EIGHT CONSIDERATIONS FOR DESIGNING STUDIES WITH AGED MICE

Abstract

JAX has male and female B6J mice from 25-78 weeks of age readily available. Aged C57BL/6J (stock # 000664) mice is a popular model for a range of therapeutic areas including, but not limited to, metabolic disorders, neurobiology, and oncology. There are many genetic and physiological similarities between aging humans and aging mice, and aged B6J mice are a great model for the biological changes that can occur during aging. Maybe you’ve been thinking about planning a study using aged mice — where do you start? What considerations might be important when working with aged mice? How might you have to adjust the experimental design to account for the difference between an aged mouse and a young mouse? Here we will discuss the top 8 considerations to know before you begin.
**#1 How many mice do you need?**

How many mice do you need to be able to see a difference between your groups? We know there is attrition of mice during aging, therefore when performing a longitudinal study you will need to consider not just how many mice are needed to start, but how many are needed at the end to ensure you have statistically relevant data. In one study, the median lifespan for C57BL/6J (B6J) mice was measured at 866 days for females and 901 days for males (Figure 1 adapted from Yuan et al. 2012, [https://phenome.jax.org/measures/23201](https://phenome.jax.org/measures/23201)).

In addition to sex differences, other studies had shown different lifespans based on factors that include whether the mouse produced litters and when mice were housed at different cage densities ([Biology of the Laboratory Mouse](https://phenome.jax.org/measures/23201), Chapter 26). Other potential causes for differences in lifespan include the environment, diet, and health status of the mice (Xie et al. 2017). What you see in your facility may not reflect what is observed in other facilities.

To account for non-aging related attrition, determine the age of mice at the end of the study and consider the average number of wildtype mice observed in other studies to survive out to that age. Calculate the percentage to determine how many extra mice you need to start with at the beginning of your studies.

**For example:** You intend to run an experiment with female B6J mice until they reach 100 weeks of age. According to the literature, B6J mice show an attrition rate of ~15% (85% of mice survive to 100 weeks). You also determine that you need measurements on 20 mice at the end of the study to have enough power to be able to determine a statistical difference between 2 groups. In this case, you will need to start with 24 mice.

\[
\text{# of mice at start \times 0.85 = 20} \rightarrow \frac{20}{0.85} = 23.5 \\
\text{ALWAYS ROUND UP!}
\]

Please note that the attrition rate in control mice may differ from your experimental groups. It is always better to overestimate the “n” needed — you don’t want to get to the end of the study having estimated 15% attrition, only to discover that your treatment group had 20% attrition. Now you don’t have enough mice to have the statistical power you need!

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**#2 When to start**

Another factor to consider is when to start your experiment. Mice are considered to be the equivalent of human ‘middle aged’ starting around 10 months of age, and elderly around 18 months of age. If you want to study a specific biomarker of aging, consider when, during the aging process, the biomarker begins to change and plan your experiments to start before this happens to fully capture the range of the changes.

**For example,** if a biomarker begins changing at 10 months of age — consider starting your studies earlier, 8-9 months of age, to capture the point at which you see the biomarker changing and its effects. If, however, your biomarker is known to change around 18 months of age, then starting your experiments at 9 months of age might be too early! You would be collecting data ~ 9 months before you might begin to identify your phenotype. That is a long time of unnecessary effort, adding a lot of animal cage costs to your budget!
How do you find when the onset of your phenotype begins? One great place to look for information on inbred strains is the Mouse Phenome Database. This database contains phenomic data sets that are contributed by investigators — allowing comparison across strains, facilities, and labs. This data can be instrumental in the context of biomarkers that might be affected by environmental changes so you can get range of “normal.” Additionally, you can look across different inbred strains to see if certain strains are more sensitive or resistant to your phenotype or biomarker.

#3 Know the health of your mice

Be aware of the expected physiological changes that occur in mice during normal aging. Mice tend to lose body fat as they age, become more fragile, and have immune system changes.

There can be changes in hematology.

For example, platelet count decreases in 78 week old B6J mice compared to 8-week old B6J mice: 800x 10³ cells/µL in 78-week old B6J females vs. 1019 x 10³ cells/µL in 8 week old female B6J mice.

There can be changes in biochemistry too.

Cholesterol increases during aging although this can be sex-dependent (females 8 weeks vs 78 week: 79 fl 9 mg/dL vs 89 fl 22 mg/dL; males 8 weeks vs 78 weeks: 100 fl 12 mg/dL vs 136 fl 29 mg/dL).

