Predictive Model for Baseline Serum Estradiol Concentration of Female Laboratory Mice

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"On our honor as University students, we have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines."

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Abstract

The purpose of this research project is to establish the baseline concentration of estradiol in female mice and develop an effective model to predict these concentrations. Estradiol, a type of estrogen hormone, plays a crucial role in regulating and developing reproductive functions [1]. Estrogen research studies have primarily focused on male mice in the past and are now considered outdated [2]. Furthermore, recent discoveries have disproved the assumption that female mice produce more variable results due to their hormonal makeup, revealing a research bias that has limited the understanding of estradiol dynamics in female mice [3]. Our research aims to contribute valuable insights to women's health, such as hormone replacement therapy, by determining the baseline estradiol concentration in female mice and improving upon existing research. The creation of a precise and predictive model for estradiol concentrations can significantly enhance the efficiency of research efforts, potentially saving valuable time and resources by reducing the need for laboratory equipment. In addition to its immediate applications, the model can have broader implications by improving the efficiency of other research projects related to estradiol, allowing researchers to rapidly estimate estradiol concentration. Furthermore, it can validate older experimentation and fill existing gaps in the understanding of the estrous cycle in female mice, which comprises four stages and plays a crucial role in reproductive processes. Our study aims to predict the baseline estradiol concentration accurately [4]. To achieve this, we will determine the baseline serum estradiol concentration in female mice and create a predictive model based on the estrous cycle stage and concentration using techniques such as vaginal flushing and cytology, as well as blood collection and analysis using the E2 ELISA techniques. Additionally, we aim to find cell area ratios for the cycle stages based on present cell types.

Keywords: Estradiol Concentration, Estrogen Hormone, Predictive Modeling, Reproductive Health, Estrous Cycle, Laboratory Efficiency, Gender Bias in Research, Animal Handling, Laboratory Bench Work, Women's Health,

Introduction

Estradiol is a naturally occurring estrogen steroid and female sex hormone. Produced by granulose cells in the ovaries, it is synthesized from cholesterol by several enzymatic reactions [5]. Compared to females, males produce small quantities of estradiol, primarily by converting testosterone. In females, estradiol is important in regulating the estrous cycle in most mammalian species, as well as the menstrual cycle in humans. Estradiol levels fluctuate significantly during the menstrual and estrous cycles, peaking just before ovulation to prepare the body for potential pregnancy. This hormone plays a significant role in regulating the female reproductive system, shaping secondary sexual characteristics like breast growth, and maintaining a healthy cycle. Estradiol is critical to various areas of reproductive health, development, and overall physiological and psychological well-being [6]. The effects of estradiol on the body are far-reaching, including influencing bone health by promoting calcium absorption, contributing to cardiovascular health by maintaining healthy cholesterol levels and promoting blood vessel relaxation, and regulating mood and brain functions [7] [8] [9].

Understanding the importance of estradiol is crucial for conducting research of the female reproductive system. However, accurately determining estradiol concentrations and cycle stages poses challenges that require attention and action. Current methodologies for predicting estradiol concentrations and cycle stages are time-consuming, labor-intensive, and require substantial lab equipment and resources. In addition, there is a notable disparity in research where more studies involve male models but not female [10] [11]. For these reasons, we need to streamline and optimize research practices to overcome these obstacles. This initiative aims to stabilize and revolutionize hormonal research, paving the way for future advancements in estradiol-related studies.

The current method for measuring estradiol in mouse serum involves commercial E2 immunoassays. In the past, the Beckman Coulter Coated-Tube (CT) RIA method was used, but it raised concerns regarding its accuracy. After an investigation, it was recently concluded that the CalBiotech EIA method is the most accurate way to measure E2 in mouse serum. However, there is a need to develop a method with enhanced sensitivity, specificity, and cost-effectiveness [12]. By creating an accurate predictive model, this technology aims to not only improve research efficiency but also offer broader applications in enhancing other studies related to estradiol. The core methods of this technology include developing an accurate tool for determining the estrous cycle stage using vaginal cytology and blood samples, quantifying estradiol concentration in mice through E2 ELISA techniques, and creating a predictive model.

The general approach involves determining the estrous cycle stage with vaginal cytology and a baseline estradiol concentration with analysis of blood serum samples, creating a predictive model to output accurate hormone concentrations, and validating that model with the experimental data collected as well as the computational data.

