

# Journal of Visualized Experiments

## Determination of reproductive competence by confirming pubertal onset and performing a fertility assay in mice and rats

--Manuscript Draft--

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| Article Type:  | Invited Methods Article - JoVE Produced Video  |
| Manuscript Number:   | JoVE58352R2  |
| Full Title:  | Determination of reproductive competence by confirming pubertal onset and performing a fertility assay in mice and rats                                      |
| Keywords:  | Pubertal onset; fertility assay; reproductive competence; preputial separation; vaginal opening; first estrus; Mouse; rodent; Rat; male; female; body weight |
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| Question   | Response   |
| Please indicate whether this article will be Standard Access or Open Access.   | Standard Access (US\$2,400)  |
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## **TITLE**

Determination of Reproductive Competence by Confirming Pubertal Onset and Performing a Fertility Assay in Mice and Rats

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## **KEYWORDS**

Pubertal onset, fertility assay, reproductive competence, preputial separation, vaginal opening, first estrus, mouse, rodent, rat, male, female, body weight

## **SHORT ABSTRACT**

Many treatments and genetic mutations impact the timing of sexual maturity and fertility. This protocol describes a non-invasive method to evaluate pubertal onset in mice and rats prior to setting up a fertility study in sexually mature animals.

## **LONG ABSTRACT**

Assessment of reproductive competence is critical for understanding the impact of a treatment or genetic manipulation on the reproductive axis, also termed the hypothalamic-pituitary-gonadal axis. The reproductive axis is a key integrator of environmental and internal input adapting fertility to favorable conditions for reproduction. Prior to embarking upon a fertility study in mice and rats, sexual maturity is evaluated to exclude the possibility that the observed reproductive phenotypes are caused by delayed or absent pubertal onset. This protocol describes a non-invasive approach to assess pubertal onset in males through the determination of preputial separation, and in females through vaginal opening and first estrus. After the confirmation of the completion of puberty and the achievement of sexual maturity, a fertility study can be initiated. The procedure describes the optimal breeding conditions for mice and rats, how to set up a fertility study, and what parameters to evaluate and determine if the treatment or gene deletion has an impact on fertility.

## **INTRODUCTION**

The transition through puberty is required to attain sexual maturity and reproductive competence. The pubertal transition and the maintenance of fertility in adulthood is regulated by the reproductive axis, also termed the hypothalamic-pituitary-gonadal axis (**Figure 1**). The timing of pubertal onset and maintenance of fertility is tightly regulated by internal as well as environmental factors to increase the chances of survival of offspring and parents<sup>1,2</sup>. This protocol provides a non-invasive approach to determine pubertal onset in mice and rats to confirm sexual maturity prior to setting up a fertility study to assess reproductive competence.

A fertility study is performed in sexually mature animals and can be initiated after the animals have gone through puberty. Prior to pubertal onset, the reproductive axis is quiescent, and the key driver of sexual maturation, gonadotropin-releasing hormone (GnRH), is released onto the pituitary in insufficient amounts to initiate puberty (**Figure 1**). Pubertal onset is a complex process that results in increased GnRH release at the median eminence. GnRH promotes luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion from the pituitary, two hormones essential for gonadal maturation and reproductive function (**Figure 1**)<sup>3-5</sup>.

Insults to the reproductive axis result in reduced fertility and can also advance or delay pubertal onset. Conditions known to influence the timing of pubertal onset and reproductive competence include the exposure to endocrine disrupting chemicals<sup>6,7</sup>, increased/decreased body weight<sup>1,8</sup>, changes in day length<sup>2,9</sup> and genetic mutations<sup>10-15</sup>.

The onset of sexual maturity is a critical step that needs to be completed prior to setting up a fertility assay. The advantages of determining pubertal onset through preputial separation, vaginal opening and first estrus, are the non-invasive characteristics of these procedures, as they do not require blood collection or sacrifice of the animal<sup>16,17</sup>.

After pubertal onset is determined, correctly setting up a fertility study will provide important information about the integrity of the reproductive axis, and usually has the second advantage of generating experimental animals for further studies (refinement)<sup>18</sup>. The fertility study setup described in this protocol can detect both minor and major deficits in reproductive competence in males and females. Key parameters evaluated include 1) time to the first litter, 2) number of litters generated in a given time frame and 3) litter size. Finally, recommendations for the type of follow up studies which can be conducted to identify the cause of fertility impairment are included.

The described protocol refers to mice and the representative data reflect work done in transgenic mice. However, all the included protocols are equally valid in rats.

## **PROTOCOL**

All methods described here have been approved by the Institutional Animal Care and Use Committee of Michigan State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

### **1. Determine Pubertal Onset**

1.1. Follow institutional guidelines for clothing, at a minimum, it is necessary to wear a clean lab coat and clean gloves. Always handle mice wearing clean gloves.

1.2. Prepare the work area by placing a pad on the table.

1.2.1. Place a clean mouse cage top with the grid facing upwards on the pad.

1.2.2. Set up a scale in the work area. Place a clean 500 – 1000 mL beaker on the scale and tare.

1.2.3. Place a sheet next to the work area to record body weight and preputial separation, vaginal opening or first estrus.

1.3. Identify the mice for the study. Perform daily inspections for pubertal onset until puberty is reached. Perform the inspection at the same time of day throughout the study. Pubertal onset can occur as early as postnatal day 11-12<sup>19</sup>, however, in most experimental setups, pubertal onset monitoring starts at ~22 days.

1.4. Place the mouse cage in the work area (Step 1.2). Open the cage and place the lid, water bottle and food holder on the table.

1.5. Determining preputial separation in male mice.

1.5.1. While holding the mouse by the tail, place it on the clean mouse cage top from Step 1.2.1. While gently holding onto the tail, let the mouse explore the grid of the cage top for ~5 s.

1.5.2. Gently pull the tail backward; this usually results in the animal moving forward while holding on to the grid with its forelegs. Approach the other hand to the skin close by the neck and ears.

1.5.3. Grasp the loose skin between the shoulders and neck with the thumb and forefinger. It is important to get a good hold of the skin, as it will prevent the mouse from turning its head around to bite.

1.5.4. Grab the skin on the back with the remaining fingers and hold it by pressing against the hand. Hold firmly without obstructing breathing, blood flow, or damaging bones, muscles or skin. Turn the hand to expose the belly of the mouse, while supporting the back of the mouse within the palm of the hand.

1.5.5. With the free hand, gently push back the skin around the penis without forcing. At preputial separation, the preputial skin slides backward exposing the *glans penis* (**Figure 2A**, arrow showing preputial separation). Record the presence or absence of preputial separation.

