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Asian elephant (Elephas maximus) vaginal vestibule microbiomes are associated with reproductive hormones and physiology

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Abstract

Reproductive microbiomes are increasingly recognized as components of reproductive health, yet their relationships with reproductive physiology remain poorly understood across animal species. This is particularly relevant to endangered species, where reproductive dysfunction can constrain conservation breeding. Asian elephants (*Elephas maximus*) represent an important example, as reproductive acyclicity is prevalent in *ex situ* females and is associated with reduced fertility and welfare factors. Here, we characterized the vaginal vestibule (VV) microbiome of 13 *ex situ* Asian elephants using full-length 16S rRNA sequencing and examined associations with ovarian cyclicity, reproductive hormones, parity, age, and VV pH. VV microbiomes were taxonomically diverse, with abundant species including *Porphyromonas somerae*, *P. canis*, and *Mycoplasmopsis equigenitalium*. *M. equigenitalium* was enriched in low-progesterone states, including non-cycling females. Microbial diversity declined with age but increased with VV pH, with independent associations of both factors with diversity. Community composition differed between cycling and non-cycling females and was more similar between mother–calf pairs than unrelated individuals. Multiple bacterial taxa were associated with progesterone, parity, age, and VV pH. These findings identify reproductive physiology and ovarian cyclicity as important correlates of reproductive microbiome variation and provide a foundation for investigating microbiome–reproduction relationships relevant to conservation breeding in Asian elephants.

Keywords: Asian elephant; ovarian cyclicity; reproductive microbiome; reproductive hormones; vaginal vestibule

Introduction

Microbiomes are critical components of animal ecology and health, playing important roles in digestion, disease prevention, endocrine function, and reproduction [1]. Although previous studies of microbiomes in wildlife have largely focused on gut communities, a

29 growing body of research has demonstrated key links between reproductive microbiomes -
30 e.g., the microbial communities in animal reproductive tracts and endocrine processes,
31 fertility, and offspring survival [2]. Microbial communities can interact with sex hormones,
32 including estrogens and androgens [3-5], thereby influencing reproductive physiology. In
33 mammals, vaginal microbiomes, in particular, are associated with estrous cycle stages [5-9],
34 pregnancy outcomes [10, 11] and fertility history [12]. In turn, vaginal communities maintain the
35 vaginal epithelium, manage inflammatory processes, combat infections, and seed neonates
36 during birth [13]. Previous research across primate species has demonstrated that the taxonomic
37 composition of vaginal microbiomes is host-specific but that functional traits may be
38 conserved to facilitate reproductive health across host species [14]. Similarly, imbalance or
39 dysfunction of the vaginal microbiome has been linked to negative reproductive outcomes
40 across multiple mammal species. Despite these insights, vaginal microbiomes remain
41 unstudied across many mammal species, with a particular lack of studies that examine
42 endocrine processes and vaginal microbiomes in tandem.

43 For endangered species under human care, conservation breeding programs are often
44 necessary to sustain diverse populations. Understanding the dynamics of reproductive
45 microbiomes and their role in reproductive success is particularly valuable for these
46 populations with managed reproduction. The Asian elephant (*Elephas maximus*) is currently
47 listed as endangered on the International Union for Conservation of Nature (IUCN) Red List.
48 Approximately one-third of the global population resides under human care, distributed
49 across zoological institutions, rescue centers, and sanctuaries in both Asian range countries
50 and Western nations [15]. Capturing elephants from the wild is no longer considered
51 appropriate or sustainable and is prohibited or strictly regulated across most Asian elephant
52 range countries. Instead, the existing *ex-situ* population is a valuable resource that can serve
53 critical roles in education, veterinary training, reproductive management, conservation

research, and public awareness. Captive elephants, however, face a distinct set of health and welfare concerns, including infectious diseases such as tuberculosis [16], metabolic disorders [17], and health issues like gastrointestinal disturbances [18] and idiopathic reproductive dysfunction affecting both males and females [19, 20]. These issues can be exacerbated by suboptimal husbandry and social conditions [21-23]. As a result, maintaining reproductive function under human care is essential for the long-term sustainability of captive elephant populations.

In female elephants, reproductive acyclicity or non-cycling, defined as the absence of normal ovarian cyclicity, is one of the most prevalent reproductive dysfunctions and a probable cause of infertility [24, 25]. This condition is typically diagnosed through immunoassay-based monitoring of reproductive hormones, including progesterone and luteinizing hormone (LH) [19, 26]. Reproductive management in elephants is particularly challenging because of their unique reproductive biology, characterized by a prolonged gestation period (approximately 22 months) [12], and relatively high rates of reproductive complications, including stillbirth and calf mortality. Together, these factors can reduce reproductive output and limit population growth. The causes of reproductive acyclicity are complex and likely multifactorial. Obesity [27, 28], metabolic dysfunction [28], stress-related factors such as seasonal variation and body condition [23, 29], and aging-related factors (such as reproductive tract pathologies) [30, 31] have all been implicated. These factors may disrupt endocrine function and ovarian activity, ultimately leading to reduced fertility. Despite the relatively high prevalence of acyclicity in captive elephants, the mechanisms underlying abnormal ovarian cycling remain poorly understood, limiting the development of effective management strategies. Progress in understanding elephant reproductive physiology is further constrained by the anatomical characteristics of the female reproductive tract. The exceptionally long vaginal vestibule (VV) makes sample collection logistically challenging,

