Multilevel community assembly of the tadpole gut microbiome

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Abstract

The assembly of local communities is likely to reflect the effects of local environmental factors associated with filters that act at larger spatial scales. Dissecting these multiscale effects remains a timely challenge that is particularly important for host-associated microbiomes. We investigated the relative roles of local selection (due to host species identity) and regional effects (due to water body identity) on the community structure of bacteria in the gut of tadpoles from three biogeographic areas and used graph theory and metanetwork approaches to explore and illustrate the distribution of bacteria across different ponds. The pond of origin, which represents a regional species pool of bacteria, was in general more important in shaping the gut microbiome of tadpoles than host species identity. The resulting metanetworks are modular and indicate relatively few species of bacteria occurring in more than one pond. Thus, each pond represents a relatively distinct species pool of bacteria available for community assembly of the tadpole microbiomes. Our findings indicate that microbiome community assembly in amphibian larvae, as in many other communities, is a multiscale process with important regional effects that constrain how local (i.e. host-dependent) filters act to influence microbiome community composition.

Introduction

The origin and assembly of host-associated microbial communities (microbiomes) is a complex process of fundamental importance in helping us understand the symbiotic relationship between hosts and their microbes [1, 2]. Various factors can play a role in the community assembly of microbiomes at multiple levels. Perhaps the most obvious is the effect of host diet on the gut microbiome, which has been shown in many organisms from insects to humans [3–6]. Other local factors include host sex, health state, and genetics [7–11]. These effects can be thought of as local 'filters' to microbial community assembly. Here we evaluate these effects as they are determined by host species identity only, recognizing that there are likely to be other more subtle effects involving inter-individual factors. However, there can also be processes that constrain which microbes are available to colonize any given host. Here, we focus on factors that determine the microbial species pool available to colonize the host and evaluate these by the identity of the site of the host community.

We view community assembly of microbiomes as being driven by metacommunity processes [12]. This recognizes that the assembly of a local community depends strongly on the species available to colonize that community, known as the regional species pool [13, 14]. From that pool, if a species is able to disperse to a given community, its persistence in that community will depend on local demographic processes and on the ability of the species to tolerate the local biotic and abiotic conditions, which is known as the local selection component of community assembly

55 [15]. Therefore, communities with identical environmental conditions could have a
56 completely different species composition if colonized by different species pools [16].
57 While most work in metacommunity ecology has focused on macroorganisms, these
58 ideas should apply equally well to microbes [1, 2, 12, 17, 18].

59 Host-associated microbial communities appear to be influenced by effects
60 related to the individual host or individual species such as diet, physiology, and
61 genotype [10, 19, 20], as well as regional and historical processes, as in the case of
62 exposure to potentially different species pools of microorganisms [21–26], or vertical
63 and horizontal transmission of the microbiome [27, 28]. Considering the importance
64 that the microbiome can have to host health and well-being, understanding the relative
65 importance of effects acting at different levels is fundamental, but studies explicitly
66 tackling that question are still lacking [29].

67 We analyzed variation in tadpole microbiomes to evaluate the effects of local
68 environmental filters by focusing on host species identity, and regional effects by
69 focusing on the locality of the water body. We assume that different microbes would be
70 differentially favored by selection associated with the physiology and diet of different
71 tadpole species, and different microbes may be present at different sites due to either
72 site-specific habitat variables or to isolation and other differences among water bodies.
73 Our studies were conducted across three different geographic locations to test if they
74 show consistent patterns. We also used graph theory and metanetwork approaches to

75 explore the degree to which each pond consists of a unique species pool, and how they
76 are connected to each other in terms of shared bacterial species.
77 The gut microbiome of tadpoles is critically important to their digestive function
78 [30], and some evidence shows gut microbiomes from the same host species are more
79 similar to each other than from different species [31]. Further, there are often multiple
80 species of tadpole per water body, which represent different hosts with different
81 characteristics that are exposed to the same pool of bacteria. Many of these tadpole
82 species can occupy a variety of different water bodies, each with potentially distinct
83 bacterial species pools, which makes them a suitable system to test questions regarding
84 the effects of environment and host identity on microbiome assembly [32]. We evaluate
85 two hypotheses: i) if the bacterial community of each pond is different across the
86 landscape, then the pond of origin would be more important in determining the gut
87 microbiome of tadpoles, i.e., the microbiomes of different species within each pond
88 would be more similar to each other than the microbiomes of the same species across
89 distinct ponds, and the metanetworks would be structured into modules according to
90 each pond; ii) if ponds have very similar bacterial communities across the landscape,
91 then the species of tadpole would be more important in determining their gut
92 microbiome, i.e., tadpoles of the same species across ponds would have more similar
93 microbiomes than tadpoles of different species from the same pond, and the
94 metanetworks would be structured into modules according to each species.
95

Methods

Study area

We sampled tadpoles from 22 water bodies located in three different localities: i) six water bodies in the Boracéia Biological Station, located in the Atlantic Forest east of the state of São Paulo, Brazil, hereafter BR; ii) nine water bodies in the David Crockett and Angelina National Forests, located in the pine forest ecosystem in eastern Texas, United States, hereafter ET; and iii) seven water bodies in the Edwards Plateau, a mix of grassland and juniper/oak woodlands in the central part of Texas, United States, hereafter CT. We classified water bodies as either lentic or lotic. For ET ponds, we did a more thorough environmental characterization quantifying in each pond the dissolved oxygen (mg/L), pH, conductivity (uS/cm), chlorophyll a (ug/L), and water temperature (^{o}C) using a Eureka multiparameter sonde, and the absence or presence of piscivorous (Bass) and/or insectivorous fish (Green Sunfish). Even though three water bodies in BR were lotic, we will refer to all of them as *ponds* for convenience.

