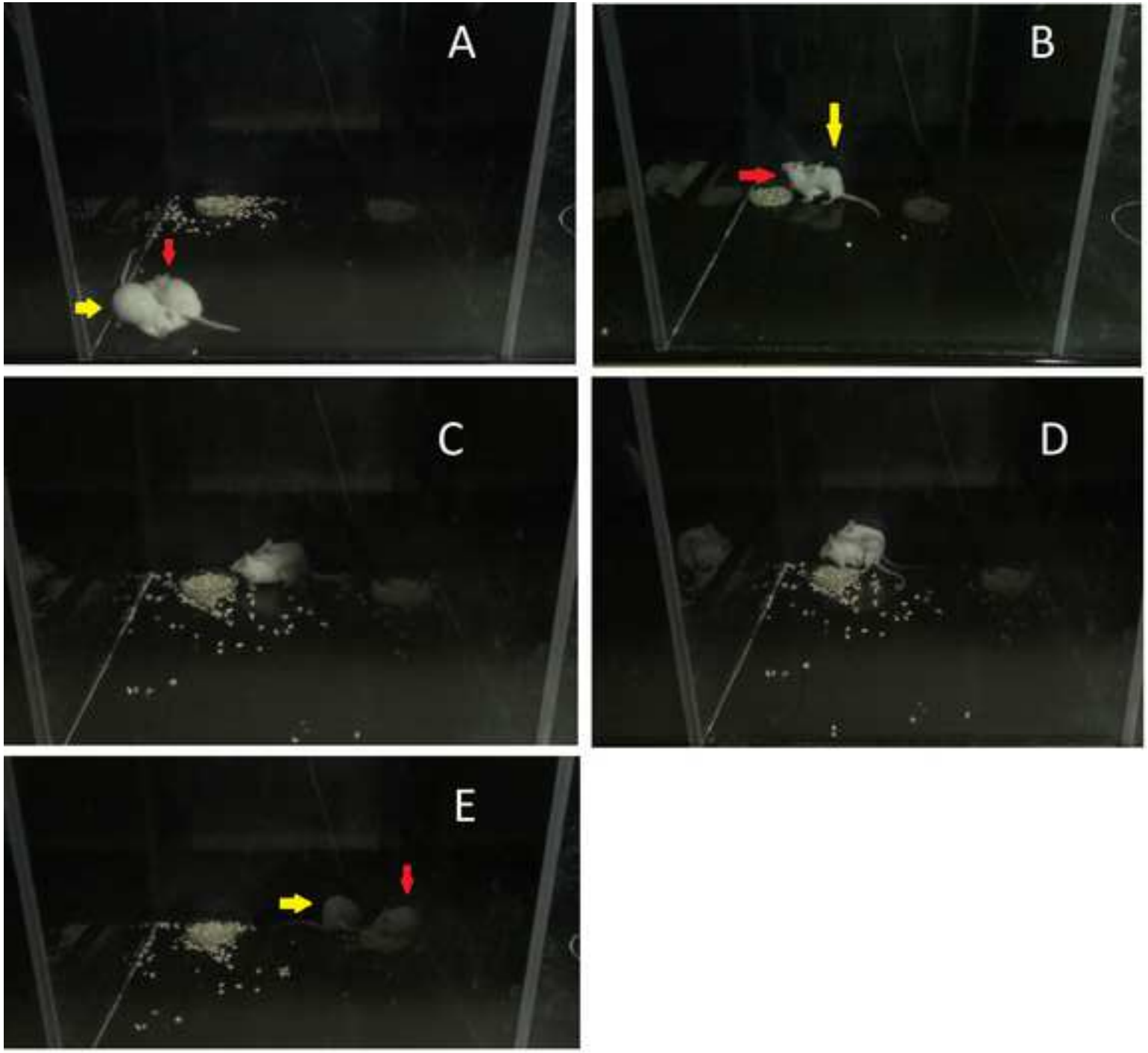

Figure3

