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Shifts in gut microbiome across five decades of repeated guppy translocations in Trinidadian streams

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An organism's gut microbiome can alter its fitness, yet we do not know how gut microbiomes change as their hosts evolve in the wild. We took advantage of a five-decade 'chronosequence' of translocated fish populations to examine associated changes in the gut microbiome. Populations of Trinidadian guppies have displayed parallel phenotypic convergence six times when moved from high predation (HP) to low predation (LP) environments. Across four drainages, we found microbiomes of fish translocated 5–6 years prior to sampling were already distinct from the microbiomes of their HP source populations. Changes in environmental conditions were most important in driving this shift, followed by phenotypic shifts in gut morphology. After 30–60 years in LP environments, microbiome composition was still distinct from native LP populations, but microbiome function was not. We found some evidence that nitrogen fixation enhanced gut nutrient absorption, but most functional shifts were not parallel across drainages. Stream-and drainage-specific signatures were present for both composition and function, despite our overall finding of consistent microbiome change across drainages. As we unravel the complexities of host–microbiome evolution in the wild, studies should consider environmental microbial colonization, host phenotypic plasticity in nature, and more realistic environmental conditions excluded from laboratory studies.

1. Introduction

The microbial community that occupies an organism's gastrointestinal tract (i.e. the gut microbiome) is crucial to organism fitness as it regulates resource acquisition [1], life histories [2] and disease resistance [3]. However, describing controls on gut microbiome assembly is difficult because gut microbiomes vary widely across individuals [4], space [5] and time [6]. Gut microbiomes are determined jointly by availability of microbial colonizers in an environment, and the filtering of those colonizers by host traits (e.g. gut morphology) or genetics. Bacteria may colonize vertically from mother to offspring [7] and horizontally through contact with the environment and food [6]. Host diet influences the gut microbiome through colonization, as food carries microorganisms into the gut, and filtering, as diet alters the gut habitat and selects for different resident taxa. But changes in morphological traits that emerge from longer evolutionary history can override colonization and filtering effects of diet. For instance, gut microbiomes of pandas, which exclusively eat leaves, are more similar to closely related carnivores than distantly related herbivores, presumably because their gut morphology evolved from a carnivorous ancestor [8].

Ultimately, microbiome composition determines its functional capacity, such as its ability to acquire or breakdown certain nutrients, or confer immunity. The microbiome may be a powerful modulator of host fitness in circumstances

when there is a mismatch between host traits and environmental demands—for example, host physiology does not allow for digestion of certain resources in a new environment, but the gut microbiome does. Classic examples of this include microbiome-mediated digestion of cellulose in termites, and of plant toxins in mammalian herbivores [9,10]. The microbiome may also help ants supplement a poor quality diet by fixing nitrogen (N) [11], and N-fixing taxa (Rhizobiales) have been found in fish guts [12].

This mismatch among environment, host traits, diet and microbiome is likely a consistent feature of hosts and their microbiomes in the wild, but impossible to capture in purely observational studies. Laboratory studies can control for genetic background by rearing populations in controlled environments, but this excludes the complex way that drivers interact in nature (e.g. behaviour and habitat interact to determine diet). We designed our study to achieve a middle-ground that aims to understand environmental and host-driven controls on gut microbiome: a 'natural laboratory' experiment where replicate wild populations of known origin have undergone parallel phenotypic evolution to novel environments [13–15]. In this system, populations of guppies (*Poecilia reticulata*) originating from high predation (HP) Trinidadian streams were introduced to guppy-free low predation (LP) sites six times between 1957 and 2009 [16–18] (Haskins, unpublished data).

A previous study showed that the microbiomes of native HP and LP populations (the 'end points' of our chronosequence) were significantly different from one another in each of two drainages. However, ecotype differences were not necessarily consistent across drainage [19], leading authors to rule out parallel evolution of gut microbiomes in guppies. Other studies have since demonstrated parallel changes in fish gut microbiomes across repeated diversifications (e.g. in stickleback, [20]), a pattern that implies that host-microbiome interactions influence host fitness. In stickleback, microbiome changes were driven by host morphological traits and diet, both of which show adaptive divergence in guppies [21–23]. We build on previous characterizations of the gut microbiome of guppy ecotypes [24], expand the sample size, and add a temporal element to understand what drives microbiome structure and function change as guppies adapt to new environments.

Variation in guppy microbiomes is likely to be shaped by several factors that differ between HP and LP environments of Trinidadian streams. First, LP environments are generally lower-order streams with less sunlight due to higher canopy cover and lower primary productivity [24]. Thus, guppies transplanted to LP environments may be exposed to a different pool of abiotically filtered stream microorganisms to colonize their gut microbiome [6]. A second reason microbiome differences may emerge is that LP guppies are forced to adopt a more omnivorous, lower-quality diet than HP guppies, because lower-resource LP sites tend to have fewer nutrient-rich macroinvertebrates available [21]. Finally, LP populations have longer guts than HP populations [22], presumably to maximize nutrient absorption and energy extraction from lower-quality food types in lower-resource LP environments [25]. A final note is that while many guppy traits have been shown to shift through rapid parallel evolution [26], diet preferences and gut length may shift through adaptive evolution, plasticity or both.

We use the guppy system to test three hypotheses about how microbiomes assemble as host populations evolve in nature. First, we hypothesize that microbiome will change gradually with time since introduction to a new environment. That is, we predict recently translocated populations will more closely resemble their source populations, to which they are more genetically and phenotypically similar, and that guppies introduced 50–60 years ago will be more similar to native LP fish in the same drainage. Alternatively, microbiomes could shift immediately, in which case all introduced populations would more closely resemble native LP fish in the same drainage. Second, while we expect some unique stream-specific signatures in the microbiome due to colonization by environmental microbes, in general we hypothesize that gut morphology and diet are the main drivers underlying temporal patterns of microbiome assembly. Third, we hypothesize that introduced populations will display parallel shifts in functions that enhance nutrient absorption in the new environment. We focused on two functions in this system: N fixation, which can enhance assimilation of low quality diet of recently translocated HP guppies, and chitin degradation, which may be lost as HP diets shifts from chitin-rich invertebrates to detritus (LP diets).