78 labor-intensive, and costly, often requiring specialized equipment and prolonged monitoring
79 [32]. Consequently, physiological data remain limited.
80 Despite these challenges, increasing attention has been directed toward the reproductive
81 tract because of its critical role in fertility and its interactions with resident microbial
82 communities. The VV represents a major interface between the external environment and the
83 reproductive tract, contributing to protection against ascending pathogens and maintenance of
84 the local reproductive environment. As a hormonally responsive tissue, the vagina undergoes
85 dynamic physiological changes across the estrous cycle. Fluctuations in reproductive
86 hormones, particularly estrogen and progesterone, can influence epithelial turnover [33],
87 mucosal immunity [34, 35], vaginal pH [36, 37] and the composition of the vaginal microbiome [38].
88 These interconnected endocrine, physiological, and microbial processes suggest that the VV
89 represents a dynamic reproductive microenvironment that may vary with ovarian function.
90 However, whether reproductive endocrine state and local VV physiology are associated with
91 reproductive-tract microbial communities in elephants remains unknown.
92 We hypothesized that VV microbial communities are associated with ovarian cycling
93 status, reproductive endocrine state, and local VV physiology in Asian elephants.
94 Specifically, we predicted that VV microbial diversity and composition would differ between
95 cycling and non-cycling females and vary across estrous cycle phases, and that microbial
96 community variation would be associated with circulating progesterone and luteinizing
97 hormone concentrations and VV pH. Given the potential influence of host characteristics on
98 reproductive microbiomes, we further examined associations with age and reproductive
99 history. Using a newly developed VV sampling approach combined with full-length 16S
100 rRNA sequencing and longitudinal reproductive monitoring, we characterized the VV
101 microbiome and tested these predictions across reproductive and physiological states. This
102 study provides the first characterization of the elephant reproductive microbiome while

investigating how microbial community variation relates to reproductive endocrine and physiological states in a long-lived endangered mammal.

Results

Circulating reproductive hormones and vaginal vestibule (VV) pH

Descriptive data for progesterone concentration, LH concentration, and VV pH are presented in Table 2 and visualized in Supplementary Fig. S1. Progesterone concentration showed no relationship with age but varied significantly across the estrous cycle phases and with reproductive history (Table 3). Across cycle phases, samples collected during diestrus had the highest progesterone concentration and a general increasing trend from proestrus through diestrus (Table 3, Fig. S1A; LMM, $F = 3.93$, $p = 0.013$). Parous females had significantly lower progesterone concentrations than nulliparous females (Table 3, Fig. S1B; LMM, $F = 6.91$, $p = 0.014$). Non-cycling elephants had progesterone concentrations similar to the proestrus phase of cycling animals (Fig. S1A). In contrast, LH did not vary significantly with reproductive history, cycle phase, or age (Table 3, Fig. S1C-D). Similarly, VV pH did not significantly correlate with any reproductive variable, age, nor either hormone (Table 3, Fig. S1E-F).

Changes in the vaginal vestibule microbiome

To first examine whether there was a shared core VV microbiome across all individuals, we examined the membership of all 37 elephant samples (Fig. 2). We identified 3,688 ASVs assigned to 362 species and 231 genera. At the genus level, *Porphyromonas* (mean = 23.3% ± standard deviation = 13.8%) and *Campylobacter* (10.8% ± 7.4%)

dominated the elephant VV microbiomes. At the species level, 25 taxa had average relative abundances greater than 1% across all taxa, with the most dominant taxa being *Porphyromonas somerae* (10.5% ± 6.8%) and *Porphyromonas canis* (8.4% ± 9.2%), followed by *Mycoplasmopsis equigenitalium* (5.3% ± 6.2%), *Campylobacter corcagiensis* (4.0% ± 4.4%), and *Treponema refringens* (3.4% ± 4.2%) (Fig. 2). Five species were abundant present in all 37 samples, suggesting a set of shared core VV microbes: *Porphyromonas somerae*, *Ezakiella massiliensis*, and unidentified species in the genera *Negativicoccus*, *Fastidiosipila*, *Christensenellaceae* R-7 group, and Family XI (order Peptostreptococcales-Tissierellales).

The diversity of elephant VV microbiomes varied across individuals and was associated primarily with age and VV pH (Fig. 3). Estrous cycle phase (Fig. 3A), reproductive history, progesterone, and LH were not significantly correlated with any of the three alpha diversity metrics, therefore, were removed from downstream models after model selection. However, elephant age was significantly and negatively correlated with ASV richness (Fig. 3B; LMM, $t = -2.16$, $p = 0.045$) and Shannon diversity (LMM, $t = -2.38$, $p = 0.038$), with the same relationship trending significant for Faith's phylogenetic diversity (LMM, $t = -2.04$, $p = 0.057$), demonstrating that older elephants had less diverse microbiomes (Fig. 3B). In contrast, there was a significant positive relationship between VV pH and ASV richness (Fig. 3C; LMM, $t = 3.21$, $p = 0.003$) and Faith's phylogenetic diversity (LMM, $t = 2.38$, $p = 0.025$), indicating greater microbial diversity at higher VV pH.

When analyzing microbiome composition, communities varied according to multiple variables. Overall, individual animals harbored rather distinct microbiomes, that clustered in coordinate space (Fig. 4A). Both Bray-Curtis (abundance-weighted; Fig. 4A) and Jaccard (presence-absence) composition correlated significantly with VV pH (PERMANOVA, Jaccard, $R^2 = 0.268$, $F = 1.071$, $p = 0.026$; Bray-Curtis, $R^2 = 0.252$, $F = 1.168$, $p = 0.036$),

suggesting that VV pH was influencing both rare and abundant microbial taxa, reinforcing the alpha diversity results. Additionally, reproductive history and age were correlated with Bray-Curtis composition (Fig. 4A; PERMANOVA, age, $R^2 = 0.362$, $F = 1.675$, $p = 0.033$; parous vs. nulliparous, $R^2 = 0.030$, $F = 1.402$, $p = 0.028$), but not with Jaccard, suggesting that those variables were predictors of abundant microbial taxa, but not the presence-absence of rare taxa. In regard to related elephants, dams and calves had lower Bray-Curtis (Fig. 4B; LMM, related vs unrelated, $t = 4.43$, $p < 0.0001$) and Jaccard (LMM, related vs unrelated, $t = 3.65$, $p < 0.001$) distances compared to unrelated individuals, indicating that related individuals had greater similarity in both taxon relative abundance and community membership.

