Effects of parental care on skin microbial community composition in poison frogs

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Abstract

Parent-offspring interactions constitute the first contact of many newborns with their environment, priming community assembly of microbes through priority effects. Early exposure to microbes can have lasting influences on the assembly and functionality of the host's microbiota, leaving a life-long imprint on host health and disease. Studies of the role played by parental care in microbial acquisition have primarily focused on humans and hosts with agricultural relevance. Anuran vertebrates offer the opportunity to examine microbial community composition across life stages as a function of parental investment. In this study, we investigate vertical transmission of microbiota during parental care in a poison frog (Family *Dendrobatidae*), where fathers transport their offspring piggyback-style from terrestrial clutches to aquatic nurseries. We found that substantial bacterial colonization of the embryo begins after hatching from the vitelline envelope, emphasizing its potential role as microbial barrier during early development. Using a laboratory cross-foster experiment, we demonstrated that poison frogs performing tadpole transport serve as a source of skin microbes for tadpoles on their back. To study how transport impacts the microbial skin communities of tadpoles in an ecologically relevant setting, we sampled frogs and tadpoles of sympatric species that do or do not exhibit tadpole transport in their natural habitat. We found more diverse microbial communities associated with tadpoles of transporting species compared to a non-transporting frog. However, we detected no difference in the degree of similarity between adult and tadpole skin microbiotas, based on whether the frog species exhibits transporting behavior or not. Using a field experiment, we confirmed that tadpole transport can result in the persistent colonization of tadpoles by isolated microbial taxa associated with the caregiver's skin, albeit often at low abundance. This is the first study to describe vertical transmission of skin microbes in anuran amphibians, showing that offspring transport may serve as a mechanism for transmission of parental skin microbes. Overall, these findings provide a foundation for further research on how vertical transmission in this order impacts host-associated microbiota and physiology.

Introduction

Host-associated microbial communities contribute to health and facilitate ecological adaptations by playing critical roles in host development, nutrition, pathogen exclusion, and immune response (Belkaid & Hand, 2014; Donald & Finlay, 2023; Gensollen et al., 2016; Mazmanian et al., 2005; Reynolds & Bettini, 2023; Sampson & Mazmanian, 2015; Von Frieling et al., 2018). Across host species, early exposure to microbes can exert lasting influences on the assembly and functionality of the host's microbiota (Al Nabhani & Eberl, 2020; Powell et al., 2014; Warne et al., 2017; Zepeda Mendoza et al., 2018). Timing of microbial contact is particularly critical in newborns and can prime community assembly through priority

effects, where species that arrive early impact establishment of later colonizers (Debray et al., 2022; Fukami, 2015; D. Sprockett et al., 2018). In many species, the transmission of host-adapted microbes is facilitated by parental care strategies, such as feeding, grooming and direct skin contact (for review see e.g. S. Wang et al., 2020 for humans, Klug & Bonsall, 2014 for animals; see also Blyton et al., 2022; D. W. Chen & Garud, 2022; Pascoe et al., 2017; D. D. Sprockett et al., 2020; Sylvain & Derome, 2017). To date, most studies investigating effects of exposure to microbiota during parental care center on humans and livestock, while vertical transmission remains largely unexplored in other classes of vertebrates (but see Kouete et al., 2023). Therefore, investigating early-life microbial colonization in other species that have dedicated parenting strategies offers opportunities to understand microbe-host interactions in a wider variety of ecologically relevant contexts.

Acquisition of host-adapted skin microbes is especially important in amphibians, which rely on healthy skin for physiological processes such as respiration, osmoregulation, immune response and barrier function (Çömden et al., 2023; Duellman & Trueb, 1986; Varga et al., 2019). The amphibian skin microbiota also serves to exclude pathogens, as the skin microbiome can confer resistance to infections by the deadly chytrid fungus *Batrachochytrium* (Alexiev et al., 2023; Harris et al., 2006, 2009; Loudon et al., 2014; Vredenburg et al., 2011; Woodhams et al., 2007), which decimates amphibian populations worldwide (Daszak et al., 2003; Luedtke et al., 2023; Wake & Vredenburg, 2008). Many studies have characterized microbe-host interactions in adult frogs in the context of chytrid infections, but few have focused on microbiomes associated with tadpoles (Fontaine et al., 2022; Santos et al., 2023; Warne et al., 2017, 2019; Weinfurther et al., 2023). Those studies that have looked at tadpoles have primarily characterized the changes in bacterial communities across development and metamorphosis (discussed e.g. in Hughey et al., 2017; Kueneman et al., 2014; Prest et al., 2018; Warne et al., 2017, 2019). Furthermore, most of this research has focused on temperate-region, pond-spawning species, where opportunities to investigate effects of parental care on community assembly are limited. These species generally lay many eggs at once into bodies of water to avoid desiccation and larvae develop without further parental contact or care. In the tropics, a warm and humid climate has favored the progression from aquatic to terrestrial reproduction, where clutches are laid on land and parents then care for their offspring (McDiarmid & Altig, 1999; Wells, 2007). Neotropical frogs show more diverse reproductive strategies where parents construct foam nests, attend clutches, defend eggs against predators, and guard, transport or feed their tadpoles (reviewed e.g. in Schulte et al., 2020, see also Crump, 2015; Delia et al., 2013; Requena et al., 2009; Warkentin, 1995). Priority effects influence community assembly of frog embryos (K. R. Jones et al., 2023, 2024) but previous studies have found no direct evidence for vertical transmission of microbes during egg attendance (Hughey et al., 2017). However, this form of offspring care does not involve direct contact between the parents and offspring as developing tadpoles in the clutch are surrounded by several layers of gelatinous jelly and a

double vitelline envelope before they hatch (see Altig & McDiarmid, 2007 and Méndez-Tepepa et al., 2023 for a review). While the vitelline layer may serve as an antimicrobial barrier in chickens (Guyot et al., 2016; Mann, 2008), studies of its role in microbial colonization of anuran embryos are currently lacking.

Parental care involving skin-to-skin contact has developed multiple times independently in Neotropical dendrobatoid frogs (Superfamily *Dendrobatoidea*), commonly referred to as poison frogs (Grant et al., 2017; Weygoldt, 1987). Closely related species in this clade display a variety of parenting behaviors ranging from egg attendance and transport of hatched tadpoles to provisioning larvae with unfertilized eggs (Crump, 1974; Ringler et al., 2023; Wells, 2007). The most widespread form of care beyond clutch attendance is tadpole transport, where parents shuttle hatched tadpoles piggy-back style from terrestrial clutches to water pools (Furness & Capellini, 2019). Transport can last multiple hours to days, where tadpoles adhere tightly to the backs of their caregivers (Pašukonis et al., 2019; Pröhl & Berke, 2001; Ringler et al., 2013, p. 201; Wells, 1980). Therefore, poison frogs provide the opportunity to evaluate the effects of parental care on offspring skin microbial community assembly. The adaptive value of parental care in this clade has received considerable attention (reviewed in Schulte et al., 2020; Summers & Tumulty, 2014), but how reproductive strategies involving skin-to-skin contact impact microbial colonization of tadpoles and whether these effects persist to later developmental stages has, to our knowledge, not been studied in any anuran to date.

The variable poison frog *Ranitomeya variabilis (Rv)* (Zimmermann & Zimmermann, 1988) is particularly well-suited to study vertical transmission of microbes during tadpole transport. This small species is diurnal, commonly found across South America and readily reproduces in captivity. Males of this species often assist their larvae with hatching (Brown et al., 2008) and carry tadpoles on their back for up to 48 hours (Lötters et al., 2007). The cannibalistic tadpoles grow up in individual pools where they feed on algae and leaf debris until they complete their development after about five months. In the natural study population located in the reserve 'Les Nouragues' in French Guiana, these frogs live on rocky outcrops, use bromeliads as a resource for reproduction and shuttle tadpoles from the oviposition site in arboreal plants to water reservoirs in leaf axils of terrestrial bromeliads (Poelman et al., 2013; Poelman & Dicke, 2007, 2008; Sarthou, 2001). This species is sympatric with other anuran species with differing parenting strategies: the poison frog *Allobates femoralis* (*Af*) shuttles tadpoles to water but transporting periods are shorter and tadpoles live in groups. The leptodactylid frog *Leptodactylus longirostris* (*Ll*) deposits eggs in ephemeral rock pools and does not exhibit parental care. These cohabiting species are potentially useful for determining if reproductive strategies impact patterns of microbial diversity of tadpoles.

In this study, we combine laboratory experiments with sampling of anuran populations in the wild to examine vertical transmission of microbes during tadpole transport and address implications for

community structure across life stages. In the laboratory, we investigated microbial colonization of hatched and unhatched *Rv* embryos and then tested the hypothesis that transporting frogs serve as a source for bacteria on tadpole skins using cross-foster experiments. We complemented these experiments with a comparative field study examining microbiomes associated with tadpoles and adults of three anuran species with varying parenting behaviors to examine how shuttling affects community composition on the skin of tadpoles in their native habitat. To our knowledge, this is the first study to document and characterize vertical transmission of microbes during parental care in any anuran.

Results

<u>Microbial colonization of a poison frog embryo occurs after hatching</u>

We used non-sterile frogs of our captive laboratory colony to determine the timing of microbial colonization of poison frog embryos. Embryos within the vitelline membrane exhibit a characteristic C-shaped posture due to the membrane's restriction on their movement and adopt a straight posture after hatching (Fig. S1A). First, we qualitatively examined bacterial presence in *Rv* egg jellies and embryos across tadpole developmental stages using a broad range PCR targeting the 16S rRNA gene. We manually separated embryos in the vitelline envelope from the surrounding jelly and processed each for DNA extraction (Fig 1A). Bacteria were detected in egg jellies across all developmental stages (formation of the dorsal lip to hatchling) but were not detected in embryos prior to hatching from the vitelline membrane (N = 12) (Fig 1B, Fig. S1B). After hatching, bacteria were detected in tadpoles that remained in the jelly and in tadpoles that were transported by their caregiver (Fig. 1B, Fig. S1B). Next, we confirmed differences in bacterial load between jelly and embryo by examining variations in 16s rRNA copy numbers using a droplet digital PCR (ddPCR) approach suited for low biomass samples (Abellan-Schneyder et al., 2021). We found that unhatched embryos contained on average 2942 copies of the 16S rRNA gene per µl (min = 742, max = 11584, std = ±3086) while jellies contained on average 6128 times as many (min = 457417, max = 66115451, mean = 18029604 ±18586471; Kruskal Wallis (KW): chi-squared = 18.6667, df = 2, $p < 0.00001$) (Fig. S1C, Table S1). 16S rRNA gene copy concentrations were on average 8310 times higher in hatched relative to unhatched embryos (min = 737340, max = 48159335, mean = 24448337 ±33532414; KW: $p = 0.0195$), but did not differ significantly from concentrations detected in the jelly (KW: $p = 0.5$) (Fig. S1C, Table S1).

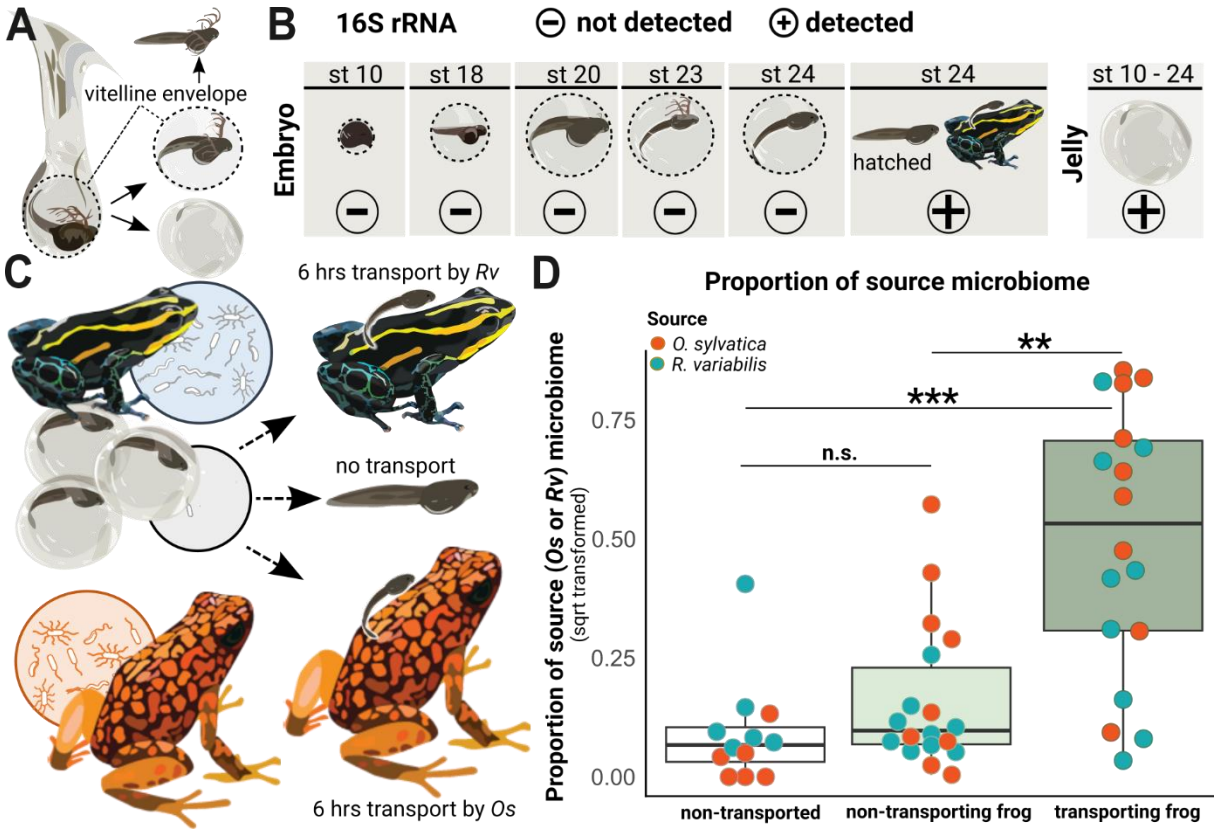

Figure 1. **Microbes colonize a poison frog embryo after hatching and are vertically transmitted during tadpole transport. (A)** Embryos in the vitelline envelope (dashed line) were manually separated from jelly using tweezers. The vitelline envelope (dashed line) containing the embryo was transferred to sterile water and opened to free the embryo. The embryo was washed in fresh sterile water before homogenization. **(B)** Detection/non detection of 16S rRNA across developmental stage. DNA was isolated from whole embryos and jellies of different developmental stages and tested for bacterial presence using a broad range PCR for near-complete 16S rRNA gene (see also Fig. S1). **(C)** After hatching, siblings of a clutch were either (1) not transported (middle arrow), (2) transported by their biological parent (upper arrow), or (3) transported by a foster poison frog of a different species (*Oophaga. sylvatica*) (lower arrow). **(D)** We performed 16S v4 specific amplicon sequencing on swabs from the transporting frogs and the skins of the transported tadpoles and used Sourcetracker to identify the sources of taxa that had been acquired by tadpoles. The function was trained on communities of adult *Rv* and *Os* that had served as caregivers. Source proportions of both species (*Os*: orange dots and *Rv*: blue dots) were determined for each tadpole (N = 24), resulting in 2 data points per tadpole. Proportions were then grouped to display either (1) proportions of the transporting species in transported tadpoles (*Rv* proportions in tadpoles transported by *Rv* and *Os* proportions in tadpoles transported by *Os*) (dark green), or (2) proportions of the non-transporting species on transported tadpoles (indicating *Rv* proportions in tadpoles transported by *Os* and *Os* proportions in tadpoles transported by *Rv*) (light green), or (3) proportions of both species in non-transported tadpoles (indicating *Rv* and *Os* proportions in non-transported tadpoles) (white). Proportions were compared with a Kruskal-Wallis test with Benjamini-Hochberg correction. Source proportions were square root transformed for plotting.

Evidence for vertical transmission of microbiome during parental care in laboratory-reared poison frogs

We used a cross-foster design to test the hypothesis that transporting frogs serve as a source of microbes for communities on tadpole skin. Siblings of the same clutch (N = 6 clutches from 2 *Rv* pairs) were randomly assigned to three groups: (1) not transported (N = 6 tadpoles), (2) transported by their biological parent for six hours (N = 9 tadpoles), or (3) transported by a heterospecific poison frog (*Oophaga sylvatica*, *Os*) for six hours (N = 9 tadpoles) (Fig. 1C). For this design, sequence-based surveys of amplified 16S rRNA genes were used to assess the composition of skin-associated microbial communities from

tadpoles and their adult caregivers (the tadpole-transporting frogs). We found that bacterial community composition on caregiver skin was primarily shaped by host species (*Rv* versus *Os*; Adonis: Bray-Curtis: $F_{1, 12} = 5.6538$, $R^2 = 0.33949$, $p = 0.001$; unweighted Unifrac: $F_{1, 12} = 2.0078$, $R^2 = 0.15435$, $p = 0.013$), allowing us to distinguish between these potential sources of tadpole-colonizing microbes (Fig. S2A). Indeed, we found that after six hours of transport, bacterial community composition on *Rv* tadpole skin was influenced by caregiver species (*Rv* versus *Os*; Adonis: Bray-Curtis: $F_{1, 12} = 1.75$, $R^2 = 0.099$, $p = 0.017$; Unifrac: $F_{1, 12} = 1.67$, $R^2 = 0.095$, $p = 0.015$). Despite their distinct compositions, we observed substantial overlap between communities of tadpoles transported by *Os* and *Rv* (Fig. S2B), possibly reflecting similarities in tadpoles' skin communities arising from a shared clutch environment after hatching until transport.

We used 'Sourcetracker' (Knights et al., 2011) to determine the relative contribution of the transporting frogs as a source of bacteria detected in tadpoles' skin after six hours of transport. Transported tadpoles (N = 18) shared higher proportions of their skin communities with the transporting species than did non-transported tadpoles (N = 6) (Kruskal Wallis $chi^2 = 19.4029$, df = 2, $p = 0.0001$) (Fig. 1D). Similarly, all but two transported tadpoles shared a larger proportion of their communities with the transporting species than with the non-transporting species ($p = 0.001$) (Table S2). Tadpoles of a clutch that were transported by *Os* always shared a higher proportion of their communities with this heterospecific caregiver than siblings that were not transported (KW: $chi^2 = 14.4696$, df = 2, $p = 0.0003$; difference in proportion: median = 39.33, min = 0.872 %, max = 68.478 %) or siblings transported by the biological parent (KW: $p = 0.015$; percentage of higher proportion: median = 37.07 %, min = 0.874 %, max = 72.97 %) (Fig. S2C, Table S2). Non-transported tadpoles were colonized by between 5 and 57 Amplicon Sequence Variants (ASV) (median = 12). We conducted a separate analysis defining non-transported tadpoles as potential microbe source to represent community proportions that were acquired in the clutch before caregiver contact (Fig. S2D). We found that transported tadpoles shared between 0 and 46.99 % (median = 13%) of their communities with non-transported tadpoles. Sourcetracker detected higher proportions of clutch-acquired microbes in tadpoles transported by *Os* (pairwise Wilcoxon test, $p = 0.02$). This likely reflects the higher degree of similarity between the source clutch and *Rv*, as the clutch remained in the *Rv* tank during development, making it harder to distinguish between these sources. Overall, our results suggest that microbes are vertically transmitted during tadpole transport in a poison frog.

<u>Tadpoles of the poison frogs *Ranitomeya variabilis* and *Allobates femoralis* host more diverse skin communities than tadpoles of the leptodactylid frog *Leptodactylus longirostris*.</u>

To investigate how vertical transmission of microbes during tadpole transport affects microbial skin communities of tadpoles in an ecologically relevant context, we studied the skin microbiome

composition and diversity of *Rv* tadpoles and adults in a natural population. To assess the broader relevance of our findings to frog species that transport their tadpoles, we also sampled two sympatric species with differing reproductive strategies: *Allobates femoralis (Af)* and *Leptodactylus longirostris* (*Ll*) (Fig. 2A). In contrast to *Rv* that transports its cannibalistic tadpoles to individual pools in bromeliads, *Af* lives in forest leaf litter and shuttles multiple non-cannibalistic tadpoles to the same pool where they grow up gregariously. *Ll* is a nocturnal leptodactylid frog inhabiting the rock savanna that does not exhibit tadpole transport but deposits eggs directly in ephemeral rock pools where the larvae grow up together (Fig. S3).

We compared bacterial communities from 137 skin swabs of adult frogs, tadpoles, and the aquatic environment belonging to the tadpoles of these three species. We sampled 44 adults and 21 tadpoles (Gosner stages 29 - 41, categorized as "medium" and "large") of the species *Rv*, ten adults and 14 tadpoles (Gosner stages 34 - 41, categorized as "medium" and "large") of the species *Ll*, and ten adult and eight tadpoles (Gosner stage 25 - 26, categorized as "small") of the species *Af* (Table S3). Additionally, we monitored the growth of 184 *Rv* tadpoles in separate bromeliad pools for up to 82 days. Out of 45 tadpoles that were monitored for over 50 days, 13 (three "small" and ten "medium") tadpoles did not advance in their sizing category, reflecting that the complete larval developmental in this population spans multiple months. Cannibalistic *Rv* tadpoles grow up in isolated pools that were sampled as single replicates when collecting the tadpole. Due to their development in groups, *Af* and *Ll* tadpoles were collected from fewer water bodies and represent a narrower developmental window (stages 34 to 41 for *Ll* and stages 25 and 26 for *Af*). If multiple tadpoles were collected from the same aquatic environment (*Af* and *Ll* tadpoles), we sampled triplicates of the respective environment.

For each species, the aquatic environment of the tadpoles displayed a higher average phylum-level diversity than the inhabiting tadpoles or adults (Table S3). As previously reported for Neotropical frogs (Hughey et al., 2017; Kueneman et al., 2016), ASVs belonging to Phylum Proteobacteria dominated the microbial community across all species and life stages (mean relative abundance: 70.9%), followed by Bacteroidota (10.3%), Actinobacteriota (4.1%), Firmicutes (4%), Verrucomicrobiota (2.3%) and Planctomycetota (1.1%) (Fig. S4). The abundance of the frog pathogen *Batrachochytrium dendrobatidis* (*Bd*) in the rock savanna was low, with detection in two samples of *Rv* adults and one *Rv* tadpole, but no positive *Ll* sample. We detected *Bd* on one adult *Af* individual collected in primary forest but not on tadpoles of this species collected in the same area. In all species, tadpole communities contained fewer taxa with known *Batrachochytrium*-growth inhibiting function than did environmental samples or adults (Table S3).

We found no significant differences in the observed ASV richness, diversity (Shannon), or evenness between tadpoles and adults of the poison frogs *Rv* and *Af*. In contrast, *Ll* tadpoles were significantly less diverse than *Ll* adults (Fig 2B, Table S4). We followed up on this finding by comparing

the diversity of tadpole-associated communities across species and found higher microbial diversity and evenness in *Rv* and *Af* tadpoles compared to *Ll* tadpoles (Fig. 2C). Differences in community diversity of tadpoles were not a reflection of habitat diversity: microbial communities associated with *Rv* aquatic environments were less diverse and even compared to those in *Af* and *Ll* habitats (Fig. 2D). These variations may therefore be linked to differing reproductive traits: *Af* and *Rv* lay terrestrial egg clutches and transport hatchlings to water, whereas *Ll*, a non-transporting species, lays eggs directly in water.

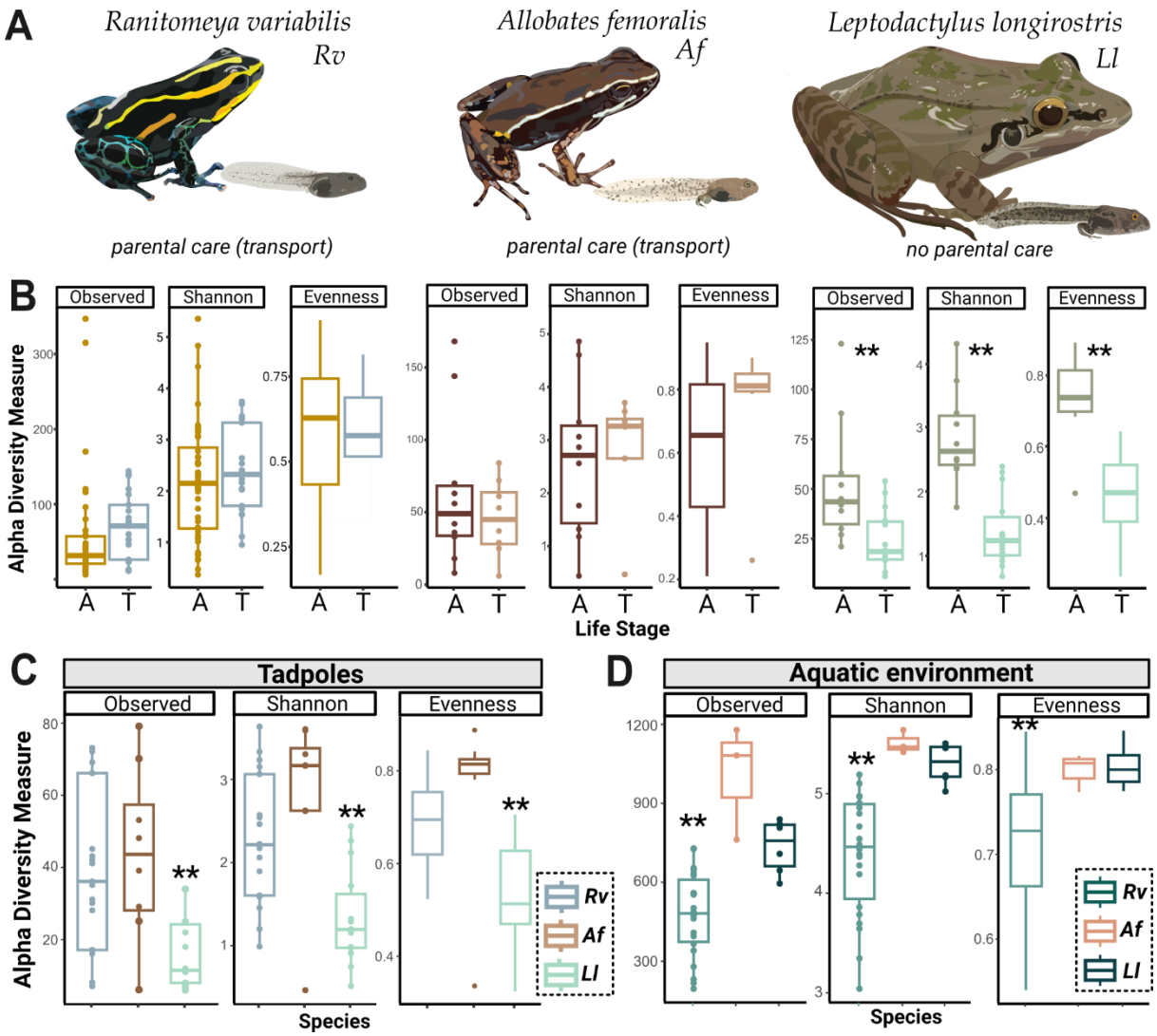

Figure 2. Tadpoles skin microbiome is shaped by their environment and is more diverse in *Rv* and *Af* compared to *Ll* tadpoles. (A) We compared the skin microbiome of three anuran species: two species of poison frogs inhabiting different habitats that transport their offspring (*Rv* and *Af*) and a leptodactylid frog *(Ll)* that deposits its eggs in water without transporting the tadpoles. **(B)** Alpha diversity measures (observed ASV richness, Shannon diversity and evenness) for tadpoles (T) and adults (A) of each species were compared. Differences were determined with an ANOVA or Kruskal Wallis test, significance ($p < 0.01$) is indicated by **. **(C)** Comparison of ASV richness, Shannon diversity, and evenness of communities associated with poison frog tadpoles (*Af* or *Rv*) and non-poison frog species (*Ll*). **(D)** Comparison of Shannon diversity and evenness of communities associated with the aquatic habitats of *Af*, *Rv*, and *Ll*. Bars in boxplots represent median values. The dataset was separately rarefied to the lowest read depth of each comparison.

Tadpole transport is not associated with higher degree of similarity between adult and tadpole skin microbiotas.

To assess how microbes transmitted during parental care shape communities associated with tadpoles, we tested the hypothesis that tadpoles of the transporting species *Rv* and *Af* would have communities more similar to conspecific adults compared to tadpoles of the non-transporting species *Ll*. We first evaluated variation in bacterial communities associated with adults, tadpoles and their microenvironment by using a principal coordinate analysis (PCoA) to construct ordinations based on Bray-Curtis dissimilarities to explore factors that influence skin community composition (Fig. 3A). Tadpoles of all species clustered separately from adults, each other, and their aquatic environment (PERMANOVA on genus level, df = 8, R^2 = 0.52909, F = 18.117, p adj = 0.001) (Fig. 3A, B, Table S5). Consistent with findings in pond-spawning anurans (Kueneman et al., 2014, 2016; Prest et al., 2018), skin communities varied significantly across life stages in all species (PERMANOVA on genus level: species: df = 5, R^2 = 0.29674, F = 11.139, p = 0.001, life stage: df = 2, R^2 = 0.23148, F = 20.331, p = 0.001). Parental care also explained some of the observed variance (PERMANOVA on genus level: df = 2, R^2 = 0.07513, F = 5.4831, p = 0.001). To further examine patterns of similarities between life stages, we compared the core communities of adults, tadpoles and their aquatic environments across a range of prevalence and abundance cutoffs (prevalence 100% or 75%; relative abundance no cutoff, ≥ 0.1% (low) and ≥ 1% (high) (Table S6). Independent of parenting strategy and species, tadpoles shared core communities of their microbiome with their environments, but not with adult individuals. Adult poison frogs never shared core taxa with the aquatic environment of their tadpoles (Fig. 3C, Table S6), even though adults were commonly found in the nurseries. In contrast, the aquatic environment contributed substantially to the skin community of adult *Ll*, which shared high- and low-abundance (≥ 0.1%) core genera with the pond water, but not with the pond-inhabiting tadpoles (Fig. 3C, Table S7).

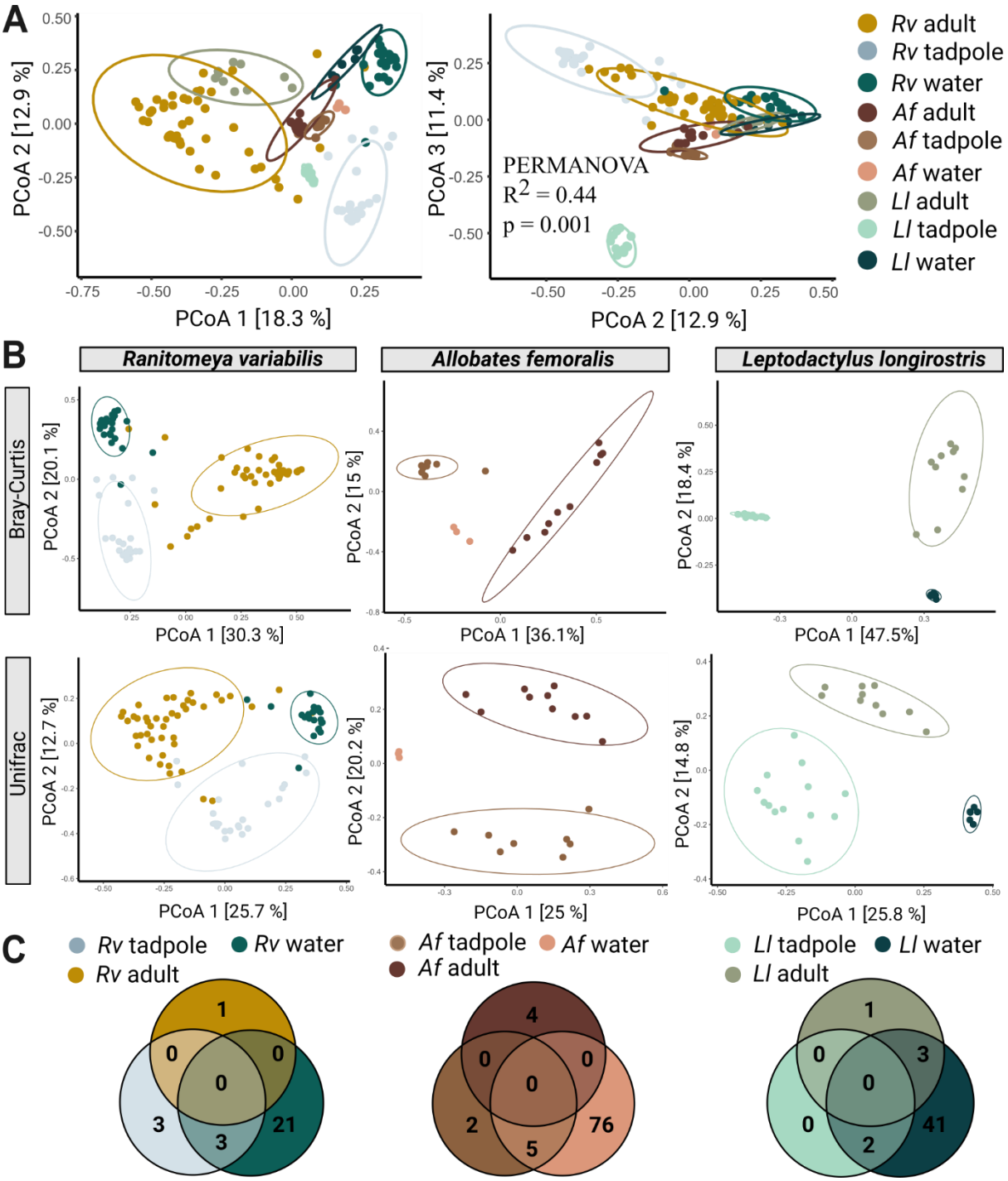

Figure 3. Species, life stage and parental care affect clustering of microbial communities. **(A)** In a Principal Coordinate Analysis constructed with Bray-Curtis distances (axis 1 and 2 on the left, axes 2 and 3 on the right) tadpoles cluster significantly differently from each other, adults, and their aquatic environment. Significances were determined with a PERMANOVA followed by a pairwise adonis posthoc test. Points in ordination plots represent the communities of each sample, circles represent confidence ellipses. **(B)** Principal Coordinate Analysis constructed with Bray-Curtis and Unifrac distances for adults, tadpoles, and aquatic environment of each species. **(C)** Number of core species (prevalence > 75%, relative abundance > 0.1%) shared between adults, tadpoles and the respective aquatic environment of each species.

To assess if microbes transmitted during tadpole transport constitute a stable part of skin communities associated with tadpoles, we compared community distances between skin communities of tadpoles and adults of the three frog species. We predicted that tadpoles and adults might have more similar skin microbiomes in the transporting species *Rv* and *Af* than in the non-transporting species *Ll*. We found no difference in community distances between tadpoles and adults in *Af* and *Ll* (iterated beta regression averaged over 999 random subsamples of similar sample size (IBR): Unifrac: $p = 0.3$). Communities associated with *Rv* tadpoles were more dissimilar from adult communities than microbiota of *Ll* tadpoles and adults (IBR: Unifrac: $p = 0.008$). Taken together, our results indicate that microbes acquired during tadpole transport do not create lasting similarities in composition between tadpoles and adults of wild populations.

<u>After 4 weeks, transported *Ranitomeya variabilis* tadpoles retain isolated members of their caretaker's core community.</u>

To test how offspring transport in *Rv* affected the community composition in tadpoles, we conducted a field experiment where we reared transported (N=10) and non-transported (N=10) tadpoles in artificial cups. Transported tadpoles were collected from the backs of their caregiver (N= 8), while we bypassed parental care in the non-transported group by transferring eggs to cups before the tadpoles hatched (Fig. 4A). Two out of ten non-transported tadpoles died before sampling. Richness and evenness of tadpole communities did not differ significantly between experimental conditions (Anova: Observed ASVs: $df = 1$, $F = 3.1981$, $p.adj = 0.2780$; Shannon: $df = 1$, $F = 1.1322$, $p.adj = 0.9093$; evenness: $df = 1$, $F = 0.0268$, $p.adj = 2.6163$), though PCoA on Bray Curtis distances revealed differences in microbiome composition attributable to the presence or absence of transport (Fig. 3B) (PERMANOVA: $df = 3$, $R^2 = 0.44$, $F = 10.402$, $p = 0.001$). Compared to transported tadpoles, non-transported tadpoles showed less overlap of genera in their microbial community with their aquatic environment (Fig. 3C). Specifically, the genera *Pelomonas*, *Rhodomicrobium* (Proteobacteria), *Mycobacterium* (Actinobacteriota) and *Candidatus Koribacter* (Acidobacteriota) primarily colonized transported tadpoles, while the genus *Limnohabitans* (Proteobacteria) was found on non-transported tadpoles. *Cetobacterium* and *Burkholderia-Caballeronia-Paraburkholderia* were found to colonize tadpoles from both experimental conditions, but at different abundances (ANCOMBC 2, $p < 0.05$) (Table S8).

