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Variable effects of captivity on microbiomes in populations of IUCN-endangered Blanding's turtles (*Emydoidea blandingii*)

Lauren Jenkins[1,7,*], **Donald T. McKnight**[2], **Matthew Parks**[3], **Nathan W. Byer**[4], **Francis J. Oliaro**[5], **Dan Thompson**[6], **Rodney Scott**[7]

[1]Nicholas School of the Environment, Duke University, Durham, NC 27708, United States
[2]Savanna Field Station, La Democracia, Belize District, Belize
[3]Department of Biology, University of Central Oklahoma, Edmond, OK 73034, United States
[4]Division of Natural Resources, Cleveland Metroparks, Cleveland, OH 44144, United States
[5]Conservation Research Department, John G. Shedd Aquarium, Chicago, IL 60605, United States
[6]Forest Preserve District of DuPage County, Wheaton, IL 60189, United States
[7]Department of Biology, Wheaton College, Wheaton, IL 60187, United States

*Corresponding author. Nicholas School of the Environment, Duke University, Durham, NC 27708, United States. E-mail: lauren.jenkins99@gmail.com

Abstract

Aims: Microbiome composition is increasingly considered in species reintroduction efforts and may influence survival and reproductive success. Many turtle species are threatened by anthropogenic pressures and are frequently raised in captivity for reintroduction efforts, yet little is known about turtle microbiome composition in either wild or captive settings. Here, we investigated trends in microbiome composition of captive and wild IUCN-endangered Blanding's turtles (*Emydoidea blandingii*).

Methods and results: We amplified and sequenced the V4 region of the 16S rDNA locus from plastron, cloaca, and water samples of wild *E. blandingii* adults and two populations of captive *E. blandingii* juveniles being raised for headstarting. Plastron, cloaca, and water-associated microbiomes differed strongly from each other and were highly variable among captive sites and between captive and wild sites. Across plastron, cloaca, and water-associated microbial communities, microbial diversity changed over time, but not in a predictable direction between captive sites. Plastron beta diversity correlated with growth rate in captive samples, indicating that external microbiomes may correlate with individual fitness.

Conclusions: Our results indicate that external and internal microbiomes vary between captive and wild turtles and may reflect differences in fitness of captive-raised individuals.

Impact Statement

Our results illustrate that microbiomes vary substantially in both wild and captive settings, complicating efforts to replicate wild microbiomes in captive conditions.

Keywords: 16S metabarcoding; captivity; conservation; turtles

Introduction

Captive breeding and headstarting (i.e. raising animals in captivity for eventual release) represent important and frequently used strategies for endangered species recovery (Seddon et al. 2007, Dolman et al. 2015). These efforts have been successful in several high-profile cases such as the California Condor and Black-footed Ferret (Bolam et al. 2021). Yet, stark differences between wild and captive settings often lead to low success in captive breeding and reintroduction efforts (Farquharson et al. 2018). Captive animals often experience altered diets, disease exposure, space use, and intra-/interspecific interactions relative to a wild baseline (Schulte-Hostedde and Mastromonaco 2015, West et al. 2019). Although these challenges are diverse, increased recognition has been paid to the critical role that microorganisms may play in either improving or impeding an individual's tolerance to captive conditions and their chances for survival in the wild (Redford et al. 2012, Bahrndorff et al. 2016, Ross et al. 2019, West et al. 2019, Diaz and Reese 2021). Host-associated microbial communities are often diverse both taxonomically and functionally, with documented associations between microorganisms and their genes (collectively termed the microbiome) (Marchesi and Ravel 2015), host evolutionary history and diet (Youngblut et al. 2019), disease prevalence (Rosshart et al. 2017, Bates et al. 2019), and overall fitness (Rosshart et al. 2017, Harrison et al. 2019, Worsley et al. 2021).

As the role of the microbiome in host health has become increasingly recognized, more refined views of captive organisms have emerged with a focus on how captive conditions alter host-microbiome interactions and influence host fitness (Martínez-Mota et al. 2020, Houtz et al. 2022). A growing body of literature has addressed questions specifically related to the impacts of captivity on host microbiome composition. For example, dietary differences may result in altered and

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more diverse gut microbiomes in captive vs. wild individuals (Metcalf et al. 2017), resulting in a mismatch between the gut microbiomes of captive animals being released and those of wild animals already in the population (Yao et al. 2019). External microbiomes may also be strongly influenced by host enclosure and captive conditions, with poorly understood consequences for the health of captive individuals (Hyde et al. 2016, Pinnell et al. 2020). These and similar results suggest that captive animals may benefit from captive conditions (e.g. diet and environment) relatively similar to wild conditions and/or an adjustment period in semi-wild conditions to gradually transition from captive to wild microbiomes.

While prior work provides useful perspective for the potential impacts of conservation headstarting and other captive-breeding efforts on microbiome change, most previous studies of captive influences on microbiome composition have focused on mammals, potentially limiting applicability to non-mammal taxa (Alberdi et al. 2021). Notably, reptiles are often headstarted in captivity and represent the third most frequently translocated taxonomic group, after mammals and birds (Seddon et al. 2014), likely reflecting the high proportion of reptiles considered vulnerable to extinction (Böhm et al. 2013, Bland and Böhm 2016). However, only recently have studies begun to focus on differences in microbiome composition between captive and wild reptiles (Jiang et al. 2017, Kohl et al. 2017, Fong et al. 2020, Sandri et al. 2020, Tang et al. 2020, Eliades et al. 2021, Filek et al. 2021).

Within reptiles, turtles are one of the groups most threatened by human activities (Böhm et al. 2013, Lovich et al. 2018) and, as a result, are frequently targeted for population management activities, including headstarting (Dodd and Seigel 1991, Germano and Bishop 2009). While a limited but growing number of studies have focused on microbiome composition in turtles (Campos et al. 2018, Parks et al. 2020, McKnight et al. 2020a, Filek et al. 2021, Parks et al. 2024), studies on the effects of captivity on turtle microbiomes are relatively few and have largely focused on marine turtles (Campos et al. 2018, Bloodgood et al. 2020, Filek et al. 2021, McNally et al. 2021), with limited published studies on freshwater turtles (Madison et al. 2018, Fong et al. 2020, Salleh et al. 2022). Given the importance of freshwater turtle conservation headstarting efforts, documenting the potential impacts of captivity on microbiome composition and corresponding fitness could provide valuable insight into best practices for manipulative conservation programs for at-risk turtle species.

In this study, we evaluated how captivity alters both internal (cloaca) and external (plastron) microbiomes of juvenile freshwater turtles, focusing on the IUCN-Endangered Blanding's turtle (*Emydoidea blandingii*), a long-lived turtle with a distribution centered around the North American Great Lakes (Ernst and Lovich 2009). Pressures posed by road mortality, habitat loss, and climate change have led to increased headstarting and translocation efforts for this species (Thompson et al. 2020, Wijewardena et al. 2023), making the evaluation of microbial effects on headstarting success particularly timely. Using data collected from both captive and wild populations of Blanding's turtles, we specifically addressed the following questions: (1) How do microbiomes differ between captive and wild turtles? (2) Do internal (cloaca) and external (plastron) microbiomes relate to one another? (3) How do the microbiomes of captive turtles change over time? (4) Is microbiome composition associated with growth and, by proxy, fitness of captive juvenile individuals being raised for headstarting? By addressing these questions, we provide new insights into the complex impacts of captivity on host-microbe interactions in turtles, which may in turn inform the design and implementation of captive-rearing and headstarting efforts for this sensitive taxonomic group.

Materials and methods

Study species and captive methods

Our study leveraged headstarting efforts conducted with Blanding's turtles from the Forest Preserve District of DuPage County (Illinois, USA) to explore microbiome associations with captive rearing in this species. Between September 2018 and May 2020, we sampled captive headstart hatchlings from partner facilities at Shedd Aquarium (Chicago, Illinois; hereafter, "Shedd") and Cosley Zoo (Wheaton, Illinois Park District; hereafter, "Cosley"). We collected turtle eggs from wild adults (within the Chicago-area region), incubated them until hatching, and raised the hatchlings in captivity for a year before releasing them into the wild.

At Shedd, we housed turtles in two separate tubs, with identifier numbers marked on their shells. We fully changed water in tubs once per week, with 30%–50% water top offs as needed throughout the week. For a full water change, we scrubbed tubs under fresh tap/hose water and rinsed the filters. At Cosley, we housed turtles in 11 separate tubs, eight of which were sampled for this project; these turtles were moved among tubs at various points throughout the project. We scrubbed, rinsed, and changed the water in Cosley tubs once per week. Additionally, at Cosley, we maintained four adult *E. blandingii* (originally hatched as part of the headstarting program) in separate containers from the juveniles.