In regard to specific bacterial taxa driving some of the above patterns, we identified multiple bacterial species that were significantly correlated with various study variables (Fig. 5; Supplementary Table S1). Among cycling elephants, two taxa were identified as varying significantly across cycle phases: *Corynebacterium pilosum* (ASV33) was enriched in the proestrus and estrus phases compared to the diestrus phase (Table S1) and *Mycoplasmopsis equigenitalium* (ASV2) was also enriched in the estrus phase compared to the metestrus phase. These two taxa show opposite patterns of enrichment across the phases, with *C. pilosum* peaking in estrus and metestrus and largely disappearing during diestrus, and *M. equigenitalium* during proestrus and decreasing throughout subsequent phases (Fig. 5). In addition, eight taxa were differentially abundant between cycling and non-cycling elephants, regardless of cycle phase (Table S1). These included *M. equigenitalium* (ASV2), which was enriched in non-cycling animals. Interestingly, two *Porphyromonas somerae* ASVs (ASV86 & ASV73) were enriched in cycling elephants whereas a different *P. somerae* ASV (ASV83) was enriched in non-cycling elephants, suggesting variation at subspecies taxonomic levels. Across elephants with varying reproductive histories (parous vs. nulliparous), three taxa –

two *P. somerae* ASVs (ASV55 & ASV105), and one *Criibacterium bergeronii* ASV (ASV134) - were enriched in the VV microbiomes of parous individuals whereas 15 taxa were enriched in nulliparous individuals, including *Peptoniphilus grossensis* (ASV145*s*, *Anaerococcuss sp.* (ASV41), and *Treponema calligyrum* (ASV96) (Table S1). We additionally identified 10 taxa associated with age, with three taxa showing a positive correlation and seven showing a negative correlation (Table S1). Only two taxa were significantly correlated with VV pH, both showing positive relationships: *Acholeplasma sp.* (ASV38) and *P. somerae* (ASV18). Finally, two taxa were negatively correlated with progesteone concentrations: The same *P. somerae* ASV (ASV18) and the dominant *M. equigenitalium* ASV (ASV2).

Discussion

This study provides the first characterization of the vaginal vestibule (VV) microbiome in Asian elephants and reveals associations between microbial community variation and reproductive endocrine and physiological states. By integrating longitudinal microbiome sampling with endocrine monitoring, estrous cycle phase classification, and local VV physiology, we show that microbial diversity and composition are associated with age and VV pH, that microbial community composition differs between cycling and non-cycling females, and that specific bacterial taxa vary with reproductive hormones and other host factors. Together, these findings reveal links between reproductive physiology and microbial community structure in a long-lived endangered mammal. Our findings also place the elephant reproductive microbiome within a broader comparative context. Consistent with studies of other wildlife species, elephant VV microbiomes were taxonomically diverse and were not dominated by a single bacterial group, contrasting with the frequent dominance of *Lactobacillus* in the human vaginal microbiome. Nevertheless, certain patterns of microbial variation found here parallel observations in humans and other animals, suggesting that

reproductive physiology may represent an important correlate of reproductive microbiome variation across mammalian hosts. Given the increasingly recognized relationships between reproductive microbiomes, reproductive health, and fertility, extending this research across wildlife species may broaden our understanding of reproductive biology and provide a foundation for evaluating the relevance of reproductive microbiomes to conservation breeding. Although the number of elephants included in this study was limited, the longitudinal design incorporated repeated microbiome sampling, endocrine monitoring, estrous cycle phase classification, assessment of local VV physiology, and full-length 16S rRNA sequencing. This integrated approach enabled an in-depth assessment of within- and among-individual microbial variation in relation to reproductive and physiological states, providing insights that would be difficult to obtain from cross-sectional sampling alone.

VV microbiome diversity was associated with both host age and local VV pH, but in opposing directions: diversity declined with advancing age and increased with higher VV pH. Notably, age and VV pH showed no correlation, indicating that the signals in VV microbiome diversity were driven by different processes. In regard to the influence of age, decreasing diversity seems to be a somewhat unique pattern. In humans, vaginal microbiome diversity decreases from puberty to adulthood but then increases through adulthood with advanced age [39]. Similarly, in cattle and domestic dogs, vaginal microbiome diversity also increases with age [12]. In domestic horses, vaginal microbiome diversity was not correlated with mare age [40] and, in black-footed ferrets, age was also not correlated with reproductive microbiome diversity for males nor females [41]. In female Asian elephants, non-cycling status and reproductive tract pathologies become increasingly prevalent with advancing age, particularly in females aged 31 to 35 [31]. Consequently, elephant managers in Thailand prioritize breeding females before this age to maximize reproductive success and reduce the risk of age-related reproductive disorders that compromise fertility [42]. Our observed pattern

of decreased VV microbiome diversity across adult animals into advanced age highlights a potentially unique dynamic in elephants and underscores the overall lack of empirical research in reproductive microbiomes in wildlife.

In contrast to the seemingly unique correlation with age, the positive correlation between VV microbiome diversity and VV pH is a consistent pattern in humans and other species [43]. In humans, low VV pH is a product of lactic-acid production by dominant *Lactobacillus* species, which limits colonization by pathogens that are less tolerant of acidic environments. Non-human primates, for example, generally harbor more diverse vaginal microbial communities with substantially lower relative abundances of *Lactobacillus* than humans [14, 44]. Captive rhesus macaques possess diverse vaginal microbiotas in which *Lactobacillus* represents only a minor component of the community [45, 46]. Here, in elephants, only five *Lactobacillus* or *Limosilactobacillus* ASVs were identified, all with abundances less than 0.002%. Other lactic-acid-producing bacteria (e.g., *Streptococcus*) were present at higher abundances but did not dominate the community. In addition, the VV pH range observed in the present study was consistent with that reported in a previous study [47]. VV pH in elephants was overall higher than in humans, suggesting that maintaining a highly acidic environment – as seen in humans – may not be the primary function of elephant VV microbiomes. Instead, it has been hypothesized that increased diversity of vaginal microbiomes is an alternative strategy that limits colonization by pathogens through competitive exclusion [48].