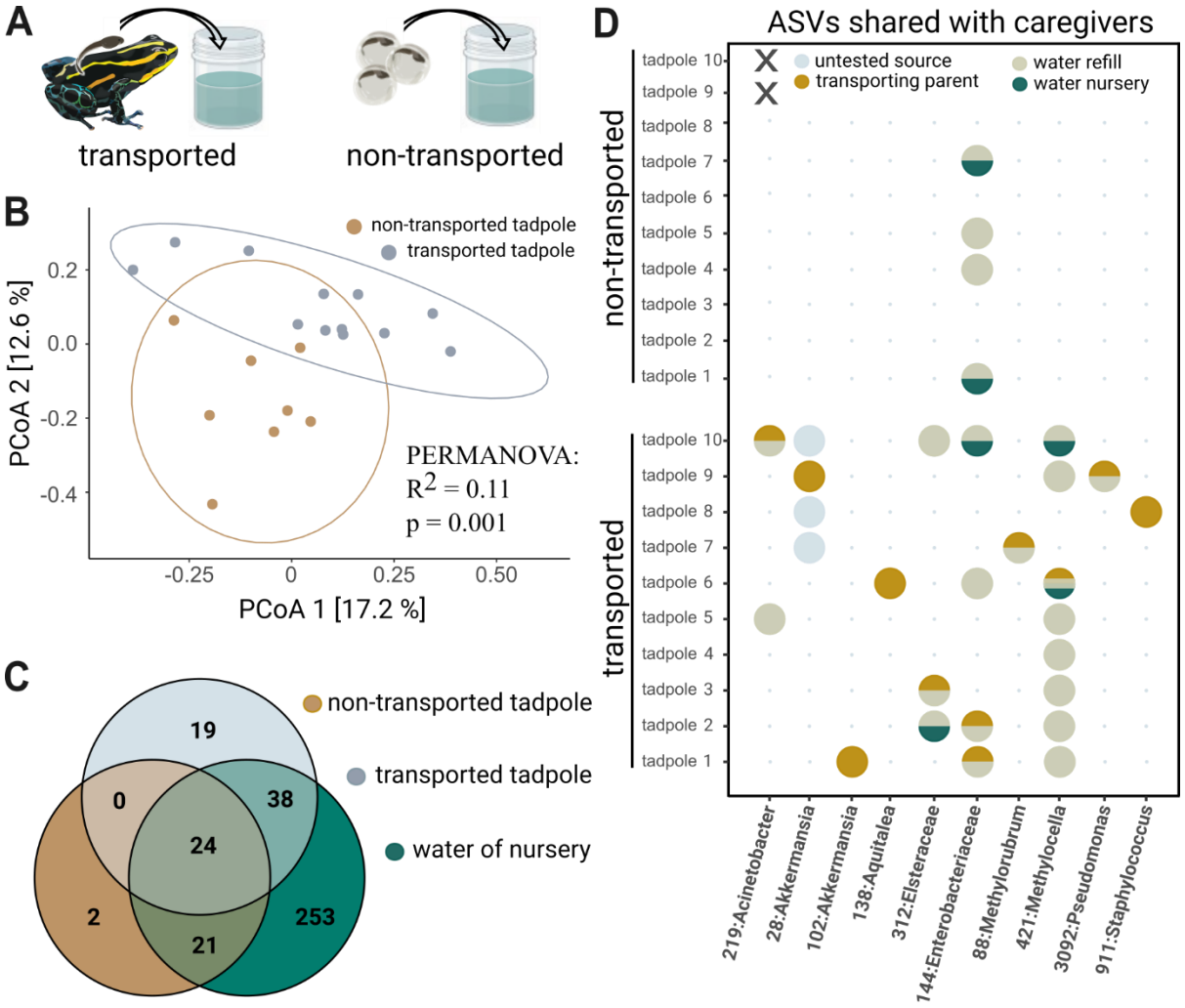

Figure 4. Tadpole transport influences community structure. (A) Tadpoles collected from the back of their caregiver ("transported") and reared in artificial cups for one month were compared to six-week-old tadpoles that hatched from eggs transferred to artificial cups and did not experience transport by adult frogs ("non-transported"). **(B)** Principal Coordinate Analysis constructed using unweighted Unifrac distances, transported tadpoles cluster significantly differently from non-transported tadpoles. Significances were determined with a PERMANOVA followed by a pairwise adonis post hoc test. **(C)** Venn diagram comparing unrarefied ASVs agglomerated on a genus level between transported tadpoles, non-transported tadpoles, and the aquatic environment. **(D)** Bubble diagram depicting the presence (circle) or absence (dot) of 10 ASV found to be shared between parents and transported tadpoles as well as their possible source ("transporting parent ", "nursery water", "refill water" or "untested"). Non-transported tadpoles that died prior to sampling are indicated by 'X'.

We further tested the hypothesis that related tadpole-adult pairs with a history of direct contact would have more similar communities than unrelated tadpole-adult pairs without such history. One month after transport, we found that skin communities of tadpoles were not more similar to the transporting parent than to unrelated adults of the population (iterated beta regression averaged over 999 random subsamples of similar sample size (N=10) (IBR): Unifrac: p = 0.27), confirming our results from the population sampling. However, a Sourcetracker analysis on unrarefied data revealed notable relative contributions of the parental skin community to the community on four transported tadpoles (tadpoles 1, 2, 6, 8) (min = 0% to max = 17%, median = 0%, IQR = 10.5) (Table S9) after one month of growth.

We followed up on this finding by directly identifying taxa shared between caregivers and the respective transported offspring without imposing an abundance cutoff. We found that 8 out of 10 tadpoles shared one or two ASVs with the frog that had transported them in relative abundances ranging from 0.02 to 19.3 % (Fig. 4D) (Table S9). Shared taxa belong to the genera *Akkermansia, Elsteraceae, Aquitalea, Methylocella, Methylobacterium-Methylorubrum, Staphylococcus, Pseudomonas* and *Acinetobacter*. Seven out of ten shared microbes constituted members of the ten most abundant ASVs found on the respective transporting frog (Table S9). A genus-level network constructed from 44 adult frogs shows that the retained genera make up a central part of the community of adult *Rv* in wild populations (Fig. S5). Finally, we determined the success of these ASVs in colonizing transported and non-transported experimental tadpoles. Even though at least six (min: 6, avg: 6.38, max: 7) of these ten ASVs were consistently present in the aquatic environment of non-transported experimental tadpoles, only one (family *Enterobacteriaceae*) successfully colonized their skin (Fig 4D, not transported tadpoles) (Table S10).

Discussion

In this study we aimed to understand the main factors influencing establishment of microbial communities associated with anuran tadpoles receiving parental care. To better understand sources contributing pioneering taxa, we first aimed to determine the ontogenetic window in which anuran embryos are colonized. Next, we tested if a specialized anuran parenting strategy, tadpole transport, transmits early colonizers. Then, we sampled natural populations of frogs to investigate if parental signatures persist into later ontogenetic stages in an ecologically relevant context. We found that substantial microbial colonization of the poison frog *Rv* occurs after embryos hatch from the vitelline envelope. Furthermore, we demonstrated through cross-fostering experiments that transporting frogs serve as a source of skin microbes for tadpoles. Next, we sampled sympatric species in a Neotropical habitat and revealed that tadpoles of poison frogs showed more diverse microbial communities than tadpoles of leptodactylid frogs. Finally, a field experiment in the wild population indicated that microbial communities of *Rv* tadpoles after four weeks of growth were not more similar to transporting caregivers than to unrelated adults, although some tadpoles did maintain isolated ASV that were shared with the transporting adults. Together, our results demonstrate that microbial colonization of a poison frog tadpole begins at an ontogenetic stage immediately preceding parental contact, that parental care in poison frogs facilitates the transfer of microbes to newly hatched tadpoles, and that signatures of these parentally transmitted microbes can persist, albeit at low prevalences, through the first month of tadpole development. This study shows that tadpole transport may serve as a mechanism to transmit host-adapted microbes, thereby filling a knowledge gap in our understanding of the function of parental care in anurans.

Early microbial colonizers have been shown to shape community structure of newborns in insects (E. W. Jones et al., 2022), mammals (e.g. Blyton et al., 2022; Kaur et al., 2022; Sprockett et al., 2018), fish (e.g. Almany, 2004), birds (e.g. Chen et al., 2020) and anurans (e.g. K. R. Jones et al., 2023, 2024) via priority effects (see Debray et al., 2022; Fukami, 2015 for reviews). Thus, determining critical developmental periods for microbial colonization of host tissue is crucial to assess which sources contribute these pioneering taxa. We detected microbes in embryos of all stages but found the abundance to be considerably lower than in the surrounding jelly. Additionally, we found that substantial colonization of *Rv* tadpoles happens after hatching from the vitelline membrane, at an ontogenetic stage immediately preceding skin-to-skin contact with the caregiver. These results underline that the opportunistic colonization of the anuran embryo is much more restricted than that of the surrounding jelly (Hayes et al., 2009; Kueneman et al., 2014; Walke et al., 2011), which harbors stable microbial assemblages (Hughey et al., 2017; Walke et al., 2011). Such restrictions might result from host-led selection as observed in marine mammals (Switzer et al., 2023) and/or a barrier function of the vitelline membrane that limits microbial access to the developing embryo. For example, the latter was described for the avian vitelline membrane, where it is mediated by antimicrobial proteins (Guyot et al., 2016; Mann, 2008; Mine & Kovacs-Nolan, 2006). The substantial microbial colonization that occurred after hatching suggests that the anuran vitelline envelope plays an important role in restricting microbial access to poison frog embryos until they are sufficiently developed to be transported by a caregiver. Those bacteria that are present in unhatched tadpoles might have been acquired prior to hatching, such as in the case of the amphibian symbiont *Oophila amblystomatis* (Kerney et al., 2011; Kim et al., 2014), which is acquired by oviductal transmission and grows on the inside of individual salamander and frog vitelline membranes. Alternatively, the vitelline membrane may function as a selective barrier, permitting the passage of certain taxa, with hosts acting as additional filter to select suitable colonizers. Future studies should therefore address the presence of antimicrobial proteins, biofilms, and immune components in the anuran embryo and vitelline membrane to shed light on the mechanisms that regulate microbial colonization of anuran embryos.

Across species, newborns might acquire bacteria not only through horizontal transfer from environmental source pools (e.g. arthropods: Douglas, 2018; Mushegian et al., 2018, amphibians: Rebollar et al., 2016, mammals: Bik et al., 2016) but also through vertical transmission during parent-offspring interactions (reptiles: Bunker et al., 2021; fish: Sylvain & Derome, 2017; humans: Wang et al., 2020). Modes of parental inheritance involve indirect transmission, like contact smearing onto the egg surface during or after oviposition, as found in European firebugs (Salem et al., 2015), and direct social acquisition, as observed in social bees (Kwong et al., 2017; Leftwich et al., 2020). Previous studies in anurans have investigated vertical transmission of microbes during parental care in the context of indirect transmission

during egg attendance without considering less widespread parenting strategies involving direct parental contact. These studies did not find evidence for parental inoculation, as hatched tadpoles supported bacterial communities very similar to adult frogs but independent of caregiver identity (Hughey et al., 2017). In contrast, we investigated vertical transmission in an anuran system where parenting involves direct skin-to-skin contact between tadpoles and their caregiver. We found that both transported and non-transported tadpoles in our cross-foster experiment shared microbial ASVs with each other, indicative of acquisition of microbes from the jelly upon hatching as demonstrated for other anuran clades (Hughey et al., 2017; K. R. Jones et al., 2024; Warne et al., 2017). After six hours of contact, transported tadpoles -but not their siblings- shared more microbes with the surrogate heterospecific frog than with the parental species, suggesting that microbes can transmit vertically during parental care in poison frogs. Thus, in this clade both clutch environment and transporting caregivers can serve as source pool for hatched tadpoles.

