This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

Module 2, Video 8: Tracking hormone fluctuations in female rodents and the influence of these fluctuations on various research-related outcomes

One argument for excluding females from preclinical research is that circulating ovarian hormones will make data from female animals more variable than data from males [1]. Additionally, there is a misconception that researchers must automatically quadruple the number of experimental animals to assess estrus-associated effects, which can be both costly and time consuming. But are these steps really needed to work with females? In this video, we will demonstrate that hormone variations occur across both sexes, contributing to behavioral and physiological variability in females AND males. We will also describe examples of WHEN experimental outcome may necessitate tracking hormone fluctuations in both males and females, and best practices for how to track hormone levels when appropriate.

Variations in hormone levels across the female infradian rythmn are well understood. But testosterone also shows variability across the day and across the lifespan [3, 4]. Factors related to group housing can effect within-cohort hormone variability in both sexes. For example, in mice, circulating testosterone levels can be (on average) 5-times higher in dominant versus subordinate males [5], leading to high variability among mice of the same age and strain housed under identical conditions [6]. Thus, data variability in males could also be related to variations in hormone levels. But does this variability skew heavier in females?

Two prominent meta-analyses of large numbers of studies in mice [7] and rats [8] showed that data variability across a number of common measures was comparable across males and females. In some instances, it was actually greater in males. Thus, in most cases, the degree of variability observed across both sexes should be accepted as natural inter-individual variability and estrous cycle assessment is not a necessity.

The shared variability across males and females doesn't mean that gonadal hormones should never be accounted for. In contrast, hormone variability should be considered equally across both sexes based on the potential to influence experimental outcomes. Later in Module 2, we will discuss how to design an experiment to consider hormone variability.

There are some documented case examples of where female hormone fluctuations do impact the results and may need to be considered when evaluating data variability. Many of these examples were discussed in Module 1 [9-37].

In males, systematic studies regarding the impact of male hormonal fluctuations on study outcomes are more limited because historically, hormone fluctuations in males were not considered a source of data variability. However, as awareness has increased, some papers have begun to stratify male animals according to their hormone status. Preliminary effects of testosterone level variations have been observed for anxiety [38, 39], depression, spatial abilities, and memory [40]. The most cited paper analyzed the effects of testosterone on anxiety in mice, showing that testosterone—either endogenous or exogenous—decreases anxiety in the elevated plus maze in a dose-dependent manner [39]. Thus, male behavioral variations may also be related to flucutations in hormone levels.

Tracking hormone levels in both males and females

Sometimes, tracking hormones is necessary and best practices should be followed.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

In females, **swab** and **lavage** are the two most commonly used methods. With swab, a cotton swab is moistened, gently inserted into the animal's vagina, and then turned and rolled against the vaginal wall before being removed. Lavage involves flushing cells from the vaginal lining by introducing a small amount of fluid into the vagina using a rounded tip disposable pipette that is gently placed at the opening of the vaginal canal. The fluid spontaneously aspirates into the vaginal canal without tip insertion. The pressure is controlled by pressing or releasing the pipette bulb.

With both techniques, extracted cells are then examined with a microscope to determine the cycle stage based on the types and morphology of the cells.

Each of these methods has PROS and CONS, and requires different levels of expertise and time. The decision of which method to use is highly dependent on the study design. Swabbing is preferred because it is the quickest and produces high quality smears [41-43].

It should also be notted that, vaginal samples need to be taken at the same time each day on consecutive days over a period of time to provide detailed information about the estrous cycle. If your experiment requires cycle tracking or must be performed during a certain stage of the estrous cycle, cycle assessment should start about a week ahead of the experiments to ensure accuracy and eliminate animals that fail to show a regular cycle.

It is also important to note that the estrous cycle is sensitive to environmental changes [44-48].

Though less commonly used, it is also possible to use a **visual identification method** [42]. However, this method eliminates the possibility of detecting transitional stages, or the intersection of two consecutive cycle stages. Further, this method is subseptible to variations in lighting.