Tadpole and microbiome sampling

In each pond we collected tadpoles with a dipnet that was swept through the pond. We then placed the larvae in a sterile plastic bag (Whirl Pak®) filled with water from their place of origin. Environmental microorganisms from water from CT and ET were sampled by filtering approximately 50 ml of water from three different places through a sterile syringe filter with a 0.45 μm cellulose acetate membrane (VWR). From

117 BR, approximately 2L of water was collected in a sterile plastic bag (Whirl Pak®), mixed,
118 and 100 mL was vacuum-filtered through a 0.45 μm cellulose acetate membrane. The
119 sediment in all ponds was sampled in three locations in each pond by removing the first
120 three centimeters with a sterile plastic straw of 1.3 cm of diameter and placed in a
121 sterile tube.

122 In the lab, tadpoles were euthanized by overdose in a solution of Milli-Q® filtered
123 water and tricaine methanesulfonate (MS 222). After the death was confirmed, each
124 individual was dissected using instruments that were cleaned in 100% ethanol and
125 flame-sterilized. The entire tadpole gut was removed and placed directly in a MoBio
126 PowerSoil® bead tube. The sediment from each pond was homogenized, centrifuged to
127 remove the excess water, and an aliquot of approximately 0.2 g was used for DNA
128 extraction. DNA from the water sample was extracted from the entire filter membrane.
129 We performed DNA extraction using MoBio PowerSoil® DNA isolation kit following the
130 manufacturer's recommendation. The same procedures without a real sample were
131 repeated to count as negative control samples, which did not have DNA when quantified
132 using Qubit™ dsDNA high sensitivity assay.

133 We amplified the V4 hypervariable region of the 16s rRNA gene (515F, 806F). CT
134 samples were amplified using the version F: GTGYCAGCMGCCGCGGTA / R:
135 GGACTACHVGGGTWTCTAAT and ET and BR the version F: GTGYCAGCMGCCGCGGTAA /
136 R: GGACTACNVGGGTWTCTAAT. The library preparation and sequencing (Illumina MiSeq
137 2x250bp) of the CT samples was realized at the Genomic Sequencing and Analysis

138 Facility (wikis.utexas.edu/display/GSAF) at The University of Texas at Austin following
139 their protocols. The library preparation and sequencing (Illumina MiSeq 2x250bp) of the
140 ET samples was realized at the Argonne National Laboratory (www.anl.gov) following
141 their protocols. For BR samples, we prepared the libraries following the Earth
142 Microbiome Project protocol (www.earthmicrobiome.org), with the exception of using
143 20uL PCR reactions and dual-index barcodes instead. Samples were sequenced in an
144 Illumina MiSeq platform (2x300 bp paired-end sequences).
145 The sequences were processed using the dada2 pipeline [33] in the software
146 Microsoft R Open v 3.5.1 [34]. Sequences from each site were processed separately.
147 Briefly, primers, adapters, and barcodes were removed and the quality profile of the
148 reads were visually inspected using the function plotQualityProfile aggregating over all
149 the samples from a site. BR, CT, and ET reads were truncated at 200 and 120, 200 and
150 180, and 200 and 160 forward and reverse reads, respectively. Sequencing errors were
151 calculated, sequences were clustered as Amplicon Sequence Variants based on the
152 DADA2 algorithm [33] and paired ends were merged. Chimeras were removed using the
153 consensus method in the function removeBimeraDenovo. Taxonomy was assigned
154 based on the Silva database v128 [35]. ASV sequences were exported to Qiime2 2018.4
155 [36], aligned using the MAFFT algorithm [37], and a phylogenetic tree was constructed
156 using FastTree [38]. We discarded all ASVs that were classified as Archaea, chloroplast,
157 mitochondria, or that were not at least assigned to bacteria. Hereafter, we will refer to
158 ASVs as species of bacteria.

159

160 **Data analysis**

161 **Diversity**

162 For each sample we constructed a rarefaction curve and compared richness and
163 Faith's phylogenetic diversity [39]. To avoid sequencing depth effects, each sample was
164 rarefied 100 times to the number of reads of the sample with the lowest coverage using
165 the R package BAT [40]. For comparison, we considered the median richness from all
166 rarefactions from a sample as our richness measure.