2. Methods

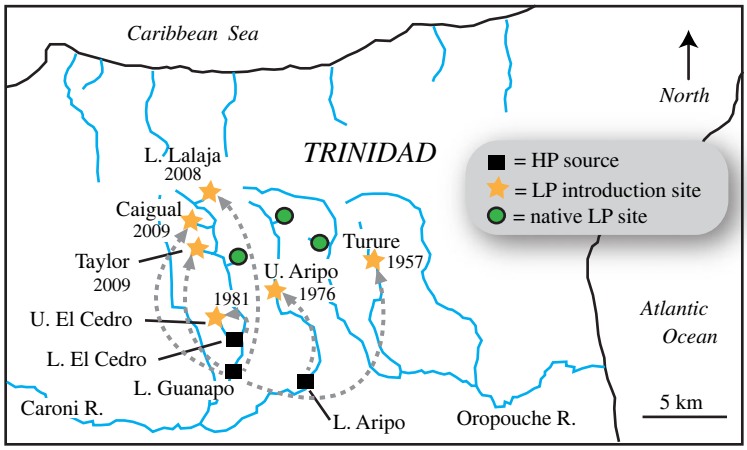
(a) Sample collection and site characterization

In the Northern Range Mountains in Trinidad, guppies originating from high predation (HP) environments have been previously transplanted into guppy-free, LP environments, and monitored as they evolved towards the LP guppy ecotype, forming one of the best examples of replicated parallel evolution in the wild [23,26,27]. Translocations occurred in six instances (1957, 1976, 1981, 2008, and for two streams in 2009) across five drainages (figure 1). In February 2014, we field-collected guppies from each of these six transplant sites, which we call 'Introductions' (LP environments where HP populations were introduced). We also collected fish from the three HP populations that served as sources for the introductions, and from three native LP populations as presumed locally adapted references (figure 1). We divided sites into four categories: HP source, LP native, old introduction (introduced 30–60 years prior to sampling), and recent introduction (5–6 years prior to sampling).

We collected 19–35 female guppies from each of these 12 sites. At each site we sampled three distinct pools that were at least 30 m apart to capture within-site heterogeneity, and only females to control for possible sex differences [28] and for comparability to a previous study in the guppy system [19]. Sampling was lower at Naranjo ($N = 19$) because rain limited sampling time and Tumbasson ($N = 21$) because of its small population size. We weighed and euthanized individuals with an overdose of MS-222 immediately after field capture, and preserved them in 95% ethanol. We measured stream pH and temperature using a handheld meter (Hanna Instruments, Smithfield, RI, USA), and recorded GPS coordinates of each pool. Fish condition was calculated as weight divided by length, which is historically used in the field, but note that more complex metrics should be considered [29].

(b) Characterization of gut length and content

In January 2015, 250 guppies preserved in ethanol at −20°C were dissected with sterile instruments in the laboratory. We recorded the fish length, weight, and presence or absence of embryos. The tubular digestive tract was removed, washed in 70% ethanol,



Site name	Site code	Drainage	Site category	Time since intro	Sample size, microbiome[b]	Sample size, gut content
Taylor	TA	Guanapo	Recent Intro (2009)	5	18	9
Caigual	CA	Guanapo	Recent Intro (2009)	5	20	9
Lower Lalaja	LL	Guanapo	Recent Intro (2008)	6	26	9
Tumbasson	TU	Guanapo	LP native	–	12	9
Guanapo	GU	Guanapo	HP source	–	25	9
El Cedro Intro	EL	El Cedro	Old Intro (1981)	33	23	9
El Cedro Source	ES	El Cedro	HP source	–	21	9
Aripo Intro	AR	Aripo	Old Intro (1976)	38	21	9
Naranjo	NA	Aripo	LP native	–	13	6
Aripo Source	AS	Aripo	HP source	–	23	9
Turure	TR	Turure[a]	Old Intro (1957)	57	25	9
Campo	CM	Campo	LPnative	–	23	9

Figure 1. Map illustrating sampling scheme for our study on the island of Trinidad, and information for each site. Six translocation scenarios are indicated by dashed grey arrows with the names of rivers and year of translocation. Black squares represent high-predation source sites and yellow stars represent translocation sites. Native LP sites (green circles from west to east) are Tumbasson, Naranjo and Campo. Table shows site and sample descriptions, with the following footnotes: [a]Guts were further divided into anterior and posterior sections for microbiome characterization. [b]Note source of Turure introductions was Guanapo HP source, thus is sometimes compared to Guanapo sites, but is not located in Guanapo drainage. (Online version in colour.)

measured for gut length with a ruler in a sterile Petri dish, and cut at the point that the gut turns 180° to delineate anterior and posterior sections. Anterior and posterior guts were preserved in ethanol and DNA was extracted within two weeks. A separate set of 105 intact guppies were shipped to Universidade do Estado do Rio de Janeiro for analysis of gut length and gut content. Upon dissection, guts were removed and preserved in 10% formalin, then weighed, photographed and measured using the software ImageJ [30]. We analysed gut content (invertebrates, detritus, diatoms and algae) using gridded slides, described previously [21], and detailed in electronic supplementary material, Appendix S1. Because invertebrate and detritus percentages were correlated, we only considered invertebrates in the statistical analysis.