In males, tracking hormones is much more difficult. There is no proper method for monitoring hormone levels in males that isn't aversive. Studies that have tracked male testosterone levels did so using blood samples, which has its own inherent challenges. Namely, only males sampled during the ultradian surge will have detectable testosterone levels [51, 52]. Other studies have inferred higher or lower levels of hormones by assessing hormone-influenced hierarchy patterns in mice via assays such as the **territory urine marking assay** [53]. But this method has not been reliably compared to blood-based testosterone levels.

As demonstrated in this video, hormone variability occurs in all rodents and should not detract from the inclusion of females. The decision to track hormone levels in both sexes should depend on whether your outcome measure is known to be influenced by hormone fluctuations or your results suggest possible sex differences. There are a number of methods in females for tracking the estrous cycle, each with their own advantages and disadvantages, while in males, the only reliable option is blood sampling.

References

1. Wald, C. and C. Wu, *Biomedical research. Of mice and women: the bias in animal models.* Science, 2010. **327**(5973): p. 1571-2. DOI

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

2. Donner, N.C. and C.A. Lowry, *Sex differences in anxiety and emotional behavior.* Pflugers Arch, 2013. **465**(5): p. 601-26. DOI
3. Coquelin, A. and C. Desjardins, *Luteinizing hormone and testosterone secretion in young and old male mice.* Am J Physiol, 1982. **243**(3): p. E257-63. DOI
4. Ellis, G.B. and C. Desjardins, *Male rats secrete luteinizing hormone and testosterone episodically.* Endocrinology, 1982. **110**(5): p. 1618-27. DOI
5. Machida, T., Y. Yonezawa, and T. Noumura, *Age-associated changes in plasma testosterone levels in male mice and their relation to social dominance or subordinance.* Horm Behav, 1981. **15**(3): p. 238-45. DOI
6. Bartke, A., et al., *Fluctuations in plasma testosterone levels in adult male rats and mice.* Endocrinology, 1973. **92**(4): p. 1223-8. DOI
7. Prendergast, B.J., K.G. Onishi, and I. Zucker, *Female mice liberated for inclusion in neuroscience and biomedical research.* Neurosci Biobehav Rev, 2014. **40**: p. 1-5. DOI
8. Becker, J.B., B.J. Prendergast, and J.W. Liang, *Female rats are not more variable than male rats: a meta-analysis of neuroscience studies.* Biology of sex differences, 2016. **7**: p. 34-34. DOI
9. Cordeau, P., Jr., et al., *Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury.* Stroke, 2008. **39**(3): p. 935-42. DOI
   Females in metestrus/diestrus had higher GFAP/astrocyte activation than males following ischemic injury. High levels of estrogen during cycling or with estrogen replacement attenuated GFAP upregulation after stroke. Males had a correlation between GFAP intensity and infarct volume while female mice did not.
10. Gonzalez-Burgos, I., M. Alejandre-Gomez, and M. Cervantes, *Spine-type densities of hippocampal CA1 neurons vary in proestrus and estrus rats.* Neurosci Lett, 2005. **379**(1): p. 52-4. DOI
    Mushroom-shaped spines on CA1 pyramidal neurons were predominate in proestrous female rats (15.1% more than in estrous). Thin spines were predominate in estrus rats (15.5% more than proestrus). Shape of spines is related to post-synaptic processing of information- thus could infelnce organization of hippocampal-activity dependent memory information.
11. Klintsova, A., W.B. Levy, and N.L. Desmond, *Astrocytic volume fluctuates in the hippocampal CA1 region across the estrous cycle.* Brain Res, 1995. **690**(2): p. 269-74. DOI
    The astrocytic volume in the CA1 of the hippocampus was significantly lower in proestrous than during estrus.
12. Qiu, L.R., et al., *Hippocampal volumes differ across the mouse estrous cycle, can change within 24 hours, and associate with cognitive strategies.* Neuroimage, 2013. **83**: p. 593-8. DOI
    MRI was used to show 2-3% hippocampal volume changes across the estrous cycle and can change over a 24h period. These volume changes were assocaited with a switch between hippocampal and striatal based navigation strategies in solving dual chocie T-maze in mice.
13. Woolley, C.S., et al., *Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons.* J Neurosci, 1990. **10**(12): p. 4035-9. DOI
    Apical dendritic spine density in CA1 hippocampal pyramidal neurons undergo cyclic fluctuations across the estrous cycle in adult female rats. 30% decrease in apical dendritic spine density over the 24h period between late proestrous and late estrous. This was not observed in CA3 pyramidal cells or dentate gyrus granule cells.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