167

168 **Species and pond effects**

169 We analyzed our data as compositional data (termed CODA) [41]. It requires a
170 centered log-ratio transformation of the read counts and therefore cannot have zeros,
171 so we added a pseudocount of one to all ASVs in all samples. We transformed the data
172 using the codaSeq.clr function from the R package CoDaSeq [42, 43]. We also analyzed
173 data that takes into account phylogenetic relatedness of microbes by using the PHILR
174 transformation from the R package philr [44]. This metric takes into account the
175 phylogenetic relationship between the ASVs, which is equivalent to the Unifrac metric
176 [45], but it considers the compositional nature of the data [44]. We then ran a Principal
177 Component Analysis (PCA) on the Euclidean distance matrix of the CODA- and PHILR-
178 transformed data to visualize the relationship between samples and tested if samples
179 from tadpoles, water, and sediment are different from each other using a Permutational

180 Multivariate Analysis of Variance (perMANOVA). If a difference was detected we then
181 used additional perMANOVAs for pairwise comparisons with Bonferroni correction.
182 To first test if either the identity of the species of tadpole or the pond of origin
183 are more related to the gut microbiome composition we used a perMANOVA to fit a
184 model with both pond and species identity to the CODA- and PHILR-transformed data
185 using the function adonis2 from the R package vegan [46]. The significance of the
186 marginal effects was tested based on 999 permutations. In addition, to test for the
187 unique effects of the species of tadpole or the pond of origin as well their shared effects
188 on the gut microbiome we performed variance partitioning and Redundancy Analysis
189 (RDA) [47] using the function varpart in the R package vegan [46]. The significance of
190 each unique component in the variation partitioning was tested using the vegan
191 function anova.cca with 1000 permutations. We repeated perMANOVA and variation
192 partitioning analyses to the data grouping the species of bacteria at Genus and Family
193 level.
194
195 **Inter-pond variation**
196 We also investigated if there was evidence for dispersal limitation in the bacteria
197 from water and sediment using multiple regression on distance matrices using the
198 function MRM from the R package ecodist [48]. We used the Euclidean distance matrix
199 based on the PHILR and CODA transformations as the dependent variable and a
200 geographical distance matrix between sites as the independent variable. In addition, to

account for environmental effects, we also include in the model a distance matrix based on the type of the pond (lentic or lotic) for BR, and a distance matrix based on the environmental variables measured for ET. The latter was a Gower distance matrix [49] created by the function dist.ktab from the R package ade4 [50]. Quantitative variables were Gower-standardized (divided by maximum minus minimum). All CT ponds were lentic and there were no other variables besides spatial distance to include in the model. For models where distance was significant in the MRMs, we applied a Partial Mantel Correlogram approach using the function mpmcorrelogram from the R package with the same name [51]. That tests, at several distance classes, in our case estimated by Sturge's rule [52], the relationship between water and sediment microbiome with geographical distance while controlling for environmental effects.

Metanetworks

We further explored the structure of three different metanetworks within each locality (BR, CT, and ET) to understand the distribution of bacteria across ponds. Shared species (here bacteria) connect different ponds in a metacommunity perspective [53, 54]. We built three bacteria metanetworks for each locality: (i) the tadpole microbiome metanetwork, composed by the bacteria living in the gut of all tadpoles within a pond, independently of the tadpole species, (ii) the water metanetwork, with bacteria found in the water, and (iii) the sediment metanetwork, composed by bacteria found in the sediment of each pond. Then, we built an a_{mn} adjacency matrix for each metanetwork in

222 which *m* corresponds to a single pond (i.e., considering bacteria from water, sediment
223 or gut of tadpoles), and *n* to each bacterium. The *mn* element represents the
224 presence/absence of species of bacteria *n* in a pond *m,* and is represented by a link in
225 the graphical representation. Therefore, each node in our metanetwork is either a
226 bacterium or a pond; when the same bacterium is found in more than one pond, there
227 is a link connecting them, forming the edges of the metanetwork.

228 To test whether ponds consist of different bacterial communities, we calculated
229 the modularity of the metanetworks using the FastGreedy algorithm [55, 56] in the
230 software Modular v 0.1 [57]. We tested the significance of the observed modularity
231 against two different null models with 100 permutations each: the Erdős-Rényi [58], in
232 which pond-bacteria interactions are connected randomly, and the 'null model 2' [59],
233 in which the probability of a pond-bacteria interaction is proportional to their number of
234 links in the observed matrix. To identify the bacteria with higher potential to connect
235 the metanetwork through shared occurrence in a higher number of ponds, we
236 calculated the degree (k) for each metanetwork using the software Pajek v. 4.10 [60].
237 Degree measures how many links each node establishes in its correspondent network.
238 For the bacteria, it represents the number of ponds in which it occurs; for the pond, it
239 represents the number of bacteria it has (from tadpoles, water or sediment). For each
240 metanetwork we also calculated the proportion of bacteria found uniquely in p_1, p_2, …,
241 p_n, where n is the total number of ponds in one locality.
242

243 **Results**

244 **Diversity**

245 We registered 19 species of tadpole in all ponds: three in CT, six in ET, and
246 eleven in BR (Table S1). After the filtering steps, there were a total of 533 777, 1 289
247 662, and 2 064 298 16s rRNA gene fragment reads for CT, ET, and BR, respectively. The
248 rarefaction curve shows that all samples reached an asymptote, even the ones with the
249 lowest number of reads (Figure S1). For richness and phylogenetic diversity measures of
250 bacteria, CT, ET, and BR samples were rarefied to 3 950, 1 791, and 3 916 reads,
251 respectively. There was no significant difference among CT samples in terms of richness,
252 but there was in terms of phylogenetic diversity, with sediment samples having the
253 highest diversity and *Rana berlandieri* tadpoles the lowest (Figure S2). Sediment
254 samples had the highest richness and phylogenetic diversity in ET and *Hylodes phyllodes*
255 the highest in BR (Figures S3 and S4). A great part of the tadpole microbiome was
256 composed by Fusobacteria, Firmicutes, Proteobacteria, and Bacteroidetes, while
257 sediment and water samples were mostly composed by Proteobacteria (Figures S5-S7).
258