1.5.6. Weigh the mouse by gently placing it in the 500-1000 mL beaker from Step 1.2.2 and record the weight.

1.5.7. Reintroduce the mouse in the housing cage by holding the beaker 0-2 cm over the cage floor and slowly tip over the beaker allowing the mouse to exit on its own. Clean the beaker with water and mild detergent and dry it off before returning it to the scale and taring the scale.

1.6. Determining vaginal opening in female mice.

1.6.1. Place a cotton ball and a bottle of sterile water in the work area (Step 1.2). Humidify the cotton ball with the sterile water and place it next to the cage top from Step 1.2.1.

1.6.2. Place the mouse cage in the work area (Step 1.2) and transfer the mouse from its home cage to the cage top (Step 1.2.1) by holding it at the base of the tail. Gently pull back the tail to encourage a forward movement of the mouse.

1.6.3. Lift the tail while supporting the hips with the free fingers. Allow the hindlimbs to maintain contact with the top of the cage. If the mouse moves too much, hold it in the hand as described in Steps 1.5.1-1.5.4.

1.6.4. Gently clean the vulva with the water humidified cotton ball from Step 1.6.1. Use a clean humidified cotton ball for each mouse. To determine vaginal opening, examine the vulva and determine whether the vagina is completely open (**Figure 2A**, female vaginal opening).

1.6.5. Record if vaginal opening has occurred. Weigh the mouse and return it to the home cage as described in Steps 1.5.6-1.5.7.

1.7. First estrus an indicator of first ovulation.

1.7.1. Place a beaker with sterile water, a labeled glass slide and a 200  $\mu$ L pipette with a clean tip in the work area (Step 1.2).

1.7.2. Hold the female as described in Step 1.6.2-1.6.3. Place the tip of the pipette containing  $\sim$ 50  $\mu$ L of sterile water at the vaginal opening.

1.7.3. Gently flush the cells from the vaginal wall by introducing and reabsorbing  $\sim$ 50  $\mu$ L of water 2-4 times. Smear the content from the pipette tip onto a labeled glass microscope slide.

1.7.4. Carefully observe the cellular morphology on a brightfield microscope using a 10X or 20X objective or let the smear airdry for  $\sim$ 1 h and then counterstain with a 0.1% methylene blue solution (dissolved in ddH<sub>2</sub>O) (**Figure 2B**). To counterstain, dip the dried slide into the 0.1% methylene blue solution for  $\sim$  30 s. Let the slide air dry for 1 h.

1.7.5. Observe the slides from Step 1.7.4 on a bright field microscope at 10X or 20X. Establish the stage of the estrous cycle (**Figure 2B**)<sup>20</sup> by observing the cell morphology which allows to distinguish the four distinct stages of the estrous cycle (**Figure 2B**).

Note: Metestrus is characterized by a mixture of cornified squamous epithelial cells and leukocytes, diestrus by leukocytes, proestrus by a mixture of leukocytes and nucleated epithelial cells, and estrus by cornified epithelial cells (**Figure 2B**)<sup>20</sup>.

1.7.6. Record the body weight and return the mouse to its home cage as described in Steps 1.5.6-1.5.7. Vaginal smears are terminated on the day when the mouse has its first estrus (**Figure 2B**).

## **2. Desirable Breeding Room Conditions**

2.1. Ensure that the breeding room has a temperature of ~20-24 °C, with 55 ± 10% humidity, and homogenous room light of an intensity of ~300-400 lux approximately 1 m above the floor<sup>21</sup>. This light intensity allows the desirable mid-cage light intensity of 25-80 lux. To test light intensity, use a light meter.

2.2. Use an automated system to control the lighting in the breeding room to ensure appropriate timing of lighting throughout the experiment. Optimal day-length conditions to assess fertility in rodents range from a 12 h day (12 h light and 12 h darkness, LD12:12), to “summer-like” conditions with 14 h of light and 10 h darkness (LD14:10)<sup>21</sup>.

### **2.3. Prepare breeding cages.**

2.3.1. Prepare a clean mouse cage with lid, water, food, bedding and nesting per breeding pair.

2.3.2. Fill out the cage cards with all the required information, including sex, date of birth, mouse strain, and the date when the mating is set up.

## **3. Fertility Study**

3.1. Identify the animals to be used for the fertility study. Ensure that the animals are sexually mature (Step 1). A typically used age range to set up a fertility study in mice is 10-16 weeks of age, an age where sexual maturation has been completed in most experimental conditions.

3.2. On a clean table, place the breeding cage prepared in Step 2.3.

### **3.3. Catch and hold the mouse.**

3.3.1. Slowly introduce a gloved hand into the mouse cage. Leave the hand in the cage for a short period of time (~5-30 s), allowing the mice to adapt to the smell of the glove and hand. Avoid hectic and jerky movements.

3.3.2. Lower the cupped hand close to the bottom of the cage and slowly approach it to the mice. They will run away. When they run over the hand, trap the tail of one mouse between the thumb and index finger.

3.3.3. Lift the mouse by the tail for 2-3 s. If the mouse needs to be held longer, hold onto the tail and place the mouse in the cupped hand maintaining the hold on the tail.

3.4. Transfer 1 male and thereafter 1 female into the breeding cage by holding them by the tail (Step 3.3). Ensure that the females are in the same estrous stage when setting up the fertility assay. Determine the estrous stage of the females by performing a vaginal smear as described in Step 1.7.

3.5. Close the cage, and ensure that the mice have food, water and nesting material. Attach the cage card holder with the cage card to the cage.

3.6. Place the breeding cage on the housing rack. Leave the breeding cage as undisturbed as possible for the full duration of the fertility study. Conduct the fertility study for 30 days. For each breeding pair, keep detailed records and note 1) the date the mating is set up, 2) the date litters are born and 3) the number of pups born, including dead and live pups. Perform litter checks daily throughout the study.

3.7. Prolong the fertility study for an additional 30-60 days if no or a weak impact on fertility is established in Step 3.6.

## REPRESENTATIVE RESULTS

The presented results are from two different transgenic mouse models where the transcription factor Ventral anterior homeobox 1 (*Vax1*) has been deleted in the whole body on one allele, here referred to as heterozygote mice (HET)<sup>13</sup>, or *Vax1* has been conditionally deleted within GnRH neurons<sup>22</sup>, here termed conditional KO (cKO). Prior to setting up the fertility study, it is important to confirm pubertal onset in all the mice.