(c) Characterization of gut microbiome composition

The adhesion and colonization of the microbiota inside fish guts may be influenced by several factors linked to the stomach, pyloric caeca and intestine portions [31]. Thus, we evaluated the microbiome in both the anterior and posterior portion of the guppy intestinal tracts. We extracted DNA from anterior and posterior sections ($250 \times 2 = 500$ total) following the manufacturer's protocol of the MoBio PowerSoil DNA Extraction kit. We submitted DNA to the Michigan State University Core Genomics Facility for Illumina sequence library construction using the Illumina TruSeq Nano DNA Library Preparation Kit. We submitted a total of 508 samples for sequencing, randomized across two lanes. Eight samples (four anterior and four posterior) were included in both lanes to serve as controls. Following the core facility's standard protocols, bacterial 16S V4 (515f/806r) Illumina compatible libraries were prepared using primers containing both the target sequences and the dual indexed Illumina compatible adapters [32]. Completed libraries were normalized using Invitrogen SequalPrep DNA Normalization plates, pooled and cleaned up using AmpureXP magnetic beads. One sample failed to amplify so 507 samples were sequenced. 16S amplicon pools were sequenced independently in a 2×250 bp paired-end format using independent v2 500 cycle Illumina MiSeq reagent cartridges (Illumina, CA, USA).

Bioinformatics pipelines are fully described in electronic supplementary material, Appendix S1. Briefly, reads were quality filtered and merged using the USEARCH pipeline (http://drive5.com/usearch/), filtered and truncated to 250 bp, clustered into operational taxonomic units (OTUs) at 97% identity level, to enable comparison to previous work [19], then classified against SILVAv123 rRNA database [33]. We removed (i) OTUs classified to Chloroplast and Mitochondria, (ii) OTUs present in less than 5 (of 507) samples, (iii) OTUs that had less than 20 reads across all samples and ((iv) samples with less than 2000 reads (electronic supplementary material, table S1). For calculating diversity metrics, we rarefied samples to 8000 so as to achieve sufficient taxa sampling but without excluding a large amount of gut samples. Sequences are submitted to NCBI SRA (Bio-Project no. PRJNA259592) and bioinformatic code is publicly available (https://gitlab.msu.edu/belldere/guppy_gut_2015).

(d) Data analysis

Our general modelling framework included site category (HP source, LP native, old introduction, and recent introduction) as a fixed effect and site as a random effect nested within drainage (figure 1), similar to previous work testing patterns among source, native and introduced populations [21,34]. We assigned drainages to reflect paired HP to LP translocations. For example, although the El Cedro is a tributary of the Guanapo River, we assigned the two El Cedro sites their own drainage because the translocation occurred within that river. Turure was assigned

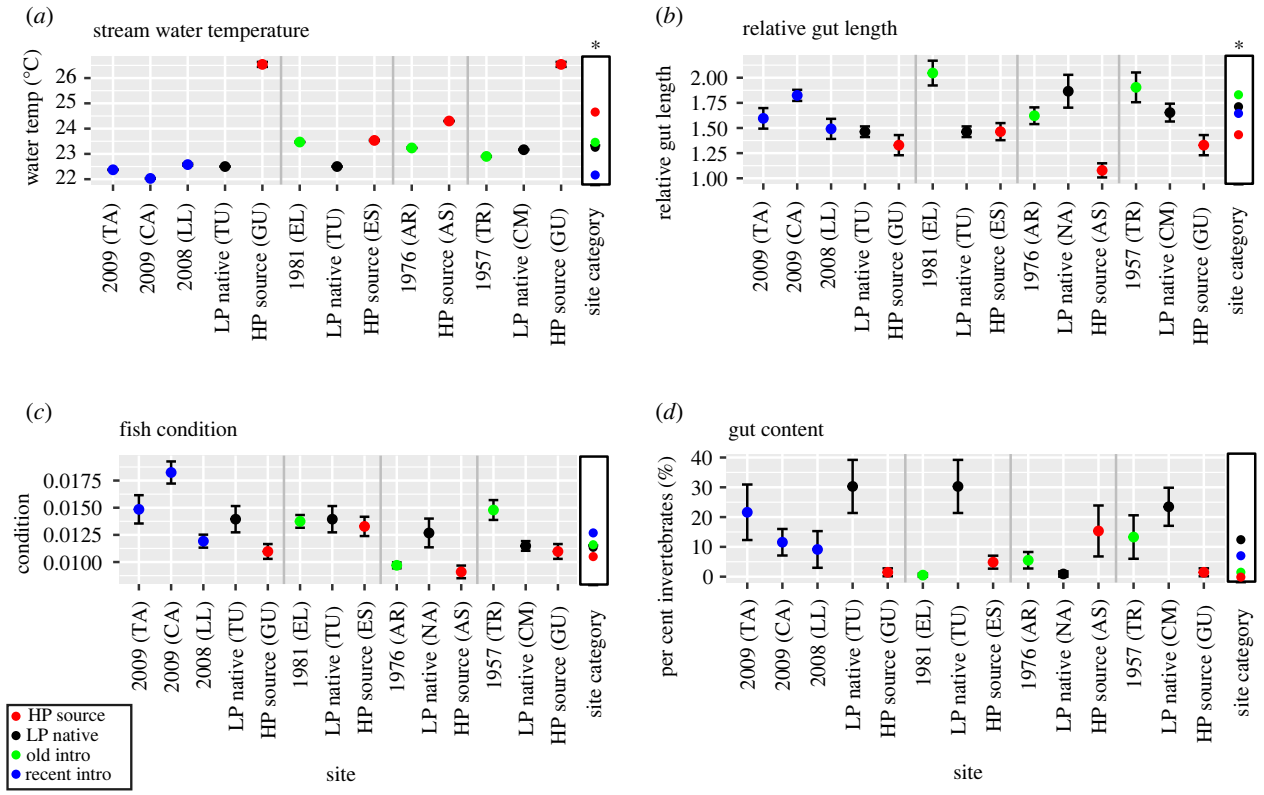


Figure 2. Stream properties, fish traits and diet for each site, as organized by drainage (sections in each graph) and site category (colours), including mean (Site category). Error bars (some hidden) show standard error and are not shown for Site Category for clarity. *Represent $p < 0.01$ for equal means across site category. (Online version in colour.)

to its own drainage (as opposed to its HP source site Guanapo) because these two sites are not physically connected. We used this model structure to test for differences among all univariate response variables (stream characteristics, fish traits, gut content) with linear mixed-effects models, after transforming response variables to meet assumptions of normality, comparing null and fixed-effect models using a likelihood ratio test.