Sample collection methodology

Sampling schedules and approaches varied between sample types, and a graphical summary of cloaca, plastron, and water sampling efforts in both captive and wild settings is shown in Fig. 1. More detailed information on sampling dates and efforts is available in Supplementary Materials S1.

Plastron and cloaca sampling, captive settings

We collected plastron and cloaca microbiome samples from captive turtles using sterile, nylon-tipped swabs, with swabbing periods lasting up to 10 s. For plastron sampling, we rinsed each turtle with sterile water prior to swabbing to remove transient bacteria (Lauer et al. 2007, McKnight et al. 2020a). We collected cloaca samples by gently inserting the tip of the swab into the cloaca and rotating it for 10 s. It was only possible to collect cloaca swabs on adults and large juveniles. Swabs were deposited in 5.0 ml cryotubes, immediately placed on ice, and stored in a freezer at −80°C.

We collected plastron samples from Shedd on two dates, one in February and another in April 2020, for 20 total captive juvenile plastron samples. At each sampling date, we sampled the same 10 turtles, and all individuals were switched between tubs. Sampled hatchlings were the offspring of three different females. We did not collect cloaca samples from turtles housed at Shedd. At Cosley, we collected plastron samples from 38 captive juvenile turtles (1–6 samples each) between September 2018 and August 2019 at 1–3-month intervals, for a total of 76 repeated samples. We also measured plastron length for these 38 turtles at ∼1-month time inter-

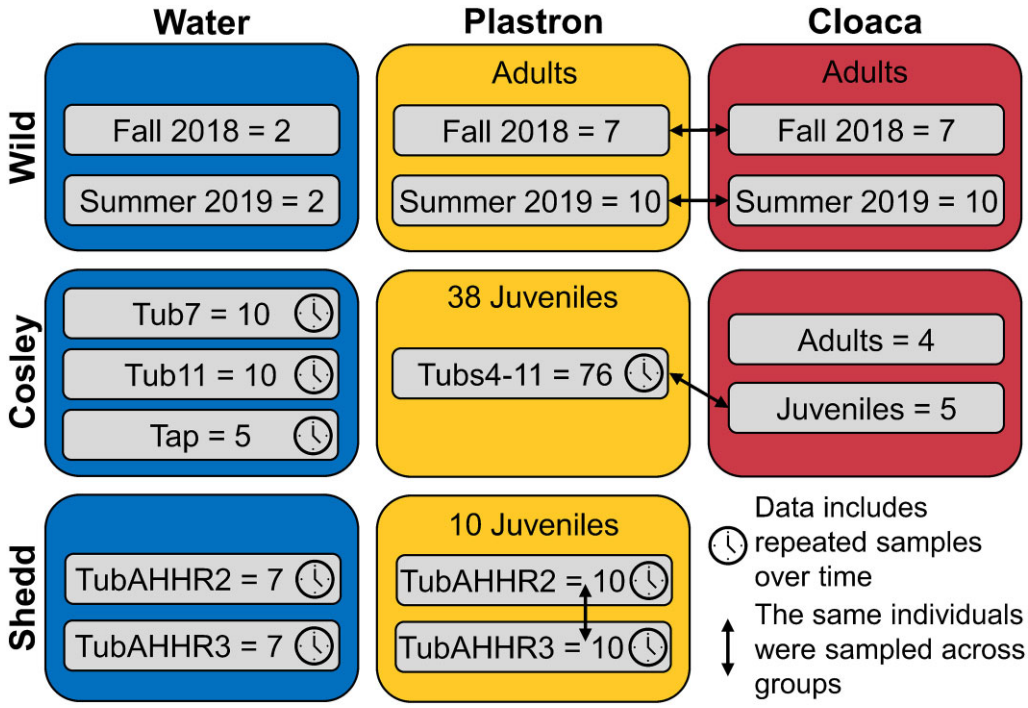

Figure 1. Diagram of sampling design. Numbers in internal boxes indicate total numbers of samples (repeated plastron samples were taken from 18 juvenile turtles at Cosley and 10 juvenile turtles at Shedd). Note that juveniles were sometimes moved among tubs. Cosley juveniles were sampled from eight different tubs.

vals between September 2018 and July 2019. Finally, we collected cloaca samples at Cosley from five juvenile turtles in August 2019 and from the four captive adults in February 2019 (Supplementary Materials S1).

Water sampling, captive settings

We collected water samples from captive enclosures in a 1-l autoclaved Nalgene bottle. We pre-filtered water samples using a sterile nylon mesh to catch large particulate matter before processing with membrane filtration. Membrane filters were deposited in a 5.0 ml cryotube, placed on ice, and stored in a freezer at −80°C. At Shedd, we collected water samples (two per tub) on four dates between February and May 2020 (14 samples total; only one per tub was available on one day). At Cosley, we collected pre- and post-water change samples from two tubs on 10 dates across November 2018, and 10 more across January–June 2019 (20 samples total). On five of these dates, we also collected samples of the tap water used to fill the tubs (Supplementary Materials S1).

Plastron, cloaca, and water sampling, wild settings

We collected 17 plastron and cloaca samples each from wild turtles captured in the northwest suburbs of Chicago; given the sensitive nature of this species in Illinois, exact locations are not provided. Sample collection protocols were as previously described, and sampling excursions occurred on five separate dates in September/October (fall) 2018 and June (summer) 2019. Near-shore water samples were likewise taken from these locations on four of the sampling dates (four samples total; Supplementary Materials S1) and processed using the same techniques described above.

DNA extraction and 16S rRNA sequencing

We extracted total DNA from swabs using the QIAGEN MagAttract Powersoil Kit and from filter membranes using the QIAGEN MagAttract Powerwater Kit, including negative control (blank) extractions with each batch of samples. We amplified the V4 region of bacterial and archaeal 16S rRNA using PCR primers 515f (Parada et al. 2016) and 806rB (Apprill et al. 2015) that were further modified to include 12 bp index barcodes on the 515f primer (Walters et al. 2016). PCRs were performed in replicate 25 µl reactions containing 12.5 µl Phusion Hot-Start Flex 2X MasterMix (New England Biolabs), 0.2 µM final concentrations of forward primer 515f and reverse primer 806rB, 2 µl of template DNA, and nuclease free water. We also prepared negative controls containing no template DNA with each set of PCR replicates. Thermal cycling conditions were as follows: 98°C for 30 s, 30 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 5 min at 72°C. PCR products were electrophoresed in 1.8% agarose gels to confirm amplification of the V4 region. We then cleaned and normalized 25 µl of each amplicon library using the SequalPrep™ Normalization Plate Kit (Applied Biosystems), followed by pooling of equal volumes of each normalized library. The pooled amplicon library was then quantified using a Qubit™ 3.0 fluorometer and Qubit™ dsDNA HS Assay Kit (Life Technologies) and prepared for sequencing using standard Illumina protocols. Paired-end sequencing for a total of 500 cycles was conducted on the Illumina MiSeq platform using custom sequencing primers described previously (Caporaso et al. 2012) with addition of 10% PhiX Control library (Illumina).

16S rRNA sequence filtering and quality control

First, we denoized sequence data using DADA2 (Callahan et al. 2016). Based on assessment of quality scores, we truncated forward reads to length of 230 base pairs and discarded reverse reads. We then filtered amplicon sequence variants (ASVs) for a minimum frequency of 100 reads across all samples, and occurrence in a minimum of three samples. We identified ASV and corresponding taxonomy using QIIME2 version 2021.2 (Bolyen et al. 2019) and the SILVA database version SSU Ref NR 99 138.1 (trimmed to represent only the 16S V4 region, positions 515 through 806; Quast et al. 2012). All hits to chloroplast and mitochondria were removed.

Additionally, we amplified and sequenced three ZymoBIOMICS Microbial Community DNA Standards (Zymo Research D6305) containing eight types of bacteria each (three Gram-negative, five Gram-positive) to evaluate the consistency and potential biases of our laboratory procedures. They showed a high level of consistency (mean Bray–Curtis dissimilarity = 0.096) and reasonably low levels of bias, with mean (across the three samples) proportion of reads per bacteria species ranging from 0.066–0.180, compared to expected values of 0.125 per bacteria species (see https://github.com/Testudinidude/BlandingsMicro for more details on these analyses). It should also be noted that *Salmonella enterica* DNA was separated into two ASVs, and an unexpected *Deinococcus* sp. was sequenced in all three mock communities (0.1% of reads in each sample) but was not sequenced in the blank. The blank only returned sequences of nine bacteria, with a total of 70 reads. This suggests a very low background level of contamination, which would have been swamped by the comparatively high yields in actual samples (10 801–60 389 reads). Therefore, we did not attempt to correct for contamination.