First, the day and weight at vaginal opening and preputial separation were established. In the HET mice, vaginal opening and preputial separation occurred at the same age and weight as in the control mice (**Figure 3A** and **F**, respectively). In contrast, vaginal opening and preputial separation were significantly delayed in cKO females and males and associated with increased bodyweight (**Figure 3B** and **G**, respectively). Pubertal onset is to some degree associated with body weight, and this is particularly true in females<sup>23</sup>. To determine if the delay in vaginal opening and preputial separation was associated with a delay in weight gain, the body weight at the average age of vaginal opening and preputial separation in controls (4.5 weeks of age) was compared to the body weight in cKO in males and females at 4.5 weeks of age. At age 4.5 weeks, female controls and cKO had comparable body weight (**Figure 3C**), whereas cKO males were slightly heavier than controls (**Figure 3H**). This indicates that the delay in vaginal opening and preputial separation in cKO is not associated with a delay in weight gain.

Vaginal opening is an early marker of sexual maturation and first estrus an indicator that the first ovulation has occurred, a marker of completion of puberty<sup>17</sup>. To determine if HET and cKO females reached first estrus at the same age and weight as controls, vaginal smears were performed daily from the day of vaginal opening until first estrus (**Figure 3E** and **3D**). In the HET mice, first estrus and the weight at first estrus was comparable between controls and HETs (**Figure 3D**). In contrast, cKO did not progress through the estrous cycle and did not have first estrus at the age of 80 days (**Figure 3E**). This delay was not due to reduced body weight (**Figure**

3E).

The HET mice with confirmed pubertal onset were used to set up the fertility study. The fertility assay was set up in 12 h light and 12 h dark conditions (LD12:12) in transgenic mice on a C57BL/6J genetic background. The mice were aged 10-16 weeks at the start of the experiment. Four groups of mice were studied: control matings (WTxWT), control males with HET females (WTxHET), HET males with WT females (HETxWT), and HET males with HET females (HETxHET). First, the number of litters generated in 3 months was determined. Control matings (WTxWT) generated an average of 2.8 litters in 3 months, which was comparable to HET males paired with control females (HETxWT mating, **Figure 4A**). HET females, whether paired with a WT male (control), or a HET male, generated significantly fewer litters in the 3 month fertility study, revealing that HET females are subfertile<sup>13</sup>. To illustrate the importance of confirming pubertal onset prior to setting up a fertility assay, reproductive competence was evaluated in cKO mice, where the males had delayed preputial separation (**Figure 3G**) and the females did not have their first estrus by 80-days of age (**Figure 3E**). The fertility study was set up >1 week after males had preputial separation, and in females at >80 days of age. During the 70-day fertility study, control matings generated an average of 2.2 litters, whereas no litters were generated from either the cKO female paired with a WT male, or the cKO male paired with a control (WT) female (**Figure 4B**), showing that these mice are infertile<sup>5, 22</sup>.

To better characterize the fertility phenotype of the HET females, we recorded the number of days it took to generate the first litter. WTxWT matings generated their first litter in an average of 22 days (**Figure 4C**), which was comparable to HET males paired with WT females. HET females paired with WT males took an average of 58 days to produce their first litter, which was similar to HETxHET matings (**Figure 4C**). However, the delay in generating the first litter of the HET females was very heterogenous, and delayed more in HETxHET matings, suggesting a semi-penetrance of the phenotype, and a contribution of the HET male to the subfertility observed in HETxHET matings<sup>13</sup>.

Evaluating the litter size can give important information about ovulation/implantation and sperm production. The average size of the first 3 litters was determined. Interestingly, all mating combinations (WTxHET, HETxWT, HETxHET) produced significantly smaller litters as compared to controls (WTxWT, **Figure 4D**), showing that both male and female HET mice are subfertile.

## FIGURE & TABLE LEGENDS

**Figure 1: The hypothalamic-pituitary-gonadal axis controls sexual maturation and reproduction.** At the apex of the reproductive axis are the hypothalamic kisspeptin neurons (yellow circles) and gonadotropin-releasing hormone (GnRH) neurons (green circles). In the juvenile period (prepubertal), kisspeptin neurons release little kisspeptin onto GnRH neurons. After pubertal onset, kisspeptin release on GnRH neurons is augmented (post-pubertal). Increased GnRH release in the late juvenile period dramatically increases luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from the pituitary, leading to gonadal maturation and reproductive function in adulthood.



**Figure 2. Determination of pubertal onset in mice.** An external marker of pubertal onset in mice is (A) preputial separation in the males, and vaginal opening in the females. Scale bar = 1 cm. (B) Example images from vaginal lavage used to determine first estrus. Slides were counterstained for 30 s with 0.1% methylene blue, airdried and observed at 20X magnification. Black arrows indicate nucleated epithelial cells, yellow arrows with black outline indicate leukocytes and white arrows indicate cornified epithelial cells. First estrus occurred on day 10 after vaginal opening.

**Figure 3. Representative data which can be generated from a pubertal onset study. (A, B)** Vaginal opening and weight at vaginal opening in the transgenic mouse models. (C) Weight at 4.5 weeks in control and cKO females. (D, E) Age and weight at first estrus. (F, G) Age and weight at preputial separation. (H) Weight at 4.5 weeks in control and cKO males. Data represent means  $\pm$  SEM. N = 5-10 per group, statistical analysis Student's-t test.  $**p<0.01$ ;  $***p<0.001$ . These figures have been modified from previous publication<sup>13, 22</sup>.

**Figure 4. Representative analysis from a fertility study of transgenic mice.** Fertility assessment of (A) Vax1 HET mice and (B) cKO was performed in virgin 10- to 16-week-old mice and the number of litters recorded in the indicated time frames. Data represent means  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test.  $*p<0.05$ ;  $***p<0.001$ . (C) The number of days to first litter (n = 5-12), and (D) average litter size of the first three litters (n = 10-13) was determined. Data represent means  $\pm$  SEM. Statistical analysis was done by Student's-t test as compared with WT x WT.  $*p<0.05$ ;  $**p<0.01$ . These figures have been modified from previous publication<sup>13, 22</sup>.

**Figure 5. Setting up a fertility assay. (A)** Example of breeding scheme and suggested number of animals required for a fertility assay. The total number of animals required for a study with 2 conditions such as a control versus KO or control diet versus high fat diet where fertility is assessed in both males and females is: 2 mice per mating (1 male x 1 female) x 8 animals per group x 2 conditions x 2 genders (to evaluate both male and female fertility) = total of 64 animals per study. If the male and female fertility study is done in parallel, one set of control x control matings can usually serve to compare both the impact on male and female reproduction. (B) When setting up a fertility assay, the mice used need to have reached sexual maturity. Different strains of mice have different reproductive characteristics and reach sexual maturity at different ages<sup>24</sup>. For information on rats, see previous publication<sup>25</sup>.