VV microbiome composition was associated with reproductive history and VV pH, while mother–daughter pairs also exhibited greater microbial community similarity than unrelated individuals. Parity is known to influence vaginal microbiomes in humans [49] and other animals [50]. Here, parity shaped both abundant and rare taxa, indicating a community-wide effect. This is further supported by our differential abundance analyses, in which

reproductive history (parous vs. nulliparous) was correlated with 18 taxa, 15 of which were enriched in nulliparous individuals. Interestingly, we also found that dam-calf pairs had more similar community compositions, a signal that was again present in both the abundant and rare taxa. This pattern has also been found in humans [51], with specific *Lactobacillus* strains being heritable across female lineages. This signal, however, was lost is the daughter was born via cesarean section, highlighting the role of vertical transmission via vaginal delivery [51]. However, mother–daughter similarity may also arise from shared environment, social contact, or host-related factors. Thus, while our findings suggest a potential maternal contribution to VV microbiome assembly, longitudinal and strain-resolved studies from birth are needed to determine whether vertical transmission contributes to the establishment and persistence of the elephant reproductive microbiome.

The identification of bacterial taxa that varied across the estrous cycle, including *Corynebacterium pilosum* and *Mycoplasmopsis equigenitalium* (formerly *Mycoplasma equigenitalium)*, suggests that the VV microbiomes of Asian elephants may be dynamically regulated by endocrine cycles. *M. equigenitalium* is known from the reproductive tracts of male and female horses, with studies reporting its identification as early as 1987 [52]. Although this taxon has been found in mares and stallions suffering from infertility and reproductive issues, it has also been identified in the reproductive microbiomes of healthy horses and donkeys [53]. In this study, *M. equigenitalium* was found to vary significantly across estrous cycle phases in a pattern that reinforces the negative correlation with progesterone: *M. equigenitalium* abundances were highest in samples collected during proestrus and those from non-cycling individuals, which are also the samples with the lowest progesterone concentrations. In Asian elephants, progesterone remains low during the follicular phase and increases following ovulation and luteinization, remaining elevated throughout the luteal phase [29, 54]. The observed distribution of *M. equigenitalium* therefore suggests that this taxon

may be associated with the low-progesterone endocrine environment characteristic of the follicular phase and non-cycling state. Although there is no evidence that *M. equigenitalium* directly utilizes or interacts with progesterone, it is possible the relationship is driven by indirect effects of reproductive hormones on the VV environment [55]. In a related species of Mollicutes, *Mycoplasma hominis*, progesterone treatment in mice eradicated the *M. hominis* [55]. In contrast, species such as *Mycoplasma genitalium* show increased colonization in response to progesterone treatment [56]. In both studies, the authors hypothesize that host-specific and environment-specific *Mycoplasma* are dependent on endocrine-mediated changes in vaginal epithelium and local immunity to adhere, colonize, and survive in the genital tract. Given the abundance of this taxon in elephant VV microbiomes, the significant correlation with progesterone, and the potential as an opportunistic pathogen, increased research on *Mycoplasmopsis equigenitalium* is warranted. Collectively, these findings suggest that estrous cycle phases and ovarian function, rather than chronological age alone, are the primary determinants of progesterone concentrations in captive Asian elephants.

Another bacterial taxon of interest is *Porphyromonas somerae*, an abundant and shared taxon across all elephant samples. *P. somerae* is largely known from human studies, where is has been identified as a member of vaginal and uterine microbiomes [57]. *P. somerae* has also been proposed as a microbial biomarker for endometrial cancer in humans, particularly uterine adenocarcinoma, with mechanistic support for *P. somerae*'s role in cellular invasion and tumor development and growth [57]. Elephants are thought to have relatively low cancer rates overall (i.e., Peto's paradox), possibly due to multiple copies of tumor-suppressing genes. Interestingly, however, a recent archival study of ex-situ Asian elephant necropsies across 80 female elephants at 15 institutions from 1988-2019 found reproductive tract neoplasms in 80% of cases, with 13% of those cases presenting with uterine adenocarcinoma [58]. This study confirmed that reproductive tract neoplasms were

surprisingly common among ex-situ Asian elephants – particularly in older nulliparous animals – and were largely underreported. Although we cannot confirm a causal link between *P. somerae* and reproductive tract cancers in elephants, the ubiquity of *P. somerae* across individuals in this study – paired with high rates of uterine/endometrial neoplasia in ex-situ Asian elephants - warrants increased investigation into reproductive microbiomes and potential links to under-reported disease processes.

Conclusions

This study establishes a baseline understanding of reproductive microbial ecology in Asian elephants and reveals previously unrecognized associations between the VV microbiome, reproductive endocrine state, ovarian cyclicity, age, and local VV physiology. Importantly, the distinct microbial profiles observed between cycling and non-cycling females indicate that reproductive acyclicity is accompanied by differences in the VV microbial environment, identifying the reproductive microbiome as an additional biological component associated with reproductive state in elephants. Together with age-associated declines in microbial diversity and reproductive state-associated bacterial taxa, these findings provide new perspectives on the biological changes accompanying reproductive aging and acyclicity in female Asian elephants.

Future studies incorporating host genetics, environmental variation, diet, social interactions, and management practices across multiple *ex situ* populations will help determine the relative contributions of host and environmental factors to reproductive microbiome variation. Longitudinal and functional investigations will also be important for determining whether microbial changes contribute to, or result from, reproductive dysfunction and disease. Collectively, our findings provide a foundation for understanding reproductive microbiome ecology in a long-lived endangered mammal and for evaluating the

potential relevance of microbial communities to reproductive health, monitoring, and conservation management of *ex situ* Asian elephant populations.