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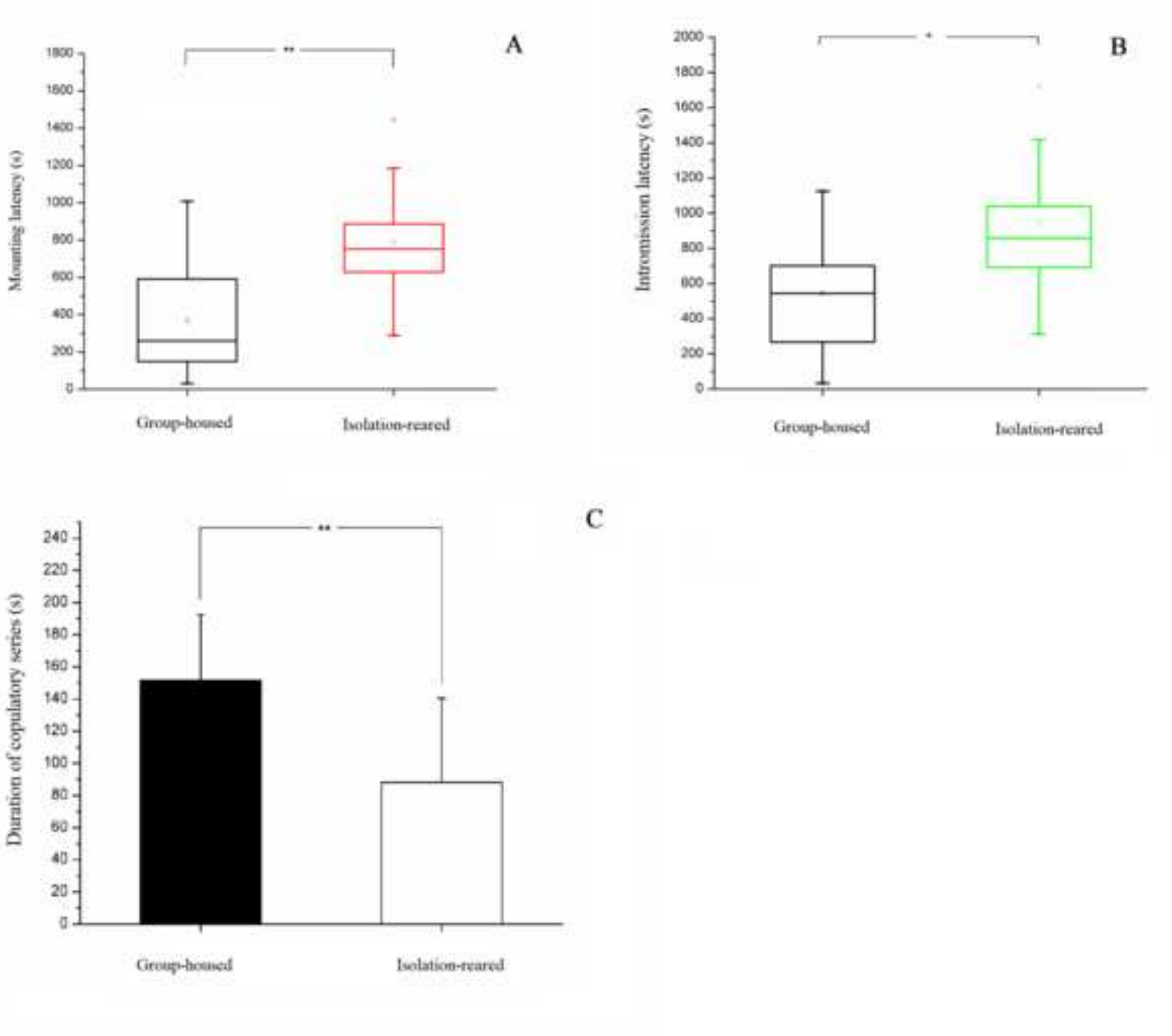