Statistical analyses

We analyzed trends in alpha and beta diversity to identify patterns within water, cloaca, and plastron data and facilitate comparisons between plastron and cloaca samples. We were broadly interested in assessing the following: (1) patterns of diversity in captive and wild microbiomes (both for turtles and water), (2) differences and similarities between internal (cloaca) and external (plastron) microbiomes for wild turtles, (3) variability in captive microbiomes through time, and (4) associations between captive turtle growth and microbiome composition. Within the context of these primary questions, we specifically addressed nine analytical priorities by applying varied subsets of our data (Table 1).

For alpha diversity, we examined richness (total count of unique ASVs per sample) and evenness (Shannon's diversity index divided by the natural logarithm of richness) separately rather than combining them into a single diversity metric (e.g. Shannon) to avoid confounding the two aspects of alpha diversity (i.e. a high Shannon diversity can be caused by high richness or high evenness). For captive juveniles, we only included the samples from the last day of sampling (i.e. the samples closest to when turtles would be released and, therefore, the most relevant for headstarting) to assess alpha diversity (except for tests looking at changes over time or associations with size or growth). For beta diversity, we used presence/absence metrics (Jaccard dissimilarities and unweighted UniFrac distances) and abundance-based methods (Bray–Curtis dissimilarities and weighted UniFrac distances). For abundance-based methods, read counts were transformed to proportions per sample (i.e. total sum normalization) prior to calculating the distances and dissimilarities (McKnight et al. 2019).

Model types and variables for each question are presented in Table 1 and detailed text descriptions of each model can be found in Supplementary Materials S2. An R markdown with complete code and details of statistical methods and results is available at https://github.com/Testudinidude/BlandingsMicro (a corresponding navigation guide is provided in Supplementary Materials S2). We performed all analyses in R (v 4.0.3; R Core Team 2022). We used linear models to analyze richness and evenness. We constructed fixed effects models using the lm function in base R and constructed mixed effects models using the lme4 package (v1.1-26; Bates et al. 2014). We checked model assumptions using QQ plots and residual plots via the performance package (v0.7.0; Lüdecke et al. 2021) and performed statistical analyses using the Anova function in the car package (v3.0-10; Fox and Weisberg 2011) with type II sums of squares. We conducted *post hoc* Tukey tests using the emmeans package (v1.5.4; Lenth and Lenth 2018). For beta diversity, we performed PERMANOVAs using the adonis2 function in the vegan package (v2.5-7; Oksanen et al. 2007). For all beta diversity tests, we adjusted *P*-values using sequential Bonferroni corrections to control the family wise type 1 error rate across the four beta diversity metrics, and adjusted *P*-values are presented in the results (unadjusted *P*-values are also available in the aforementioned GitHub repository). We also performed beta diversity analyses that were constricted to the family level; these results were generally similar and are also presented in the GitHub repository.

Many models included date as a covariate. Unless otherwise specified, date was included as a continuous variable (numeric) and was scaled to *Z*-scores by subtracting the mean and dividing by the standard deviation. We also note that in 2018, wild turtles were sampled in the fall, whereas in 2019, they were sampled in the summer. Therefore, season and year are confounded for wild turtles, and any models using season or year as a variable cannot distinguish them. They are noted as "year (season)" throughout.

Although rarefaction curves suggested that sufficient read depth had been achieved, there was a strong positive association between read depth and richness, even after rarefying the data. Therefore, we used the original data (not rarefied) and included the $\log_{10}$ of the read depth as a covariate in all models (log transforming the read depth resulted in better model fits).

Results

In the following sections, we address the taxonomic composition of sampled plastron, cloaca, and water communities, and the results of statistical tests as described in Table 1. The outcomes of statistical tests are summarized in Fig. 2, and representative figures are subsequently provided throughout this section. Complete details and results of statistical tests are available here: https://github.com/Testudinidude/BlandingsMicro.

Diversity and composition of microbial communities

Overall, sampled microbial communities across captive and wild sites were highly diverse. Both water samples and plas-

Table 1. Study aims and questions addressed, as well as model structures.

Aims/questions	Sites	Predictors	Reponses	Model
(1) Captive vs. wild effects				
1a. Do microbiomes differ between captive and wild plastrons?	Cosley, Shedd, wild	Site/tub # or year	Beta diversity	PERMANOVA
1b. Do microbiomes differ between captive and wild cloaca?[a]	Cosley, wild	Site or year	Beta diversity	PERMANOVA
1c. Do water microbiomes vary between captive and wild sites?	Cosley, Shedd, wild	Site / tub # or wetland + date (categorical; random)	Alpha diversity	Linear
1d. Do tub and tap water microbiomes differ at captive sites?	Cosley	Sample type (tub or tap) / tub # + date	Alpha diversity	Linear
			Beta diversity	PERMANOVA
(2) Internal vs. external microbiomes (wild turtles)				
2a. Are there correlations between individuals' cloaca and plastron microbiomes?[b]	Wild	Year (categorical) + cloaca alpha diversity	Plastron alpha diversity	Linear
		Cloaca beta diversity	Plastron beta diversity	Mantel
2b. Are there differences between individuals' cloaca and plastron microbiomes?[b]	Wild	Sample type * year (categorical) + turtle ID	Alpha diversity	Linear
			Beta diversity	PERMANOVA
(3) Time effects in captivity				
3a. Do captive plastron microbiomes change over time and vary among individuals?[c]	Cosley, Shedd	Tub # + date + turtle ID	Alpha diversity	Linear
			Beta diversity	PERMANOVA
3b. Do captive tub (water) microbiomes change over time?	Cosley, Shedd	Tub # + date[d]	Alpha diversity	Linear
			Beta diversity	PERMANOVA
(4) Associations between microbiomes and growth				
4 Are captive plastron microbiomes associated with plastron length or growth?[e]	Cosley	Growth or plastron length + turtle ID (random) + date_tub # (random)see[c]	Alpha diversity	Linear
			Beta diversity	PERMANOVA

Alpha diversity = the same model structure was used to test richness and evenness. All models include read depth (log10 transformed), except for correlations between cloaca and plastron samples. Random = a mixed effects model was used, and the previous variable was included as a random intercept (note that all variables were fixed for the PERMANOVAs). For predictor variables, + = main effect, * = interaction, / = the second variable was nested in the first. Date was entered as a continuous variable unless otherwise specified.

[a]Cloacal samples were not collected from Shedd. Made pairwise comparisons among captive juveniles, captive adults, wild adults in 2018, and wild adults in 2019 (a sequential Bonferroni correction was applied).

[b]Paired plastron and cloaca samples were only collected from wild individuals.

[c]At Cosley, only individuals with at least three samples were included, and a random intercept that combined date and tub # was included to account for individuals shuffling among tubs.

[d]At Cosley, we included an additional variable noting whether the sample was collected before or after a water change.

[e]Only individuals with ≥3 samples were used (no Shedd or wild turtles had ≥3 samples).

tron samples featured over 1300 ASVs each (combined wild and captive sites), while nearly 700 ASVs were recovered from cloaca sampling of Cosley and wild turtles combined. The distribution of dominant ASVs was largely consistent across samples within specific combinations of sample type (plastron, cloaca, water), age category (juvenile, adult), and collection site (Cosley, Shedd, wild) (Fig. 3).

Cosley and wild turtle cloaca samples were dominated by Bacteroidia (phylum Bacteroidota) and Gammaproteobacteria (Proteobacteria), while Firmicutes were variably present across captive and wild samples, and Spirochaetota were only present in wild samples (Fig. 3). Weeksellaceae was the most prevalent family in both Cosley and wild cloaca samples, accounting for 22% and 11.3% of sequence reads, respectively, while Neisseriaceae (13.6% and 3%, respectively) was also fairly common from both locations (Table 2).

Plastron samples were consistently dominated by several classes of bacteria, including Alphaproteobacteria (phylum Proteobacteria), Bacteroidia (Bacteroidota), Blastocatellia (Acidobacteriota), Deinococci (Deinococcota), and Gammaproteobacteria (Proteobacteria) (Fig. 3). At the family level, Deinococcaceae was the most common taxon recovered from both Cosley (30.2% of sequence reads) and Shedd (18.8% of sequence reads) plastrons, and second only to Weeksellaceae on wild plastrons (26.5% vs. 21%). Weeksellaceae, Blastocatellaceae, and Sphingomonadaceae were also common to plastrons across all three sites, with the exception that Blastocatellaceae was rare on wild plastrons (<1%) (Table 2).