14. Woolley, C.S. and B.S. McEwen, *Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat.* J Neurosci, 1992. **12**(7): p. 2549-54. DOI
Synaptic density in the stratum radiatum of the hippocampus CA1 region in adult female rats fluctuates as levels of ovarian steroids change across the estrous cycle. Low levels of estradiol correlated with lower synapse density and high estradiol correlated with higher synapse density. These changes can occur over a 24h period between proestrou and estrous (32% decrease).
15. Broestl, L., et al., *Ovarian Cycle Stages Modulate Alzheimer-Related Cognitive and Brain Network Alterations in Female Mice.* eNeuro, 2018. **5**(6). DOI
Ovarian cycle stages significantly modulate Alzheimer's disease (AD)-related alterations in neural network patterns, cognitive impairments, and pathogenic protein production in a knockout mouse model of AD. Female AD-knockout (hAPP) spent more time in estrogen-dominant cycle stages and these worsened AD-related network dysfunction and cognitive impairments. These effects were attenauted by progesterone-dominant stages and gonadectomy.
16. Marrocco, J. and B.S. McEwen, *Sex in the brain: hormones and sex differences.* Dialogues in clinical neuroscience, 2016. **18**(4): p. 373-383. DOI
Review highlighting that the entire brain is affected by gonadal steroids with subtle sex differences. It covers plasticity of the adult brain, sex hormone actions beyond the hypothalamus, developmental programming of sex differences, hippocampus response to stressors, estrogen and the aging hippocampus and prefrontal cortex.
17. Duclot, F. and M. Kabbaj, *The estrous cycle surpasses sex differences in regulating the transcriptome in the rat medial prefrontal cortex and reveals an underlying role of early growth response 1.* Genome Biol, 2015. **16**: p. 256. DOI
Transcriptome sex differences and their interaction with estrous cycle in adult medial prefrontal cortex of female rat. Supports the multi-level reorganization of synaptic fucntion across the estrous cycle, highlighting the role of early growth response 1 binding in controlled synapse-related genes varying within females.
18. Jaric, I., et al., *Chromatin organization in the female mouse brain fluctuates across the oestrous cycle.* Nat Commun, 2019. **10**(1): p. 2851. DOI
Neural chromatin organization in the the female ventral hippocampous of the mouse fluctuates with the estrous cycle. Finding chromatin organizational changes associated with the transciptional activity of genes important for neuronal function and behavior (anxiety behavior and related brain structures). Implicating immediate-early gene product and Egr1 as part of the mechanism mediated cycle-dependent chromatin transciptional changes.
19. Marrocco, J., et al., *A sexually dimorphic pre-stressed translational signature in CA3 pyramidal neurons of BDNF Val66Met mice.* Nat Commun, 2017. **8**(1): p. 808. DOI
In the CA3 hippocampal neurons females had greater gene expression activation after actue stress than males. In a stress-sensitive BDNF Val66Met mice, heterozygous females had impaired spatial memory, regardless of acute stress but this was not observed in ovariectomized females. No cognitive impairment were observed in any male mice.
20. Yagi, S., et al., *Sex and estrous cycle differences in immediate early gene activation in the hippocampus and the dorsal striatum after the cue competition task.* Horm Behav, 2017. **87**: p. 69-79. DOI

Proestrous rats were more likely to choose a place strategy than non-proestous or male rats in the cue-competition training in the Morris water maze. Female place strategy users exhibited greater zif268 expression while male place strategy uses had greater cFos expression compared to all other groups in the CA3 of the hippocampus. Cue strategy users had greater expression of cFos in the dorsal striatum than place strategy users.