We assessed statistical differences in community structure across site categories using permutational analysis of variance (PerMANOVA) on the unweighted (presence-absence) and weighted (incorporates abundance) Unifrac distance matrices using phyloseq [35] and vegan [36] in R [37]. Permutations were robust to the order of factors, which included gut position, stream treatment, and site, and were constrained by drainage and site as random effects, as above. We also tested another model that treated site and drainage as fixed effects so we could better understand the relative influence of categorical variables on microbiome variation. We examined the effect of site category on posterior gut microbiomes (all sites), in LP sites alone (LP natives and introductions) and pairwise, within each drainage, adjusting *p*-values using Bonferroni correction.

We quantified the variance in community composition explained by environmental variables (stream temperature, which correlated to stream pH, $r^2 = 0.42$, $p < 0.001$), gut length, and gut content using distance-based redundancy analysis [38] (dbRDA, electronic supplementary material, Appendix S1), with drainage as a random effect. Gut content and length analysis was performed on a subset of fish distinct from the subset for which gut microbiome was sequenced, from which we calculated population-level mean diet. We thus used population-level means for all predictor variables (diet, stream characteristics and gut length) in dbRDA and variance partitioning. This did not affect conclusions; the direction and relative influence of factors remained the same when gut length and abiotic conditions (which had very little within-population variation) were aggregated by population, like gut content. Since the microbiome from HP source was so distinct, we repeated all variance partitioning and dbRDA without HP source to examine factors controlling microbiome in LP environments. We also examined the influence of geography by testing drainage as a fixed effect (electronic supplementary material, Appendix S1). We calculated all mean pairwise distances between samples in a site, focusing on mean distance between an HP source population and its respective sites. We quantified betadispersion and richness on rarefied data.

We used PICRUST2 to predict the functional profiles of microbial communities based on their 16S rRNA gene sequences, which is improved from the first version but has limitations [39,40]. After removing 373 of 12 028 OTUs that did not align well and 20 samples above 2.0 Nearest Sequenced Taxon Index (NSTI), we normalized each predicted gene copy by OTU abundance. We then assessed changes in predicted gene family abundance for 2390 Enzyme Classification (EC) groups across samples using many of the compositional analyses described above (db-RDA, variance partitioning, distance, richness). We also tested whether predicted gene copy number differed across sites for two functions: Nitrogenase (EC:1.18.6.1) and Chitinase (EC:3.2.1.14).

3. Results

Environmental properties across HP and LP environments varied mostly as expected based on what is already known about Trinidadian streams. Namely, lower elevation, high-predation (HP) sites had higher mean stream water temperature (HP: 24.87°C, ±0.08 versus LP: 22.82°C, ±0.03, figure 2*a*) and pH (HP: 7.5 ± 0.03, LP: 6.7 ± 0.03) compared to low-predation sites ($p < 0.01$ for both comparisons), which include LP native, old and new introductions. Fish morphology also fit expectations: fish in LP environments had longer gut lengths