A power analysis was performed to determine the smallest sample size of mice needed to maintain a level of significance. The data to perform the analysis was obtained from the study, Hormonal Fluctuations during the Estrous Cycle Modulate Heme Oxygenase-1 Expression in the Uterus [13]. Estradiol concentrations from the two most statistically different sample groups, the Estrus stage group, and the Metestrus stage group, were used in the analysis. The alpha was set to 0.05 with a power of 0.8, which produced a result of n=9 per group, with a total of 18 mice in the study. With budgeting considerations, sixteen mice were used in this study. More specifically, there was a sample size of four mice per four groups for the four-cycle stages.

Results

Lasso Regression Model of Estradiol Concentration

Using the optical density data obtained from the ELISA, the estradiol concentration values were calculated, and a spreadsheet was made containing the weight of the mice, mouse identification, week, cycle stage, and estradiol concentration. A Python script was made in Jupyter notebook to do exploratory data analysis. A scatter plot was made to analyze correlation between weight and cycle stage, and it was found that there was no significant correlation between the two variables (Fig. 1).

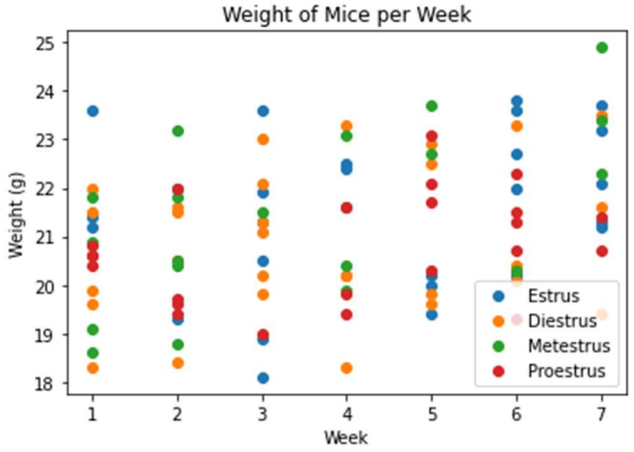

Fig. 1. Correlation of weight of mice and their cycle stage. The weight of the mice is plotted over seven weeks and grouped by the estrus cycle stage they were in. There is no clear correlation seen between these two variables and the weight is widely varied in each group.

Bar graphs were made to view the distribution of mouse estradiol concentrations for the four estrous cycle stages (Fig. 2). The distribution was widely varied so when the lasso regression model for the concentrations and cycle stage was created, it was not accurate, and a proper model was not able to be created.

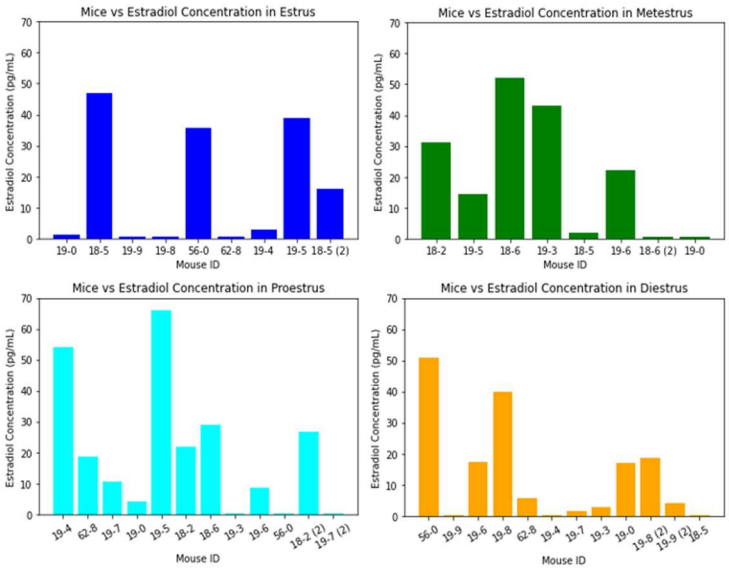

Fig. 2. Distribution of Estradiol Concentration in Each Cycle Stage. The estradiol concentrations were graphed for each estrus cycle stage to view the distribution. Mice ID are noted on the x-axis. Some mice were in the same stage on different blood collection days and these mice are specified on the x-axis. The bars that show extremely low concentration values were truly low values and some were due to there being less serum sample for that mouse during the ELISA.