Body composition can change with aging, with changes in weight and percent body fat. One important consideration is that although in general, weight increases with age, it also becomes more variable. At 8 weeks of age, the range of normal weights can be minimal — but by 78 weeks the range in weights can be quite large (Figure 2). Basic physiological data is available for both young (8 & 16 weeks) and aged (24-78 weeks) C57BL/6J mice on the Jackson Laboratory website.

**Figure 2**

Weight increase with age but also shows an increased variability. Groups of 30 male and 30 female C57BL/6J mice were weighed monthly. Mice were fed a diet containing 6% fat (LabDiet® 5K52 formulation).
Recent data from The Jackson Laboratory have shown that aged mice have changes in their immune system composition, with significantly more Treg cells than young mice and males show an increase in B cells (Figure 3). By 18 months both male and female mice also have a significant decrease in naïve CD4 and CD8 T-cells and a significant increase in effector and effector/memory CD4 and CD8 T-cells compared to mice that are 2 months of age (Figure 4). These changes may affect how well the aged mouse immune system responds to new pathogens or microorganisms.

Some more things to consider:
• Where will the mice be housed?
• How will you monitor their welfare?
• Do aged mice need additional monitoring compared to young mice?

One of the best things you can do before you start your study is to talk to your facility staff and clinical veterinarians. Discuss the data you intend to collect, what you consider to be the necessary endpoints for your studies and determine the best method to monitor the health and welfare of the mice. Additionally, you will need to have approval of your IACUC before you can begin your experiments, so having those conversations early-on will help ensure that everyone is on the same page and has the same goals.

Figure 3
B6 J mice show an age-dependent increase in Tregs and other differences as seen in spleen flow cytometry. Percentage of viable spleen cells from C57BL/6 J mice at 2 months and 18 months of age. Horizontal bars indicate the mean. Aged females had fewer T cells (p = 0.0383, mean difference was 5.1%) and more Tregs (p < 0.0001; mean difference was 10.3%) compared to 2-month old females. Aged males had more B cells (p < 0.0001; mean difference was 13.9%) and more Tregs (p = 0.0129; mean difference was 5.4%) compared to 2-month old males. The data was analyzed separately by sex, using 2 way ANOVA with Sidak’s multiple comparisons correction.

Figure 4
Spleen flow cytometry shows an age-dependent decrease in naïve T cells and an increase in effector/effector memory T cells in aged C57BL/6 J mice. Percentage of viable spleen cells expressing indicated markers: Naïve (CD44 Low, CD62 High), Effector/Effector Memory (CD44 High, CD62 Low), Central Memory (CD44 High, CD62 High), Activated Effector (CD44 Low, CD62 Low). Horizontal bars indicate the mean. The decline in naïve CD4 & CD8 T cells was significant in aged males & females (p < 0.0001 for all 4 comparisons). The increase in effector/effector memory CD4 & CD8 T cells was significant in aged males and females (p < 0.0001 for all 4 comparisons). The increase in central memory CD8 T cells was significant in aged males and females (p < 0.0001 for both comparisons). CD8 Activated effector CD8 T cell populations increased in aged females (p < 0.0001). The data was analyzed separately by sex, using 2 way ANOVA with Sidak’s multiple comparisons correction.
#4 How will you identify individual mice?

Any time you embark on a longitudinal study, carefully consider how to identify the individual mice for the duration of the study. Some methods of identification may be more difficult to use long-term, so it is essential to determine the best method before you begin. Ear punches or notches tend to be popular because they are cost effective, but as mice age, they can fight (particularly males) causing ears to get torn or scratched, which can make identification of the individual mice difficult. A more sophisticated approach is microchipping, but this can be expensive and may become dislodged. In Table 1 we summarize some of the commonly used methods of subject identification, and consideration for their use in aged mice.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>WHAT IT IS</th>
<th>PROS</th>
<th>CONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear punch</td>
<td>A hole or notch punched in the ear</td>
<td>• Under 3 weeks of age does not require anesthesia</td>
<td>• May get torn from fighting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cost-effective</td>
<td>• Could be difficult to read in older mice</td>
</tr>
<tr>
<td>Ear tag</td>
<td>Metal tag with a number</td>
<td>• Each mouse has a clear number</td>
<td>• Can fall out, or be torn out from</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fighting or grooming</td>
</tr>
<tr>
<td>Microchip</td>
<td>Subcutaneous microchip</td>
<td>• Each microchip has a unique identifier that can be read</td>
<td>• May require sedation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• Not recommended for newborns.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Chips can be dislodged</td>
</tr>
<tr>
<td>Tattoo</td>
<td>Tattoo on tail, toe, or ear</td>
<td>• Does not require anesthesia in neonates</td>
<td>• If performed on adult mice, may</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>require sedation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• May fade over time</td>
</tr>
</tbody>
</table>

Table 1 Commonly used methods of subject identification.