Methods

1. Animals and management

The study involved thirteen sexually mature female elephants aged 14 to 65 years (35.5 ± 16.3 years). All elephants were housed at the National Elephant Institute (NEI), Forest Industry Organization, Lampang, Thailand (latitude 18º 21.60′E and longitude 99º 14.92′E) under the same feeding and management protocol. This study was conducted from May 2024 to December 2024.

The general background information of the elephants included in this study is presented in Table 1. Nutritional management was standardized for all elephants, which were housed and fed under the same regimen. Each elephant received a daily diet equivalent to approximately 6.0% of body weight, with continuous access to water. Feeding primarily included Napier grass (*Pennisetum purpureum*) and pangola grass (*Digitaria eriantha*), while treats such as tamarind (*Tamarindus indica*), bananas, and other seasonal and natural foods were provided sparingly. Each morning (8:00 to 9:00 AM), the elephants were walked within the NEI. From 9:00 AM to 3:00 PM, they participated in daily tourist activities, such as riding or show activities (Table 1). During downtime, they were fed as usual. After work, from 4:00 to 6:30 PM, the elephants were restrained with long 20–30-meter tethers in the forest to forage freely. At night, all elephants were housed in individual enclosures, separated from males.

2. Swabbing of the vaginal vestibule

A collection method specific to this study was developed for the first time. To ensure accurate sampling and avoid contamination of fluids from undesired locations, especially

352 vulvar areas. A sterile double-guarded swab (length 745 mm, IMV Technologies, France)
353 covered with a sterile sanitary sheath was employed to collect vaginal vestibule (VV)
354 samples.

355 VV samples were collected every other week (bi-weekly), as illustrated in Fig. 1.
356 During each procedure, the elephants were restrained in a standing position with all four
357 limbs securely restrained to ensure stability and safety. The vulvar area and approximately 20
358 cm of the vestibule were thoroughly scrubbed and flushed with normal saline and neutral
359 soap to minimize contamination. Following cleaning, a veterinarian wore sterile surgical
360 gloves and carefully and gently inserted a sterile double-guarded swab into the vulva (Fig.
361 1a). The swab was advanced approximately 50–60 cm to reach the targeted vestibular region
362 (Fig. 1b). Once localized, the assistant extended the swab from its protective guard, carefully
363 breaking the sanitary sheath to expose the sterile tip. The swab was gently rotated against the
364 vestibular wall for at least 30 seconds, ensuring thorough contact in three distinct directions
365 to collect an adequate sample (Fig. 1c, d). The swab was then retracted back into the sterile
366 guard sheet to protect the integrity of the sample and prevent external contamination during
367 removal. If contamination looked possible, the swab was discarded, and the elephant was re-
368 swabbed. The head of the mahout team monitored the elephants' condition to prevent any
369 undue discomfort and ensure consistency, accuracy, and adherence to the standardized
370 sampling protocol. Non-cycling elephants were sampled twice during the study period.

371 Each sampling session included the collection of three VV swabs for microbiome,
372 cytology, and then pH analyses. A total of 37 VV microbiome swabs were collected,
373 comprising 29 samples from nine normal-cycling elephants and 8 samples from four non-
374 cycling elephants. Two negative controls were included for microbiome analysis to identify
375 potential contamination: (1) a sterile swab exposed to ambient air during sample collection
376 and (2) a sterile swab rubbed over the external vulvar region, away from the vestibular

sampling site. All microbiome and negative control swabs were immediately placed in a chilled icebox and transferred to a −80°C freezer within 1 h of collection.

3. Microbiome DNA extraction, 16S rRNA gene (V1-V9) library preparation, and sequencing

DNA was extracted from thawed swabs using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. All extractions were stored at –80ºC until DNA library preparation. The 16S rRNA gene (regions V1-V9) was amplified using the Kinnex™ 16S rRNA kit according to the manufacturer's protocols. The targeted PCR followed these cycling conditions: 95 °C for 3 minutes, followed by 20 cycles of 98 °C for 20 seconds, 57 °C for 30 seconds, and 72 °C for 75 seconds, with a final extension at 72 °C for 5 minutes. The 16S rRNA amplicons were pooled and purified using SMRTbell cleanup beads.

The pooled DNA library with different primer sets (1,700 bp) was concatenated using a 12 Kinnex primer mix (Primer mix A-LQ) and amplified under the following cycling conditions: 98 °C for 3 minutes, followed by 9 cycles of 98 °C for 20 seconds, 68 °C for 30 seconds, and 72 °C for 90 seconds, with a final extension at 72 °C for 5 minutes. The 12 Kinnex PCR products were circularized into a SMRTbell library using Kinnex enzymes, ligase, and barcoded Kinnex terminal adapters. The quality and quantity of the SMRTbell library were evaluated using a DeNovix QFX Fluorometer and QIAxcel Advanced (Qiagen, Hilden, Germany), respectively. Sequencing of the SMRTbell library was performed on the PacBio Sequel IIe platform following the manufacturer's protocols.