Water samples, including source tap water, featured high proportions of Actinobacteria (phylum Actinobacteriota) (Cosley tap and tubs, wild), Alphaproteobacteria (Cosley tap and tubs, Shedd tubs), Clostridia (Firmicutes) (tap water and Shedd tubs, primarily), and Gammaproteobacteria (Cosley and Shedd tubs, wild) (Fig. 3). Cosley tap water was strongly dominated by Mycobacteriaceae at the family level (59.6% of

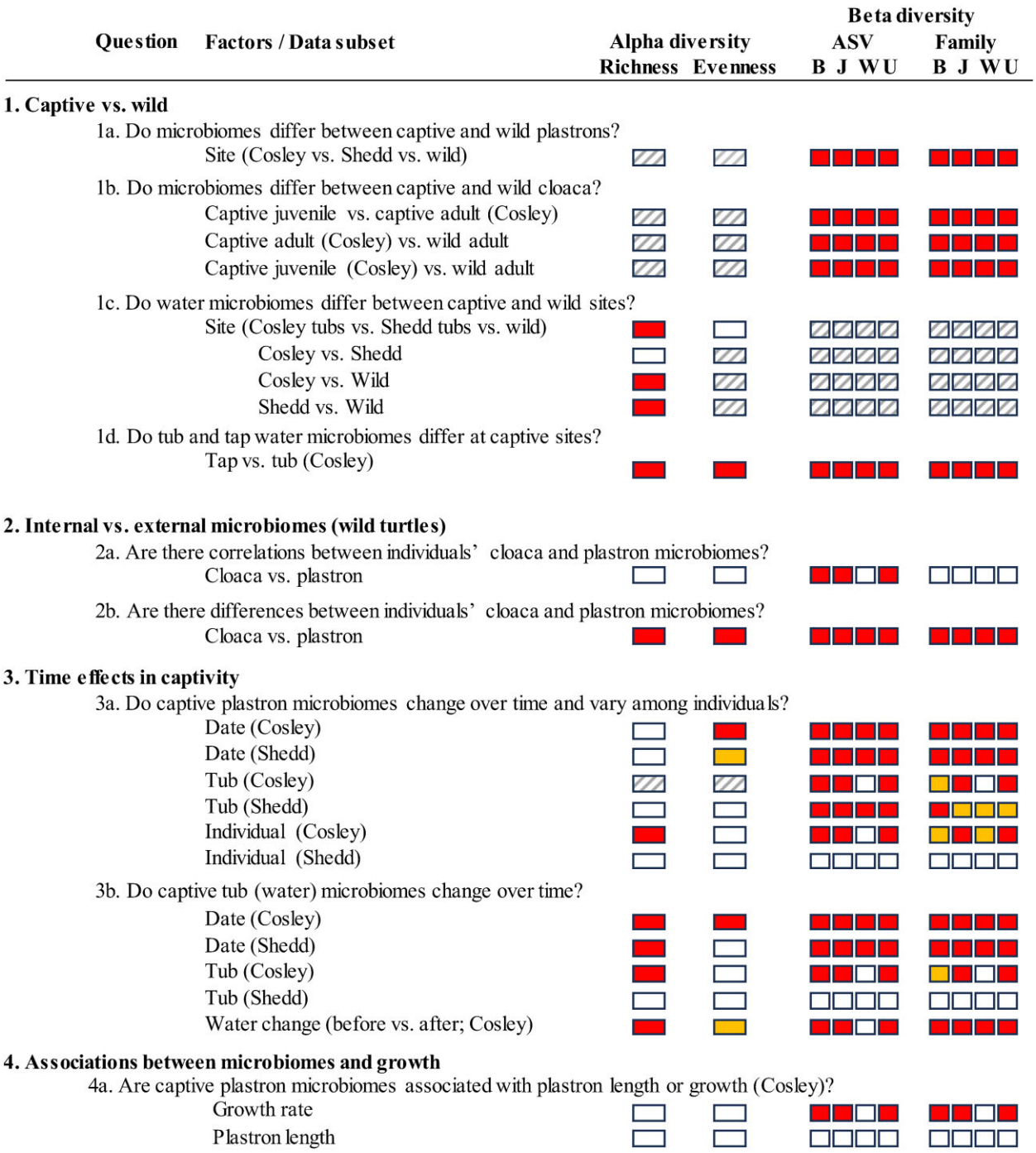

Figure 2. Diagram of statistical analyses conducted for each question outlined in Table 1. Red boxes indicate significant findings ($P < .05$), orange boxes indicate marginally significant findings ($0.05 < P < .10$), white boxes indicate nonsignificant findings ($P > .10$), and hashed boxes indicate cases where analyses were not conducted (typically due to low sample sizes for comparisons). For beta diversity comparisons, B = Bray–Curtis, J = Jaccard, W = Weighted Unifrac, and U = Unifrac distances.

sequence reads), with substantial proportions of Sphingomonadaceae (14.2%) and Xanthobacteraceae (11.5%). In Cosley tubs, Tannerellaceae were most prevalent (10.8%), although Mycobacteriaceae (5.3%), Sphingomonadaceae (7.7%), and Microbacteriaceae (7.3%) were also somewhat common. No families reached above 10% abundance in sequence reads for Shedd tub water, although Cyanobacteriaceae (8.4%), Sphingomonadaceae (4.5%), and Tannerellaceae (4.0%) were somewhat common. Wild water samples were dominated by Microbacteriaceae (36.6%) and Sporichthyaceae (10.2%) (Table 2).

Aim 1. Captive vs. wild effects

Turtle microbiomes differed between captive and wild turtles, as well as between both captive sites. At the level of ASV, samples appeared strongly differentiated between captive and wild turtles for both cloaca and plastron microbiomes (Cosley

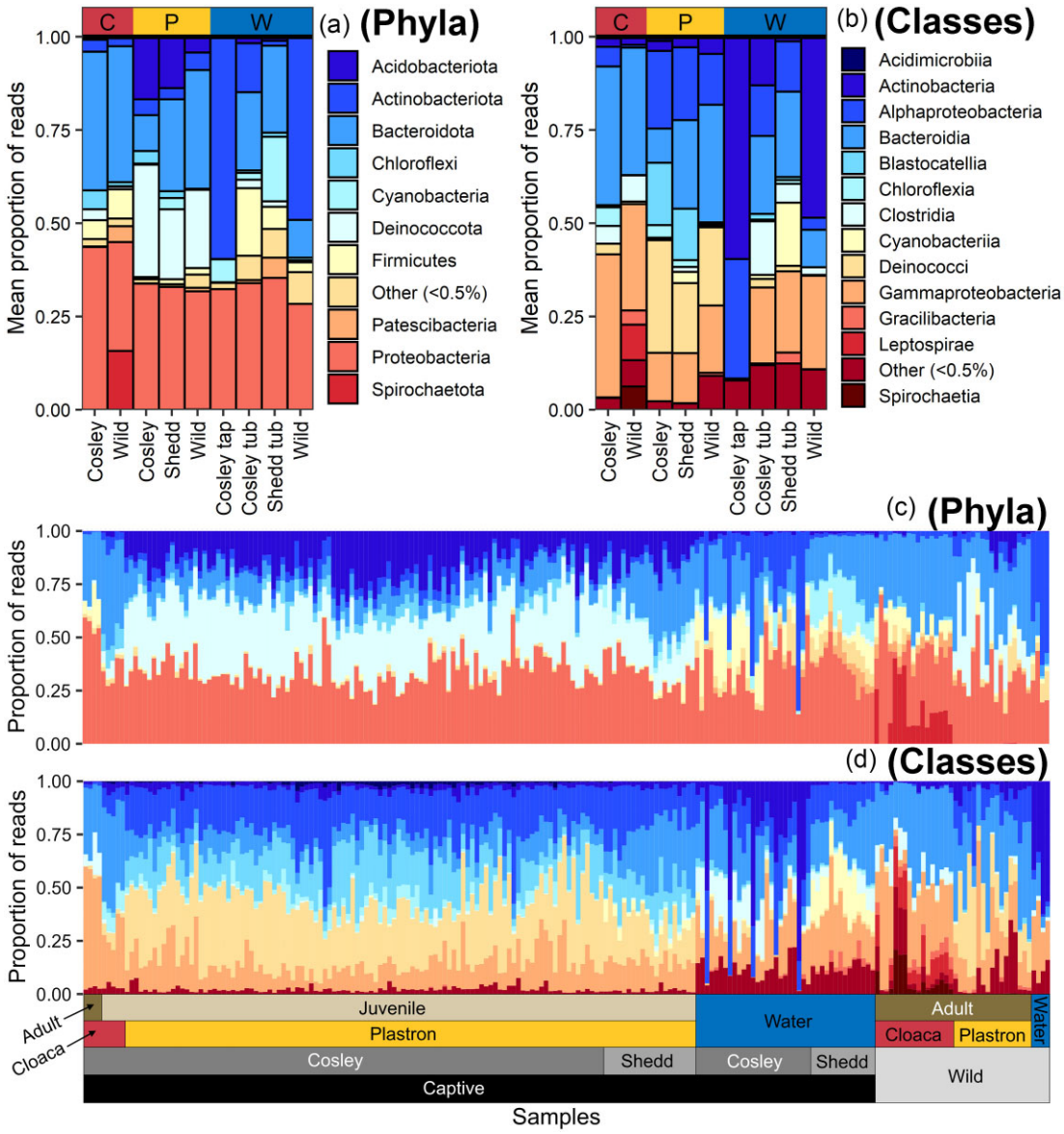

Figure 3. Stacked barplots showing the proportion of reads belonging to each phylum (a and c) and class (b and d). (a) and (b) showed the proportions averaged for each category (after standardizing the read depth), and (c) and (d) show the proportions for each sample. In (a) and (b), C = cloaca, P = plastron, and W = water. Only phyla or classes that comprised >= 0.5% of all reads are shown as unique colors (the remainder are combined in the "other" category). In the Cosley water samples, the five samples that are dominated by Actinobacteria and Alphaproteobacteria were from the tap water used to fill tubs. Stacked bar plots at the order and family levels are available in the Supplemental Information.