## DISCUSSION

The overall wellbeing of the mice is critical for a successful fertility assay<sup>21</sup>. When performing a fertility assay, it is important to not physically check on the mice every day as this can cause stress. Further avoid frequent cage changes, as these are also stressful. Ideally cage changes will be done no more than 1-2 times per week. Light exposure during the dark phase negatively impacts breeding in nocturnal rodents. Do not turn on lights in the breeding room during the dark hours. If entry to the room is required during the dark hours, use dim red light illumination. Another stressor is the presence of excessive odors, vibrations or noise in the vivarium for all or parts of the study. To help alleviate stress and improve mouse wellbeing and breeding, give the mice nesting material or other types of enrichment. Nest building is an important behavior in

mice and will increase breeding success. The nests also allow the mice a place to hide and play. Healthy and well-nourished animals breed well, therefore it is important to provide *ad libitum* access to high quality food and water. If breeding pairs start to exhibit symptoms of disease, such as dermatitis (frequent in C57BL/6J), dental malocclusion or other, exclude these mice from the study. In addition, if the study is being done in immunodeficient mice, it is key that the mouse room is kept clean to preserve fertility.

To appropriately set up a fertility study, it is important to decide if the study will be performed in virgin mice or proven breeders as well as carefully select the mice used for control matings. Control matings should be run in parallel with the experimental breeders. This is imperative as unknown or unanticipated stressors and changes in the environment can cause changes in breeding rate. Control matings are preferentially composed of litter mates to the experimental mice (**Figure 5A**). This is particularly important when the fertility study is done in mice with mixed genetic background due to the great variations in breeding characteristics between mouse strains (**Figure 5B**).

Identifying a subfertility phenotype can be challenging due to the prolonged length of the study, and the need to analyze additional parameters to reveal its cause. The HET mouse in the representative data is a good example of a modest subfertility phenotype (**Figure 4**), and the extended analysis required to identify the male subfertility. As shown in the **Representative Results (Figure 3 and 4)**, normal onset of puberty (**Figure 3A, D, and F**) can be associated with both male and female subfertility (**Figure 4A, C, D**). In this case, the mice were only sub-fertile, and thus the length of the study was extended to 120 days. This is important, as a shorter fertility study (*e.g.*, 60 days) would not have been able to reveal the phenotype. The male subfertility was modest, and first revealed when HET males were paired with HET females, which also were subfertile (**Figure 4A**). Indeed, the subfertility of HET males was confirmed when evaluating the average size of litters sired (**Figure 4D**). Follow up studies confirmed the male subfertility and revealed a poor sperm quality in the HET males, with an ~80 % reduction in motile sperm<sup>13</sup>.

When performing a fertility and pubertal onset study in mice with a major delay in pubertal onset (>5 days), it is important to consider the different factors which regulate pubertal onset<sup>26</sup>. Although vaginal opening and first estrus most often happen concomitantly in rats, first estrus in mice usually occurs ~10 days after vaginal opening<sup>25, 27</sup>. Therefore, it is recommended to ensure that both vaginal opening and first estrus are established when working in both rats and mice<sup>18, 28</sup>. Pubertal onset is linked to metabolic status and body weight in females and to a minor degree in males<sup>1, 7, 9, 29</sup>, and as such, a treatment or genetic mutation slowing animal growth will in some cases result in a delayed pubertal onset. To determine if the delay/advancement in pubertal onset may be associated with rapid/slow growth, it is important to weigh the mice daily during the assessment of pubertal onset. As seen in **Figure 3B and C**, as well as **G and H**, the delay in pubertal onset was not associated with reduced body weight, but was a true delay in pubertal onset, as confirmed by gonadal histology and circulating hormone levels<sup>22</sup>. One of the major limitations of using preputial separation and vaginal opening as markers of pubertal onset is that both will often happen eventually over time due to mechanisms other than increased activity of the reproductive axis<sup>18, 30, 31</sup> (**Figure 1**). An example of this is the delayed vaginal opening in the

cKO females (**Figure 3B**). The cKO females never become fertile due to an absence of ovulation<sup>22</sup> (**Figure 3E**). Indeed, further studies evaluating hormonal levels and gonadal histology of the cKO mice demonstrated that these mice never completed puberty and were infertile<sup>22</sup> (**Figure 4B**). This shows the limit of using external markers to determine pubertal onset. Completion of puberty and reaching sexual maturity requires increased GnRH release and activation of the reproductive axis (**Figure 1**), and to fully confirm sexual maturity both gonadal morphology and circulating hormone levels need to be evaluated<sup>22,31</sup>. In males, measuring testosterone, and evaluating seminal vesicle size and testis morphology and size in addition to sperm quality will confirm puberty was achieved<sup>16,22,32</sup>. In females, measuring progesterone and estrogen levels in combination with uterine and ovarian weight and morphology will confirm the completion of puberty<sup>17,19,22,33</sup>.

Litter size can impact both gestation length and age at pubertal onset. Most mouse strains have a gestation period of 18-22 days. However, if the mother is expecting a large litter, the gestation period tends to be reduced<sup>34,35</sup>. On the other hand, if the mother is nursing a previous litter, this can result in an extension of the gestational period<sup>5</sup>. Pups in large litters also tend to have delayed pubertal onset. This is caused by their growth being slightly delayed<sup>1,9,34,35</sup>. If a fertility study is performed and one of the mouse groups (control or experimental) produces very large litters, it is recommended to homogenize litter sizes, also termed culling, allowing the studied litters to have comparable sizes.

Reproduction can only be assessed *in vivo* due to the multiple organs involved in reproduction (**Figure 1**) and the extensive feedback mechanisms regulating the reproductive axis<sup>3</sup>. Certain aspects of reproductive competence can be rapidly evaluated through a vaginal plugging assay, where the capacity of the male to mount the female and leave a physical plug in the vagina is observed<sup>13,36</sup>. Although this is a useful approach to assess male sexual behavior, this type of study does not allow the detection of minor problems in reproduction, nor does it assess maintained fertility for a long period of time or resulting litter size and parental behavior. The protocol described here presents numerous advantages over a plugging assay, the major being the capacity to identify both minor and major impacts on reproductive competence in males and females (**Figure 4**).