4. *Bioinformatics of DNA sequence data*

Raw DNA sequence data were analyzed using a bioinformatic pipeline in RStudio (R v.4.5.0) and QIIME2 (v.amplicon.2025.4) tailored to PacBio single-end long reads. Briefly, we used dada2 in R to remove sequence primers, quality filter and denoise reads, remove chimeras, and assign taxonomy to amplicon sequence variants (ASVs) using the Silva species-level full-length 16S dataset (v.138.2). We additionally used QIIME2 to generate a phylogenetic tree using a dereplicated representative sequence file. In R, we combined the cleaned ASV table, taxonomy, phylogenetic tree, and metadata using the phyloseq package [59]. We used the decontam package [60] to remove putative contaminants using the prevalence method based on negative controls, resulting in the removal of 198 ASVs. Additional filtering included the removal of ASVs with fewer than 10 reads assigned, those that were not assigned to a bacterial phylum, and those that were identified either as chloroplasts or mitochondria. After filtering, one sample was omitted from downstream analysis due to insufficient sequencing depth (1,849 reads). Rarefaction curves demonstrated sufficient coverage across all other samples, with a minimum depth of 10,887 (mean sequencing depth = 50,514). To characterize microbiome structure, we calculated metrics of bacterial diversity (alpha diversity; ASV richness, Shannon diversity, and Faith's phylogenetic diversity) and composition (beta diversity; Jaccard and Bray-Curtis dissimilarity).

5. Blood sampling, enzyme immunoassays and vaginal vestibule pH measurement

On the same day as each VV swab, a blood sample (~6 mL) was collected. Additionally, blood samples were taken weekly throughout the sampling period from the ear veins by veterinarians at NEI, resulting in 384 blood samples. Following collection, the blood samples were allowed to clot at room temperature and then centrifuged at 2,000 × g (3500 rpm) for 5 min to obtain serum. The obtained serum samples were stored at –80ºC for further

analysis. Enzyme immunoassays (EIAs) were used to measure serum progesterone concentrations following previously established protocols [29, 61]. Serum LH concentrations were also determined using an EIA method [61, 62]. The intra-assay and inter-assay coefficients of variation (CVs) for both high and low controls were maintained at less than 10% and 15%, respectively. VV pH measurements were conducted simultaneously with blood sampling, following established protocols from our previous study [47].

6. Experimental design and statistical analyses

The analytical dataset comprised 37 VV microbiome samples, including 29 samples from nine normal-cycling elephants and eight samples from four non-cycling elephants. Microbiome samples from normal-cycling elephants were assigned to estrous cycle phases using an integrated assessment of VV cytology, VV pH, and endocrine profiles (progesterone and LH concentrations). Accordingly, the 29 microbiome samples were classified into four estrous phases: proestrus (n = 8), estrus (n = 7), metestrus (n = 6), and diestrus (n = 8), while samples from non-cycling elephants were analyzed as a separate group (n = 8). These classifications were subsequently used to investigate associations between the VV microbiome and reproductive status, hormone concentrations, and host characteristics. Individual progesterone baselines were established for each elephant to distinguish luteal from follicular phases[63], with LH surges used to support identification of the follicular phase. Weekly endocrine profiles and individual progesterone baselines were used to classify females as normal-cycling, characterized by 14–18-week progesterone cycles, or non-cycling throughout the sampling period [64].

To investigate patterns in elephant VV microbiomes, in relation to reproductive metrics and hormone concentrations, we used a combination of linear mixed-effect models, PERMANOVAs, and differential abundance analyses. We first used linear mixed-effect

models (LMM) to characterize variation in hormonal concentrations (progesterone and LH), VV pH, and microbiome diversity. All models included individual elephant as a random effect to account for repeated sampling. Model fixed effects initially included animal age, VV pH, reproductive history (parous vs. nulliparous), cycle phase (proestrus, metestrus, estrus, diestrus, or non-cycling), progesterone concentration, and LH concentration. We used model selection by removing non-significant variables and comparing AIC values to identify the best-fit model for each dependent variable. For both hormones, the final model included cycle phase, reproductive history, and age. For VV pH, none of the variables were significant for any model combination so we reported the full model. Our final model for microbial diversity included animal age and VV pH as fixed effects. Our PERMANOVA models were similarly analyzed to identify the model with the best fit, which included age, VV pH, and reproductive history, with individual as a random effect. Additionally, we used LMMs of pairwise beta diversity distances to investigate whether related elephants (dams and calves) had more similar community composition compared to unrelated individuals. Finally, we used Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2) [65] to identify specific taxa that were significantly correlated with estrous cycle phase, reproductive history, age, VV pH, progesterone, or LH.

Tables:

Table 1| Background metadata for elephants in this study. BCS = Body condition score.

ID	Cycling status	Age	BCS	Reproductive history
029	Normal	45	4.0	Parity 1
032	Normal	29	4.0	Parity 1
028	Normal	41	4.0	Parity 1
041	Normal	42	4.5	Parity 1
066	Normal	16	3.5	Parity 1
144	Normal	16	3.5	Nulliparous
M4	Normal	15	3.5	Nulliparous
103	Normal	14	3.5	Nulliparous
039	Normal	45	4.0	Parity 2
007	Non-cycling	65	4	Parity 3
002	Non-cycling	50	4	Parity 1
025	Non-cycling	48	4	Parity 1
036	Non-cycling	36	3.0	Nulliparous

Table 2 | Descriptive data of luteinizing hormone (LH), progesterone concentration, vaginal vestibule (VV) pH, in non-cycling elephants and normal-cycling elephants across different estrous cycle phases. Values are presented as mean ± SD.

Phases/status	**N**	**LH (ng/mL)**	**Progesterone (ng/mL)**	**VV pH**
Diestrus	8	322.4 ± 92.8	1.6 ± 3.5	6.3 ± 0.8
Estrus	7	352.6 ± 75.0	0.9 ± 0.7	7.1 ± 1.0
Metestrus	6	364.2 ± 85.6	3.2 ± 5.7	6.9 ± 1.3
Proestrus	8	369.9 ± 246.5	1.3 ± 0.9	7.1 ± 1.3
Non-cycling	8	1087.2 ± 1701.1	0.2 ± 0.2	6.6 ± 1.1

Table 3 | Statistical results of linear mixed-effect models on hormone concentrations – progesterone and luteinizing hormone (LH) – and vaginal vestibule (VV) pH. Bold values denote significance ($p<0.05$).