and Shedd vs. wild) and between captive sites for plastron microbiomes (Fig. 4a, b). Of all ASVs found on the plastrons of wild adults or captive juveniles from either captive-rearing site (1330), only 128 (9.6%) were found on all three groups of turtles (Fig. 4b). Likewise, 413 (63.4%) of the ASVs and 74 (41.2%) of the families found on wild adult plastrons were not found on any captive juvenile plastrons, and the majority of plastron-associated ASVs from juvenile turtles at each captive site were not found on wild adults (Fig. 4b). Plastron ASVs for Cosley and Shedd were also more frequently shared than wild ASVs, supporting greater commonalities in plastron microbiomes between captive settings (Fig. 4a). Cloaca results were similar, with 340 (73.8%) of the ASVs and 81 (53.3%) of the families from wild adults not occurring in either captive adults or captive juveniles from Cosley (Fig. 4a). There were also large microbial differences between captive adults and captive juveniles. Overall, plastron samples were more similar to the local captive environment than were cloaca samples, as a larger proportion of plastron ASVs were shared with water samples across all sites combined (Fig. 4c).

Beta-diversity also differed among sites. For plastrons, we recovered significant differences in beta diversity among all sites (wild, Cosley, and Shedd) for all four distances/dissimilarities (all $F_{2,32} > 9.5$, all $P < .001$; ordination in Fig. 5c). Likewise, for all metrics, beta diversity in cloaca samples was significantly different in all pairwise comparisons of Cosley juveniles, Cosley adults, wild adults (2018), and wild adults (2019) (all $P < .016$; ordination in Fig. 5e; note that cloaca samples were not available from Shedd, and plastron samples were not available from captive adults).

Patterns in alpha diversity were variable for turtles in captive and wild settings, but difficult to test due to incom-

Table 2. Abundant bacterial families across sample types and locations.

Cloaca		Plastron			Water			
Cosley	Wild	Cosley	Shedd	Wild	Cosley tap	Cosley tub	Shedd tub	Wild
Weeksellaceae (Bacteroidota) (22%)	**Weeksellaceae (11.3%)**	**Deinococcaceae (Deinococcota) (30.2%)**	**Deinococcaceae (18.8%)**	**Deinococcaceae (21%)**	**Mycobacteriaceae (Actinomycetota) (59.6%)**	Mycobacteriaceae (5.3%)	Mycobacteriaceae (<1%)	Mycobacteriaceae (1.8%)
Neisseriaceae (Pseudomonadota) (13.6%)	Neisseriaceae (3.0%)	**Blastocatellaceae (Acidobacteria) (16.5%)**	**Blastocatellaceae (11.5%)**	Blastocatellaceae (<1%)	**Sphingomonadaceae (Pseudomonadota) (14.2%)**	Sphingomonadaceae (7.7%)	Sphingomonadaceae (4.5%)	Sphingomonadaceae (<1%)
		Weeksellaceae (Bacteroidota) (4.2%)	**Weeksellaceae (16.2%)**	**Weeksellaceae (26.5%)**	**Xanthobacteraceae (Pseudomonadota) (11.5%)**	Xanthobacteraceae (<1%)	Xanthobacteraceae (<1%)	Xanthobacteraceae (<1%)
		Sphingomonadaceae (Pseudomonadota) (9.9%)	**Sphingomonadaceae (11.3%)**	Sphingomonadaceae (6.3%)	Tannerellaceae (Bacteroidota) (<1%)	**Tannerellaceae (10.8%)**	Tannerellaceae (4.0%)	Tannerellaceae (<1%)
					Cyanobacteriaceae (Cyanobacteria) (0%)	Cyanobacteriaceae (0%)	**∗Cyanobacteriaceae (8.4%)**	Cyanobacteriaceae (0%)
					Microbacteriaceae (Actinomycetota) (0%)	Microbacteriaceae (7.3%)	Microbacteriaceae (<1%)	**Microbacteriaceae (36.6%)**
					Sporichthyaceae (Actinomycetota) (0%)	Sporichthyaceae (0%)	Sporichthyaceae (<1%)	**Sporichthyaceae (10.2%)**

Bacterial families indicated in bold represent > 10% of sequence reads for a sample type/location. Bacterial families not in bold are included for comparison within a sample type. Associated phyla for families are shown in parentheses in the first column for each sample type; percentages of sequence reads for sample type/location are shown in parentheses for all sample types/locations. ∗Cyanobacteriaceae is included for Shedd tub water as the family closest to reaching 10% of sequence reads in that sequence pool.

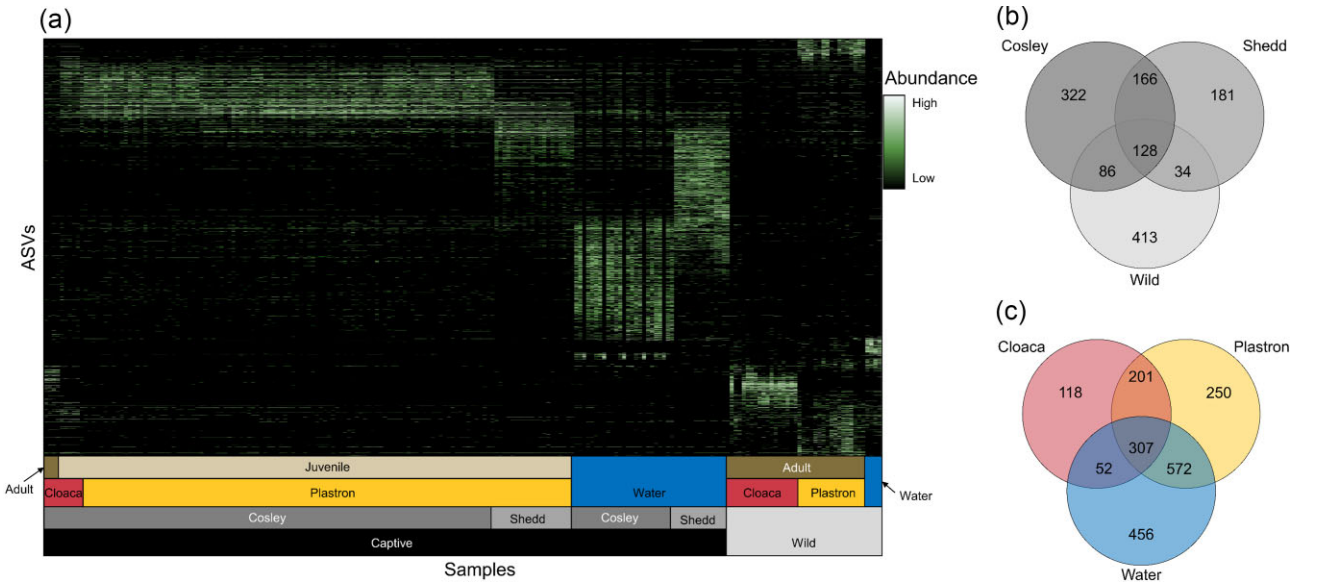

Figure 4. On the left (a), a heatmap of ASV-level abundance, with experimental factors denoted below. On the right, overlap in plastron sample ASVs between captive-rearing facilities and wild animals (b) and overlap in ASVs between body sections and water samples, regardless of whether samples came from captive or wild animals (c). In the Cosley water samples, the five samples appearing as vertical black lines with few reads were the tap water samples.

plete sampling across factor levels. However, suggestive trends are apparent in boxplots (Fig. 6) and are briefly described here. First, wild adults showed differences between years (seasons) with higher plastron richness and evenness in fall 2018 (Fig. 6c, d) and higher cloaca richness and evenness in summer 2019 (Fig. 6e, f). Captive juvenile plastrons (at both sites) generally had lower richness and evenness compared to fall 2018 wild adults, but similar levels to summer 2019 wild adults (Fig. 6c, d). In contrast, cloaca richness was generally higher for captive juveniles than wild adults of either year (Fig. 6e). Cloaca richness and evenness of captive adults were substantially lower than the richness and evenness of both captive juveniles and wild adults (Fig. 6e, f).