Table Click here to access/download;Table;Table1.xlsx

| | Isolation-reared | Group-housed | *t*/*t*′ | *p* |
|---|---|---|---|---|
| N | 30 | 15 | | |
| Mounting latency [a] | 788.70 ± 262.77 | 365.03 ± 288.65 | -3.87 | 0.002 |
| Intromission latency | 937.30 ± 369.87 | 542.94 ± 352.40 | -2.75 | 0.015 |
| Ejaculation latency | 16.58 ± 9.78 | 17.37 ± 13.03 | -0.2 | 0.845 |
| Post-ejaculatory mount latency | 173.00 ± 89.84 | 192.87 ± 106.91 | 0.58 | 0.565 |
| Duration of copulatory series | 88.27 ± 52.40 | 151.65 ± 40.87 | 3.44 | 0.002 |
| Number of mounts [b] | 2.4±2.0 | 3.3±3.3 | 1.09 | 0.282 |
| Number of intromissions | 20.1±12.9 | 22.6±12.3 | 0.58 | 0.564 |
| Number of copulatory series | 7.0±4.3 | 9.3±4.6 | 1.55 | 0.131 |

Mean ± SD; [a] unit is second (s). [b] unit is counts.

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

| Name of Material/Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Choral hydrate | Reagent Co.,Ltd | 20160225 | |
| Coated VICRYL Plus Sutures | Ethicon, Inc. | missing | |
| Estradiol benzoate | J&K Scientific Ltd. | L930Q170 | |
| Ethanol absolute | Beijing Chemical Works Co., Ltd | 20160715 | |
| Ibuprofen (Children's Motrin) | Shanghai Johnson & Johnson Co., Ltd | 160629478 | |
| Isoflurane | RWD Life Science Co., Ltd | 217180501 | |
| Lidocaine | Hebei Tiancheng Pharmacreutical Co.,Ltd | 1170506107 | |
| Male and female CD-1 mice | Vital River Beijing | SCXK（京）2013-0023 | |
| Olive oil | | | |
| Penicillin sodium | North China Pharmaceutical co.,Ltd | F5126420 | |
| Progesterone | J&K Scientific Ltd. | LR50Q07 | |
| Sony digital camera | Sony Corporation | HDR-CX290E | |
| Test box | | | DIY |
| ThinkStation Computer | Lenovo | S/N PCOGLQKG | |
| Vaporizer for Isoflurane | RWD Life Science Co., Ltd | E05904-009M | |

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**Editorial comments:**

Note that some changes have been made to better fit JoVE format and correct some grammatical and usage errors.

1. Formatted per JoVe guidelines (e.g., spaces between every step/substep) the highlighted portion of the protocol exceeds 2.75 pages. Please reduce the length of the highlighted portion.

Aw: As per suggestion, the length of the highlighted portion has been reduced accordingly.

2. 2.1: 5% isoflurane is likely too high a concentration for anesthesia; please check.

Aw: Isoflurane 4~5% has been used for induction whereas 1~2% for maintenance of anesthesia. We added this information in line 203.

3. 3.1: If this is to be filmed, please at least describe what 'no estrus cycle change' looks like.

Aw: I removed the highlight mark. I think it is hard to film. No estrus cycle change is decided by vaginal smear test [1], which has been published in JoVE journal. Provide with a reference.

[1] McLean, A. C., Valenzuela, N., Fai, S. & Bennett, S. A. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp. 10.3791/4389 (67), e4389, (2012).

4. 6.4/7: If the behaviors seen and their timing are to be shown in the video, they should be highlighted and/or discussed more in the protocol. Note that, for section 7, all steps/substeps should be no more than 4 sentences and should have at least one instruction (i.e., in the imperative).

Aw: I removed the highlights of 6.4, 6.6, and 6.7. For section 7, the description has been revised.