Fertility decreases with age, and in addition the female estrous cycle becomes longer and irregular during aging<sup>5,28</sup>. It is therefore recommended to start a fertility study in young and sexually mature animals. A typically used age range to set up a fertility study is 10-16 weeks of age. However, it is important to be familiar with the specific reproductive characteristics of the mouse strain used in the study, as major differences in reproduction and sexual maturity exist between different mouse or rat strains (**Figure 5B**). Additional information about reproductive characteristics can be found in the JAX Mouse Phenome Database <https://phenome.jax.org/>. Performing a longer (>4 month) fertility study has the advantage of being able to detect early reproductive decline, where the mice might breed normally for the first 1-2 months of the fertility assay, but then have an early decline in reproduction starting before 6 months of age.

#### ACKNOWLEDGMENTS:

I thank the authors contributing to the initial work which is the basis of this publication. Thanks to Aitor Aguirre, Genevieve E. Ryan and Erica L. Schoeller for help preparing the manuscript. Thanks to Jessica Sora Lee and Austin Chin for technical assistance with the manuscript. H.M.H. was supported by Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health under Award Number R00HD084759.

#### DISCLOSURES:

The author has nothing to disclose.

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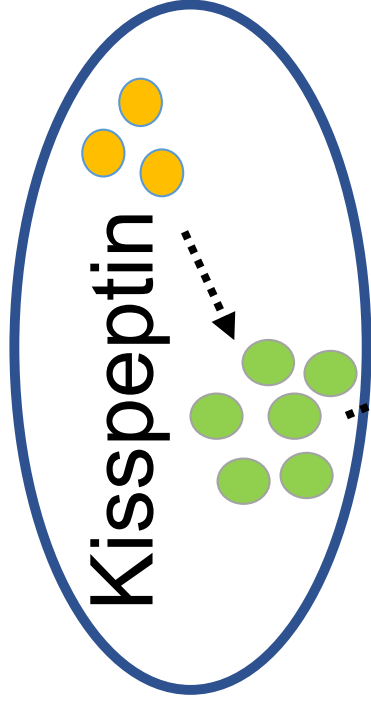
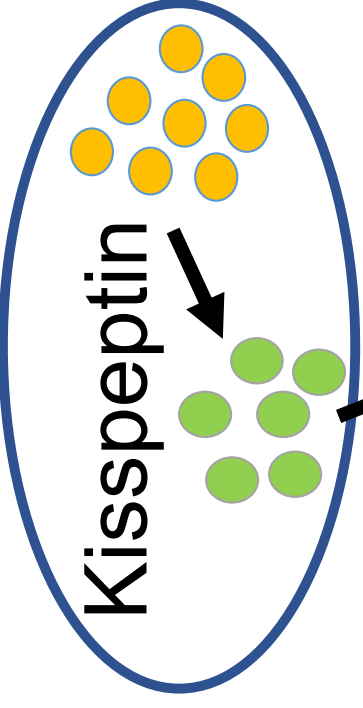
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# Post-pubertal

# Prepubertal

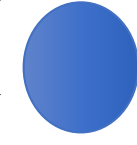
Hypothalamus



GnRH

GnRH

Pituitary



LH

LH

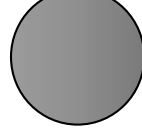
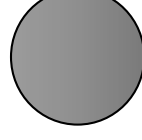
FSH