259 **Species and pond effects**

260 The composition of the tadpole gut microbiome is different from both water and
261 sediment samples in all localities (Figure 1, Figure S8, Table S2). Also, in most cases,
262 tadpole gut microbiome samples cluster more with other samples from the same pond
263 than with samples from the same species from a different pond (Figure 1, Figures S8-

264 S10). However, within a pond, there can be separation by species and, in fact, the
265 perMANOVA shows that both species identity of tadpole and pond of origin are
266 significant predictors of the tadpole gut microbiome in all localities when using either
267 PHILR- or CODA-transformed data, except for CT CODA, in which species identity was
268 only marginally significant (Table 1). In CT and ET, pond of origin was a stronger
269 predictor of the tadpole gut microbiome than tadpole species identity. The overall
270 results are consistent over all the taxonomic levels considered (Tables S3-S6).
271 For CT and ET, the pond of origin explained considerably more of the variance in
272 tadpole gut microbiome than the species of tadpole (Figure 2). For BR, the tadpole
273 species explained a slightly higher proportion of the variance in their gut microbiome.
274 However, most of the variation in that environment is explained by the joint effects of
275 tadpole species and pond of origin, i.e., the variation that could not be separated in
276 unique components (Figure 2). Again, the overall results are comparable across all the
277 taxonomic levels considered (Tables S7-S8).
278
279 **Inter-pond variation**
280 Only the bacterial communities of water from CT were significantly related to the
281 spatial distance between ponds (Table S9). However, that was the only dataset where
282 environmental data is not available and could not be accounted for, allowing for the
283 possibility that such distance effects are due to spatially structured environmental
284 factors. Partial Mantel Correlograms showed that, for both CODA- (p_{adj} = 0.044) and

285 PHILR-transformed data (p_{adj} = 0.022), there is a spatial signal only for the first distance
286 range, from 174 to 6 223 m. ET water and sediment communities were significantly
287 related to environmental variables for PHILR-transformed data and BR water
288 communities were significantly related to both PHILR- and CODA-transformed data
289 (Table S9).
290
291 **Metanetworks**
292 All metanetworks are significantly modular according to the two null models
293 used ($p < 0.001$ for all null models; Figure 3, Figure S11). In most cases, each module
294 corresponds to a single pond (Figure 3, Figure S11 and Table S10). Metanetworks of
295 water and sediment are more modular than those of tadpoles for all localities (Figure 3,
296 Figure S11). In all cases, the vast majority of bacteria occur in a single pond (k = 1),
297 varying from 63.2% in East Texas to 91.3% in Brazil (Figure 4, Figures S12-S16). Only 0.1
298 to 1.1% of bacteria were present in all ponds (Figure 4, Figure S12-S16). From those, for
299 BR and ET they were mostly from the phyla Bacteroidetes and Proteobacteria and
300 Orders Bacteroidales and Desulfovibrionales. CT had only three bacteria present across
301 all ponds. From the ponds' perspective, their degree, which corresponds to the number
302 of unique links they have in the metanetwork, varied from 118 to 615 for water, 90 to
303 478 for sediment, and 241 to 2 751 for bacteria from tadpoles (Figures S17-S19).
304

305 **Discussion**

306 We found that microbial community assembly of tadpole microbiomes was
307 structured by processes happening at different levels and broadly resemble findings
308 from macroorganisms [1, 15, 61–63]. At higher levels, there are inter-pond differences
309 in microbial communities due to site-specific variables, isolation, and/or stochasticity. It
310 was not our goal to understand the drivers of inter-pond differences, which is ultimately
311 responsible for the different bacterial species pool tadpoles are exposed to. However, in
312 some cases, for the ponds where we had environmental data there is a relationship
313 between environmental variables and the bacterial community of water and/or
314 substrate. In these cases, each pond could act as a regional scale environmental filter for
315 bacteria. For CT, we found some evidence for a spatial correlation in bacterial
316 communities among nearby ponds, which could indicate dispersal limitation structuring
317 the pond communities, but that was the only dataset for which we did not have
318 environmental variables. Nevertheless, independently of the causes of the differences
319 among ponds, each one of them constitutes a unique species pool of colonizers for the
320 microbiomes of tadpoles. As tadpoles develop, the community assembly of their
321 microbiome is restricted by that species pool of microbes. Each tadpole comprises a
322 smaller-scale filter to a subset of the microbes within the species pool. Therefore, even
323 if similar habitats (same species of tadpole in our case) are exposed to different species
324 pools, they will not end up with the same microbial community.

The composition and diversity of the gut microbiome of tadpoles can be variable across species. The microbiome of tadpoles from this study is slightly different than previous studies. Proteobacteria and Firmicutes dominated in other studies [31, 64–66] but here we found that Fusobacteria were also important. Alpha and phylogenetic diversity were also mostly higher in our study than in previous ones [31, 64–66]. The knowledge about the gut microbiome of tadpoles is still limited to a few species and quantitative comparisons of diversity across studies is problematic because of differences in sampling procedures, sample processing, data cleaning, and the algorithms and databases. However, overall differences in microbiome composition could be due to the exposure of tadpoles to different pools of colonist microbes.

The pond of origin of the tadpoles was more important in determining their gut microbiome than the species of the tadpole in both of the Texas locations. We assume the same species of tadpole has similar requirements in terms of microbes and/or constitutes a similar patch for colonization by microbes. There were only a few bacteria in common across all ponds in all three localities, therefore each pond constitutes a unique bacterial species pool from which the tadpoles can be colonized. In Brazil, the pond of origin and species of tadpole showed similar relationships with the gut microbiome of tadpoles. However, most of variation in Brazil was due to variation that could not be separately attributed to either because of the uniquely distinct tadpole faunas of ponds.

345 The limited and unique pool of bacteria tadpoles are exposed to and the higher
346 similarity within a pond can be visualized on the metanetworks of bacteria from water,
347 sediment, and tadpole gut. The metanetworks are modular and each pond constitutes a
348 unique community of bacteria. The vast majority of bacteria occur only in a single pond,
349 and just a few are present in all of them. In fact, for water and sediment, the maximum
350 number of bacteria found in all ponds was only eight. That number would probably
351 increase if more sediment or water was sampled. On the other hand, we likely sampled
352 all species of tadpoles from each water body and the proportion of bacteria from their
353 gut found in all water bodies is still very low (0.1-1.1%) despite similar phyla found in all
354 of them. The major bacteria in tadpole guts belong to orders Bacteroidales and
355 Desulfovibrionales, which do not overlap with the most widespread taxa found in water
356 or sediment. It is common to not find much overlap between bacteria from environment
357 and gut of aquatic organisms [67, 68]. Likewise, we show that the gut microbiome of
358 tadpoles is distinct from both water and sediment from the ponds suggesting that the
359 gut constitutes a strong environmental filter that favors certain taxa. Desulfovibrionales,
360 for example, is a group of sulfur-reducing bacteria that is mostly anaerobic [69].
361 Therefore, at least certain parts of the digestive tract of tadpoles might be anaerobic
362 [31, 70]. Pryor and Bjorndal [30] detected significant levels of fermentation in the
363 hindgut of bullfrog tadpoles. It is unknown to which extent other species of tadpoles are
364 hindgut fermenters. For those that are, even though they might not have a core

365 microbiome, at least not at fine-scale taxonomy levels, there may be a functional core
366 gut microbiome.

367 In terrestrial and aquatic environments, the importance of the regional species
368 pool in shaping the microbial communities can be noticed even when not tested
369 directly. In many species, from insects to humans, individuals likely exposed to the same
370 regional species pool, such as being from the same environment, have more similar
371 microbiomes [21–26]. There are many difficulties in quantifying and testing how
372 regional effects affect community assembly [29, 62], but comparing results with
373 predictions from theory can lead to important insights. If hosts from different
374 environments have similar microbiomes it could indicate that (i) there is not much
375 difference in the regional species pools between environments, (ii) the hosts move
376 between species pools, or (iii) there are other methods for microbiome acquisition such
377 as transmission from conspecifics. For example, Kueneman *et al*. [71] and McKenzie *et*
378 *al*. [32] studying the skin microbiome of tadpoles found that the identity of the species
379 was the strongest predictor of the skin microbiome, with the water body of origin
380 explaining additional [71] or no variance at all [32]. Vertical transmission of the
381 microbiome is unlikely in amphibians [72], and they probably acquire their microbiome
382 from the environment at every generation [73]. Therefore, the results from Kueneman
383 *et al*. [71] and McKenzie *et al*. [32] indicate there is at least a partially shared species
384 pool of microbes across their sampling sites.

385 The enormous intra and inter-specific variation in the microbiomes of different
386 species of animals and plants can be at least partially explained by differences in
387 regional species pool during the community assembly of the microbiome. The gut
388 microbiome of animals can be stable over time [63, 74] and can also be resistant to
389 invasions and colonization by other bacteria [75–77]. Therefore, if the community
390 assembly of individuals of the same species occurred while they were exposed to
391 different species pools, historical effects can cause lasting effects in the development of
392 the microbiome [78]. Moreover, recent efforts to manipulate the microbiome of plants
393 and animals to improve health and/or increase productivity might not be as effective if
394 the manipulation is done only at the level of the individual host, i.e., at the local scale.
395 As stated by Chase [16] "if local and regional processes determine the community
396 composition, then both processes need to be restored to achieve the desired
397 community". Our results indicate that the same logic is valid for microbes.
398 In summary, we showed that local assembly of host-associated bacterial
399 communities are affected by regional scale processes, more specifically changes in the
400 regional species pool of colonizers. The inter-pond variation in bacterial community can
401 be due to stochastic, historical, spatial, and/or environmental processes or even the
402 result of local evolution [79]. Nevertheless, microbiome assembly, as any other
403 community, is a multiscale process. Microbial and microbiome ecology could be better
404 linked to ecological theory by considering the multiscale dynamics of community
405 assembly [12, 17, 80, 81]. Because it is much easier to quantify effects at the scale of the

individual host or host species, i.e., local effects [29, 62], the role of regional effects is probably underestimated. Regional effects, however, appear to be a fundamental piece of the community assembly puzzle that can be used to understand variation in microbiomes.