5. Please remove the embedded tables and upload as separate .xls/.xlsx files, 1 per table (i.e., 2 in total).

Aw: Thank you for the reminder and the file have been uploaded as per suggested format.

6. Figure 1: The surgery is still shown without the use of sterile drapes; perhaps you could zoom in on the incision site and not show the table at all?

Aw: Thank you for the kind reminder. I have revised the figure 1. But I hope to be able to take a new picture for display in the formal article when we make video product.

7. Table 1/Figure 3/Results: The p-value shown for intromission latency in the text and table (0.015) still contradicts the value indicated in the Figure (<0.01).

Aw: The error has been corrected.

8. Table 2: Could you discuss the lack of normality a bit in the Results?

Aw: Previously, I treated the number of mounts, intromission, mating (now it is copulatory series) as discrete data, because the occurrence is small. Hence, median and inter-quartile ranges were used to describe the data. Non-parametric test was used to test the hypothesis. This time, I consulted a statistician and he told me that these indexes can be treated as continuous variables in our case. Therefore, I tested the distribution and homogeneity of variance of data. The results show as follows: the distribution is normal and the variances are equal. Student-t test is used to compare the differences of three index between isolation and control mice. No statistically significant differences are found between the two groups in terms of number of mounts, intromission, and copulatory series. The results has been revised in the revised manuscript.

**One-Sample Kolmogorov-Smirnov Test**

| 组别 | | | number of mounts | number of copulatory series | number of intromissions |
|---|---|---|---|---|---|
| control | N | | 13 | 13 | 13 |
| | Normal Parameters[a,b] | Mean | 3.31 | 9.31 | 22.62 |
| | | Std. Deviation | 3.301 | 4.644 | 12.299 |
| | Most Extreme Differences | Absolute | .242 | .252 | .185 |
| | | Positive | .227 | .144 | .185 |
| | | Negative | -.242 | -.252 | -.125 |
| | Kolmogorov-Smirnov Z | | .873 | .907 | .666 |
| | Asymp. Sig. (2-tailed) | | .430 | .383 | .767 |
| isolation | N | | 26 | 25 | 25 |
| | Normal Parameters[a,b] | Mean | 2.38 | 6.92 | 20.08 |
| | | Std. Deviation | 1.981 | 4.396 | 12.929 |
| | Most Extreme Differences | Absolute | .269 | .163 | .188 |
| | | Positive | .269 | .163 | .188 |
| | | Negative | -.242 | -.097 | -.116 |
| | Kolmogorov-Smirnov Z | | 1.373 | .815 | .940 |
| | Asymp. Sig. (2-tailed) | | .046 | .520 | .340 |

a. Test distribution is Normal.

b. Calculated from data.

**Group Statistics**

| | 组别 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| number of mounts | control | 13 | 3.31 | 3.301 | .916 |
| | isolation | 26 | 2.38 | 1.981 | .389 |
| number of intromissions | control | 13 | 22.62 | 12.299 | 3.411 |
| | isolation | 25 | 20.08 | 12.929 | 2.586 |
| number of copulatory series | control | 13 | 9.31 | 4.644 | 1.288 |
| | isolation | 25 | 6.92 | 4.396 | .879 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| number of mounts | Equal variances assumed | 1.733 | .196 | 1.092 | 37 | .282 | .923 | .845 | -.789 | 2.635 |
| | Equal variances not assumed | | | .928 | 16.456 | .367 | .923 | .995 | -1.181 | 3.027 |
| number of intromissions | Equal variances assumed | .076 | .785 | .583 | 36 | .564 | 2.535 | 4.350 | -6.287 | 11.358 |
| | Equal variances not assumed | | | .592 | 25.538 | .559 | 2.535 | 4.280 | -6.271 | 11.342 |
| number of copulatory series | Equal variances assumed | .050 | .824 | 1.559 | 36 | .128 | 2.388 | 1.532 | -.719 | 5.495 |
| | Equal variances not assumed | | | 1.531 | 23.265 | .139 | 2.388 | 1.559 | -.836 | 5.612 |