21. Tada, H., et al., *Estrous Cycle-Dependent Phasic Changes in the Stoichiometry of Hippocampal Synaptic AMPA Receptors in Rats.* PloS one, 2015. **10**(6): p. e0131359-e0131359. DOI
In female rats, a hippocampus-dependent contextual fear-learning task drives GluA2-lacking Ca^{2+}-permeable AMPA receptors into the hippocampal CA3-CA1 synapses during all periods of the estrous cycle except proestrous (high estrogen). This did not occur in ovariectoized female rats, only when estrogen was present. Long-term potentiation following the task was prevented during the proestrous period, but did occur during the estrous cycle.
22. Spencer-Segal, J.L., et al., *Distribution of phosphorylated TrkB receptor in the mouse hippocampal formation depends on sex and estrous cycle stage.* J Neurosci, 2011. **31**(18): p. 6780-90. DOI
TrkB phosphorylated at tyrosine 816 (pTrkB) in the hippocampal formation examined under normal low and high circulating estradiol. During high circulating estradiol BDNF expression, TrkB signaling, and synaptic plasticity are enhanced. pTrkB-ir was in axons, axon terminals, dendrites, and dendritic spines of neurons in the hippocampus, majorly localized to presynatpic profiles. pTrkB-ir was also abundant in microglia. Axonal, glial pTrkB-ir, and pTrkB-ir in the CA1 stratum radiatum were more abundant in high-estradiol states (proestrous females) than low-estradiol states (estrous, diestrous females and males).
23. Warren, S.G., et al., *LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats.* Brain Res, 1995. **703**(1-2): p. 26-30. DOI
The degree of long-term potentiation was greater in female rats during the afternoon of proestrous (indicated to have highest number of synapses) relative to males and females in other stages.
24. Bi, R., et al., *Cyclic changes in estradiol regulate synaptic plasticity through the MAP kinase pathway.* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13391-5. DOI
Endogenous estrogen produces tonic phosphorylation of extracellular signal-regulated kinase 2 (ERK2)/MAP kinase throughout the female rat brain and an increase in tyrosine phosphorylation of NR2 subunits of N-methyl-D0aspartate (NMDA). Cycling changes in estrogen levels during the estrous cycle of female rats is associated with corresponding changes in the levels of activation of ERK2, state of tyrosine phosphorylation of NR2 subunits of NMDA receptors, and the magnitude of long-term potentitaion in the hippocampus.
25. Carrier, N., et al., *The Anxiolytic and Antidepressant-like Effects of Testosterone and Estrogen in Gonadectomized Male Rats.* Biol Psychiatry, 2015. **78**(4): p. 259-69. DOI
Treatment of gonadectomized males with testosterone or estradiol had antidepressant-like effects associated with a substrantial overlap in transciptional regulation (synaptic plasticity- and mitogen-activated protein kinase pathway-related genes). Chronic inhibition of aromatase within the dentate gyrus blocked the protective effets of testosterone.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