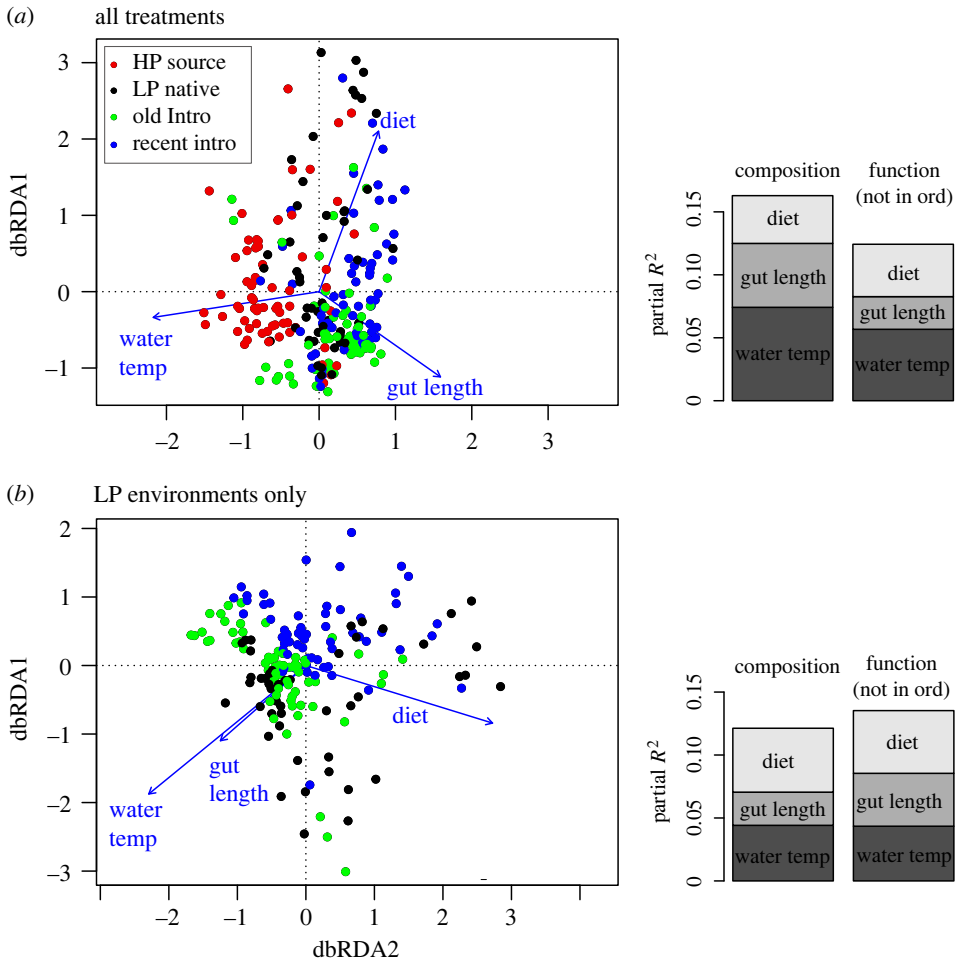


Figure 3. Distance-based redundancy analysis based on the composition of bacterial guppy gut microbiomes (weighted Unifrac) in high and in LP streams, where HP fish (HP source, red) have been introduced either 30–60 years ago (Old Intro, green) or 5–6 years ago (Recent Intro, blue), and evolved to resemble native LP fish (black). (*a*) shows all site categories and (*b*) excludes HP source sites, showing sites in LP environments (LP native, old intro and recent intro). Arrows show the predictor variables that explain changes in composition based on dbRDA, and length corresponds to dbRDA R^2. Right barplots show the partial R^2 variance partitioned for diet, gut length and water temperature (grayscale). In the electronic supplementary material, you will find ordinations and barplots for all distance metrics (figures S4 and S5), drainage-specific ordinations (figure S6), dbRDA statistics (table S4) and PerMANOVA results (tables S2 and S3). (Online version in colour.)

relative to body size (figure 2*b*). Site-specific variation overwhelmed significant effects of site category on fish condition (weight/length, figure 2*c*; electronic supplementary material, figure S1E) and gut content (as measured by percent invertebrates, figure 2*d*; electronic supplementary material, figure S1E). Contrary to expectations, LP fish guts had more invertebrates at the time of sampling, whereas HP fish had diets richer in detritus, despite their shorter guts.

After pre-filtering steps, we identified 12 068 bacterial OTUs in 485 guppy gut microbiome samples (rarefaction curves, electronic supplementary material, figure S2). Microbiomes in anterior and posterior sections showed only slight differences (see electronic supplementary material, Appendix S2), so we focused analyses on the posterior gut microbiome, which may be less transient than the anterior gut community. Site category had a significant effect on gut microbiome community composition (PerMANOVA $p = 0.001$, figure 3; electronic supplementary material, tables S2 and S3), and explained more variation than site and drainage when the latter were treated as fixed effects (electronic supplementary material, table S2). Patterns varied depending on whether we considered relative abundance (figure 3*a*), presence-absence (electronic supplementary material, figure S4) or gene-inferred functional profile (electronic supplementary material, figure S4).

Gut microbiomes in LP environments (Introductions and LP native) were generally quite dissimilar from their respective HP source populations (figure 4; electronic supplementary material, figure S4). However, Introduction sites and LP native sites were not necessarily similar to one another (electronic supplementary material, figure S5 and Appendix S2). Stream water temperature (which correlated closely to stream pH) was generally the most important factor driving composition and function (figure 3; electronic supplementary material, figure S5), but gut length was also consistently important. Geography (Drainage) also contributed to variation in microbiome at levels comparable to continuous variables, but did not account for all of their contributions (electronic supplementary material, Appendix S2). The importance of diet increased when explaining differences across LP environments only, and in explaining microbiome function (electronic supplementary material, figure S5). Mean microbiome richness (mean number of species within individual guts) was significantly lower in native populations (HP source and LP native) and higher in introductions (figure 5*a*). Richness was positively correlated to relative gut length ($r^2 = 0.43$, $p < 0.001$), and fish length, as previously observed [41,42], and was not explained by higher diet diversity [42].

Gut communities were dominated by Proteobacteria, Actinomycetes and Firmicutes, similar to other fish microbiomes

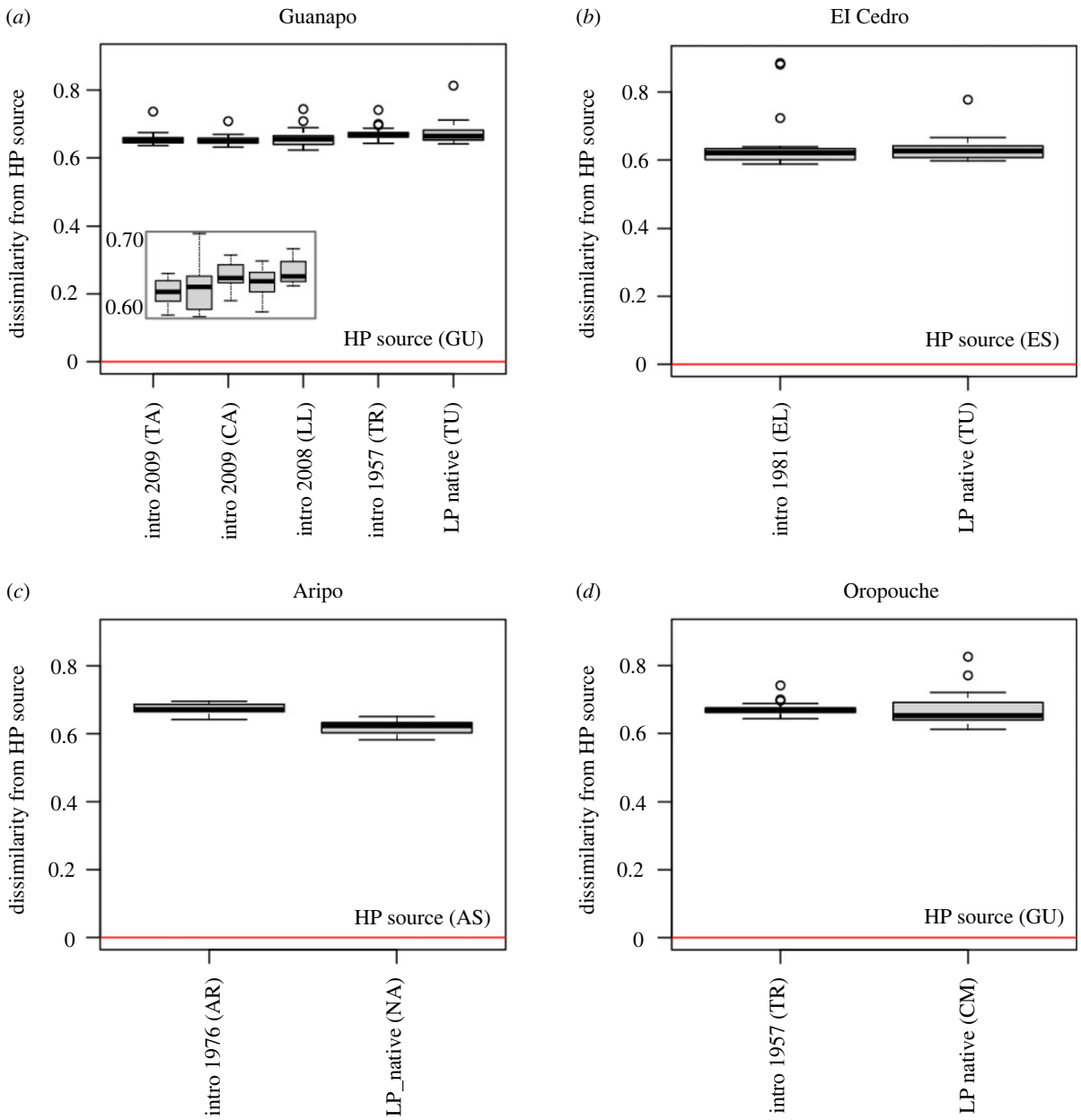


Figure 4. Dissimilarity (unweighted Unifrac distance) of the gut microbiome of each population compared to the gut microbiome of its HP source population (shown by a red line at 0). Higher values are more dissimilar to HP source. Inset of (*a*) shows finer scale axes. See electronic supplementary material, figure S7 for mean pairwise distances of all sites. Note that TR is sourced from the Guanapo HP site but because they are both in Oropouche drainage, serves as a comparison to Campo. (Online version in colour.)

[12], and previously identified dominant phyla in guppies [19]. Five of the 10 most abundant taxa across the entire dataset were from the order Rhizobiales, an order that contains N-fixing bacteria. The total Rhizobiales abundance (figure 5*b*; electronic supplementary material, table S5), as well as the abundances of these dominant taxa (figure 5*c*), tended to be highest in the old introduction streams, and lowest in HP source streams. Mycobacterium, a genus that contains pathogens, was also abundant across sites, and highest in Recent introductions (electronic supplementary material, table S5).

Predicted functions per sample was significantly higher in Introduction sites compared to native (figure 5*d*), a trend similar to compositional richness. Neither predicted nitrogenase nor chitinase gene abundance significantly differed across site categories (figure 5*e,f*), and both showed high site to site variation (electronic supplementary material, figure S8). Nitrogenase gene abundance had a weak but significant correlation with fish condition ($p = 0.040$, $r = 0.12$), as did number of functions ($p = 0.008$, $r = 0.18$). Chitinase gene abundance was weakly correlated to percent invertebrates in the gut (data not shown, $p = 0.020$, $r = 0.18$). Looking across all functions, of the 2390 enzyme classification pathways we characterized, 370 had significantly different gene abundance across site categories ($p < 0.05$), and only 35 after adjusting for multiple comparisons (electronic supplementary material, table S6).

4. Discussion

(a) Temporal progression of gut microbiome after host is introduced to a new site

We investigated variation in the guppy gut microbiome along an evolutionary 'chronosequence' of time since introduction to a novel predation regime. Previous work has documented