FSH

Gonads

Immature gonads

Mature gonads



● GnRH neuron

● Kisspeptin neuron

← Little neuropeptide/hormone release

← High neuropeptide/hormone release



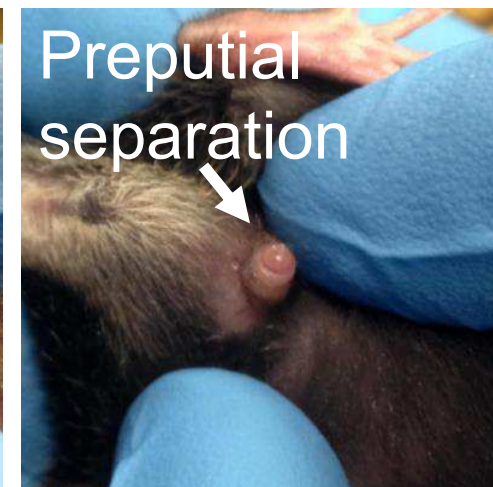
# A

## Male Preputial separation

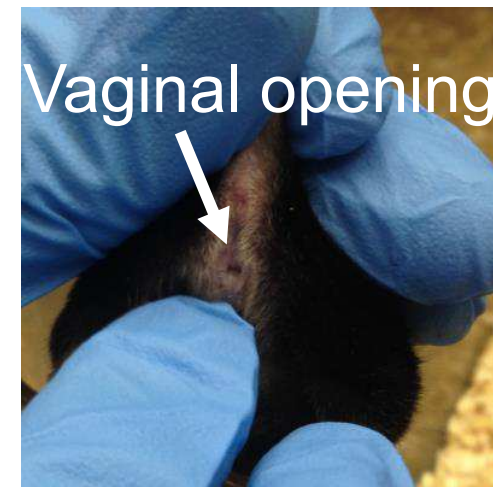
### Prepubertal



### Pubertal onset

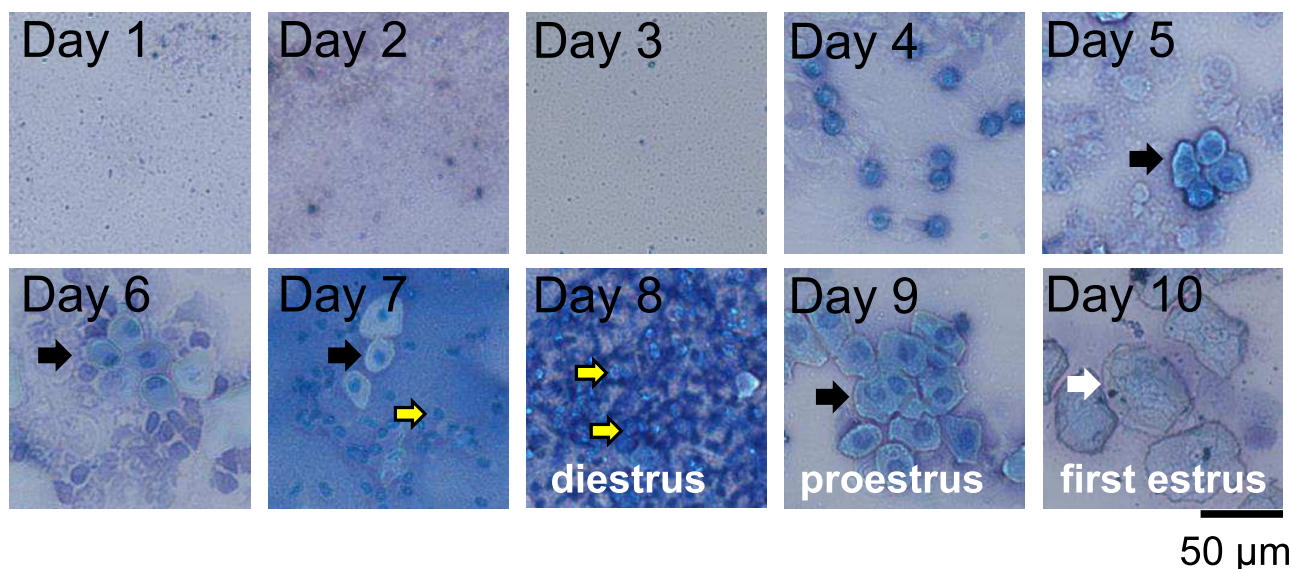


## Female Vaginal opening

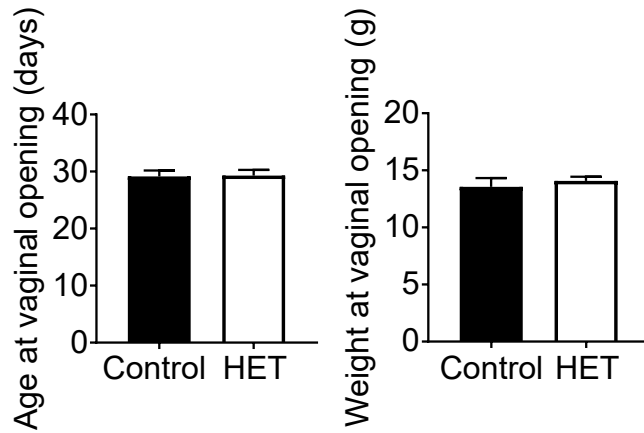
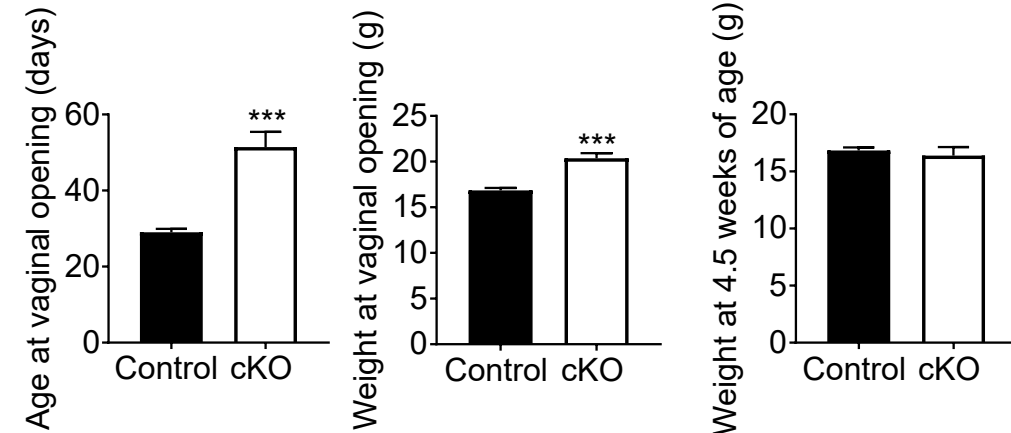
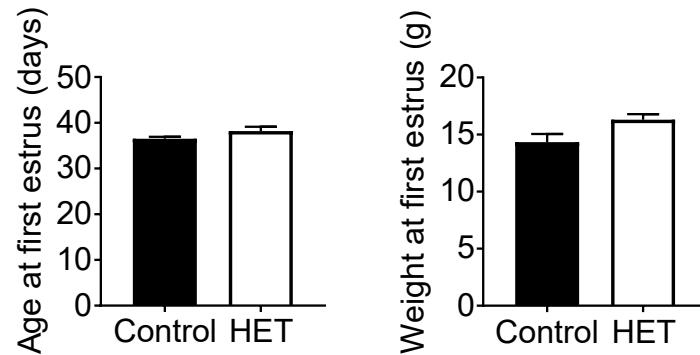
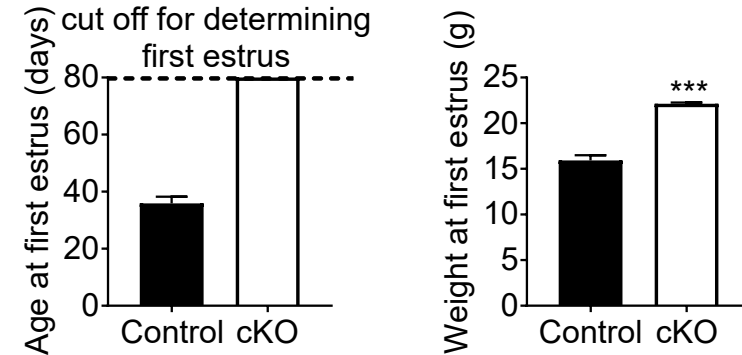
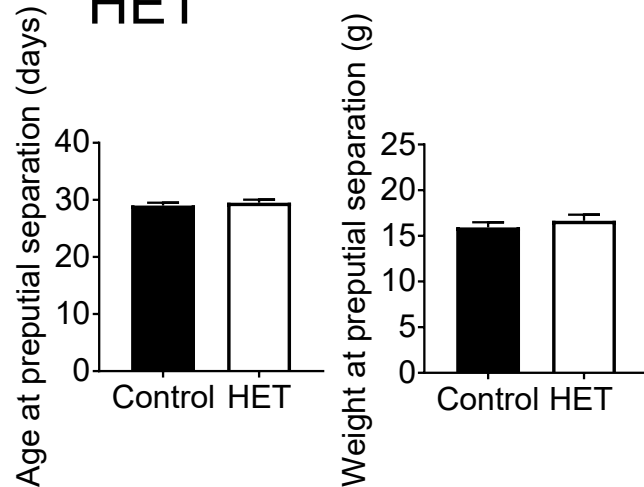
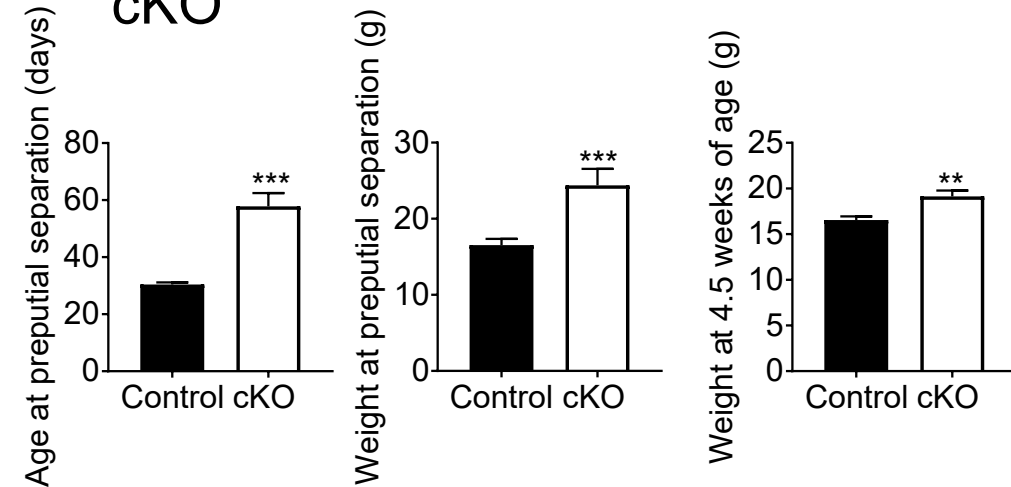