Variation in the source proportions that transported tadpoles acquired from transporting frogs suggests that additional factors modulate the efficacy of vertical transmission in poison frogs. Evidence for duration-dependent microbial transmission is seen in humans, where recent research highlights a cumulative contribution of the paternal microbiome to the assembly of infants' microbial communities (Dubois et al., 2024). In anurans, the time that hatched tadpoles spend in contact with jelly-associated microbes or in skin-to-skin contact with parents might therefore affect the success and stochasticity of colonizers that are transmitted during parental care. These transport logistics vary between and within anuran species (Altig & McDiarmid, 2007; McDiarmid & Altig, 1999) and are closely linked to trade-off decisions of the caregivers, which often need to balance territory defense, mating, and caring for offspring (Pašukonis et al., 2019; Ringler et al., 2013; Wells, 2007). For instance, *Rv* males often assist tadpoles with hatching by tearing the capsule apart with their rear legs to initiate transport (Brown et al., 2008), but have also been observed to pick-up free-swimming tadpoles (Schulte & Mayer, 2017). Likewise, *Af* tadpoles have been observed to remain in the jelly for several days before being transported (Peignier et al., 2022; Ringler et al., 2013). Parentally transmitted microbes might thus constitute pioneering taxa in an unoccupied niche if caregivers assist with hatching or encounter established assemblages as in the latter scenario. Prolonged parental care has been shown to enhance the generational transmission of symbiotic microorganisms in burying beetles (e.g. Körner et al., 2023) and thus, duration of the skin-to-skin contact during tadpoles transport might influence microbial acquisition in a time-dependent manner. How transport durations which range from a few hours to several days depending on the species (Lötters et al., 2007; McDiarmid & Altig, 1999; Pašukonis et al., 2019) affect stochasticity of transmitted microbes remains unexplored. Collectively, our findings suggest that early life microbes are introduced to the poison frog host in a mixed transmission mode, where community members can be sourced environmentally from the

egg jelly and socially from a caregiving frog. Recent evidence from Kouete et al. documented the first case of vertical transmission in amphibians, observed in subterranean caecilians that protect and feed their offspring (Kouete et al., 2023). To our knowledge, this is the first study that extends evidence for vertical transmission during parental care to the order of anurans.

To investigate if parental signatures persist into later ontogenetic stages, we tested the hypothesis that a history of direct contact would result in more similar skin communities between tadpoles and caregivers relative to unrelated adults. Based on previous findings which documented that priority inoculation of tadpoles increased the relative abundance of some beneficial inoculates (K. R. Jones et al., 2024), we hypothesized that vertical transmission manifests in persistent associations with parentally acquired microbes. Similar to Hughey et al., we found that vertical transmission did not lead to a higher degree of similarity between caregiver and tadpole skin microbiota relative to unrelated adult-tadpole pairs. Likewise, communities associated with adults and tadpoles of transporting species were no more similar than those of non-transporting species. While tadpoles do acquire caregiver-specific microbes during transport, these results suggest that most of these microbes do not persist on the tadpoles' skin long-term. A similar pattern of convergence in the composition of tadpole-associated communities has been observed previously in wood frogs inoculated with gut microbes from bullfrogs (Warne et al., 2019). Interestingly, as documented in fruit flies (Cox et al., 2014), effects of early microbial disruption on physiology and disease susceptibility of the anuran host persisted even after community structures converged (Warne et al., 2019). Therefore, it is possible that vertically acquired microbes shape communities of poison frogs in different ways than by gaining abundance priority on tadpole skin. For example, they may durably imprint the immune system (Fallet et al., 2022; Gensollen et al., 2016) and alter host metabolic phenotypes (Cox et al., 2014; Sommer & Bäckhed, 2013). Additionally, isolated ASVs contributing to central genera of communities on adults persisted on some tadpoles in varying abundance. There is clear evidence that low-abundant keystone taxa, despite their rarity, can act as drivers of compositional changes (Han & Vaishnava, 2023). For instance, low abundance ASVs drive compositional changes in the hindgut of subterranean termites after dietary alterations (Benjamino et al., 2018) and confer resistance to Salmonella-induced colitis in mice (Herp et al., 2019). Moreover, despite their abundance in the water of all tadpole nurseries, most of the ASVs that tadpoles shared with caregivers failed to colonize non-transported hosts. These results suggest that vertical transmission may be a more effective mode of transmission of host-adapted microbes compared to horizontal transmission from the environment. Whether vertically transmitted microbes shape the anuran immune response and if retaining certain ASVs allows them to become dominant community members after metamorphosis remains to be investigated.

We found that the higher diversity of communities associated with tadpoles of species that exhibit offspring transport was not linked to the diversity found in tadpoles' aquatic habitat but might instead be associated with other life history traits. Although skin alkaloids are found on many adult poison frogs including *Rv* and influence skin community composition (Caty, Alvarez-Buylla, et al., 2024), they are not present on adult *Af* or on tadpoles of any species included in this study (Villanueva et al., 2022) and are thus unlikely to cause the observed differences in diversity. It is possible that oviposition strategy or physiological characteristics like skin pH or abundance of antimicrobial proteins (Conlon, 2011; Faszewski et al., 2008; Kueneman et al., 2014) contributed to increased diversity in *Rv* and *Af* relative to *Ll* tadpoles. Alternatively, transport might have been co-opted in an evolutionary context to increase microbial diversity in tadpoles and shape immune recognition and health in the anuran host in a manner comparable to humans (Al Nabhani & Eberl, 2020; Donald & Finlay, 2023; Gensollen et al., 2016). Increased microbial diversity is linked to better health outcomes, including improved immune function, lower disease risk, and enhanced overall fitness in other vertebrate classes. For example, higher diversity of gut microbial communities is associated with enhanced exploratory behavior in songbirds (Florkowski & Yorzinski, 2023) and improved pathogen resilience in fish (de Bruijn et al., 2018). Previous research in frogs has linked higher diversity of tadpole skin communities to enhanced parasite resistance later in life (Knutie et al., 2017; Warne et al., 2019). Further studies across more transporting and non-transporting species are needed to assess the role of transport in shaping diversity of tadpole-associated communities. Moreover, the low chytrid infection-rate in the studied natural populations limits the ability to draw conclusions about differences in disease susceptibility within and between species or life stages in our dataset.

Elucidating the key factors that select for parental care and account for the diversity of care strategies across various species remains a challenge in the field of evolutionary and behavioral ecology (Balshine, 2012; Clutton-Brock & Scott, 1991; Gross, 2005; Royle et al., 2012). Microbiota management is documented to actively drive, rather than merely accompany sociality and parenting behavior in other organisms like carrion feeding insects (Biedermann & Rohlfs, 2017; Körner et al., 2023) and has recently been proposed as a factor driving the evolution of paternal care in vertebrates (Gurevich, 2020). Even though commensal skin microbiota undoubtably play a pivotal role in the health and pathogen defense of amphibians, it remains poorly understood how complex parental care strategies in this class aid microbial transmission. This is especially surprising as amphibians have recently been identified as the most threatened class of vertebrates (Daszak et al., 2003; Luedtke et al., 2023; Wake & Vredenburg, 2008) due to their susceptibility to globalization-related threats like climate change and disease-spread. Our observation that anuran parental care transmits microbes fills a knowledge gap about the function of

parental care in this class and highlights the need for further studies across more anuran species with contrasting reproductive strategies.

Methods

Captive-bred animals

Captive-bred *Rv* of our laboratory colony are kept non-sterile and reproduce year-long. Males attend the terrestrial clutch of 3-5 eggs and transport larva to water when they reach Gosner stage 24-25 where they hatch from the vitelline membrane. *Rv* were housed in pairs and provided with horizontally mounted film canisters as egg deposition sites, and film canisters filled with water (treated with reverse osmosis R/O Rx, Josh's Frogs, Owosso, MI) for tadpole deposition (Goolsby et al., 2023).