Strong differences in water-associated communities among sites (wild, Cosley, and Shedd) were apparent in both heatmaps (Fig. 4a) and ordination plots (Fig. 5b). Water samples also had a greater proportion of unique ASVs (compared to plastron and cloaca samples) across all sites (Fig. 4c). Richness of captive tub and wild water samples differed significantly among sites ($\chi^2 = 88.8, P < .001$), but evenness did not differ significantly ($\chi^2 = 3.4, P = .187$; Fig. 6a, b). The difference in richness was driven by the captive sites, which had significantly higher richness than wild samples (both $P < .001$) but were not significantly different from each other ($P = .145$). Nevertheless, all sites were highly differentiated in community composition. Across wild, Cosley, and Shedd sites, the following numbers (and percentages) of ASVs were unique (compared to other water sources): Cosley (tubs) = 441 (51.5%), Shedd (tubs) = 365 (46.0%), wild = 155 (73.1%). Indeed, out of all the water-associated ASVs from any site, only 31 (0.02%) were present at all three sites. Results at the family level were somewhat less pronounced, but still showed strong differences. Additionally, within Cosley, the water microbiomes between tubs differed significantly for richness ($F_{1,15} = 8.4, P = .011$) and beta diversity (for every metric other than weighted unifrac), but not for evenness ($F_{1,15} = 2.1, P = .165$). In contrast, within Shedd, tubs were not significantly different for richness ($F_{1,10} = 1.2, P = .297$), evenness ($F_{1,10} = 0.2, P = .670$), or beta diversity (all $F_{1,10} < 1.2$, all $P > .992$).

Aim 2. Internal vs. external microbiomes (wild turtles)

Although our dataset did not have paired cloaca-plastron samples for captive turtles, we were able to assess cloaca-plastron correlations in wild turtles. We did not recover a significant correlation for richness ($F_{1,13} = 0.9, P = .357$) or evenness ($F_{1,13} = 1.2, P = .292$) between plastron and cloaca communities. However, beta diversity was significantly positively correlated between plastron and cloaca communities at the ASV level (but not the family level) for all distances/dissimilarities (all Mantel's $r > 0.331$, all $P < .023$) except for weighted Unifrac distances (Mantel's $r = -0.112$, $P = .594$). For wild adults, plastron communities were significantly richer than cloaca communities ($F_{1,16} = 15.4, P = .001$) (Fig. 6a). There was a significant interaction with year (season), but plastron communities had higher richness than cloaca communities in both years (seasons). The comparison of mean evenness was driven by a strong interaction between sample type and year (season) ($F_{1,16} = 18.9, P < .001$). Plastron evenness was slightly higher than cloaca evenness in 2018 (fall), but lower than cloaca evenness in 2019 (summer) (Fig. 6d, f). Beta diversity between plastron and cloaca samples was significantly different for all distances/dissimilarities (all $F_{1,16} > 10.1$, all $P < .001$; ordination shown in Fig. 5a).

Aim 3. Time effects in captivity

Within the Cosley juvenile plastron samples, richness was significantly influenced by individual ($\chi^2 = 32.6, P = .013$); however, there was no consistent change over time ($\chi^2 = 0.0, P = .830$), with some individuals showing increases in richness while others showing decreases. Evenness of plastron microbial communities, in contrast, increased significantly over time ($\chi^2 = 6.4, P = .011$), but no significant effect of individual

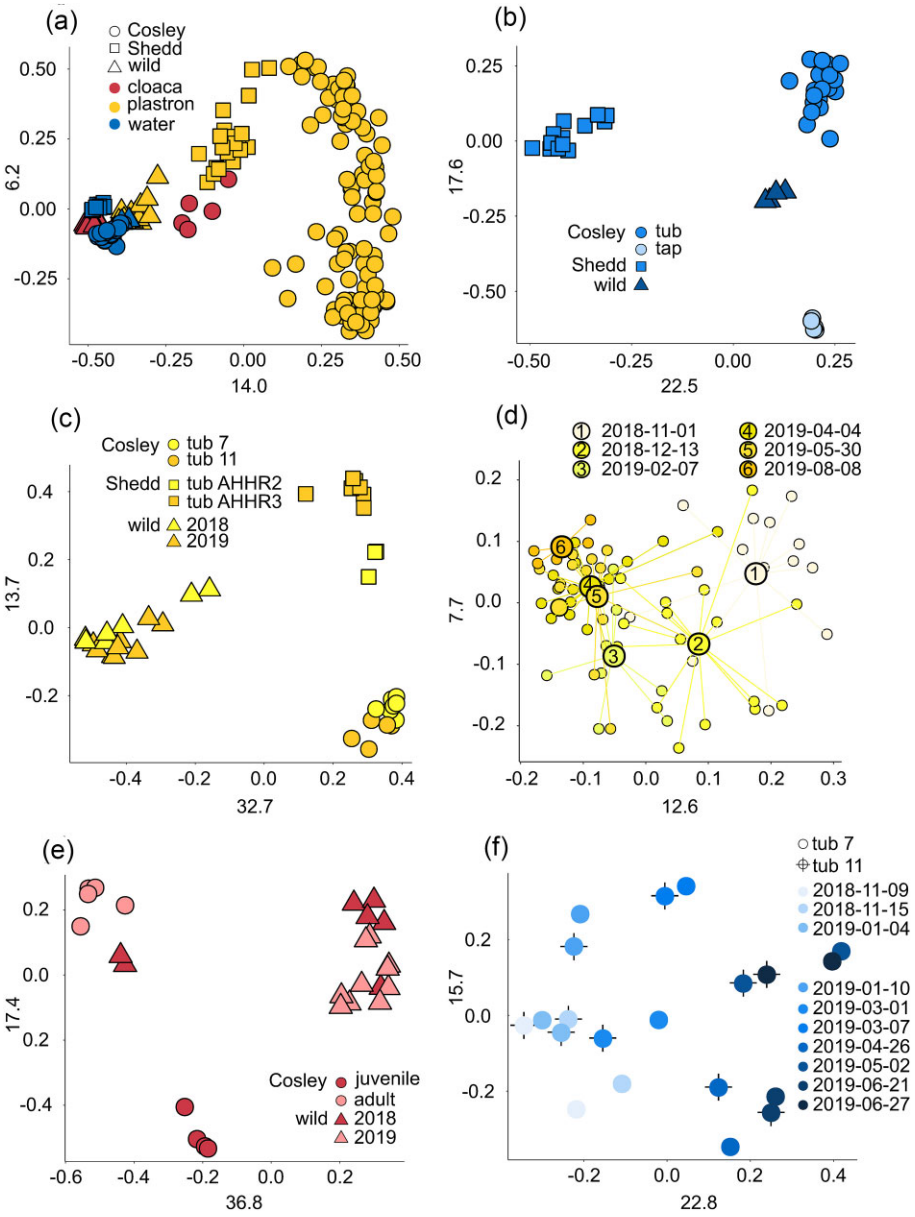

Figure 5. Ordination plots for beta diversity comparisons of sample type, site/source, and year. All plots shown are for Bray–Curtis dissimilarities at the level of ASV. Plots for additional metrics and at the family level are available at https://testudinidude.github.io/BlandingsMicro/. (a) Overall patterns of beta diversity across sample types and locations. (b) Differences in water sample diversity by site and source. (c) Plastron diversity by site, tub, and year. (d) Differences in plastron diversity of Cosley juvenile turtles across sampling dates; numbered circles are centroids of distribution for each sampling date. (e) Cloaca diversity for Cosley and wild samples by age and year. (f) Water diversity for Cosley tubs by date and tub.

was recovered ($\chi^2 = 13.7$, $P = .686$). For Shedd juvenile samples, neither richness or evenness were significantly influenced by time (richness: $F_{1,6} = 1.4$, $P = .278$; evenness: $F_{1,6} = 4.5$, $P = .078$) or individual (richness: $F_{9,6} = 0.9$, $P = .576$; evenness: $F_{9,6} = 0.5$, $P = .799$), although small sample sizes (ten individuals over two dates) made it difficult to draw strong conclusions.