26. D'Souza, D. and M. Sadananda, *Estrous Cycle Phase-Dependent Changes in Anxiety- and Depression-Like Profiles in the Late Adolescent Wistar-Kyoto Rat.* Ann Neurosci, 2017. **24**(3): p. 136-145. DOI
Female adolescent wistar rats in proestrous-estrous demonstrated reduced anxiety levels, increased risk taking and decreased risk assessment. The Wistar Kyoto model of adolescent depression did not cycle dependent changes in anxiety levels, risk taking or risk assessment.
27. Mora, S., N. Dussaubat, and G. Diaz-Veliz, *Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats.* Psychoneuroendocrinology, 1996. **21**(7): p. 609-20. DOI
Behavioral indicies of anxiety vary across the estrous cycle and low light has an anxiolytic effect, which is more senstive during estrous and proestrous.
28. Ramos-Ortolaza, D.L., et al., *Ovarian hormones modify anxiety behavior and glucocorticoid receptors after chronic social isolation stress.* Behav Brain Res, 2017. **328**: p. 115-122. DOI
Isolation increased depressive-like behaviors relative to paired housing in female rats, regardless of estrous stage. In the estrous phase females had decreased anxiety behavior accompanied by a decrease in clucocorticoid receptor expression in the dentate gyrus and CA3 region of the hippocampus.
29. Kaur, S., et al., *Sex Differences and Estrous Cycle Effects of Peripheral Serotonin-Evoked Rodent Pain Behaviors.* Neuroscience, 2018. **384**: p. 87-100. DOI
Female Sprague-Dawley rats in proestrous and estrous exhibited greater and/or longer pain behaviors compared to males and ovariectomized females or those in diestrous. There were no sex differences or estrous cycle effects of seratonin-evoked edema or seratonin content in inflamed hindpaws. Local pretreatment with a seratonint 2A receptor atagonist blocked seratonin-evoked thermal hyperalgesia and edema.
30. Vinogradova, E.P., D.A. Zhukov, and A.S. Batuev, *The effects of stages of the estrous cycle on pain thresholds in female white rats.* Neurosci Behav Physiol, 2003. **33**(3): p. 269-72. DOI
On day 3, females in diestrous and estrous showed sharp increases in shudder and paw-shuffling thresholds to electical pain. These thresholds were unchanged in females in proestrous and metestrous.
31. Alonso-Caraballo, Y. and C.R. Ferrario, *Effects of the estrous cycle and ovarian hormones on cue-triggered motivation and intrinsic excitability of medium spiny neurons in the Nucleus Accumbens core of female rats.* Horm Behav, 2019. **116**: p. 104583. DOI
Found that intrinsic excitability of NAc MSNs and conditioned approach are enhanced in female obesity-prone vs. obesity-resistant rats. These effects were driven by greater MSN excitability and conditioned approach behavior during metestrus/diestrus vs. proestrus/estrus in obesity-prone but not obesity-resistant rats, despite similar regulation of food intake and food motivation by the cycle in these groups. Estradiol and progesterone treatment reduced conditioned approach behavior in obesity-prone and outbred Sprague-Dawley females.
32. Calipari, E.S., et al., *Dopaminergic dynamics underlying sex-specific cocaine reward.* Nat Commun, 2017. **8**: p. 13877. DOI
Estrous cycle-dependent mechanism for controlling increased cocaine reward in females. During estrous the ventral tegmental area dopamine neuron activity is enhanced and drives post translational modifications at the dopamine transporter to increse the ability of cocaine to inhibit its function, an effect mediated by estradiol.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

33. Lacy, R.T., et al., *The effects of sex, estrous cycle, and social contact on cocaine and heroin self-administration in rats.* Psychopharmacology (Berl), 2016. **233**(17): p. 3201-10. DOI
During estrous females increased their cocaine-mediated breakpoints by ~17% but remained consistent with males. Heroin self-administration decreased by 70% during proestrous in females regardless of housing condition.

34. Frick, K.M., et al., *Sex steroid hormones matter for learning and memory: estrogenic regulation of hippocampal function in male and female rodents.* Learn Mem, 2015. **22**(9): p. 472-93. DOI
Estrogen is a crucial regulator of hippocampal morphology , plasticity, and memory in both male and female rodents.

35. Kulkarni, K.H., et al., *Effects of estrogen and estrus cycle on pharmacokinetics, absorption, and disposition of genistein in female Sprague-Dawley rats.* J Agric Food Chem, 2012. **60**(32): p. 7949-56. DOI
Estrogen and estrous cycle effects on pharmacokinetics and absorption in female rats. Rats in the proestrous group had reduced oral bioavailability of total genistein than those in metestrous or ovariectomized (basal/low level of estrogen).

36. Robinson, D.L., L.J. Brunner, and R.A. Gonzales, *Effect of gender and estrous cycle on the pharmacokinetics of ethanol in the rat brain.* Alcohol Clin Exp Res, 2002. **26**(2): p. 165-72. DOI
Effects of estrous cycle on pharmacokinetics of ethanol in the rat brain. Elimination of ethanol was ~15% faster in females than males in both brain and blood. Females also exhibited faster absorption and distribution than males. These parameters did not vary across the estrous cycle.

37. Sheppard, P.A.S., E. Choleris, and L.A.M. Galea, *Structural plasticity of the hippocampus in response to estrogens in female rodents.* Molecular brain, 2019. **12**(1): p. 22-22. DOI

38. Fernández-Guasti, A. and L. Martínez-Mota, *Anxiolytic-like actions of testosterone in the burying behavior test: role of androgen and GABA-benzodiazepine receptors.* Psychoneuroendocrinology, 2005. **30**(8): p. 762-70. DOI

39. Aikey, J.L., et al., *Testosterone rapidly reduces anxiety in male house mice (Mus musculus).* Horm Behav, 2002. **42**(4): p. 448-60. DOI

40. Celec, P., D. Ostatníková, and J. Hodosy, *On the effects of testosterone on brain behavioral functions.* Frontiers in neuroscience, 2015. **9**: p. 12-12. DOI

41. Gonzalez, G., *Determining the Stage of the Estrous Cycle in Female Mice by Vaginal Smear.* Cold Spring Harb Protoc, 2016. **2016**(8). DOI
Swabs are the quickest and prodcue high quality smears.