Cell Area Ratios of Estrus Cycle Stages

The mounted microscope slides made during the vaginal cytology procedure were imaged using the THUNDER Imager. The images were loaded into the HALO software and a 3x3 image section of each image was analyzed using a classifier trained to identify the three cell types present during the estrous cycle (Fig. 3).

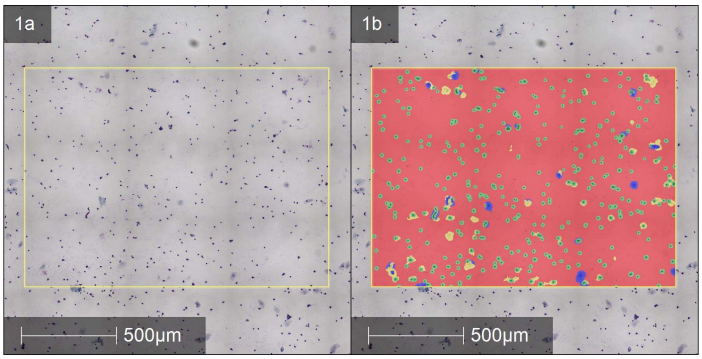

Fig. 3. HALO Analysis of Cell Types. (1a) A 3x3 frame is selected as the region for analysis and is outlined by a yellow rectangle. (1b) Classifier is used to analyze the selected region. The red region is the background area, blue region is the nucleated epithelial cells, yellow region is the cornified epithelial cells, and green region is the leukocytes.

Cell areas for each cell type were obtained for each image and cell ratios for each cycle stage were calculated using a Python script in Jupyter notebook. The table of the cell ratios can be seen below (Table 1). Box plots were created to view the distribution of the ratios as well (Fig. 4).

Cell Area Ratio Ranges (mm^2)	Proestrus	Estrus	Metestrus	Diestrus
Nucleated Epithelial	0.0471-0.070	0.0262-0.241	0.0962-0.160	0.0208-0.185
Cornified Epithelial	0.628-0.698	0.355-0.743	0.253-0.528	0.0790-0.180
Leukocytes	0.244-0.303	0.0871-0.458	0.373-0.594	0.680-0.819

Table 1. Cell Area Ratio Ranges for the Estrous Cycle Stages. The different cell types have different ranges for the area they take up in a 3x3 frame region in a microscope slide based on what stage the mice are in.

Discussion

Purpose of Baseline Collection

The reasoning for conducting baseline collection is to establish a reference point for the normal levels of estradiol in the absence of experimental manipulation. Baseline levels provide a control against which changes in estradiol levels can be compared following experimental interventions or treatments. They also help understand the normal physiological range of estradiol in female mice. This is crucial for interpreting the significance of any deviations caused by an experiment. Female mice additionally experience cyclical changes in hormone levels due to their estrous cycle. Knowing the baseline levels allows for

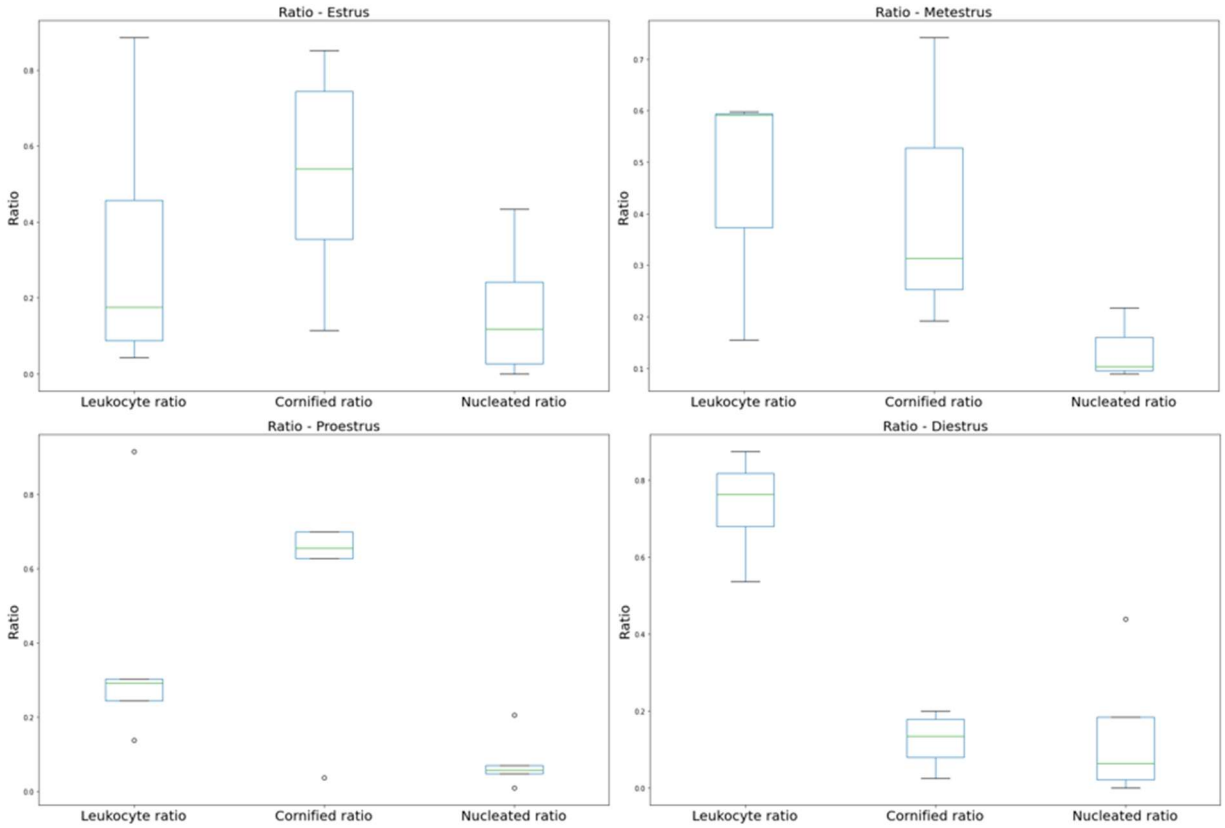

Fig. 4. Box Plots of Cell Area Ratios for Cycle Stage. Box plots showing the ranges of the areas each cell type took up in the microscope slides. Each estrus cycle stage contains three box plots.

accounting for these natural fluctuations when analyzing the effects of variables. There can be individual differences in hormone levels among mice, and baseline data helps to identify and control for this variability, ensuring that results are due to the treatment rather than any pre-existing differences. Lastly, baseline collection is used to validate the methods employed for measuring estradiol concentrations, ensuring that the assays are sensitive and accurate enough to detect changes from the baseline. This was used for the E2 ELISA assay to measure these changes.

Vaginal Flushing

When conducting this procedure, the animal handlers had to consider the fluctuations that arose from repeatedly flushing the same mouse multiple times a day and throughout the week. This repetition resulted in the collection of lower amounts of new cells available, which might lead to inaccurate results when determining cycle stage in cytology. To circumvent this problem and allow for the replenishment of new cells, flushing was performed on each mouse only once per day, deemed necessary. In addition to this potential issue, it was important to consider the technicalities in vaginal flushing. When flushing the vaginal area, it was important to avoid collecting tissue from the vaginal wall as this leads to tissue damage. Equally important was the need to ensure that the micropipette was not inserted too deeply in the vaginal area, as this causes further tissue damage and potentially induces pseudopregnancy.

Blood Collection

During the blood collection process, the animal handlers had trouble trying to collect blood at certain times. Some of this was due to lack of experience for this procedure, or the mice being more dehydrated than expected. At other times, the mice showed an adverse reaction to the isoflurane, so the blood collection had to be paused. Some of the mice were breathing heavily (gasping) and were immediately taken off isoflurane and placed on a heating pad in an oxygenated area to recover. The isoflurane was adjusted from 2.00% to 1.75% at times to prevent the adverse reaction from reoccurring. Additionally, for heavier mice (>20 grams), handlers considered using 2.00% isoflurane levels. Originally, there were 16 mice for experimentation, but during the experiment, 3 of the mice died – one due to collecting too much blood, and two due to adverse reactions to the isoflurane. This resulted in loss of some data and affected the distribution of serum samples collected for each cycle stage each week. In response to these adverse effects, handlers also began providing saline to mice <20g to ensure hydration levels were normal and healthy from blood collections. Blood collection was halted during week 7 as there was an inadequate supply of blood collection tubes so less concentration data was available to build our model on. Handlers tried to collect at least 200 µL of blood, but sometimes the volume collected was lower than expected value due to the issues previously mentioned. Thus, the bench team needed to consider this by running a singlet when analyzing blood using the ELISA kit.

Vaginal Cytology

Although vaginal cytology is an uncomplicated process, some human error occurred. As cycle stage was determined through visual inspection, an incorrect conclusion on the cycle stage was made. Some of the slides were in between cycle stages, so it was difficult to pinpoint which stage the mouse was in. This had a negative impact on our concentration results and led to errors in the predictive model.

Giemsa Staining

The Giemsa staining was carried out effectively with only minor problems. Once the slides were fixed with methanol after vaginal flushing, the methanol was evaporating at a quicker rate than expected due to the microscope slides not being placed in hydration chambers during the fixation period. This was later corrected for the rest of the microscope slides. This had affected the results of the visual inspection of cell types present. Having a short time for methanol fixation can lead to cells being washed off during the later steps of the procedure. This led to inaccurate identification of the estrous cycle stage.

ELISA Technique and Concentration Data

The first ELISA was conducted after four weeks of data collection, but due to human error, the multichannel pipette could not be used for the time-dependent steps of the ELISA. Thus, we had two people simultaneously pipetting for the time-dependent steps to ensure that the steps were completed on time and accurately. This, however, did not work as well as a multichannel pipette, and was reflected in the optical density values given by the spectrometer. The optical density values for the calibrators were not used to create a standard curve as values were inaccurate; thus, sample serum optical densities were unable to be used, as well.

The second ELISA was conducted for the rest of the weeks of data collection and was done without any problems. When running the well plate through the spectrometer, the calibration values were ideal, and their duplicate values were mostly similar so a standard curve could be created. The serum sample values were also good, however, all the samples that were run in duplicate were the same so when the concentration values were determined, the standard deviation and covariance were 0 for each sample which is a source of concern. There were no possible sources of error found when looking into the causes of this, and since the duplicate calibrators' optical densities were not the same, the spectrometer was also proven to be working correctly.

Predictive Modeling

The identification of cycle stage during the vaginal cytology procedure was done by visual inspection of the cell types and how prominent each cell type was on the microscope slides. This was used to determine which mice will have blood collected. Some of the microspore slides were hard to distinguish the correct stage so a reasonable conclusion had to be made based on the cell types viewed. As some of the blood collections were based on these conclusions, the concentration values can be associated with the wrong cycle stage leading to the distribution of the concentrations for each cycle stage to be inaccurate. As the error was made in the early stages of data collection, it compounded, leading to the predictive lasso regression model being inaccurate.

Image Classification

As the predictive model did not meet expectations, image classification was done to see if cell area ratios for each cell type could be used to interpret estrous cycle stage. The classifier used in the HALO software to analyze the images was trained on identifying the background area and the different cell types in the images, but it had trouble with doing so for some of the cell cycle stages. For example, in some of the diestrus images, the leukocytes were seen as nucleated epithelial cells or were counted as part of the background area. Due to this, the classifier was retrained to identify the cell types in those stages increased the identification accuracy as was seen when the images were reanalyzed. The software also had trouble identifying the cornified epithelial cells as the boundary between that cell type and the background was extremely blurry and was not easily distinguishable. The classifier was also trained to identify these cells more accurately so correct this error. As the classifier was trained multiple times, overtraining it became a slight concern as the analysis was being done, however, this was not able to be corrected as there was a limitation on the experimental timeline. Overall, the diestrus and metestrus stages had ratios aligned with the cell types known to be prominent in those stages. The estrus stage was partially aligned, although it was seen that there was also an abundance of leukocytes which is incorrect. This may be due to the problems stated above. Image analysis produced different conclusions to researcher visual inspection for the proestrus stage. According to the ratios, nucleated epithelial cells took up the least area when this cell type should be the majority. As there were problems with the visual inspection, the image classifier can be used to correct the cycle stage identification for the stages that the classifier was accurate for. This can help the estradiol concentrations match with their cycle stages.

Future Directions

The study should be performed a second time with a larger sample size to ensure that the results are significant. In future experimentation, more images with an even distribution of cycle stages will allow for a more accurate distribution of cell area ratio ranges for each cycle stage. This increases the validity of the ranges, as currently, ranges are based on a small set of images for each set. In addition, the number of images for each stage are not the same which introduces bias into the ratio calculations. The correlation between estradiol concentration and cell area ratios can also be found through further experimentation to better understand the estrous cycle. Improving classifier training in the HALO software will allow for a more accurate identification of the three cell types, and thus more accurate cell area ratio results. New training would involve inputting more data into the classifier by identifying more cell types from a large set of cytology images. A particular focus would be selecting more nucleated epithelial cells which was found to be more difficult for the AI to distinguish when running the classifier.

Materials and Methods

The study was performed within the M3 Laboratory at the University of Virginia, under IACUC-approved protocol, and all work was performed in an AAALAC-accredited facility. Mice were placed on heating pads when under anesthesia to ensure homeostasis and observed when taken off anesthesia to ensure they were fully awake and exhibited normal behavior before returning to the vivarium.

Animals

Sixteen 7 weeks old C57BL/6J female mice at intake were used in our experiment. We had 4 cages with 4 mice each, for a total of 16 mice. Mice were fed a "Soft Food" diet to ensure their hydration was normal after procedures. Each mouse was tagged on the right ear, where ear tags were replaced if scratched off onto the left ear.

Shuffling of Mice

At the beginning of data collection, the sixteen mice were placed in different cages than their siblings in a process called shuffling. Shuffling is done for several reasons in research labs, even when all mice are females. Female mice tend to exhibit more stable exploratory behavior than males, despite hormonal fluctuations, and this stability is important for consistent results. Female mice can also exhibit aggressive behavior towards each other, especially in stressful environments and confined spaces, and separation from siblings can reduce the risk of this. Lastly, by housing the mice separately researchers can better adhere to guidelines and ensure the welfare of the animals [14].

Randomizing the placement of the female mice increases the variability within a study, which is vital for generalizing the results. This improves the quality of data collected by ensuring that each subject is exposed to a similar range of conditions, thus reducing confounding variables. Shuffling allows for more controlled studies by minimizing the influence of social hierarchies or dominance that could affect the outcome of the research. It also prevents any potential biases that may arise from environmental and genetic similarities among siblings. By shuffling them, researchers ensure that any observed effects are due to the experimental conditions rather than familial traits [15].

Vaginal Flushing

A vaginal flushing procedure was performed on the mice to collect the cells from the vaginal canal (Fig. 5). To begin, the mouse was placed in an anesthesia chamber delivering 1.75-2% of isoflurane anesthesia. Once anesthetized after approximately 1-2 minutes, tweezers were used to gently squeeze the foot to check if the mouse was reactive. If un-reactive, the mouse was then placed in a nose cone tube delivering isoflurane, as well, for another 1-2 minutes to ensure full sedation. The ear tag ID number was recorded, and the mouse was weighed, and returned to the nose cone; weight was recorded. Reactivity was determined for a second time using the tweezers before beginning the procedure. A maximum of 200 µL (minimum 150 µL) of room-temperature PBS solution was drawn into a micropipette and the mouse was placed in a supine position for this procedure. The vaginal opening was located, and the micropipette tip was gently inserted no more than 1 cm into the vaginal wall. The area was gently aspirated with the solution, being careful not to damage the area and collect tissue from the wall. Flushing was done 5 to 6 times, where an opaqueness to the solution was ideal as this indicated the correct location was flushed and that cells were gathered. The solution was then put on a labeled glass slide and spread across the slide to ensure an even distribution of cells. When all specimens were collected, slides were left to dry in the fume hood for staining analysis.

Vaginal Cytology

Giemsa Staining

Dried cells were fixed with methanol (Fig. 5). The dry fixed slides were then stained using a Giemsa stain solution. A 1:10 ratio of filtered Giemsa stain and PBS was applied to each slide, which was set for 1 minute. The slides were then rinsed with water and allowed to dry before being mounted for analysis. This method of staining is quick and gives a basic view of the different cell types. It colors each of the cell types and does not require laser microscopy, simplifying the process of analysis.

Microscopy

The estrous cycle stage of the mouse models was determined using vaginal cytology to analyze the proportions of cells in the vaginal canal and determine with which stage they are consistent. Three types of cells were examined and identified during the estrous cycle: leukocytes, cornified epithelial cells, and nucleated epithelial cells. The stained slides were analyzed under the microscope using a 20x objective, which allowed for the identification of the proportion of the cells. The entire length of the slide was analyzed as opposed to a small area as cell types and numbers will vary in different areas on the slide. Under the microscope, leukocytes presented as darkened small circles, cornified epithelial cells as larger, more transparent, and lacking a nucleus, and nucleated epithelial cells were similar in appearance to cornified epithelial cells except they also contained a darkened nucleus. Slides containing samples from mice in the Diestrus stage should show a predominance of leukocytes. Slides taken from the Proestrus stage should show a predominance of nucleated epithelial cells and slides taken from the estrus stage should show a predominance of cornified epithelial cells. Finally, slides taken from mice in the metestrus stage should show an equal number of leukocytes, cornified epithelial cells, and nucleated epithelial cells.

Blood Collection

Measuring estradiol concentrations from blood samples was done by calculating average absorbance values from the samples. Samples were collected from the submandibular vein. This technique was selected due to its ease of execution, requiring minimal training given our time constraints, while also providing the least amount of discomfort for the mouse compared to more invasive measures. Additionally, this region allowed for an adequate amount of blood to be obtained, where a minimum of 200 µL of blood was required to generate 100 µL of serum,

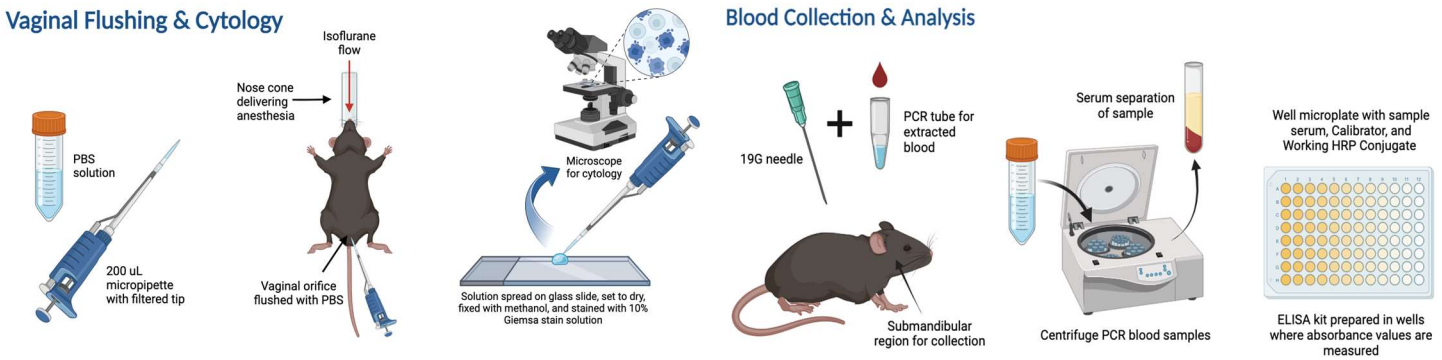

Fig. 5. Approach for Vaginal Flushing & Cytology and Blood Collection. Details the methods for vaginal flushing, vaginal cytology, and blood collection. These procedures were used to identify estrous cycle stage and collect blood serum samples.

which is the minimum serum volume needed to run the ELISA for analysis. It is important to note that blood was not collected more than once from each mouse for the week, following ACUC protocol, as well as not collecting more than 10% of blood based on the mouse's body weight. Additionally, fluids were given to mice <20g (saline) to ensure hydration levels were at normal levels after blood collections.

Sub-Mandibular Vein Blood Collection (Fig. 5)

Similar to the vaginal flushing procedure, the mouse was placed in an anesthesia chamber delivering 1.75-2% of isoflurane anesthesia. Once anesthetized (~1-2 minutes), tweezers were used to determine reactivity of the mouse. If unresponsive, the mouse was moved to a nose cone tube delivering the anesthesia for another 1-2 minutes to ensure anesthetization. The ear tag number was observed, and reactivity was determined again before beginning the procedure. Due to the length of the procedure (> 5 minutes), paralube was applied to the mouse's eyes to lubricate the area. The mouse was turned on its left side in a C-shape position to allow for easier blood draw. After locating the submandibular region (behind the jawbone), a 19G needle was quickly inserted into the skin and blood was collected in a Lithium Heparin tube based on previously recorded bodyweight. If more blood was required, the area was re-pierced on the same side or the other side. Once the correct amount of blood was collected, pressure was quickly applied over the blood collection site with a piece of gauze to stop further bleeding.

Once the blood was obtained, the tubes were set aside to clot for 30 minutes. They were then centrifuged at a speed of 3,400 rpm for 90 seconds, to allow the serum to be separated. The serum was extracted using a pipette and transferred to another test tube. All the samples were stored in a deep freezer at -80º C until the execution of the ELISA.

ELISA Technique

ALPCO Estradiol ELISA kit was utilized to conduct the immunoassay. Two new calibrators - 5 pg/mL and 10 pg/mL - were created from the A (0 pg/mL) and B (20 pg/mL) calibrators provided in the kit. 50 µL of each calibrator (A-H) were added adjacently in columns 1-2 of the well plate. 50 µL of Control 1 and Control 2 were added adjacently in the first two rows of columns 3-4. 50 µL of collected serum samples were added to the remaining wells. From here the instructions provided with the ALPCO Estradiol ELISA kit were followed. The plate was analyzed on a spectrophotometer at a 450 nm range, and data was output as optical density. The average absorbance values for each set of calibrators and samples were calculated from optical density, and a standard curve of the mean absorbance vs. the concentration of the standard was plotted. Using the mean absorbance value for each sample, the corresponding concentration data from the calibration curve was collected and consolidated into a CSV file.

Lasso Regression Model

A lasso regression model was chosen for the estradiol concentration data as this model has the capacity for feature selection meaning that it only uses a few variables [16]. The dataset for this experiment does not contain many variables so this model is apt for this experiment. The model would take in initial baseline serum concentration value and estrous cycle stage, and it would output the estradiol concentration. The model was made in Jupyter notebook using Python.

Image Classification

Cell area ratios for each cell type in the estrous cycle stages were calculated using image classification. This was found so that it can be used as another way to identify the estrous cycle stage.

THUNDER Microscope

The Leica THUNDER microscope was used to image microscope slides. Each slide was placed in the imager and the associated software was opened on a computer. The slide was focused on a dense region of cells and an 8x8 region of frames were imaged. Six focus points were selected in the 8x8 region, and these focus points were adjusted to ensure the frames were not blurry. A cell closest to each focus point was used as a reference to ensure it could focus the frames correctly. Once the focus map was completed, the frames were merged and saved as a TIFF file. The merged TIFF file was later run through the HALO software.

HALO AI Software

One representative image from each cycle stage (proestrus, estrus, metestrus, diestrus) was used to create a Random Forest classifier to train the AI in this software in deciphering between the background and the 3 cell types. Using the selection tool, nucleated epithelial cells were color-coded as blue, cornified epithelial cells as yellow, leukocytes as green, and the background area as red. If cells were not individual, or separated, in the image, clusters of cells were all selected as one. Background selection involved anything that was not clearly a cell, including any blurry/opaque areas on the image.

The merged TIFF files were opened in the software. Each slide was analyzed by right-clicking on the file and selecting analyze. The classifier created above was selected when prompted and the 3x3 region was analyzed. The results were displayed in the results tab, and these were copied into an Excel spreadsheet which was used to calculate the cell area ratios.

Cell Area Ratios

The cell area ratios were calculated using a Python Script in Jupyter notebook. The Pandas python package and Matplotlib.pyplot sub-module was used to write the script.

End Matter

Author Contributions and Notes

K.P. and M.J. performed vaginal flushing; K.P. and M.J. performed blood collection; R.T, S.S., and G.L performed blood serum collection; R.T., S.S, and G.L. performed Giemsa staining, cycle identification, and ELISA; R.T. imaged microscope slides; K.P. and R.T. performed image classification using HALO; R.T. wrote Python Scripts; R.T. analyzed data and created cell area ratios; and R.T, K.P., M.J., S.S. and G.L. wrote the paper.

The authors declare no conflict of interest.

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