Beta diversity of plastron communities for Cosley juveniles changed significantly over time (date) for all distances/dissimilarities (all $F_{1,48} > 9.6$, all $P < .001$) and differed by individual turtle (all $F_{17,48} > 1.5$, all $P < .001$) and tubs (all $F_{7,48} > 1.6$, all $P < .001$) for all distances/dissimilarities except weighted Unifrac distances (individuals: $F_{17,48} = 1.3$, $P = .109$; tubs: $F_{7,48} = 1.3$, $P = .173$; Fig. 5d). In other words, the microbial composition of the turtles' plastrons collectively shifted over time, but there continued to be a distinct signature of individual turtles and tubs.

Beta diversity of Shedd juveniles was also significantly associated with date (all $F_{1,7} > 4.1$, all $P < .001$) and tub (all $F_{1,7} > 1.9$, all $P < .044$), but not with individual (all $F_{9,7} < 1.2$, all $P = .904$; Fig. 5c). The lack of effect of individual was likely influenced by limited sampling per individual turtle (two samples per turtle).

In Cosley tubs, richness of water communities decreased significantly over time ($F_{1,15} = 20.4$, $P < .001$), while a significant richness increase was observed in Shedd tubs over time ($F_{1,10} = 19.7$, $P = .001$). We also note that richness was significantly higher in Cosley tubs after water changes com-

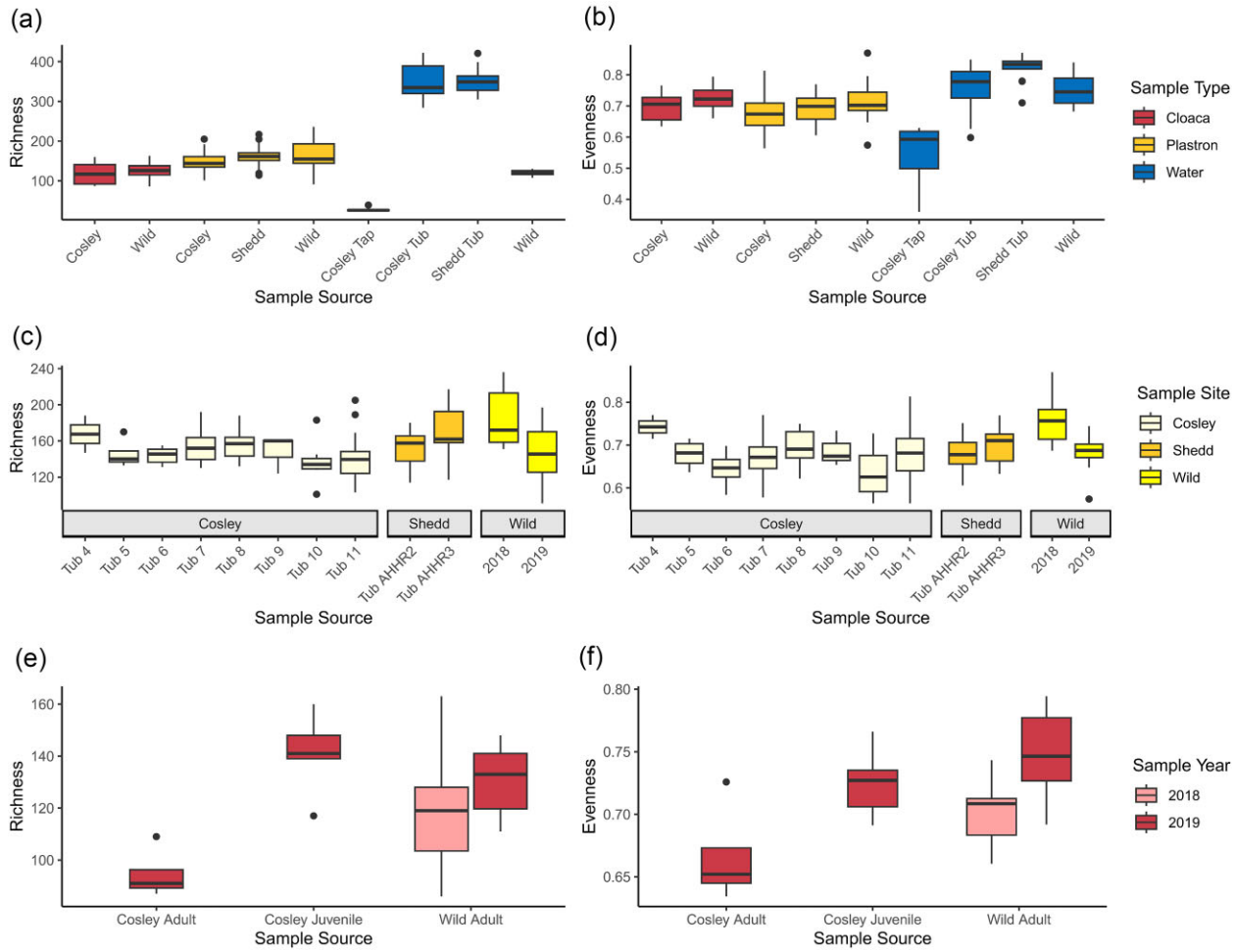

Figure 6. Variation in alpha diversity richness (a, c, and e) and evenness (b, d, and f) by sample type (cloaca, red; plastron, yellow; water, blue) (a, b), site (Cosley, Shedd, or wild) (c, d), and year (2018 or 2019) (e, f). Note that (c) and (d) display only plastron richness and evenness, whereas (e) and (f) only display cloaca richness and evenness.

pared to before water changes ($F_{1,15} = 17.5$, $P < .001$), which could indicate a disruption of settled microbes during water changes due to physical agitation. Evenness in Cosley tub water communities decreased significantly over time ($F_{1,15} = 7.5$, $P = .015$), but evenness did not significantly change over time in Shedd tub water communities ($F_{1,10} = 0.3$, $P = .591$). The beta diversity of water samples also changed significantly over time based on all distance/dissimilarity metrics for both Cosley (all $F_{1,15} > 3.2$, all $P < .001$) and Shedd (all $F_{1,10} > 3.3$, all $P < .004$).

Aim 4. Associations between microbiomes and growth

Neither richness nor evenness were significantly correlated with plastron length (richness: $\chi^2 = 1.9$, $P = .164$; evenness: $\chi^2 = 0.0$, $P = .844$) or plastron growth rates (richness: $\chi^2 = 1.7$, $P = .193$; evenness: $\chi^2 = 0.0$, $P = .989$) for captive (Cosley) juvenile turtles. Plastron growth rate was, however, significantly associated with beta diversity for all distances/dissimilarities (all $F_{1,48} > 2.4$, all $P < .021$) except weighted Unifrac distances ($F_{1,48} = 1.6$, $P = .163$). Plastron length was not significantly associated with beta diversity (all $F_{1,48} < 1.9$, all $P = .318$).

Discussion

We compared plastron, cloacal, and water microbial communities associated with the Blanding's turtle (*E. blandingii*) between captive and wild individuals and settings, as well as across time and age groups. This is the first study to encompass all of these comparisons for a freshwater turtle species and should provide useful insights for captive-based husbandry and conservation efforts in Blanding's turtles, as well as other freshwater turtle species.

Diversity and composition of microbial communities

As with previous studies of freshwater turtle-associated microbiomes, we recovered highly diverse communities associated with shells and cloaca of captive turtles (Fig. 3). Additionally, water microbial diversity increased over time from tap water sources, unexpectedly exceeding the richness of water samples from wild release sites (Fig. 6). Although the majority of our samples were from captive settings, we can nonetheless report some similarities in microbiome compositions to previous reports on wild turtles and their environments. For example, we recovered high frequencies of several phyla from our plastron surfaces (Pseudomonadota, Baterioidota, Deinococcota) also reported as common from plastrons of wild fresh-

water red-eared sliders (*Trachemys scripta*) and western pond turtles (*Emys marmorata*) in the United States (Parks et al. 2020, White et al. 2023) and Krefft's river turtle (*Emydura macquarii krefftii*) in Australia (McKnight et al. 2020a). Similarly, high frequencies of Bacteroidota and Pseudomonadota in Blanding's turtle cloaca samples are reflective of similar results in western pond and Krefft's river turtles (McKnight et al. 2020a, White et al. 2023). In particular, the prevalence of these two phyla in the cloacal samples of marine loggerhead turtles (*Caretta caretta*) (Filek et al. 2021) suggests that the turtle cloacal microbiome is somewhat consistent across distantly related species at least at higher taxonomic levels. At the same time, we recovered numerous differences in both alpha and beta diversity between captive and wild turtles and settings, as well as within captive locations (discussed below). This recapitulates previous findings that captive microbiomes may be substantially differentiated from wild microbiomes in turtles (Fong et al. 2020), even under short-term captivity periods (Filek et al. 2021). Our findings are also consistent with broad patterns in captive vs. wild animals ranging from amphibians (Kueneman et al. 2022) to birds (Oliveira et al. 2020) to primates (Clayton et al. 2016).