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628

629 **Tables**

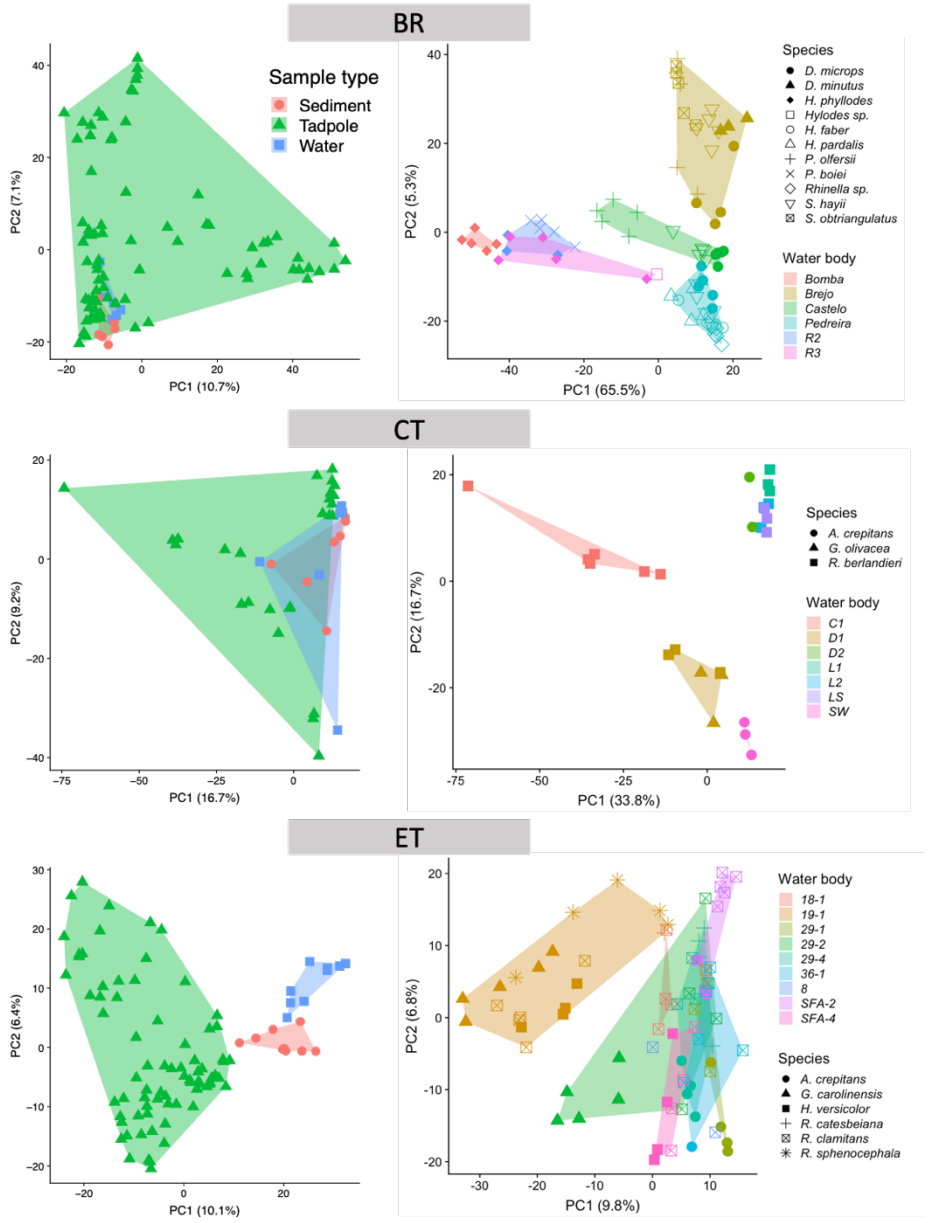
630 Table 1. Results of a Permutational Analysis of Variance (perMANOVA) testing if the
631 species of tadpole or the pond of origin are related to the gut microbiome composition
632 of tadpoles using the bacteria as Amplicon Sequence Variants (ASVs). Models were
633 fitted to CODA and PHILR transformed data. The significance of the marginal effects was
634 tested based on 999 permutations. BR = Brazil, CT = Central Texas, ET = East Texas.
635

		PHILR			CODA		
		R^2	F	p	R^2	F	p
BR	Water body$_{(4,80)}$	0.08	3	0.005	0.1	2.7	0.001
	Species$_{(9,80)}$	0.15	2.5	0.002	0.16	2.1	0.001
CT	Water body$_{(5,25)}$	0.48	6	0.001	0.41	3.6	0.001
	Species$_{(1,25)}$	0.05	2.8	0.010	0.04	1.8	0.056
ET	Water body$_{(8,76)}$	0.27	4.7	0.001	0.24	3.2	0.001
	Species$_{(5,76)}$	0.18	5.1	0.001	0.12	2.6	0.001