		F	p
Progesterone	Estrous cycle phase	3.9	0.01
	Reproductive history	6.9	0.01
	Age	0.7	0.40
LH	Estrous cycle phase	0.1	0.98
	Reproductive history	0.1	0.76
	Age	2.3	0.14
VV pH	Estrous cycle phase	1.1	0.39
	Reproductive history	1.8	0.21

	Age	4.4	0.07
	Progesterone	0.9	0.37
	LH	1.2	0.29

478

479 **Figures**



480

481 **Fig. 1** | Illustration of the procedure used for swabbing samples from the vaginal vestibule of
482 elephants for microbiome analysis: a) Insert a sterile double-guarded swab into the vulva; b)
483 Advance the swab 50–60 cm to the sampling area; c) Extend the swab from its guard by
484 breaking the sheath to expose the sterile tip; and d) Rotate the swab against the vestibular
485 wall for 30 seconds in three directions for thorough sampling.
486

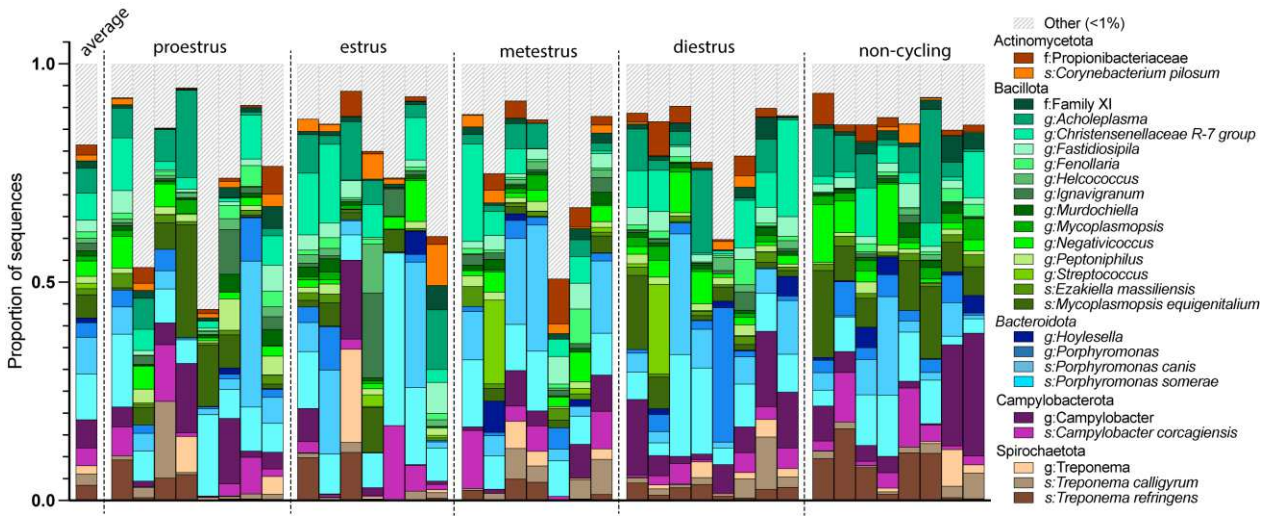


Fig. 2 | The relative abundance of bacterial taxa – species level or above – present in the vaginal vestibule microbiomes of Asian elephants. Colors denote taxa that represent >1% of sequencing reads and the category "Other" is a conglomerate of all taxa that account for <1% of sequencing reads. The "average" bar represents the mean relative abundance across all samples, while each subsequent bar represents a single sample. Samples are grouped by the estrous cycle phase (proestrus, estrus, metestrus, diestrus) and non-cycling individuals.