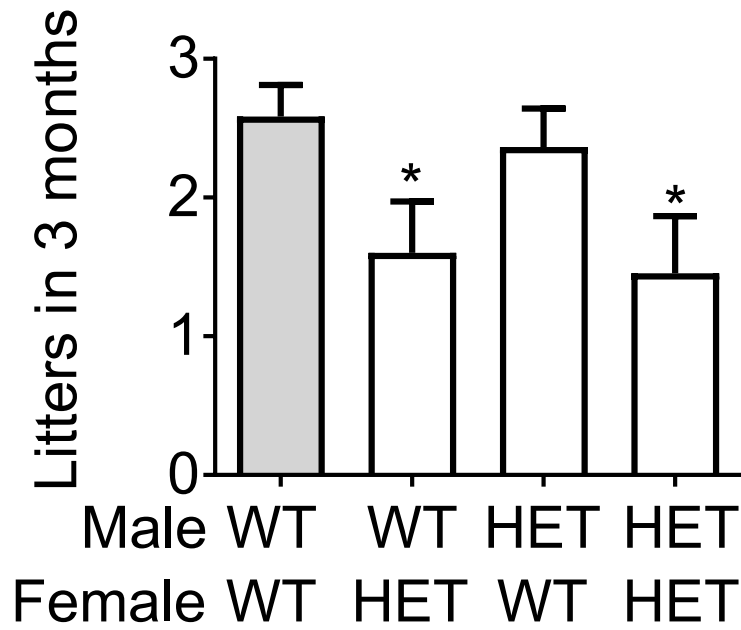
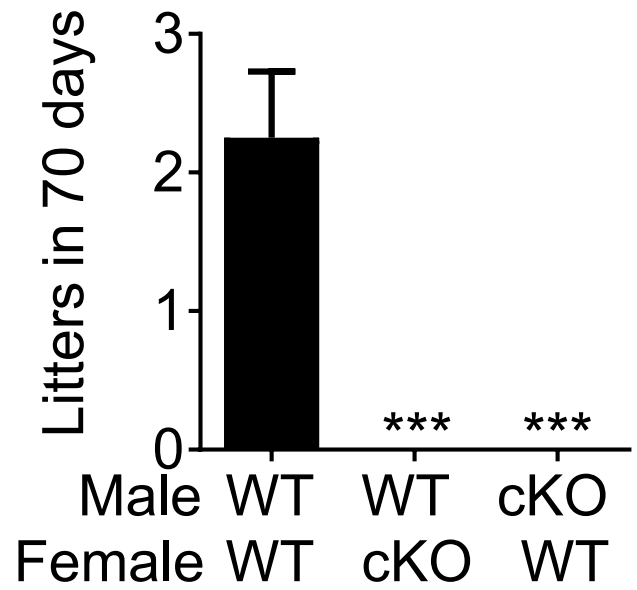
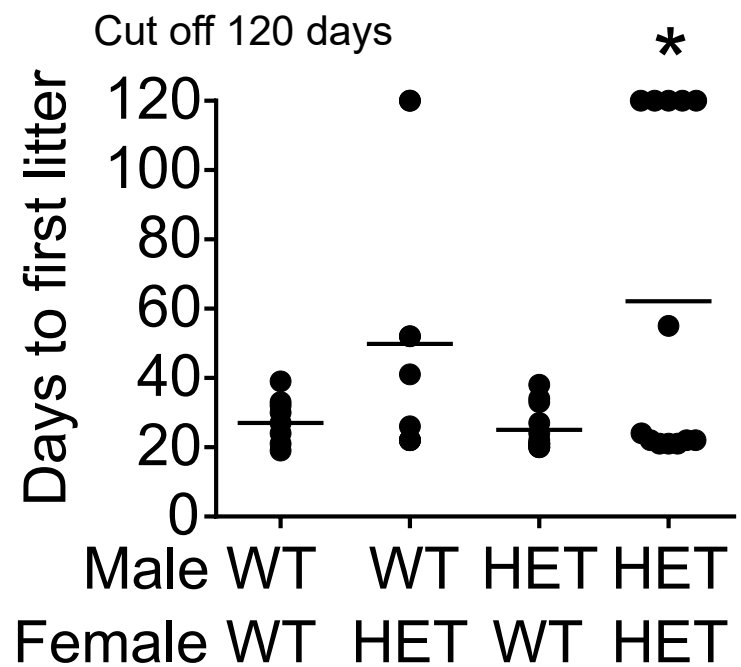
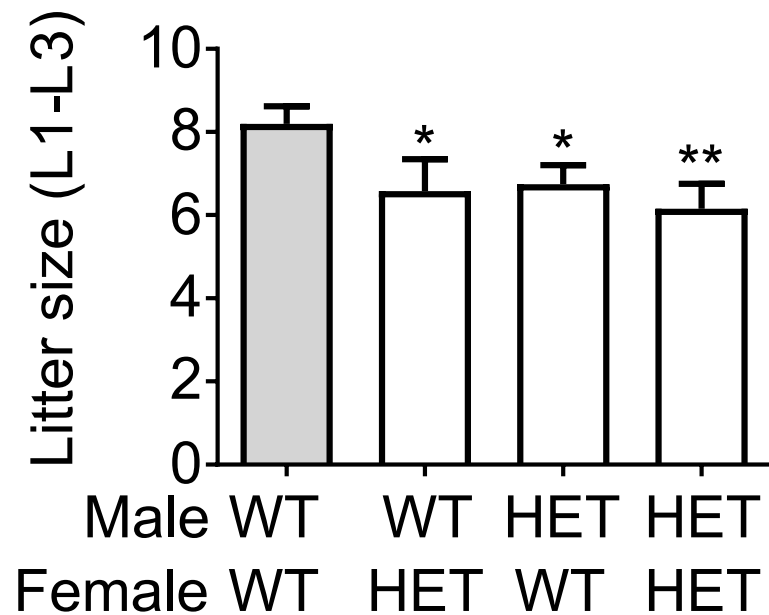
# B