636

637

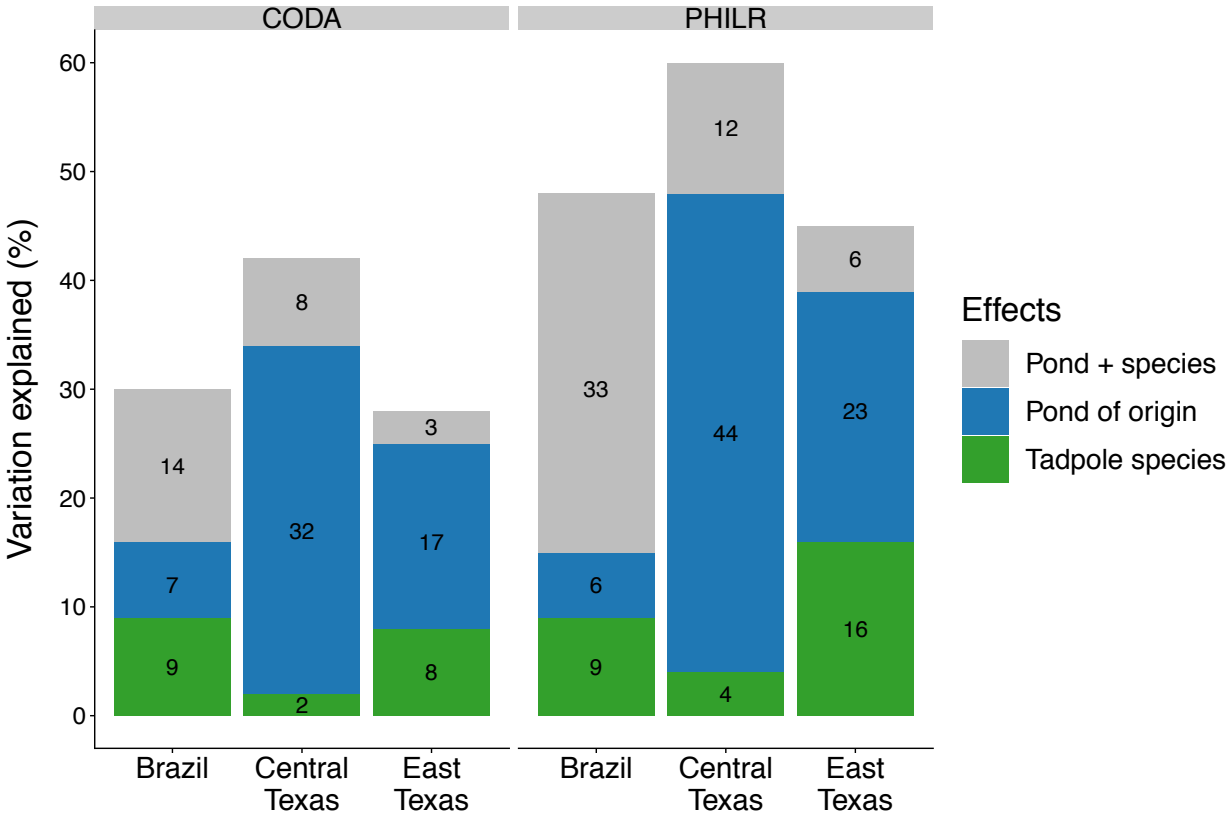
638 **Figures**



639

640 Figure 1. Plot of the first two axes of a Principal Component Analysis on CODA
641 transformed microbiome data (amplicon sequence variants) from tadpole samples from
642 Brazil (BR), Central Texas (CT), and Eastern Texas (ET). Panels on the left show sediment,
643 water, and tadpole samples. Panels on the right show samples from tadpoles only. Left
644 and right panels are results from separate analyses. Shapes represent different species
645 of tadpoles and colors represent water body of origin. See Table S1 for abbreviations.
646

647



648

649 Figure 2. Results of a variation partitioning analysis testing the unique effects of the
650 species of tadpole or pond of origin as well their shared effects on the tadpole gut
651 microbiome considering the bacteria as Amplicon Sequence Variants (ASVs) for CODA-
652 and PHILR-transformed data. The effects of pond of origin and species of tadpole were
653 all significant ($p < 0.05$) based on 1000 permutations, except for the shared component,
654 which cannot be tested.
655

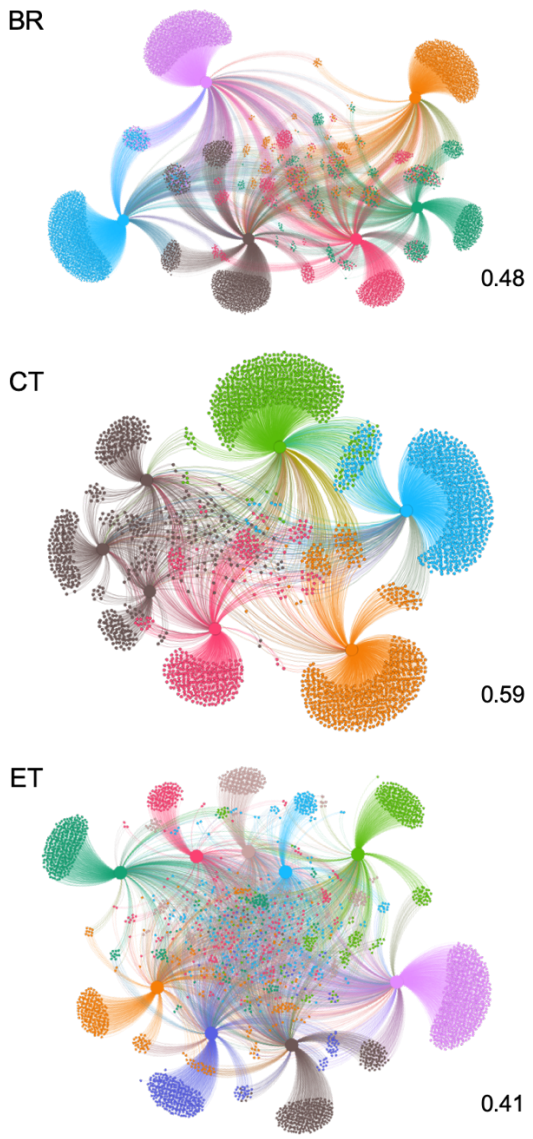


Figure 3. Microbiome metanetworks of the shared bacteria (amplicon sequence variant - ASV) found in the gut of tadpoles. Larger nodes represent each water body for each locality, *i.e.*, Brazil (BR), Central Texas (CT), and East Texas (ET). Smaller nodes represent a unique bacterium that can be found in a single water body (link between smaller and larger nodes) or shared among ponds thus connecting them. Numbers on the bottom-right are the modularity values for each metanetwork. Distinct colors represent different modules which, with a few exceptions, correspond to a single water body (Table S10).

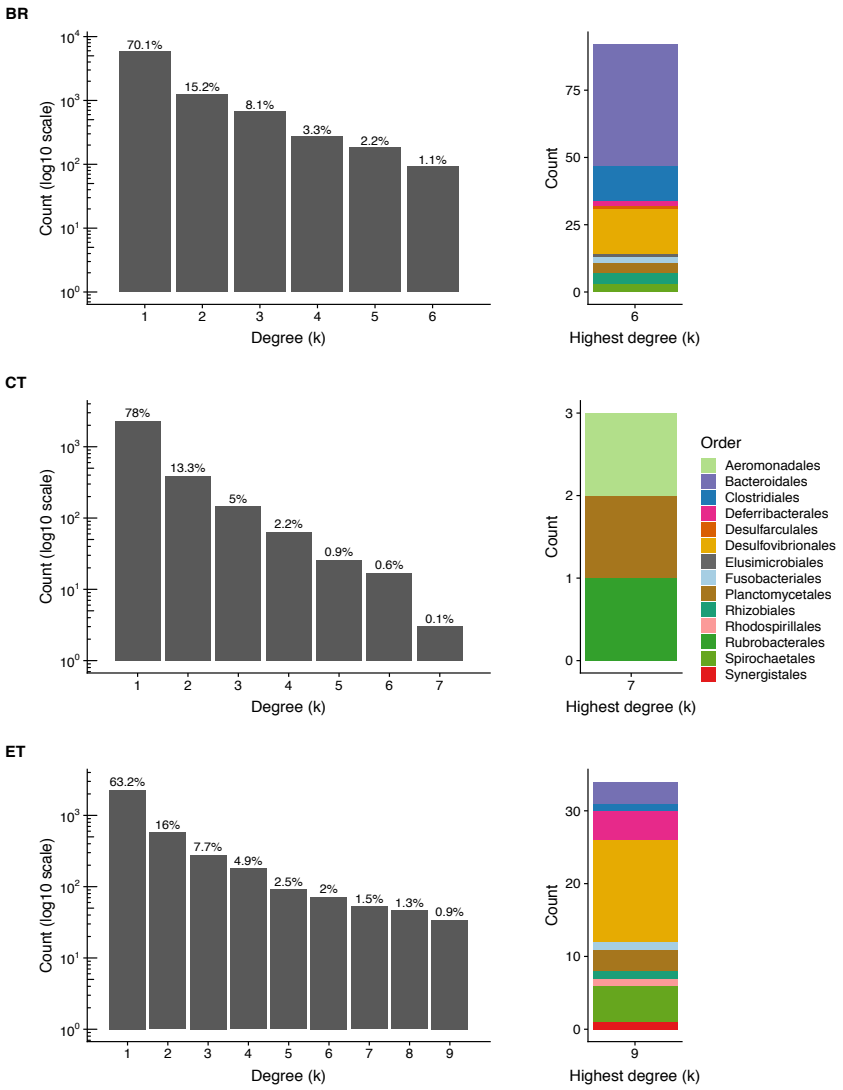


Figure 4. Number of bacteria from the gut of tadpoles for each degree (k) on the left and Order of bacteria present in the highest degree on the right. Each degree represents one pond. Bacteria found in only one pond are represented in the degree category 1. Likewise, bacteria present in tadpoles across all ponds are represented in the highest category of each histogram. The Order of bacteria present in tadpoles across all ponds from each locality is shown on the barplot on the right. Same color across barplots represent the same Order. See Figures S11-S16 for water and sediment metanetworks. BR = Brazil, CT = Central Texas, ET = East Texas.