#5 Differences between males and females

Just as in young mice, aged mice have sexually dimorphic traits. Data from the International Mouse Phenotyping Consortium (IMPC) shows that across more than 2,000 knock-out mouse strains, over 14,000 wildtype mice and more than 40,000 mutant mice, a large proportion of phenotypes have been found to be influenced by sex, although this dimorphism is more common in continuous rather than categorical traits (56.6% vs. 9.9%, Karp et al. 2017). Importantly, they found that the sexually dimorphic traits were reproducible across multiple centers, with less than 10% of the dimorphic traits showing opposing effects.

It is essential to understand the differences you might see between sexes for the traits you are interested in. Aged male C57BL/6J mice have a progressive glucose intolerance that is more pronounced than in aged B6J females (Figure 5).
To study metabolic changes in aged mice, it might be tempting to choose to study only males because they have a more progressive difference with age. NIH guidelines suggest “Adequate consideration of both sexes in experiments and disaggregation of data by sex allows for sex-based comparisons and may inform clinical interventions. Appropriate analysis and transparent reporting of data by sex may, therefore, enhance the rigor and applicability of preclinical biomedical research.” (NOT-OD-15-102 “Consideration of Sex as a Biological Variable in NIH-funded Research”). Where possible, always analyze both sexes and be sure to use appropriate statistical testing to account for sex as a biological variable.

#6 Which assays are most appropriate for your study?

Careful consideration of the assays you plan to use for aged mice is necessary to ensure meaningful results, including which tests will yield the most accurate information for the phenotype you wish to study. Aged mice may not be able to perform in specific tests reliably.

**Example A:** Aged C57BL/6J mice can have decreased visual acuity which may preclude using certain tests that require the use of visual cues. Before using an assay that requires visual cues, first test the visual capabilities of the mice using an optokinetic function test (Rizzo et al. 2018). Consider alternative tests that can be performed in mice with and without visual acuity. The spontaneous alteration test is used to assess spatial working memory and is one that can be performed in mice with reduced visual acuity (Rizzo et al. 2018).

**Example B:** An assay of sensory and fine motor ability is the adhesion removal test, which consists of putting a small adhesive sticker on the top of the mouse’s head and then tracking the time it takes for the mouse to remove the sticker. However, alopecia, which is common in aging mouse populations on a B6 background, can confound the assay (Rizzo et al. 2018).

An important consideration for any studies involving surgical procedures on aged mice is the use of anesthesia. An impairment of the renal, cardiac or hepatic system, which can present in older mice, may make the mice more sensitive to anesthesia. Some anesthetics may work better in older animals (Gargiulo et al. 2012 ILAR). Consider alternatives for tests that do not require anesthesia (see Table 2).
The order in which tests are performed matter and should be decided ahead of time. Some tests are sensitive to the order in which they are performed; it is typically recommended to start with the least invasive and finish with the most invasive tests (McIlwain et al. 2001). Studies have shown that previous tests can affect the results of later tests, even when the mice are given time to recover in between testing (McIlwain et al. 2001).

### #7 Assay validation and statistical analysis

In any study, it’s essential to validate your assay. Make sure the technicians are trained and the equipment works before you test the efficacy of novel treatments. The goal is to validate using a control population before you invest in your treated group. In aged studies, this validation is equally important, but even if you have previously validated your assay in young mice, re-validate using a control aged group. This can be difficult with aged mice — it takes time to age mice to be used for validation, and all that has to be done before the experiment starts.

For some assays, such as tests that assess cognitive function, scopolamine can be used for validation. Scopolamine can mimic the cognitive deficits seen in aged mice. However, scopolamine may not be an appropriate method of validation for all assays (i.e., visual acuity or smell). Another option