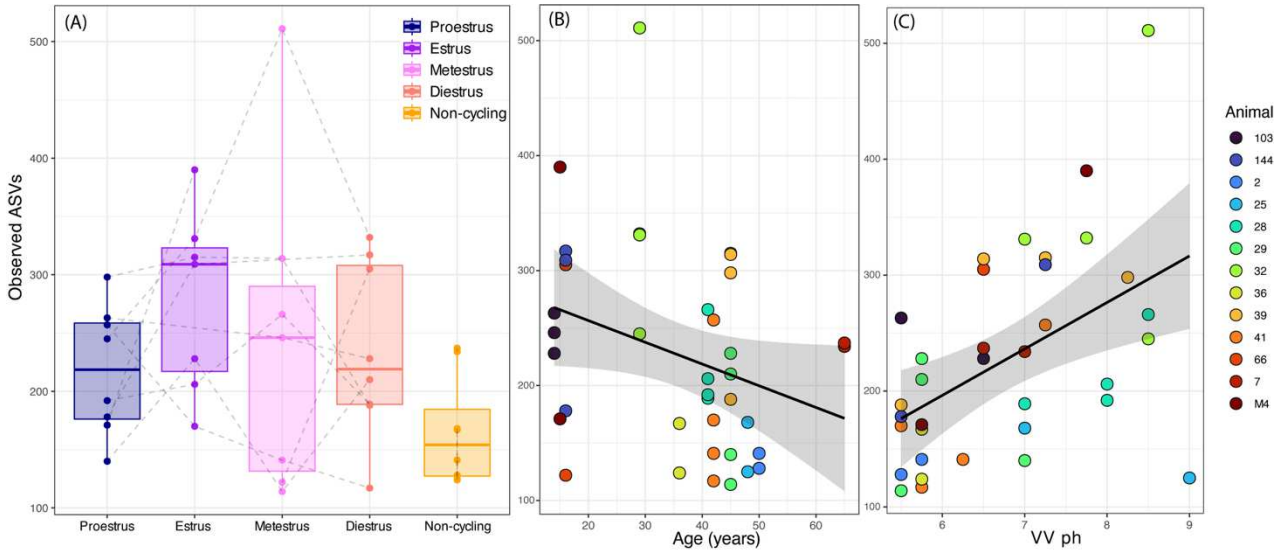
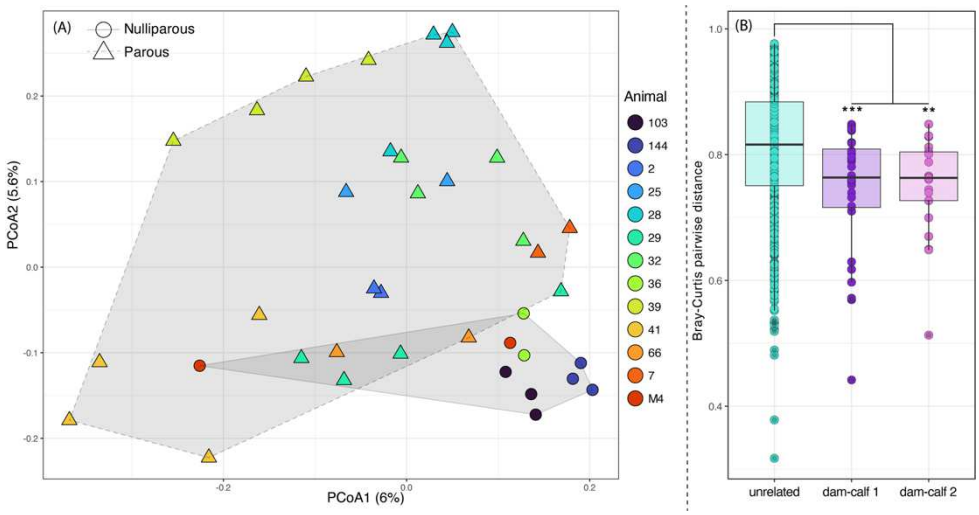
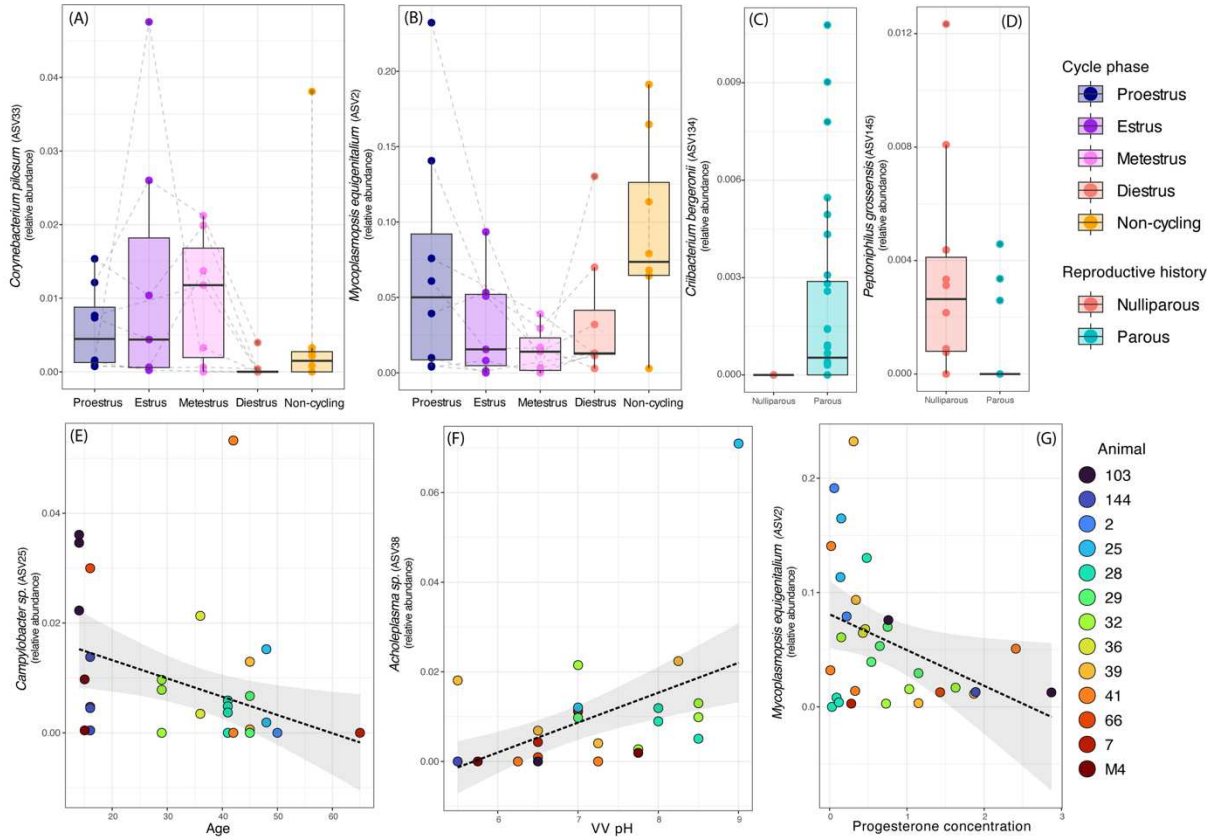


Fig. 3 | The diversity (observed ASVs) of elephant vaginal vestibule (VV) microbiomes (A) across reproductive cycle phases (including non-cycling individuals), and in correlation with (B) age and (C) VV pH.



506
507 **Fig. 4** | The composition (beta diversity, Bray-Curtis dissimilarity) of elephant vaginal
508 vestibule microbiomes (A) represented in coordinate space, colored by individual and
509 grouped by reproductive history (nulliparous vs. parous). (B) Pairwise dissimilarity
510 comparisons between samples from unrelated elephants and between samples from two dam-
511 calf pairs. Statistical analyses of linear models, ***p<0.001, **p<0.01.
512
513



514 **Fig. 5** | Relative abundance of taxa in elephant vaginal vestibule (VV) microbiomes that were
515 found to vary significantly (A-C) across estrous cycle phases, (D, E) between cycling and
516 non-cycling individuals, and with (F) VV pH, (G) age, and (H) progesterone concentrations.
517
518

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