Double-click to activate

```
SORT CASES  BY group.
SPLIT FILE LAYERED BY group.
NPAR TESTS
  /K-S(NORMAL)=FS5 FS8 FS6
  /MISSING ANALYSIS.
```

**Reviewers' comments:**

**Reviewer #2:**

Manuscript Summary:

The revised manuscript " Assessment of Sexual Behavior of Male Mice" by Zi-Wei Liu with co-authors sounds better now and present a fully described method which would be helpful for researchers to replicate this behavioral set up to study sexual behavior in laboratory mice.

Major Concerns:

none

Minor Concerns:

none

**Reviewer #3:**

Manuscript Summary:

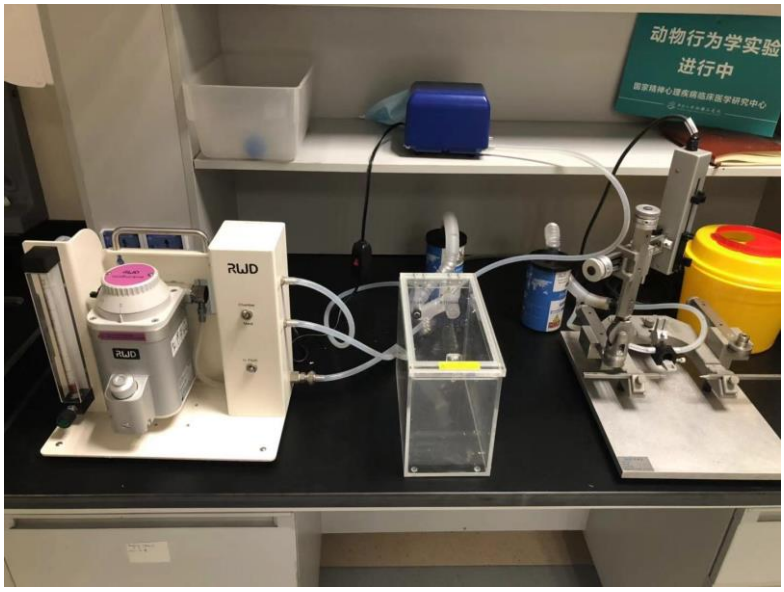
The aim of this study is to present the standard procedure for measuring male sexual behavior in laboratory setting. The manuscript includes the information on preparing ovariectomized female stimuli for hormone-induced estrus ovariectomized female model and assessing the mating behavior of male mice.

Major Concerns:

1. In this revision, authors claimed that they used isofluorane anesthesia. However, the mouse shown in Fig. 1 is same as one in original manuscript. I am not convinced that they really used inhalation anesthesia in this study.

Aw: We had not taken a photograph record in the formal experiment. This picture was taken in a practice experiment. The mouse in the figure1 was anesthesia by

pentobarbital sodium (there is no buying channel now). We do have isoflurane and the apparatus for anesthesia showed as following. I will take a more clear picture for display in the formal article when we make formal video product




2. Authors should specify that the "isolation" is the post-weaning isolation starting at PND 23.

Aw: I added the following information of “Male CD-1 mice were randomly assigned into the isolation-reared group (IS, one mouse per cage, n = 30) and group-housed group (GH, five mice per cage, n = 15). The mice underwent isolation rearing from postnatal day 23 to day 93. Then, both groups of mice assessed for sexual behavior” in line 422 to 425.

3. Is there any reason to employ i.p. injection for EB and P administration? Why not s.c. injection?

Aw: Most of the past studies recommended using s.c injection in sexual behavior test of the rat. Based on our experience, repeatedly s,c injection with oil often forms a lump at the injection site. The position of s.c. injection is usually chosen posterior to neck on the dorsal side of the female mouse where the male mouse used to put head during copulation. A lump in the back mentioned location would change the shape of body of female mice, which may affect the behavior of male mice. The lump size has a bigger impact on mouse than rat due to the different body sizes. In order to reduce the possible impact of experimental manipulation. I speculate that i.p. inject produce a small lump and its position is on the abdomen of female which results in little effect on the male’s behavior. Hence I employed i.p. injection instead of s.c. injection. However, the drug absorption is quicker in i.p, injection than that of s.c. injection. The difference between i.p, and s.c. injection on sexual behavior is still needed to be explored in the further study.