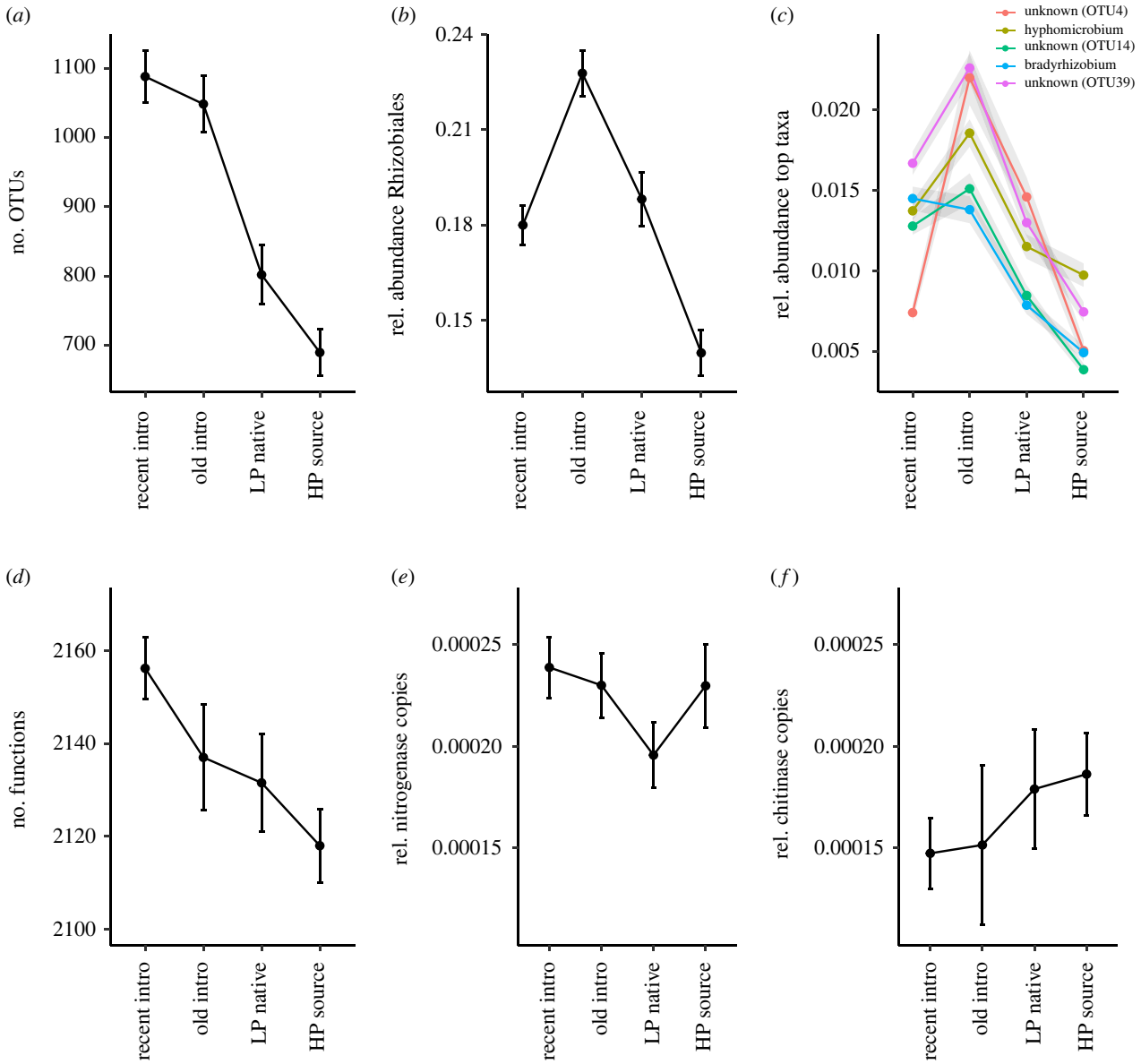


Figure 5. Gut microbiome richness (*a*), mean relative abundance of order Rhizobiales (*b*), relative abundance of five Rhizobiales species (categorized by genus, if known) that were part of the ten most abundant OTUs across all treatments (*c*), mean number of metabolic functions detected (*d*), nitrogenase copies (*e*) and chitinase copies (*f*). Bottom row panels are predicted from 16S copies using PICRUST2. Other community diversity metrics are shown in electronic supplementary material, figure S3, most abundant OTUs listed in electronic supplementary material, table S5, and site-level nitrogenase and chitinase are show in electronic supplementary material, figure S8. (Online version in colour.)

parallel evolution in a suite of genetically based host traits [26]. Since we thought microbiome-shaping traits (diet, gut morphology) would follow a similar trajectory [21,43], we first hypothesized that the microbiomes of recent introductions would be similar to those of HP host populations, and the microbiomes of older introductions would resemble typical LP populations. We found some evidence for our hypothesis: microbiomes of fish introduced into new (LP) environments were significantly different from HP source fish, and this shift occurred in each of the four drainages. We also found that drainage had a significant effect on microbiome, but unlike a previous study, these stream-specific signatures did not overwhelm microbiome differences associated with site category [19]. This suggests that with this larger sample size, gut microbiome can show some level of parallelism across drainage, even though stream-specific factors are no doubt present.

Even though microbiome did shift along the chronosequence, our findings deviated from our first hypothesis in two important ways. First, changes in gut microbiome did not occur gradually as a function of time since introduction. Rather, guppy microbiomes translocated to LP environments just 5–6 years before sampling (approx. 15–18 guppy generations) were already quite different from their source (HP) fish (figure 4), despite the fact that their genetic background remained similar to their HP source populations [34]. This surprisingly rapid microbiome change was associated with both a change in environment as well as rapid divergence in phenotypic traits, making it difficult to tease apart the cause (discussed below).

Second, as gut microbiome composition shifted, it did not necessarily change to resemble those of LP native populations. Gut microbiomes of introduced populations remained significantly different from those of LP natives even when they were in an LP environment for more than 50 years (electronic supplementary material, table S3). We expected microbiomes of introductions would become more similar to those of LP natives, as many traits have evolved to resemble these ecotypes [22,23]. Interestingly, when we examined microbiome *function*, we found it was not significantly different

among introduced and LP native microbiomes (electronic supplementary material, table S3), discussed more in third section). Future studies should consider using other metrics to assess convergence (see [20]), rather than statistical significance of differences (or lack thereof).

(b) Drivers of microbiome composition and function

Our second hypothesis was that gut morphology and diet were stronger drivers of microbiome composition than abiotic conditions in a stream, due to the strong pressure a host exerts on its microbiome. In contrast to these expectations, we found that abiotic stream characteristics (temperature and pH) most strongly predicted gut microbiome, though we could not disentangle abiotic conditions and gut length, which both changed within 5–6 years after translocation. Temperature and pH are known drivers of microbial community composition in freshwater environments [44], so these factors may determine the taxa that can colonize the gut. While we cannot test this without characterizing the stream microbial community, a previous study found that only a small proportion of taxa in guppy guts were present in stream water [19], suggesting abiotic factors or geography could determine the presence/absence of taxa, but other factors determine abundance. Abiotic variables may also shape gut microbiome through other processes, such as by determining the types of prey present, which introduce unique colonizers when consumed [5,45].

Gut length was also an important driver of microbiome composition and function, shifting quickly from the typical HP phenotype to more closely resemble the LP phenotype, even in recent introductions. While adaptive evolution of longer guts in LP environments has been shown to occur quickly in this system [46], the rapid change in gut length may also reflect some amount of phenotypic plasticity. Zandonà *et al*. [22] previously showed that differences between HP and LP guppy gut lengths are smaller during the wet season when HP populations are more omnivorous. This trait may be plastic in other organisms, particularly under fluctuating resource availability [47,48], since digestive tissues are costly [49]. At the same time, we know that guppy gut length is not entirely plastic: a previous study showed that differences in HP and LP gut length were maintained even after receiving distinct diets for 10 weeks in the laboratory [19]. In sum, although we cannot attribute the observed reduction in gut length to host evolution versus plasticity without a common garden, we find that phenotypic change in this trait, no matter the mechanism, can shape microbiomes.

Since both stream properties and gut length had already shifted in the most recent introductions by the time of sampling (5–6 years in LP environment), we could not parse out which of these factors were initially a stronger driver of gut composition. Future studies could ask how the microbiome impacts host colonization of new sites by capturing the generation immediately after translocation or invasion and characterize environmental microbiomes, like those in water and on prey. Experiments are also needed that control for geography, which contributed to microbiome variation in our study, and co-varied with environmental conditions. In addition to geographical distance, microbiome variation could have been driven by variables we did not measure, such as parasite load (high in three of the four drainages we sampled [50]), age, and sex [28], which may or may not covary with geography.

Diet played a smaller than expected role in driving microbiome composition. There are two explanations for this. First, diet may be a weaker driver of microbiome than gut morphology and stream. Indeed, previous work showed that HP and LP fish (with different gut morphologies) retain different microbiomes even when fed the same diet [19]. Also, at broad taxonomic scales, microbiome divergence tracks diet primarily in accordance with evolution of digestive traits (e.g. hindgut to foregut) [51]. Gut content may primarily affect microbiome, and particularly taxon abundance and function, when gut morphology is held constant, or when gut content matches aggregated diet.

A second explanation is that the gut content we captured may not have reflected typical HP and LP diets due to time of sampling [22,24]. While this may have prevented us from capturing divergence in diet due to predation regime (and weakened diet as a driver of category-associated microbiome), this variation is still notable, since seasonal diet shifts could be equally powerful in altering gut microbiomes. As our understanding of microbiome divergence begins to extend beyond controlled studies, it will be important to capture the complexity of diet in the wild, which may be temporally dynamic [52], influenced by complex behaviours, and not easily characterized by single axes induced in the laboratory.

(c) Shifts in microbiome function

Our final hypothesis was that certain functions that could impact fitness in new environments would vary across the translocation time series. Both gene-inferred function (i.e. PICRUST) and taxonomy-inferred function have significant limitations, so we restrict our investigation to tests in two specific functions (chitinase and nitrogenase) for which we had *a priori* hypotheses based on diet differences between HP and LP environments [21,22], and encourage findings to be used as hypotheses that can be subject to more robust tests.

N fixation (enabled by the nitrogenase gene) can enhance nutrition of low-quality diets [11], which may occur when HP guppies are translocated to LP environments. Interestingly, five of the ten most abundant OTUs in the entire dataset belonged to a single order, Rhizobiales, which contain many N fixers. Rhizobiales are abundant in the guts of many fish species [12], and decrease as diets become more carnivorous in fish [12]. In our study, abundant Rhizobiales in LP populations (particularly old introductions, figure 5*b*,*c*) could increases fitness of introduced populations relying on poor quality diets. Indeed, the significantly more abundant Rhizobiales species in the introduced El Cedro population (electronic supplementary material, figure S8) could explain why these fish were in better condition than their LP native counterparts (figure 1*c*), despite a lower quality diet (figure 1*d*). While gene-inferred nitrogenase function did correlate to fish condition, suggesting there is potential for this function to affect fitness (with limited inference), we did not find support that nitrogenase gene abundance changed in parallel across drainages (i.e. no effect of site category, figure 5*e*; electronic supplementary material, figure S8). Rhizobiales abundance may not have aligned with nitrogenase gene abundance because the Rhizobiales we identified do not fix N. On the other hand, PICRUST-inferred gene abundance does not always correspond to rates [53], and may miss uncharacterized N fixers.

We expected chitinase degradation genes to decrease in introduced populations as LP fish consume fewer invertebrates. Chitinase gene abundance was significantly correlated to percent invertebrates in the gut, supporting our prediction, but

like nitrogenase, gene abundance did not differ across site categories. It may be that these functions more closely followed diet, which varied more at the stream level and within populations, than the site category level, or that chitinase gene estimates are also limited by existing databases [53].

When considering gut microbiome function as a whole, we did not detect functional differences between LP natives and introduced populations, but compositional differences were maintained. While we again note that lack of significant difference does not equal convergence, this discrepancy may be explained by LP sites having similar dominant taxa, some of which have similar functions but different species identities. Indeed, functional redundancy is known to be high in microbiome communities, including gut microbiomes [54,55].

It is also notable that introduced populations had significantly greater richness (figure 5*a*), and more unique functions (figure 5*d*), than either HP or LP natives. This may be because introduced populations had more sources of microbiome transmission (vertical transmission from source population as well as horizontal transmission from the new LP environment, with the latter a stronger contributor). It also could suggest that host filtering processes can change after introduction to a new environment. Regardless of the mechanism, future studies could ask whether greater microbiome richness increases opportunities for introduced populations to select for novel or rare microbiome functions.

5. Conclusion

By studying the microbiome in a chronosequence of guppy evolution, we revealed that the gut microbiome composition and function rapidly diverges from microbiomes of source populations with shifts in gut morphology and stream environment, which covaried with geography. Microbiomes of translocated populations ultimately may converge with LP native populations in terms of function, but retain stream-specific signatures in composition. We also found nutrient acquisition microbiome functions correlated to fish condition, but did not change in parallel across drainages. In contrast to much of our knowledge of host-microbiome evolution based on macro-evolutionary patterns and laboratory studies, our study offers a window into host-microbiome changes in real-time in wild populations, where environment and traits covary, and both plastic and genotypic changes will influence microbiome composition.

Data accessibility. Sequences are submitted to NCBI SRA (Bio-Project no. PRJNA259592) and bioinformatic code is publicly available (https://gitlab.msu.edu/belldere/guppy_gut_2015).

Non-sequence data are available on Dryad: https://doi.org/10.5061/dryad.73n5tb304 [56]. Electronic supplementary material is available online [57].

Authors' contributions. S.E.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation, visualization, writing—original draft, writing—review and editing; E.Z.: data curation, formal analysis, funding acquisition, methodology, supervision, validation, writing—review and editing; J.R.A.: data curation, formal analysis, methodology, validation, writing—review and editing; S.F.: conceptualization, project administration, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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