<table>
<thead>
<tr>
<th>PHENOTYPE</th>
<th>ASSAY</th>
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<tbody>
<tr>
<td>Anxiety</td>
<td>Open Field (^1)</td>
</tr>
<tr>
<td>Neuromuscular</td>
<td>Grip Strength (^1)</td>
</tr>
<tr>
<td>Motor Coordination</td>
<td>Rotorod (^1)</td>
</tr>
<tr>
<td></td>
<td>Adhesion Removal — confounded by hair loss (alopecia or barbering — common in aged B6) (^1)</td>
</tr>
<tr>
<td>Visual Acuity</td>
<td>Optokinetic Function Test (^1)</td>
</tr>
<tr>
<td>Age-Related Changes in Smell</td>
<td>Olfactory Discrimination (^1)</td>
</tr>
<tr>
<td>Body Composition, Diet, Exercise</td>
<td>Frailty Assessment (^1)</td>
</tr>
<tr>
<td></td>
<td>Wheel Running (^1)</td>
</tr>
<tr>
<td>Cardiovascular Disease</td>
<td>Electrocardiogram — requires anesthesia (^2)</td>
</tr>
<tr>
<td></td>
<td>Echocardiogram — requires anesthesia (^2)</td>
</tr>
<tr>
<td>Immune Function</td>
<td>FACs sorting (^3)</td>
</tr>
<tr>
<td>Hearing</td>
<td>Auditory Brainstem Response — invasive and requires anesthesia (^1)</td>
</tr>
<tr>
<td></td>
<td>Acoustic Startle — non-invasive (^1)</td>
</tr>
<tr>
<td>Memory</td>
<td>Spontaneous Alternation — does not require visual acuity (^1)</td>
</tr>
<tr>
<td></td>
<td>Novel Spatial Recognition — requires visual cues (^1)</td>
</tr>
</tbody>
</table>

\(^1\) Rizzo et al. 2018; \(^2\) Doevendans et al. 1998; \(^3\) The Jackson Laboratory
is to purchase mice that are already aged (available up to 78 weeks) — this can be expeditious and allow you to validate your assay in a model that more closely mimics the population that you intend to use.

In addition to validating assays before running experiments, it is also essential to have a plan in place for data analysis. Having the proper controls for biological variables is important (i.e. sex). However, you may need to control for other variables as well, such as body weight — as mice age and their body weight become more variable. For tests such as grip strength, results should be normalized to body weight (Rizzo et al. 2018). It is always a good plan to consult a biostatistician before planning your experiments to determine the number of mice required to have the power necessary to determine a statistical difference. This is true of any study, but especially in a longitudinal study — you don’t want to get to the end of a yearlong study and find out that you don’t have enough data points!

#8 Consider your experimental endpoint

Last but not least, it is crucial to consider the health monitoring of aging colonies and experimental endpoints. Be sure to consult veterinary staff to discuss how to monitor the health of your aging mouse colony with attentiveness to the welfare of the mice. It will be essential to continue to monitor for these changes, not just to control for your experiments, but also for the welfare of the mice on study.

It is vital to have a clearly defined set of endpoints to properly plan for your studies. An endpoint is the point for the mouse when the study is over — whether because you have collected the data that is necessary for your research, or because of the accumulation of health issues from aging means the mouse needs to be euthanized. If you are studying the rate of tumor growth, then the endpoint might be when a tumor reaches a certain size. Similarly, when studying lifespans, the endpoint might be the natural, spontaneous death of the mouse. It is essential to always consider animal welfare in addition to the scientific goal of your studies — checking with your facility IACUC is the best way to ensure humane animal welfare endpoints are met.

However you decide on an endpoint, have a way of assessing the health of the mouse. Frailty and body conditioning assessments are a common method of general health assessment. Frailty is defined as “weakness, slowness, low activity, and poor endurance” (Toth 2018). There are multiple ways to assess frailty, but the most studied in mice is the clinical frailty index (CFI, Toth 2018, Rizzo 2018). Frailty in mice is similar in humans. For example, frailty scores increase with age in both mice and humans, though humans have greater variability in their frailty scores across populations (Toth 2018).

Another method of assessing the welfare of mice is to use body weight and temperature. Studies have shown that across different strains, a decline in body weight and temperature can be predictive of death, even in the absence of indications of morbidity (Toth 2018). By monitoring the weights and temperatures of mice in your study, you might be able to anticipate the point at which a mouse might be nearing its natural endpoint.

Aged B6J mice have the potential to model aging in humans along a vast spectrum of age-related diseases. Although much of the data described came from the aged C57BL/6J mice, many of these tips will apply to working with aged mouse strains in general. Careful planning and consideration of the unique requirements of aged mice will help ensure that you can collect data that is both significant and reproducible. Keeping in mind these types of studies have the potential to be long term, careful planning ahead of time will set your research up for success.

To learn more about Aged B6J from JAX visit www.jax.org/aged-b6.
References:


