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Impacts of the Reproductive Cycle on the Microbiome: Lessons from Peromyscus maniculatus

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Impacts of the Reproductive Cycle on the Microbiome: Lessons from *Peromyscus maniculatus*

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ABSTRACT This low-cost pilot study performed in an animal model attempts to answer the question of whether the estrous cycle affects skin bacteria population size and diversity. In the study, deer mice (*Peromyscus maniculatus)* are models because the University of Northern Colorado maintains a colony at the animal research facility, and the rodents have a conveniently short reproductive cycle. Vaginal swabs and flushes were collected for two full estrous cycles to obtain cells for determination of the phase of the cycle and flora samples to examine diversity and population size. We witnessed the vaginal flora fluctuate throughout the entire cycle with most peaks occurring at, or around, metestrus. These results may demonstrate that deer mice, and likely other mammalian species, experience fluctuation in their microbiota during the reproductive cycle. An expansion of the study could include an examination of transmitters of disease in males and females, even humans, to determine if fluctuation in microbiota affects selection.

IMPORTANCE It has been demonstrated that skin bacteria activities attract vectors. However, previous studies have not used human females due to the fear that the reproductive cycle may affect the results. This gap in research affects half of the human population; therefore, it is important to investigate how the female reproductive cycle impacts normal flora. Our results may have important implications on vector attraction and disease epidemiology. Lastly, the study can be used to distinguish invading microbes from residents on *P. maniculatus.*

Several studies found that blood-feasting vectors are attracted to the odor produced by human skin bacteria (1,2,3). In Verhulst et al. (2018) study, the authors pointed out that human female participants were not included in their research because they believed the menstrual cycle probably affects the skin microbiota, natural bacteria inhabitants. If that is true, then that means half the human population, the female population, may have a completely different relationship with these vectors. Therefore, it is important to understand how the menstrual cycle affects skin microbiota population size and diversity.

However, one cannot understand the matter so simply. First, one must consider the many difficulties controlling all human variables. For example, diet, medical issues, and shower routine and regularity, are variables that must be standardized to ensure validity in the results. After all the variables are accounted for, one must determine if the change in skin flora, if any, affects mosquito attraction. In addition, one must consider what factors of the reproductive cycle may cause the fluctuation, if any at all.

To understand the effects of the human menstrual cycle on skin bacteria and the implications of the effects, several experiments need to be conducted with the aid of financial assistance, the approval of the Institutional Review Board (IRB), and years' worth of time. After

thorough background research, no one appears to be investigating the matter. At this time, people seem more interested in what attracts the vector, an important matter on its own. However, if one sex is affected differently by the vector, that too merits attention. Therefore, we proposed this pilot study before much time and money was spent; we studied the effects of the estrous cycle on vaginal skin bacteria diversity and population size, in deer mice (*Peromyscus maniculatus*).

For the study, deer mice were an ideal candidate to begin research on the topic. *P. maniculatus* were easily accessible and controllable at this institution, the University of Northern Colorado (UNCO), for they are already bred and cared for here. In addition, the usage of deer mice allowed us to postpone the use of humans, until one is certain there might be effects experienced by the reproductive cycle. The last main benefit, but also pitfall, of utilizing *P. maniculatus* is their short estrous cycles. The estrous cycle, or the reproductive cycle of non-primate mammals, is very similar to the primate menstrual cycle, except it does not involve the shedding of the uterine wall. Since the cycles are not identical, the data is not completely applicable to humans. However, the animal model's four to six day long cycle is significantly shorter than any primate's cycle that could last up to 37 days (4,5). With such short sequences, it was possible to see the variation in the natural flora of the skin in a time efficient manner.

LITERATURE REVIEW

Microbiota. A study by Kumar et al. (2019) aimed to understand how well isolated human skin bacteria co-aggregate, or amass, with each other. The researchers identified ten species from seven genera of bacteria. The study concluded that "*Staphylococcus haemolyticus* had the highest coaggregation partners" (p. 1). The article is important because it provided a list of bacteria found on human hands and the partners they co-aggregate with, which is useful to understand transmission of bacteria. Importantly to this research, it provided a list of natural flora

found on human hands that might be relatable to our research. In particular, the list offered some insight on the differences between human and mice microbes.

Tavakkol et al. (2010) study investigated the skin flora of mice living in Specific Pathogen Free (SPF) conditions and concluded that Staphylococci was the most common species. However, 20 other species were also identified. No biases were apparent, if one ignores the fact that female mice were not included in the study. This article offered a great background of what skin bacteria are naturally found on mice, which was vital to our project. In addition, the study exemplifies the overlap of flora between humans and mice. Indeed, both mammals share at least two species of bacteria. It granted us with a reference of what bacteria we should expect on our research subjects.

Available methods. Festing and Altman (2002) provided numerous suggestions that might be helpful to scientists of a wide range of disciplines who seek to improve research development. In addition, the article pointed out key features in experimental design and their importance.

A study by Caligioni (2009) detailed how to determine the estrous phases of mice, a key method used in the research project. The process of determining the phase of the estrous cycle in mice includes the following:

> "Place the tip of plastic pipette, filled with PBS or saline (~10μL), into the vagina. Flush the vagina gently three to five times with same PBS/saline solution (fig 4). Collect final flush in pipette tip. A volume of 10 μL of saline solution allows collecting sufficient material for observation of vaginal cytology. Place final flush containing vaginal fluid on a glass slide. Observe unstained material under light microscope with a 10× objective" (p. 3-4).

These methods simplified the process of utilizing mice as a model animal. The article was important because it provided a guide to develop our own method of determining the estrous phase of our mice in the study.

Vector attraction. Verhulst et al. (2011) study demonstrates that certain species of mosquitoes are attracted to certain bacteria. The results of this study are important because it could potentially lead to new methods of protecting against mosquitoes. The article demonstrates the connection between skin microbes and mosquitoes. In addition, it indirectly exemplifies the biases of using an abundance of Caucasian males over any other race and sex. Such biases cause a significant gap in research, especially as one considers that flora various per person.

A study by Zhang et al. (2015) aimed to investigate the attraction factor of agr-based quorum sensing (QS), which is bacteria's ability to respond to population density by *Staphylococcus epidermidis* to *Aedes aegypti,* a yellow fever virus-carrying mosquito species. The article concluded that blood feeders (the equipment used to feed the vectors) with bacteria attract more mosquitoes. In addition, the *S. epidermidis* with normal functioning QS attracted far more mosquitoes than the *S. epidermidis* that could not quorum sense. The study provides information on how mosquitoes make decisions in host selection and is relevant to our work because it provides reasons that may contribute to the attraction of mosquitoes. Overall, the study implies that mosquitoes can sense when there is a chemical imbalance in a host. This could be a factor that affects females differently throughout their reproductive cycle.

Another study by Verhulst et al. (2018) investigated the similarities of primates' skin microbiota and odor profile to that of people. The article concluded that apes and humans have similar odor profiles. However, humans lack diversity on their skin flora, when compared to nonhuman primates. The authors believe this might be due to humans' hygienic practices and

their lack of hair. The research is important because it targets a variable that attracts one of malaria's vectors. The paper's lack of human female usage due to the unknown effects of the menstrual cycle led to the inspiration behind our research project.

Purpose. This study aimed to understand how the estrous cycle affects the skin flora's population size and diversity, in *P. maniculatus* in order to gain a better understanding of how the reproductive cycle may affect components that may attract vectors. The articles detailed in the literature review served four purposes in our research. First, some of the research articles ascertained that skin bacteria do indeed play a role in mosquito attraction (1,2,3). Second, some studies exemplified the lack of female participants, which creates a gap in our knowledge of effects (1,2). Third, some articles provided a reference of normal flora found on humans and mice (6,7). Fourth, the remaining papers provided background information essential to developing and conducting this research (4,8).

RESULTS

Design. In order to address our research question, we conducted an observational study. Vaginal swabs of *P. maniculatus* were inoculated on Tryptic Soy Broth (TSB) growth medium in the laboratory. The mice derived from the UNCO animal facility where there is an established colony. Four females between the ages of 6 to 8 months old were randomly chosen and placed in individual cages for observation and swabbing. Vaginal flushes were used for cytological examination.

Analysis. After data collection and processing, we organized the data into tables. The tables hold data for each mouse for each of their sampled cycles. The tables display the types of bacterial forms observed during each phase of the estrous cycle for each mouse (Table 1-8). Asterisks in

the tables signify the abundance of each form, relative to its fellow microbes. The bottom of the tables shows the total of forms observed.

The focus of the analysis was on the number of bacterial cell morphologies in each phase of the estrous cycle (Graph 1). The largest number of forms observed in proestrus was 9; the smallest number of forms was 4. After taking all the totals in proestrus, the mean was 5.7. In estrus, the largest number was 8, while the smallest number was 3. The mean of estrus was 4.83. On two occasions, the mice were between the stages of estrus and metestrus during cultivation. There, the largest number of forms was 7 and the smallest number was 4 with a mean of 5.5. In metestrus, the largest value was 7, the smallest value was 4, and the mean was 6. Some bacteria were cultivated between the metestrus and diestrus phases. Here, the largest number of forms seen was 7, the smallest number of forms was 3, and the mean was 4.75. The last phase, diestrus, was observed to have at the most 7 forms of bacteria and at the least 3 forms with the mean of 4.66. Graph 1 represents the data from this analysis and was used for determining trends throughout the estrous cycle.

In addition, we created a graph to see the abundance of particular forms (Graph 2). Only four forms were inputted: gram-positive rods, gram-negative rods, gram-positive tetra coccus, and gram-negative tetra coccus. Bacteria of this appearance were selected because they seemed promising to test if abundancy varied throughout the estrous cycle. We utilized the asterisks in the tables to quantify relative abundancy. We recorded if the form was observed and how abundant it was in each phase of the deer mice's cycles. We quantified the mean for each form in each phase of the reproductive cycle.

Gram-negative rods received a mean in proestrus of .4; the mean of it in estrus is .33; the mean of it in metestrus is 1.2; the mean of it in metestrus to diestrus is .5; the mean of it in

diestrus is .66. Gram-positive rods in proestrus have a mean of .7; it has a mean of .66 in estrus; it has a mean of .8 in metestrus; it has a mean of .5 in metestrus to diestrus; it has a mean of .77 in diestrus. Gram-positive tetra coccus received a mean in proestrus of .8; the mean of it in estrus is .5; the mean of it in metestrus is 1; the mean of it in metestrus to diestrus is .5; the mean of it in diestrus is .66. Gram-negative tetra coccus in proestrus have a mean of 0; it has a mean of .33 in estrus; it has a mean of .4 in metestrus; it has a mean of .5 in metestrus to diestrus; it has a mean of 0 in diestrus.

Table Legend	
(*)	Relatively not common
(**)	Relatively common
(***)	Relatively abundant

Table 1: Deer mouse 1, Week 1

Proestrus	Estrus	Estrus into Metestrus	Metestrus	Diestrus	Diestrus 2 (3/6/20) Day 4
Gram + Staphylococcus (**) Gram + streptococcus (**) Gram + staphylo rods (**) Gram – diplococcus (*) Gram + diplococcus (***) Gram + single coccus (*) Gram + tetra coccus (*) Gram + diplo rods (**)	N/A	Gram + Staphylococcus (**) Gram + Streptococcus (**) Gram + strepto rods (**) Gram + single coccus (*) Gram + diplo rods (**) Gram - diplococcus (***) Gram - single coccus (*)	N/A	Gram + Staphylococcus (**) Gram + Streptococcus (**) Gram + diplococcus (**) Gram – diplococcus (**)	Long gram – rods (***) Gram - strepto rods (**) Gram + staphylococcus (**) Long gram + rods (***) Gram – diplococcus (**)
Number of forms: 8	Number of forms: N/A	Number of forms: 7	Number of forms: N/A	Number of forms: 4	Number of forms: 5

Table 2: Deer mouse 2, Week 1

Proestrus	Estrus	Metestrus to Diestrus	Metestrus	Diestrus	Diestrus 2 (3/5/2020) Day 3
Gram + Streptococcus (**) Gram - diplo rods (**) Gram + diplococcus (**) Gram + Staphylococcus (**) Gram + strep rods (**)	N/A	Gram + Staphylococcus (**) Gram + Streptococcus (**) Gram + diplo rods (**) Gram + tetra coccus (**) Gram + diplococcus (**) Gram – diplococcus (**) Gram + long rods (**)	N/A	Gram + diplo rods (***) Gram + streptococcus (**) Gram + staphylococcus (**)	Tiny gram – single coccus (***) Gram + diplo rods (**) Gram - diplo rods (**)
Number of forms: 5	Number of forms: N/A	Number of forms: 7	Number of forms: N/A	Number of forms: 3	Number of forms: 3

Table 3: Deer mouse 3, Week 1

Proestrus	Estrus	Metestrus	Metestrus to Diestrus	Diestrus
Gram – diplococcus (**) Gram + Staphylococcus (**) Gram + diplococcus (***) Gram + diplo rods (**)	Gram + single Coccus (**) Bacillus (**) Gram + Diplococcus (***) Gram + diplo rods (**) Gram + Staphylococcus (**) Gram + streptococcus (**) Gram - diplo rods (**)	Gram + Staphylococcus (**) Gram + Streptococcus (**) Gram – Tetra coccus (**) Gram – rods (**) Gram - diplo rods (**) Gram + diplo rods (***)	gram - diplo rods (**) Gram - tetra coccus (**) Gram + staphylococcus (**) Gram + diplococcus (**)	N/A
Number of forms: 4	Number of forms: 7	Number of forms: 6	Number of forms: 4	Number of forms: N/A

Table 4: Deer mouse 4, Week 1

Proestrus	Proestrus 2 (5/6/20) Day 4	Estrus	Metestrus	Diestrus
Gram + Staphylococcus (**) Gram + Streptococcus (**) Tiny gram + rods (**) Gram + diplococcus (**) Gram + diplo rods (**) Gram – diplococcus (***)	Gram + Staphylococcus (**) Tiny gram + coccus (**) Gram + diplo rods (**) Gram + diplococcus (**)	Gram + Streptococcus (**) Gram + Staphylococcus (**) Gram + rods (*)	N/A	Long skinny gram – rods (**) Small gram + staphylococcus (**) Gram – diplococcus (**) Gram + streptococcus (**) Gram + diplo rods (***) Gram + staph rods (**)
Number of forms: 6	Number of forms: 4	Number of forms: 3	Number of forms: N/A	Number of forms: 6

Table 5: Deer mouse 1, Week 2

Proestrus	Estrus	Metestrus	Metestrus 2 (3/15/20) Day 5	Diestrus
Gram + diplococcus (**) Gram + staphylococcus (**) Gram + streptococcus (**) Gram + tetra coccus (**) Bacillus (*)	Gram + Strep rods (**) Gram + diplo rods (**) Gram + streptococcus (**) Single gram + rods (**) Gram + staphylococcus (**) Gram + tetra coccus (*)	Gram + staphylococcus (**) Gram + streptococcus (**) Gram + tetra coccus (**) Gram + strepto rods (**)	Gram + staphylococcus (**) Gram + streptococcus (***) Gram + strepto rods (**) Gram + diplococcus (**) Gram + medium rods (*) Gram - medium sizes rods (***) Gram + tetra coccus (***)	Gram + tetra coccus (**) Gram + staphylococcus (**) Gram + diplococcus (**)
Number of forms: 5	Number of forms: 6	Number of forms: 4	Number of forms: 7	Number of forms: 3

Table 6: Deer mouse 2, Week 2

Proestrus	Estrus	Metestrus	Metestrus to Diestrus (3/14/20) Day 4	Diestrus
Gram - diplo coccus (**) Gram - long rods (**) Gram - staphy rods (**) Gram + diplococcus (**) Gram – diplococcus (**)	Gram + staphylococcus (**) Gram + strep rods (**) Gram + skinny rods (**)	Gram + diplo rods (***) Gram - diplo rods (**) Gram + staphylococcus (**) Gram + diplococcus (**) Gram + streptococcus (**) Gram - single coccus (**) Gram + streptococcus (**)	Gram + staphylococcus (**) Gram – long rods (**) Gram + diplo coccus (**)	Gram + tetra coccus (**) Gram + staphylococcus (***) Gram + medium size rod (bacillus) (**) Gram + diplococcus (*) Gram + staphylococcus (**)
Number of forms: 5	Number of forms: 3	Number of forms: 7	Number of forms: 3	Number of forms: 5

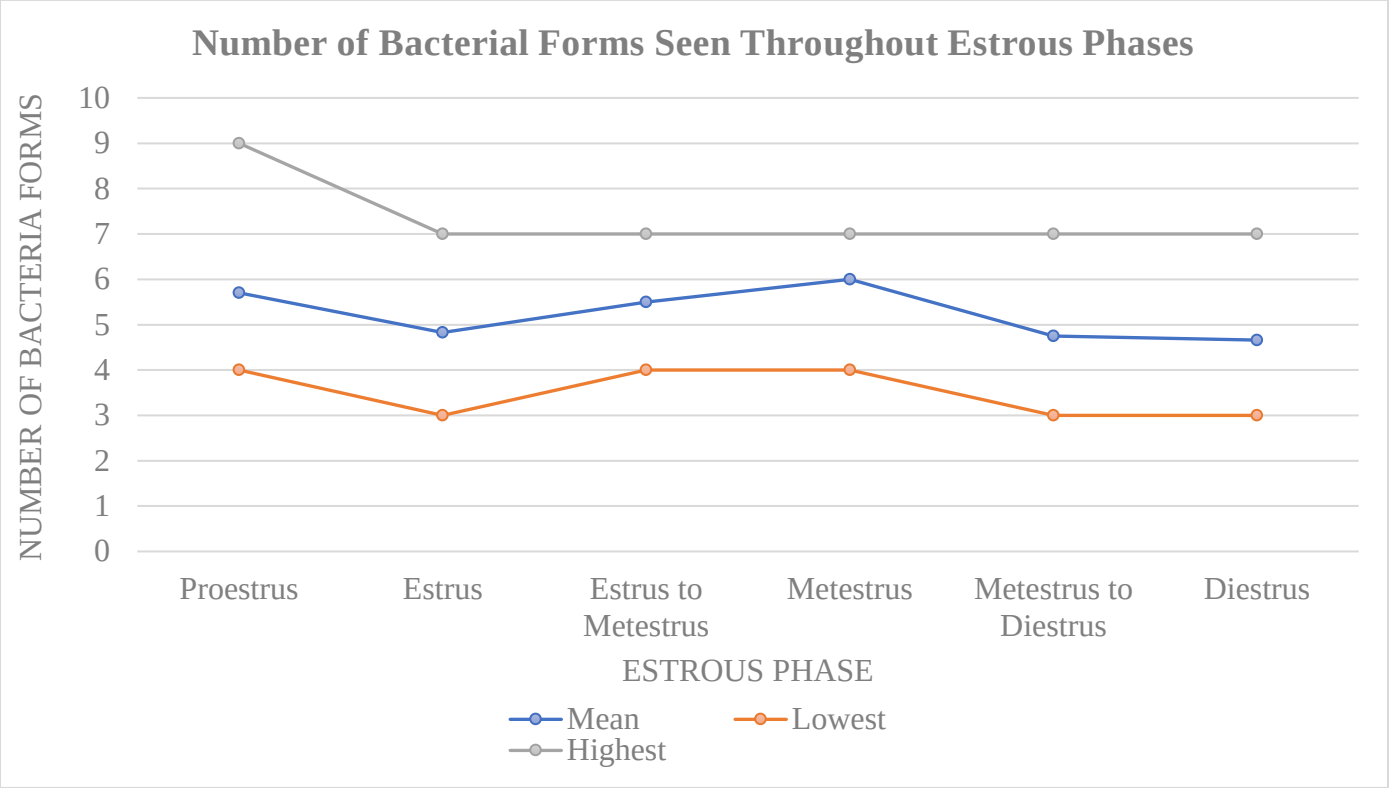
Table 7: Deer mouse 3, Week 2

Proestrus	Estrus	Estrus to Metestrus	Metestrus	Diestrus	Diestrus 2 (3/15/20) Day 5
Gram + tetra coccus (**) Gram + diplococcus (**) Gram + streptococcus (**) Gram + staphylococcus (**) Gram - single spirillum (*)	N/A	Gram + diplococcus (**) Gram - diplo rods (**) Gram + staphylococcus (**) Gram + streptococcus? (**)	Lactobacillus (***) Gram + diplo rods (bacillus?) (**) Gram – long rods (***) Gram - diplo rods (**) Gram + staphylococcus (**) Gram + streptococcus (**)	Gram – diplococcus (***) Gram + streptococcus (**) Gram + staphylococcus (**) Gram + diplococcus (**) Gram - diplo rods (**) Gram + medium diplo rods (**) Gram – medium rods (*)	Gram + staphylococcus (**) Gram + diplococcus (**) Gram + streptococcus (**) Gram + diplo rods (**) Gram + tetra coccus (**) Gram - diplo rods (*)
Number of forms: 5	Number of forms: N/A	Number of forms: 4	Number of forms: 6	Number of forms: 7	Number of forms: 6

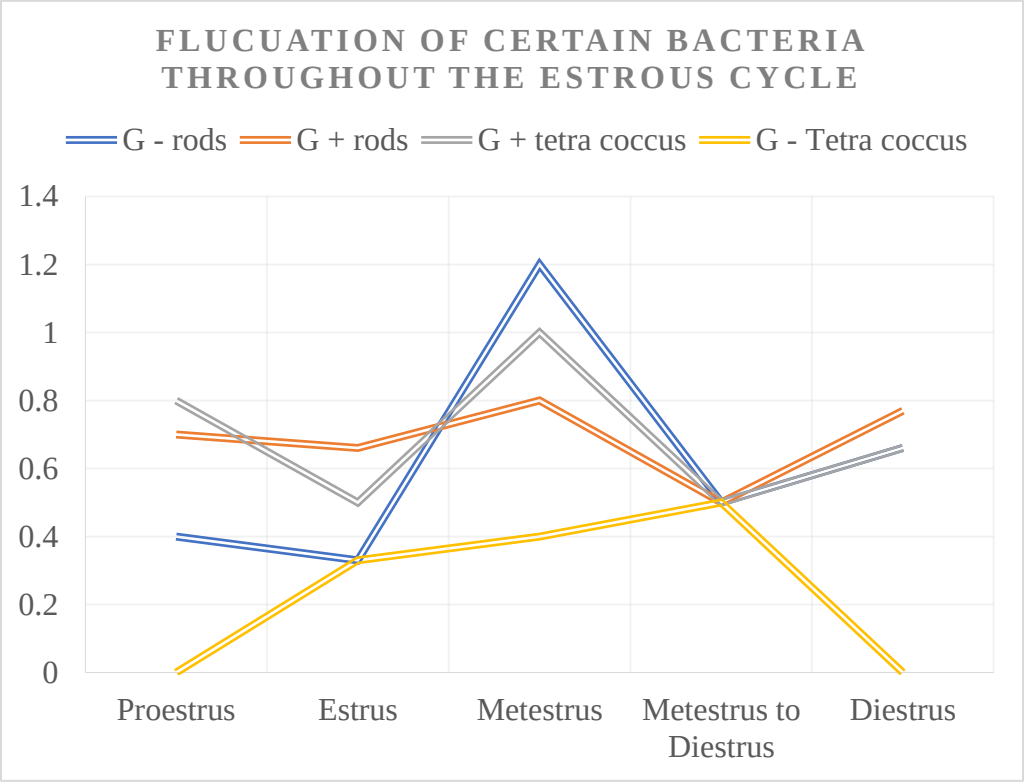
Table 8: Deer mouse 4, week 2

DM4 WEEK2						
Proestrus	Proestrus 2 (3/14/20) Day 4	Estrus	Estrus 2 (3/15/20) Day 5	Metestrus	Metestrus to Diestrus	Diestrus
Gram + diplococcus (**) Lactobacillus (***) Gram + diplo rods (**) Gram + staphy rods (**) Gram + strepto rods (**) Gram + staphylococcus (**) Gram – diplococcus (**) Gram + tetra coccus (*) Gram + streptococcus (**)	Gram - medium size rods (**) Gram – diplococcus (**) Gram + staphylococcus (**) Gram + streptococcus (**) Gram + tetra coccus (**) Gram + medium size rods (***)	Gram + medium rods (**) Medium size gram - rods (**) Gram + staphylococcus (**) Gram + streptococcus (**)	Gram + streptococcus (***) Gram + diplococcus (**) Gram - tetra coccus (**) Gram + strepto rods (**) Gram + staphylococcus (**) Gram + tetra coccus (**)	N/A	Gram + diplo rods (**) Gram - diplo coccus (**) Gram + streptococcus (**) Tiny gram + staphylococcus (**) Gram – Diplo rods (***)	N/A
Number of forms: 9	Number of forms: 6	Number of forms: 4	Number of forms: 6	Number of forms: N/A	Number of forms: 5	Number of forms: N/A

Graph 1: Number of Bacterial Forms Seen Throughout Estrous Phases



Graph 2: Fluctuation of Certain Bacteria Throughout the Estrous Cycle



DISCUSSION

Discoveries. The study provided much insight. First, previously described methods birthed the development of methods for obtaining cells and microbe samples from deer mice confirming the ability to determine estrous phase in this animal model (4). Second, some bacteria were identified by their cell morphologies and arrangements, which granted us a better idea of the diversity of the normal flora on the rodents. Third, Graph 1 suggests that microbiota does indeed fluctuate throughout the estrous cycle of the deer mouse. We expected fluctuation since hormonal variation is common in the reproductive cycle and hormones can affect available sugar for microbial metabolism (9). Almost all the peaks in microbial diversity happen at, or around, metestrus (Graph 1). In particular, the mean line demonstrates two peaks in the cycle, one in

proestrus and another in metestrus. We find the time periods of the peaks interesting because proestrus and metestrus possess the most diverse cell types in the vaginal opening. Since it appears that fluctuation of microbe diversity occurs throughout the estrous cycle, then that might cause changes in odor production, quorum sensing, and other bacterial activities. That, in turn, may affect vector meal selection. Lastly, Graph 2 hints at the possibility that certain bacteria vary in abundancy throughout the estrous cycle. Most interestingly, the phase with the most diversity in microbiome, metestrus, experiences a boost in abundancy in almost all the selected forms. In addition, it appears that some microbes are present in only some of the phases, in this case it is gram-negative tetra coccus. The variation in abundancy may have similar effects in meal selection like those caused by diversity.

Limitations. The study had some huge limitations. First, the pandemic of COVID-19 caused the closure of the University of Northern Colorado, which prevented the collection of more data and full analyzation of bacterial samples. The analysis of bacterial samples using differential media genetic analysis was essential for determining population size of each microbe as well as fluctuations over different stages in the estrous cycle. Second, additional time and funding would be needed to accurately determine the species diversity of bacteria using metagenomic DNA sequencing techniques, which is the sequencing of many genomes at once to facilitate the determination of diversity. Under these circumstances, diversity can only be evaluated by distinguishing the physical features of the microbes after Gram staining. In addition, there are potential limitations in bacterial diversity analysis from cross contamination of fur, urine, and feces. This might explain the limited amount of bacterial diversity fluctuation observed between phases. To counter the issues, we could experiment with minor modifications to the collection methods to ensure the animal cannot move in a way that will contaminate the sample. Perhaps,

allowing the anesthetic to work on the mouse longer may sufficiently prolong their anesthesia in order to acquire the samples without the mouse moving. Though, one must be extremely careful and observant in order to prevent unintended death of the rodent. Perhaps, simply ensuring a sturdy grasp of the scruff will suffice in preventing the animal model from moving. An ideal modification is accustoming the mice to being handled in order to minimize their fright, which may decrease their movement, urination, and defecation. Lastly, the significance or reproducibility of the data cannot be tested at this time, making our results just observations at this point.

CONCLUSION

There is an obvious gap in knowledge associated with how skin bacteria activities affect human females in regard to blood-sucking vector attraction. The gap exists due to experimental design fallacies and the presumption that females' reproductive cycle affects the normal microbiota, and, therefore, would interfere with vector selection studies. This project sought to gather data that may transform the presumption into a certainty. Though, due to the scope of the research, time restrictions, monetary restrictions, and ability to control variables in human research, deer mice were used as animal models to see if fluctuation in bacteria population size and diversity can be observed during the estrous cycle.

After cultivating, processing, organizing, and analyzing, the data, we determined that some fluctuation is seen throughout the estrous cycle. It appears that proestrus and metestrus experience a peak of biodiversity. In addition, the results suggest that the abundancy of certain bacteria fluctuate throughout the cycle, as well. At the end, the results may demonstrate that it is reasonable to think the reproductive cycle will affect the skin flora in *P. maniculatus,* which may possibly be used in vector selection studies in the future. However, due to our many limitations

in this study, more research is needed to ensure that our observations are supported. Future analysis will include the conduction of a similar study with the addition of DNA analysis and calculation of population size using density screening and colony morphology. If that follow-up analysis supports our initial findings, we plan to conduct a study to observe if vectors prefer female mice during specific estrous phases or prefer to feed on males versus females.

MATERIAL AND METHODS

Animal Assurance. All animal work was conducted with the consent of the University of Northern Colorado's Institutional Animal Care and Use Committee (IACUC protocol # 2002C-AH-DM-23). The animal facility at the University of Northern Colorado cares for the animals in a controlled environment. There, only professional, experienced, and authorized personnel have access to the mice. In addition, the animals only interact with people during times of feeding and cage-cleaning. The authors were responsible for the health of the deer mice used for the study, which involved regularly conducting check-ups on the subjects.

Materials. To conduct the study, various microbiology and animal husbandry materials were used. In order to safely handle the animals, we needed latex gloves, leather gloves, Isoflurane, cotton balls, and a plastic container with a lid. In order to acquire and harvest the microbes, we needed 1x DPBX, sterile polyester tipped applicators, test tube racks, nutrient broth, nutrient agar plates, and a microbiology incubator. In order to facilitate the inspection of bacteria, we needed micro pipettes, adjustable volume pipette, pipette tips, microscope slides, a compound microscope, inoculating loop, wax pencil, bacteria incinerator, alcohol torch, crystal violet stain, distilled water, Gram's iodine, ethyl alcohol, safranin stain, bibulous paper, immersion oil, lens paper and cleaner, and parafilm.

Procedures. Every day, at around the same time for 4 or 5 days in a row, vaginal swabs and flushes were collected for bacteria and cells, respectively. To do this, mice were anesthetized in a plastic container using a small amount of Isoflurane on a cotton ball. Mice were removed from the container as a soon as they showed signs of anesthesia. Immediately after, mice were handled by the scruff of their neck and holding of their tail. Sterile polyester tipped applicators dipped in 1x DPBS were used to gently swab the vaginal opening. Precautions were taken to limit cross contamination with fur, urine, and feces. Swabs were then immediately placed into TSB and subsequently placed into an incubator at 37˚C for 24 hours. Pipette 10 microliters (ul) of 1x DPBS solution to gently flush the vagina. The 10ul volume was pipetted up and down until the liquid became clouded with cells. Collected solution and cells were then released onto a microscope slide. Wet mounts, created by placing a cover slip over the cytology sample, were examined under the microscope. Estrous phase was determined by analyzing the cells using Caligioni's (2009) article as a reference.

After incubation, bacterial cultures grown in TSB were Gram stained utilizing traditional techniques and examined under oil emersion to determine Gram orientation and cell morphologies. Traditional techniques were also used to perform streaks for isolation in Tryptic Soy Agar (TSA) for each tube of bacteria. The plates incubated for 24 hours at 37˚C. Afterwards, plates were parafilmed and placed in the refrigerator.

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APPENDIXES

Chart 1: Examples of Cell Morphologies Throughout the Estrous Cycle

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