Aim 1. Captive vs. wild effects

Both plastron and cloaca microbiomes differed between captive and wild turtles. Microbial community beta diversity differed significantly in all our comparisons across both plastron and cloaca communities, including between wild and captive turtles, and between juvenile and adult captive turtles (Fig. 5a, c, e). Similarly, both cloaca richness and evenness differed between captive adults (both lower) vs. wild adults (both higher) (Fig. 6e, f). Adding yet more complexity, the differences between captive juvenile plastron samples and wild adult plastron samples changed between years (seasons) (Fig. 6c). Counter to expectations, we recovered richer microbial communities in captive water samples compared to two wild wetland release sites (Fig. 6a).

We also detected differences in microbiomes across the two captive-rearing facilities (Cosley and Shedd), with a majority of ASVs (both plastron and water) found exclusively in single sites (Fig. 4). The simplest explanation for this difference likely lies in underlying husbandry practices between the two facilities, possibly attributable to feeding practices and differences in densities of animals within enclosures (Dallas and Warne 2023). Although differences in water source between the captive sites could be implicated as well, it is important to note that alpha and beta diversity differed significantly between tap and tub water samples at Cosley, with substantially lower alpha diversity in tap water than tub water (Fig. 6a); thus, it is unlikely that core differences in underlying water supply produced the observed patterns in water microbiomes. Additionally, for Cosley water microbiomes, both alpha and beta diversity differed among tubs, suggesting that even stochastic effects and subtle differences within a rearing facility can influence captive-associated microbiomes.

Taken together, these data support previous findings (Diaz and Reese 2021) that captive conditions influence microbial community membership for captive individuals, often (but not always) leading to decreased diversity in microbial communities. Microbial trends in captive vs. wild environments are likely to be nuanced, and it may not be possible to fully control or predict host microbiomes by controlling their captive environments. As such, an attempt at controlling environmental microbiomes may not directly translate to the diversity patterns seen in captive turtle microbiomes.

Aim 2. Internal vs. external microbiomes (wild turtles)

When considering variation in turtle microbiomes across body sections, we detected significant differences in both alpha and beta diversity between cloaca and plastron samples of wild turtles. These differences are at least partly reflected in the dominant taxa recorded from each source, with cloacal samples strongly dominated by Bacteroidea and Gammaproteobacteria and plastron samples dominated by Alphaproteobacteria, Bacteroidea, Blastocatellia, Deinococci, and Gammaproteobacteria. Whereas the cloaca microbiome likely reflects a mixture of both externally contributed and internal gut microbiomes, the dynamics of plastron microbiomes are likely influenced by diverse factors, including algal colonization and ecdysis (scute-shedding), that may contribute to differing microbiomes between body sections (McKnight et al. 2020a, Kuschke 2022). While these differences likely reflect contrasts in functional roles in plastron and cloaca microbiota, our dataset is limited in taxonomic resolution, which in turn limits our ability to robustly assess the functional characteristics of microbial communities. Future studies utilizing longer or multiple barcoding loci would likely provide useful insight into the functional roles and capacities of these communities.

Aim 3. Time effects in captivity

While beta diversity of plastron samples changed over time for captive juveniles (Fig. 5d), plastron richness at both Cosley and Shedd did not change significantly over time, and evenness changed over time only at Cosley. Given that individuals age over time, this may indicate that the effects of time may be intertwined with growth and age. We isolated this relationship to investigate age as a factor in microbial community diversity and composition by comparing juvenile and adult captive individuals. At Cosley, captive juveniles, and adults had significant differences in cloaca richness, evenness, and beta diversity (plastron samples were not available for adults). Additionally, both captive juveniles and adults differed from wild individuals in cloaca beta diversity (Fig. 5e). These differences indicate that aligning the microbiomes of captive and wild individuals may prove challenging across different age groups of captive individuals. This, in turn, may necessitate shifting husbandry strategies as animals age to favor consistency in microbial communities of captive and wild individuals.

Captive water communities saw significant changes in microbiome composition over time, but the direction of change was not consistent between the captive sites. While the Cosley water samples decreased in richness and evenness over time, Shedd water samples increased in richness, and evenness remained unchanged. Both captive sites followed similar trends of changing beta diversity over time (Fig. 5f). Given that individuals rotated among tubs throughout our sampling period, microbial communities may have been interconnected by a shared flow of microbes between individuals moving between tubs. At Shedd, time, rather than individual tub, was a significant factor in beta diversity differences, whereas at Cosley both time and individual tub were significant fac-

tors (extended analyses are available here: https://github.com/Testudinidude/BlandingsMicro). This could be attributed to differences in experimental design at each captive site, as Cosley utilized 11 different tubs (eight of which were sampled for juvenile turtle microbiomes and two of which were sampled for water microbiomes) and Shedd only utilized two tubs.

Aim 4. Associations between microbiomes and growth

Growth of individuals was also correlated in our data set with changes in the plastron microbial communities of captive turtles at the Cosley site. While neither growth rate nor plastron length was significantly associated with plastron community richness or evenness, growth rate was significantly related to plastron community beta diversity. Further and more extensive sampling of additional microbial communities (cloaca and other internal communities) would be helpful in clarifying the strength of this association between growth and microbial beta diversity. It would also be useful to directly determine the direction of this relationship (i.e. do factors related to faster growth affect the microbiome, or do certain microbiomes aid individuals in growing more rapidly, or both). In turtle head-start programs, size of released turtles can have a disproportionate effect on post-release survival in wild conditions, so a better understanding of this relationship could be crucial for reintroduction efforts (Tetzlaff et al. 2019). It is unclear whether additional sampling would reveal differences in internal microbiomes associated with growth and fitness, as previously reported for Chinese three-keeled pond turtles (*Chinemys reevesii*) and invasive red-eared sliders (*T. scripta*) (Qu et al. 2020). Based on Qu et al. (2020), it is possible that these differences would be more clearly seen in fecal samples compared to cloacal swabs.

Conclusions and future directions

There is growing interest in the role of microbiomes in head-starting and captive rearing efforts, and there is a valid concern that captivity could alter host microbiomes in ways that negatively affects animals' fitness or survival post-release. Ideally, captive rearing conditions should be designed to encourage the establishment of microbiomes that are similar to those found on wild hosts or act to maximize host fitness either prior to or after release into wild conditions (Diaz and Reese 2021, Dallas and Warne 2023). However, our results highlight some of the inherent difficulties in this approach. Captive microbiomes varied among rearing locations and, in some cases, tubs and individuals within a given location. Further complicating matters, the microbiomes of wild turtles were different at the two sampling periods, suggesting that microbiomes may fluctuate over time. Thus, wild microbiomes are a moving target, and designing rearing conditions that replicate wild microbiomes presents substantial challenges. It is worth noting that at no point did our captive microbiomes align with the wild microbiomes; nevertheless, there may be strategies that can at least bring them closer to alignment.

There is still much work to be done, and we recommend the following: First, for species such as *E. blandingii*, more work is needed on wild individuals to properly understand the natural range of microbial variation and the impacts of microbiome composition on individuals' fitness. Factors such as sex, season, age, and microhabitat should be explored. This work should also include additional microbial groups such as fungi and algae, which may interact with bacterial communities (McKnight et al. 2020b) as well as being important on their own. Second, more experimental work is needed to understand the sources of variation in captive microbiomes and develop methods for standardizing rearing conditions such that predictable microbial outcomes can be expected. Third, and building on the first two points, methods for facilitating the growth of captive microbiomes that fall within the normal range of wild microbiomes should be tested. This may include approaches such as temporarily rearing juveniles with wild adults or supplying juveniles with water and substrates from wild habitats. Finally, work is needed to understand both how the microbiomes of captive-reared individuals shift post-release (i.e. do they quickly shift to match the microbiomes of wild individuals) and whether differences in the microbiomes at the time of release ultimately result in differential survival or fitness. Our data provide a starting point in addressing these recommendations based on a headstarting system for one, at-risk turtle species, yet these are open questions that ideally will be resolved across diverse taxa to maximize the success of assisted release conservation programs.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

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Author contributions

Lauren Jenkins (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Donald T. McKnight (Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Matthew Parks (Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Nathan W. Byer (Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Francis J. Oliaro (Conceptualization, Formal analysis, Funding acquisition, Investi-

gation, Methodology, Project administration, Resources, Software, Supervision, Writing – review & editing), Dan Thompson (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing), and Rodney Scott (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing)

Data availability

The data underlying this article are available in the National Center for Biotechnology Information BioProject PRJNA826108 at https://dataview.ncbi.nlm.nih.gov/object/PRJNA826108?reviewer=v15hkples2bbhmpb1llmbt1qsn. Annotated code is available here: https://testudinidude.github.io/BlandingsMicro/.

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