Besides, before the test of sexual behavior, we examine the receptivity of

female mouse by whether female mouse accepts genital insertion of a male mouse three and more times.

4. In this revised manuscript, authors defined "Number of mounting" as the number of mounting times before the first ejaculation with and without intromission, "Number of intromissions" as the total number of intromissions before the first ejaculation, "Total number of mounting" as the total number of mounting during the observation period, and "Total number of mating" as the total number of mating (including mounting, intromission, ejaculation, and refractory period) during the observation period. If so, how is it possible that the median for "Number of intromission" become higher than the median for "Number of mounting", "Total number of mounting", or "Total number of mating".
I would also like to know 1) the means for these data, 2) how many mice in each group showed each behavior, and 3) how many mice in each group ejaculated.
Aw: I update more concise definitions of mounting, intromission, and copulatory behavior in the revised manuscript. Mouse would keep a mounting posture during the whole sex process until ejaculation completion. However, there may be one to more than 100 intromissions before ejaculation [1], which indicated male mice would make repeated attempts of intromission meanwhile keeping the mounting posture. Hence, the number of intromission could be higher than the number of mounts.

1. The staff of the Jackson laboratory. Biology of the Laboratory Mouse, Second Edition, Chapter 11. Dover publications, inc., New York, (2007).

Besides, I added the means of each behavior, the sample size in Table 1 in the revised manuscript. As for the last question, there 24 of 30 mice ejaculated in the social isolation group determined by the behavioral performance and examined the residual semen or a copulatory plug at the vaginal open of female mice. In the control group, 13 of 15 mice ejaculated.

Minor Concerns:
1. Six weeks old seems to be too young for ovariectomy. Did they properly react to the EB & P treatment?
Aw: According to the book of *Biology of the Laboratory Mouse*, Parkes ( 1925) stated that his albino mice were usually mature by 7 weeks of age, whereas Engle and Rosasco ( 1927) reported a median age of 35 days and a range of 28 to 49 days also in an albino strain [1]. Six weeks old mice seems to be sexual mature. In our study, we ovariectomized the female at postanal 6 weeks, and healing period extended for about three weeks. When female mouse employed in the test, they were gain sexual maturity. They showed a good sexual receptivity in the tests.

1. The staff of the Jackson laboratory. Biology of the Laboratory Mouse, Second Edition, Chapter 11. Dover publications, inc., New York, (2007).

**Reviewer #5:**

Manuscript Summary:

Acceptable for publication, no suggestions.

Major Concerns:

None

Minor Concerns:

Authors described the methods in detail and very well. Nonetheless, there are a few suggestions for improving their manuscript:

Line 189. Should read "observation of mouse movement"

Aw: Thank you for the reminder. Corrected it.

Line 208. Authors indicate that habituation occurs twice a day for 2 days. Does this mean 4 times? How long in between each day?

Aw: Each mouse habituated for 30 mins every day and habituated for twice in total. Habituation was conducted in two consecutive days preceding the testing day.

Line 216. Suggested wording: Place the testing mouse in the center of the open field box, allowing…

Aw: The error has been corrected.

Line 219. Is female placed in the center of the open field box as well?

Aw: Yes.

Methods do not indicate clearly if new and naïve females are used every time.

Aw: No. all female mice are sexually experienced. I specified that the eligibility of an estrus female is determined by the female accepting genital insertion of a male mouse 3 and more times in the NOTE of 3.3. I added the following description of “Female mouse accepted mating can be not repeated employed in anther sexual behavior test in one day.”in line 340 - 341.