42. Byers, S.L., et al., *Mouse estrous cycle identification tool and images.* PLoS One, 2012. **7**(4): p. e35538. DOI
Visual method for determining proestrous or estrous in C57BL/6J, CByB6F1/J, and BALB/cByJ. Also covers vaginal cytology cycle identification in all three species.

43. Caligioni, C.S., *Assessing reproductive status/stages in mice.* Curr Protoc Neurosci, 2009. DOI **Appendix 4**: p. Appendix 4I.
The aim of this unit is to provide guidelines for quickly and accurately determining estrous cycle phases in mice via visual, smears via lavage.

44. Peña, C.J., et al., *Early life stress alters transcriptomic patterning across reward circuitry in male and female mice.* Nat Commun, 2019. **10**(1): p. 5098. DOI

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

Early life stress in postnatal females is distinictly represented in the brain's transcriptome in the vetnral tegmental area, nucleus accumbens, and prefreontal cortex.

45. Kokras, N., et al., *Forced swim test: What about females?* Neuropharmacology, 2015. **99**: p. 408-21. DOI
Female Wistar rats exhibited higher levels of immobility than males and the sex difference was removed with antidepressant treatment. Lower levels of head swimming were observed in females than males. Treatment with high dose sertraline enhanced climbing practicularly during proestrous and diestrous females.
46. Kokras, N., et al., *Behavioral sexual dimorphism in models of anxiety and depression due to changes in HPA axis activity.* Neuropharmacology, 2012. **62**(1): p. 436-45. DOI
Adrenalectomized male rats treated with corticosterone replacement exhibited less anxiety-like behavior compared to intact males. Corticosterone replacement in males increased swimming and reduced climbing behavior in the foced swim test (no immobility differences). In contrast, adrenalectomy and corticosterone replacement did not have an effect on the female behavioral response. Females were on average more active and had less anxiety-like behavior than males while exhibiting higher depressive-like symptomatology.
47. Meziane, H., et al., *Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies.* Genes Brain Behav, 2007. **6**(2): p. 192-200. DOI
The performance of BALB/cByJ female mice varied significantly depending on the phase of the estrous cycle in the open field, tail flick and tail suspension tests, the behavior of C57BL/6J females, with the exception of the tail suspension performance, remained stable across all four phases of the estrous cycle. Irrespective of the estrous cycle, there were also stain differences in all behavior paradigms.
48. Manzano Nieves, G., et al., *Early Life Stress Delays Sexual Maturation in Female Mice.* Frontiers in molecular neuroscience, 2019. **12**: p. 27-27. DOI
Early life stress of limited bedding speeds male mautration of the hippocampus and increased risk for depressive-like pathology and attentional disturbances in female mice. Early life stress in female mice also delayed puberty and impacted measures of estrous cycling. No effect of estrous cycle stage was found on anxiety-like behavior for either contorl or early-life stress mice.
49. Fu, X.Y., et al., *Effects of chronic unpredictable mild stress on ovarian reserve in female rats: Feasibility analysis of a rat model of premature ovarian failure.* Mol Med Rep, 2018. **18**(1): p. 532-540. DOI
50. Dalla, C., et al., *Chronic mild stress impact: are females more vulnerable?* Neuroscience, 2005. **135**(3): p. 703-14. DOI
51. Wu, M.V. and J. Tollkuhn, *Estrogen receptor alpha is required in GABAergic, but not glutamatergic, neurons to masculinize behavior.* Hormones and behavior, 2017. **95**: p. 3-12. DOI
52. Heywood, L.H., *Testosterone levels in the male laboratory rat: variation under experimental conditions.* Int J Androl, 1980. **3**(5): p. 519-29. DOI
53. Lehmann, M.L., et al., *Urine scent marking (USM): a novel test for depressive-like behavior and a predictor of stress resiliency in mice.* PLoS One, 2013. **8**(7): p. e69822. DOI