Supplementary Materials S2

The following section provides additional details of the statistical models used in our analyses. These methods were outlined in Table 1 in the main text, and the numbers and letters used here for each question correspond with that table. The code, diagnostic plots, and full outputs are available in the GitHub repository (https://github.com/Testudinidude/BlandingsMicro), and the Table of Contents paths for the appropriate sections in the R markdown are shown.

All PERMANOVAs were performed via the adonis2 function in the vegan package (Oksanen et al. 2007). Mixed effects models were run using the lme4 package (Bates et al. 2014) and significance was assessed via the Anova function in the car package (Fox and Weisberg 2011) with a type II sums of squares.

1). Captive vs. wild effects

1a. Do microbiomes differ between captive and wild plastrons?

We used PERMANOVAs to compare the beta diversity of plastron communities of wild adults with those of headstarted turtles at Cosley and Shedd. To avoid pseudoreplication, for headstarted turtles, we only used samples from the last day that samples were collected. We chose this date because it was the most reflective of what the headstarted turtles' microbiomes would be upon release, and therefore the most relevant point of comparison between captive and wild turtles. Because captive turtles came from two tubs each within Cosley and Shedd, while wild turtles were captured over two years, we included site (Cosley, Shedd, wild) in the model, with either tub number or year nested in site. We did not run statistical analyses on alpha diversity, because there were clear interactions, and the limited sample sizes within factors made statistical inference problematic.

R markdown path:

6 Plastron
6.3 Shedd vs Cosley vs wild
6.3.5 Beta diversity
6.3.5.1 All sites
6.3.5.1.2 PERMANOVAs

1b Do microbiomes differ between captive and wild cloaca?

We used PERMANOVAs to compare the beta diversity of cloacal communities of wild adults with those of headstarted turtles at Cosley. Captive samples were only available from Cosley, and they were only collected at the end of the study (so there was no pseudoreplication). Data were also available from captive adults that had been raised at Cosley. Wild data were available from two seasons (years). Because of the non-orthogonal nature of the data, we ran six tests on possible groups, while controlling the large number of tests by adjusting the P values using the sequential Bonferroni method. The six tests were: wild 2018 vs. wild 2019, Cosley juvenile vs. Colsey adult, Cosley adult vs. wild 2018, Cosley adult vs. wild 2019, Cosley juvenile vs. wild 2018, Cosley juvenile vs. wild 2019.

R markdown path:

5 Cloaca

5.1 Compare wild, captive, age

5.1.4 Beta diversity

5.1.4.2 PERMANOVAs

1c Do water microbiomes vary between captive and wild sites?

We used mixed effects models to compare alpha diversity (separate models for richness and evenness) between water samples at captive and wild sites. Only tub samples were included from the captive sites. Richness or evenness were included as the response variable, and read depth (log10 transformed) and specific location (tub number or wetland) nested in general location (Cosley, Shedd, or wild) were included as fixed effects. Date was included as a categorical random effect (i.e., we wanted to account for multiple samples collected on the same date, but we were not testing whether conditions changed over time). We did not further analyse these data with a PERMANOVA out of concern that the complex structure of the data would produce unreliable results. However, heatmaps and ordination plots strongly suggest differences among the sites.

R markdown path:

4 Water

4.3 Alpha diversity: sites

4.3.1 Richness

4.3.2 Evenness

1d Do tub and tap water microbiomes differ at captive sites?

At Cosley (where water samples were available from both tubs and tap water), we compared the alpha and beta diversity of tubs and tap water samples. Samples were collected on five days, with one sample from each of two tubs (taken after a partial water change) and one sample from the tap water used to fill the tubs. For richness and evenness, we used linear models, with tub number nested in sample type (tub or tap), date, and read depth (log10 transformed) as the response variables. We used the same model structure for PERMANOVAs.

R markdown path:

4 Water

4.5 Cosley

4.5.1 Tap and tubs

4.5.1.2 Richness

4.5.1.3 Evenness

4.5.1.4 Beta diversity

4.5.1.4.2 PERMANOVAs

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

2a. Are there correlations between individuals' cloaca and plastron microbiomes?

We focused on wild turtles for correlations of plastron and cloaca communities because they were the only group with a sufficient sample size of both sample types. Both sample types were collected from each individual. For richness and evenness, we used linear models with plastron alpha diversity as the response and year (categorical) and cloacal alpha diversity as the predictors. For beta diversity, we used a Mantel test, with plastron beta diversity as the response, and cloacal beta diversity as the predictor.

