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(Grant Number: 5 R25 GM133017-03),
awarded to Cohen Veterans Bioscience
(Principal Investigator: Chantelle Ferland-Beckham, PhD)

Module 2, Video 9: Logistical considerations for behavioral experiments incorporating males and females and sex-specific behavioral readouts for commonly employed behavioral tests

Lab animal behavior has been studied for many years since Pavlov's work on conditional reflexes in dogs in the 19th century. However, much of this work has been exclusively conducted in males [1]. In preclinical research, behavioral tests are largely used to improve understanding of the central nervous system and test treatments for common diseases. Unfortunately, many commonly used behavioral tests [2-4] were also developed using only male rodents and thus need to be interpreted more cautiously when females are used.

In this video, we will discuss how to design behavioral experiments in both sexes. We will also show two examples highlighting how males and females can use different behavioral strategies in the same behavioral test [5].

Logistically, there are six important factors to consider when designing a behavioral experiment:

1. First, as discussed in Video 8, it isn't always necessary to measure gonadal hormones. But if the experiment necessitates it, hormone levels should be determined after behavioral testing to avoid stressing the animal. One exception is when the behavior only occurs at a specific point in the animal's cycle, such as lordosis [6-8].
2. Second, the size of the equipment may also need to be adjusted because males are larger than females. For example, typical adult male rats weigh between 300-500 grams, whereas adult female rats weigh between 250-300 grams. Similar dimorphic size differences are found in other species and should be considered across multiple logistical aspects of experimental design.
3. Third, food restriction is often necessary in behavioral experiments as hunger is a common motivator. But due to their different sizes, males and females may require different levels of food restriction. Food deprivation should be calculated based on a 10–20% reduction in body weight for each animal.
4. Fourth, female rats are also more active than males. Tests that depend on the activity may detect fewer behavioral changes in females than in males [9, 10].
5. Fifth, when using both males and females, cleaning behavioral equipment between animals becomes particularly important as the smells left behind on the apparatus from conspecifics may change the behavioral outcome.
6. And sixth, the smells of conspecifics are important inside the testing room. For example, female mice show more rearings in proximity to male urine [11]. Similarly, male mice will explore and vocalize more in the presence of female urinary scents [12, 13]. Two

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exceptions are operant testing and food motivation tasks where reward/motivation factors overcome these effects. Auditory cues show similar effects.

When using both males and females, behavioral test should be run in separate batches, or blocks of both sexes alternated throughout the testing day. This helps control for non-gonadal circulating hormones that fluctuate throughout the day. Males and females can also be tested on different days but this could introduce potential environmental changes that cannot be controlled for statistically. We recommend running a subgroup of each sex on each day of behavioral testing, as described in the study design video.

As mentioned previously, most behavioral tests were designed using only males. But a number of behavioral tests show sex-specific differences including fear conditioning [5], spatial learning [14-16], running behavior, and pain sensitivity [17]. Interpreting animal behavior in the lab requires considering both **what** the situation you have placed the animals in means to *them* and **how** their response reflects differences in the needs of each sex.

One well-documented example involves how male and female rodents respond to fear. In the typical rodent fear-conditioning task, animals are presented with a tone paired with a slight electric shock to the foot. This pairing normally induces a fear response, usually measured by freezing behavior.

However, Rebecca Shansky's lab proved that females also show fear using another behavior, called darting, which is a brief, high-velocity movement. Her lab found that approximately 40% of females exhibited darting. Most males, however displayed the typical freezing behavior, with only 10% of males exhibiting darting. Further, both behaviors could be extinguished over time, and females that darted exhibited better extinction memory than non-darters [18]. Darting was not associated with the estrus cycle while freezing-based extinction is better during proestrus, when estrogen levels are higher [19]. Mechanistically, males show morphology changes in prefrontal amygdala circuitry but females do not even though both sexes split into high and low freezing groups. This example highlights how two separate behaviors can both indicate fear learning and memory, but through different molecular mechanisms. This is further covered in Module 1.

Spatial learning strategies are another example of differences in the underlying mechanism but the same outward behavior. Rats can be trained on a T-maze to find a reward located in a specific arm. Once they have learned the place of the reward during training, the maze is rotated 180 degrees and their ability to find the reward during a probe trial is assessed. Males predominantly use a place strategy to find the reward. When females were assessed on the

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same task they tended to perform worse, suggesting poorer learning and memory. Donna Korol's work showed that females predominantly find the reward using another strategy, called response strategy [14, 15, 20]. Comparing females to the male standard—place strategy—caused researchers to incorrectly conclude that females did not learn well in spatial tasks. The discovery of the response strategy in females showed that males and females both learn, but through different strategies. Similar observations have been observed in the Morris Water maze [21].

Considering the potential influence of sex on both how we test animals and how we interpret behavioral results is necessary. Comparing females to a male standard when observing animal behavior may cause researchers to unintendedlly interpret the results of behavioral tests as a sex difference when females may just be employing a different strategy.

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