Line 253. How long does the post-ejaculatory period last? Authors indicate a brief time line of other sex behavior components and that seems helpful to give an idea to inexperienced investigators.

Aw: I added following information of “Mouse sexual behavior exists strain differences. For instance, ejaculation latencies ranged from 594 to 6943 seconds, and the numbers of intromissions ranged from 5 to more than 100. Post-ejaculation latencies ranged from 17 to 60 min. However, the introduction of a novel female can decrease this time interval. In some cases, male ejaculating on the first intromission with the new female.” In line 63 -67

Line 289: Suggested to change "time to initiate sex" to "time to initiate sexual

behavior"
Aw: The error has been corrected.

Line 322: Suggested modification from "is better" to "is less detrimental"
Aw: The error has been corrected.

Line 372. "Sex" can be changed for "sexual behavior"; "Sexual native" to "sexually naïve?
Aw: The error has been corrected.and thanks again.

**Reviewer #6:**
Manuscript Summary:
OK

Major Concerns:
1. The introduction does not mention that authors will analyze two population of animals: reared in isolation and group housed. There is information about this manipulation on sexual behavior that authors ignore.
Aw: Thank you for your kind reminder. I re-write the introduction to focus on the measurement of sexual behavior in male mice. Besides, I hope to present an example of sexual behavior. Meanwhile, social isolation can reduce sexual behavior in male mice. Hence, I just want to use it as an example of sexual behavior test, based on our research experience.

2. The reader is confused on whether the main goal of the manuscript is to describe the methods followed to observe sexual behavior in this species or to understand how isolation affects the expression of this behavior. The reader finds isolated reared groups in Results tables for the first time in the manuscript.
Aw: I re-write the introduction to focus on the measurement of sexual behavior in male mice. This time, I hope this revision makes the topic of the manuscript clearer.

3. The manuscript fails to mention the literature reviews on male sexual behavior that consider several species and various strain of mice. For example, Meisel and Sachs or Hull and Rodríguez Manzo.
Aw: I added this information and references in the introduction of the revised manuscript.

4. Authors have to check carefully their interpretations. For example: "Sex is a learning process" is too strong. In many species, like mouse, sex is an innate behavior although modified by experience and other factors. Lordosis is a reflex.
Aw: Thank you for your kind reminder. I have revised this description.

Minor Concerns:

5. Why did authors decided to use rectangular (instead of cylindrical) arenas for their observations?

Aw: In order to avoid the light refraction result from curved edge of cylindrical. Light refraction would blur the image of video. Curved edge is not suitable for video camera to record the action of mice. Rectangular arenas are more easy to observe mouse behavior from a lateral view. Moreover, rectangular arenas provide more stable phenotype and the rectangular edges are preferred to stay by normal mice. In contrast, circular arenas are continuous and mice do not find any safe corner.

6. Why did they selected the 12h of the dark cycle while all authors choose the first hours of the dark phase?

Aw: Female mice should not be bred before 50 days of age. They are continuously "polyestrous," which means that they come into heat at fairly regular intervals (every 4-5 days) throughout the entire year until they are bred. The period during which the female is receptive to the male and allows breeding is about 12 hours and usually occurs at night.

https://www.animalhospitals-usa.com/small-pets/mice-and-rats/mice-rat-reproduction.html

Aw: The first hours of the dark phase is right. I have revised the description in line 309.

7. Figure 2 does not show latencies. Please check citations in the text.

Aw: The error has been corrected.

8. The same group has published figure 3 earlier? Please explain.

Aw: Yes, this figure has been published in *Frontiers in Behavioral Neuroscience*. Here, I just want to use it as an example to present what the data of sexual behavior look like, which is acceptable by the JoVE journal as far as I know. In addition, I have received the permission of copyright owner and the journal to reuse this result.

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I hope I have addressed your question. Please let me know if you have any other questions or concerns.

Best regards,
Gean
--

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