## Staging estrous slides from the day of vaginal opening to determine first estrus





**A** Vaginal opening HET**B** Vaginal opening cKO**D** First estrus HET**E** First estrus cKO**F** Preputial separation HET**G** Preputial separation cKO

**A****B****C****D**

A

Control matings

Examples

Number of animals

Experimental matings

Examples

Number of animals

Male control matings

Control male x Control female

Control diet male x control diet female

8 males + 8 females = 16 mice

Male experimental matings

Knock-out male x control female

High fat diet male x control diet female

8 males + 8 females = 16 mice

Female control matings

Control male x Control female

Control diet male x control diet female

8 males + 8 females = 16 mice

Female experimental matings

Control male x knock-out female

Control diet male x high fat diet female

8 males + 8 females = 16 mice

B

| Strain    | Matings generating litters | Reproductive age | Average litter size |
|-----------|----------------------------|------------------|---------------------|
| B57/BL/6J | 84 %                       | 6.8 weeks        | 7.0                 |
| BALB/cJ   | 47 %                       | 8.0 weeks        | 5.2                 |
| 129/SvJ   | 75 %                       | 7.9 weeks        | 5.9                 |
| CBA/CaJ   | 96 %                       | 6.4 weeks        | 6.9                 |

| <b>Name of Material/ Equipment</b> | <b>Company</b>     |
|------------------------------------|--------------------|
| Sterile Cotton Balls               | Fisher             |
| Surface protector                  | Fisher             |
| Light meter                        | VWR                |
| Methylene blue                     | Sigma-Aldrich      |
| Microscope Slides                  | Genesee Scientific |
| Optimouse rack with cages          | AnimalCare systems |
| Water Bottle Basket                | AnimalCare systems |
| Filtered Cage Tops                 | AnimalCare systems |
| Optimice Standard Feeder           | AnimalCare systems |
| Cage Card Holder                   | AnimalCare systems |
| Cage Cards                         | AnimalCare systems |
| Bottle Assambley                   | AnimalCare systems |
| Bed R'Nest Nesting                 | The Andersons      |
| 1/8" Corn Cob bedding              | The Andersons      |
| Standard mouse chow                | Teklad             |
| Scale                              | VWR                |
| Polypropylene Beaker               | Fisher             |

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Author(s): Hanne M Hoffmann

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Dear Dr. Wu,

Thank you for the additional comments on my manuscript JoVE58352R1. I have addressed all the comments and updated the materials sheet.

Best regards

Hanne Hoffmann,

### **Editorial comments:**

**Q1.** Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**R1.** We have thoroughly revised the manuscript and corrected spelling mistakes etc.

**Q2.** For in-text referencing, please put the reference number before a comma or period.

**R2.** Punctuation has been placed after references.

**Q3.** Please use h, min, s for time units.

**R3.** International units have been used throughout .

**Q4.** Step 1.7.5: Please write this step in imperative tense.

**R4.** The step has been reworded as follows.

Observe slides from step 1.7.4 on a bright field microscope at x10 or x20. Establish the stage of the estrous cycle (Figure 2B)<sup>20</sup> by observing cell morphology which allows to distinguish the four distinct stages of the estrous cycle (Figure 2B); metestrus is characterized by a mixture of cornified squamous epithelial cells and leukocytes, diestrus by leukocytes, proestrus by a mixture of leukocytes and nucleated epithelial cells, and estrus by cornified epithelial cells (Figure 2B)<sup>20</sup>.

**Q5.** 3.7.1-3.7.3: Please write these steps in imperative tense.

**R5.** This section has been revised as follows:

3.6 Place the breeding cage on the housing rack. Leave the breeding cage as undisturbed as possible for the full duration of the fertility study. Conduct the fertility study for 30 days. For each breeding pair keep detailed records and note 1) the date the mating is set up, 2) the date litters are born and 3) the number of pups born, include dead and live pups. Litter checks are done daily throughout the study.

3.7 Prolong the fertility study for an additional 30-60 days if no or a weak impact on fertility is established in step 3.6.

**Q6.** Line 239, 267: Please remove all headers in Representative Results.

**R6.** All headers have been removed.

**Q7.** Line 345, 366, 382, 412, 422: Please remove all headers from Discussion.

**R7.** All headers have been removed.

**Q8.** Line 348-364: Please do not use a list.

**R8.** The list has been removed and the section has been written as a paragraph.

The overall wellbeing of the mice is critical for a successful fertility assay<sup>21</sup>. When performing a fertility assay it is important to not physically check on the mice every day as this can cause stress. Further avoid frequent cage changes, as these are also stressful. Ideally cage changes will be done no more than 1-2 times per week. Light exposure during the dark phase negatively impacts breeding in nocturnal rodents. Do not turn on lights in the breeding room during the dark hours. If entry to the room is required during the dark hours use dim red light illumination. Another stressor is the presence of excessive odors, vibrations or noise in the vivarium for all or parts of the study. To help alleviate stress and improve mouse wellbeing and breeding, give the mice nesting material or other types of enrichment. Nest building is an important behavior in mice and will increase breeding success. The nests also allow the mice a place to hide and play.

Healthy and well-nourished animals breed well, it is therefore important to provide *ad libitum* access to high quality food and water. If breeding pairs start to exhibit symptoms of disease, such as dermatitis (frequent in C57BL/6J), dental malocclusion or other, exclude these mice from the study. In addition, if the study is being done in immunodeficient mice it is key the mouse room is kept clean to preserve fertility.

**Q9.** Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

**R9.** Table of Equipment and Materials has been revised.

**Q10.** Please revise the table of materials to include all essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

**R10.** Table of Equipment and Materials has been revised and equipment has been included.