R markdown path:

7 Cloaca x plastron
7.1 Correlations
7.1.2 Richness
7.1.3 Evenness
7.1.4 Beta diversity
7.1.4.2 Mantel

2b. Are there differences between individuals' cloaca and plastron microbiomes?

We focused on wild turtles for comparisons of plastron and microbiome communities because they were the only group with a sufficient sample size of both sample types. Both sample types were collected from each individual. For richness and evenness, we used linear models with sample type, year (categorical), read depth (log10 transformed), and turtle ID as the predictor variables (including an interaction between sample type and year). Richness or evenness were the response variables. PERMANOVAs followed the same model structure.

R markdown path:

7 Cloaca x plastron
7.2 Comparisons of mean values
7.2.2 Richness
7.2.2.2 Statistics
7.2.3 Evenness
7.2.3.2 Statistics
7.2.4 Beta diversity
7.2.4.2 PERMANOVAs

3). Time effects in captivity

3a. Do captive plastron microbiomes change over time and vary among individuals?

For these analyses, we ran separate tests for Cosley and Shedd, and at Cosley, we only used turtles with at least three data points (samples were collected over six dates, but not all individuals were sampled on each date). At Shedd, samples were only collected on two dates, which limited inferences (samples sizes were also small).

At Cosley, we examined richness and evenness using linear mixed effects models with date (continuous), read depth (log10 transformed), and turtle ID as the fixed effects (an interaction term was included between date and turtle ID). We included a random intercept that combined date and tub # to account for individuals shuffling among tubs. The PERMANOVA was structured identically, except the random intercept was included as a fixed term because adonis2 does not allow a mixed effects structure.

At Shedd, we followed the same model structure, except there was no random effect (thus we used a fixed effects model), because all turtles made a single switch between tubs (thus allowing us to model that using the tub number and date terms).

R markdown path:

6 Plastron
- 6.1 Cosley headstarts
  - 6.1.3 Richness (individuals and time)
    - 6.1.3.2 Statistics
  - 6.1.6 Evenness (individuals and time)
    - 6.1.6.2 Statistis
  - 6.1.9 Beta-diversity (individuals, time, and growth)
    - 6.1.9.2 PERMANOVAs
- 6.2 Shedd
  - 6.2.1 Richness
  - 6.2.2 Evenness
  - 6.2.3 Beta diversity

3b. Do captive tub (water) microbiomes change over time?

We looked for changes in the water microbiomes over time separately at Cosley and Shedd. At both sites, we assessed alpha diversity using linear models with tub number, date (continuous), and read depth (log10 transformed) as the predictor variables. At Cosley, two samples were taken per date (one before and one after partial water changes); therefore, we included an additional term noting whether the sample was before or after a water change. PERMANOVAs followed the same structures as the linear models.

R markdown path:

4 Water
- 4.5 Cosley
  - 4.5.2 Tubs and dates
    - 4.5.2.1 Richness
    - 4.5.2.2 Evenness
    - 4.5.2.3 Beta diversity
- 4.6 Shedd
  - 4.6.1 Richness
  - 4.6.2 Evenness
  - 4.6.3 Beta diversity

4). Associations between microbiomes and growth

4a. Are captive plastron microbiomes associated with plastron length or growth?

For these analyses, we only used Cosley turtles, because they were the only ones with sufficient data. Additionally, we only used individuals with at least three data points. For alpha diversity, we used linear mixed effects models with growth (change in plastron length per day since the previous measurement) or size (plastron length) as the fixed effect, and turtle ID and a term combining date and tub number as the random effects.

PERMANOVAs were structure identically except the random intercept was included as a fixed term because adonis2 does not allow a mixed effects structure.

R markdown path:

6 Plastron

6.1 Cosley headstarts

6.1.4 Richness (growth)

6.1.5 Richness (size)

6.1.7 Evenness (growth)

6.1.8 Evenness (size)

6.1.9 Beta-diversity (individuals, time, and growth)

6.1.9.2 PERMANOVAs

Literature Cited

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2014) Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:14065823*.

Fox, J. and Weisberg, S. (2011) Multivariate linear models in R. *An R Companion to Applied Regression Los Angeles: Thousand Oaks*.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J. and Suggests, M. (2007) The vegan package. *Community ecology package* **10**, 719.