Microbial colonization of embryos and egg jelly

We used individually packed sterile transfer pipets (Samco™, Thermo Scientific, 7.7 mL, cat # 202-1SPK) with a cut tip to move eggs and embryos. All tools were treated with 8.25% Sodium Hypochlorite (CloroxPro) and wiped down with 70% Ethanol before use and between tissues and tadpoles. The experimenter always wore gloves that were changed between individuals and disinfected with 70% Ethanol before handling frogs.

Triplicates of five different developmental stages (Gosner, 1960) before (stages 10, 19, 23, 24) and after hatching from the vitelline membrane (stages 24, 25) were collected from 3 different breeding pairs. We manually separated the jelly from the egg sac with autoclaved forceps, a procedure commonly done in preparation for microinjections. The embryo in the vitelline membrane was transferred to a small petri dish with sterile, filtered deionized water and opened using forceps. Tadpoles were transferred to a new petri dish with clean water twice to remove transient bacteria from the skin and were then euthanized and homogenized. Organic matter was removed from the jelly, and both jelly and tadpole tissues were flash frozen and stored at -80 °C until DNA isolation. Isolation of DNA was performed as detailed below, and 1 µL of isolated DNA was used for PCR amplification of the full length 16S rRNA region (~1500 bp) with 1 µL of 10 µM forward 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTAYGACTT-3') (Fredriksson et al., 2013), 12.5 µL of OneTaq Hot Start Quick Load polymerase (New England Biolabs) and 9 µL water. We followed an amplification protocol with denaturation for 30 s at 94 °C followed by 30 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 58 °C and extension for 2 min at 68 °C and a final extension period of 5 min at 68 °C. To determine the

presence or absence of amplification, we visualized 5 µL of each PCR product on a 1% Agarose gel (90mV for 90 min) with 2 µL of 1kb GeneRuler for sizing.

Quantitative analysis of 16S rRNA copy numbers with droplet digital PCR (ddPCR)

Quantification of prokaryotic concentration for each sample was determined by ddPCR for all samples. Sample DNA were serially diluted 1:10 in sterile nuclease-free water to dilute the concentration of any PCR inhibitors using a liquid handler (Agilent, Agilent Velocity 11 Vprep). 16S rRNA concentrations from embryonic tissue are reported from undiluted DNA as its concentration was approaching the limit of detection in diluted reactions. Universal 16S rRNA primers (331F/797R) and 16S rRNA HPLC-purified FAM probes were used, as previously described (Langenfeld et al., 2021). Primer-probe mixture was created by mixing forward primer, reverse primer, and probe 1:1:1 for a total volume of 0.264uL per reaction. Each ddPCR reaction was composed of 11uL of ddPCR Supermix for probes (Bio-Rad, 1863024), 0.264uL of primer-probe mixture, 4.736uL of sterile nuclease-free water and 6uL of sample, for a total of 22uL/reaction. As a positive control, equal amounts of NIST Microbial Pathogen DNA Standards for Detection and Identification (NIST, RM8376) components A through R were combined in equal amounts. Each ddPCR plate included a positive control, NIST mixture, and negative control, sterile nuclease-free water, in quadruplicate. ddPCR reactions were performed with the QX200 AutoDG Droplet Digital PCR system (Bio-Rad). PCR amplification was performed with the Bio-Rad T100 thermocycler using the following program: 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds and 56°C for 1 minute and 72°C for 2 minutes, followed by 1 cycle of 4°C for 5 minutes and 95°C for 5 minutes with a ramp speed of 2°C/second at each step. Amplified reactions were quantified using a ddPCR reader. Thresholds were set for each ddPCR reaction based on the negative and positive control rain plots generated by the QX Manager Software, 2.1 Standard Edition (Bio-Rad). All reactions had greater than 15,000 accepted droplets. 16S rRNA copies per droplet were calculated by taking the natural logarithm of the ratio of accepted droplets to negative droplets for each reaction. Values were corrected for droplet volume and ddPCR reaction volume to calculate total 16S rRNA copies per reaction. We further calculate and report the total 16S rRNA copies per µL by accounting for the sample ddPCR volume, dilution factor, and DNA extraction volume. Differences in copy number concentrations detected in jellies, hatched and unhatched tadpoles were determined using a Kruskal Wallis test with p-values adjusted for multiple comparisons using Benjamini Hochberg correction (R Core Team, 2023) and followed by a Dunn test.

Vertical transmission of bacteria

Siblings of one clutch were randomly assigned to one of three treatments: (1) no transport, (2) transport by the biological caregiver, or (3) transport by a heterospecific surrogate frog. We chose the poison frog *Oophaga sylvatica* as foster frogs because adults are docile, parental care in this species involves transporting tadpoles, and skin microbial communities differ from *Rv*. Tanks of *Rv* breeding pairs were monitored for clutches of at least three eggs daily. Embryos assigned to group one or three were placed into a petri dish in a separate tank without access to adults when tadpoles reached a developmental stage close to hatching (Gosner stage 22) to bypass transport by the biological father. The remaining eggs in the parental tank were monitored with security cameras (Wyze v3, Goolsby et al., 2023) that notify the user of any movement in the canister. After tadpole pickup by the biological father, all water canisters for tadpole deposition were removed from the tank to standardize transport time to 6 hours for both groups. Siblings of group three were transferred to a new petri dish, hatched using a sterile brush and directly transferred to the back of a surrogate frog (as described in Pašukonis et al., 2017) that was caught in a fresh plastic bag and rinsed with sterile water. The surrogate frog was placed into a plastic containment (Sterilite 16428012) with moist, autoclaved paper towels for the duration of the experiment. After 6 hours, the transporting biological father and the surrogate frog were caught in a fresh plastic bag. Each tadpole was removed from the back and washed twice to remove transient bacteria by transferring them to new petri dishes with 10 ml sterile water. Non-transported tadpoles were removed from the jelly and washed in a similar way. Each frog was rinsed with sterile water, and a skin swab of their back was collected using a sterile Puritan applicator (Puritan Medical Products, 25-206 1PD BT) (Caty, Alvarez-Buylla, et al., 2024; Caty, Vasek, et al., 2024). All tadpoles were euthanized, their skin was collected, and skin and applicators were stored at -20 °C until DNA extraction. We chose to sample whole skin instead of swabs to detect small amounts of transferred bacteria on and in the skin.

Field site and natural populations

The natural study site of *Rv* and *Leptodactylus longirostris* (*Ll*) is situated on top of the mountain 'Inselberg' in vicinity to the Centre Nationale de la Recherche Scientifique managed research station (4°5’ N, 52°41’ W) within the Nature Reserve ´Les Nouragues´ in French Guiana (Bongers et al., 2011). Patches of Clusia trees are separated by bare granite rocks and exposed to extreme environmental conditions (e.g. temperature oscillations between 18°C - 75°C (Sarthou, 2001). *Rv* and *Ll* (IUCN Conservation status: Least Concern) inhabit the granite outcrop of the study site, *Af* inhabit forest floors of primary terra-firme forests surrounding the campsites 'Inselberg' and 'Saut Pararé' (4°02’N/52°41’W) in the Nouragues reserve.

Field study species, reproductive strategies and life history

Ranitomeya variabilis (Rv) (Zimmermann & Zimmermann 1988; CITES Appendix II, IUCN Conservation status: Least Concern) is a small diurnal poison frog (Family *Dendrobatidae*) with male uniparental care that lives largely arboreal and uses bromeliads as a resource for reproduction. Adult frogs defend territories, are polygamous and typically lay clutches of 3-4 eggs into small arboreal bromeliads (*Catopsis berteroniana*) that are abundant on Clusia trees in the rock savanna. Male frogs transport hatched tadpoles to individual water bodies that form in the leaf axils of the large terrestrial tank bromeliads (*Aechmea aquilega*). The cannibalistic tadpoles grow up in individual pools of ~80 ml (Poelman et al., 2013) where they feed on algae and leaf debris until they complete their development after about 3 - 5 months (Poelman & Dicke, 2007).

Allobates femoralis (Af) (Boulenger, 1884; CITES Appendix II, IUCN Conservation status: Least Concern) are polygamous diurnal poison frogs (Family *Aromobatidae*) that inhabit the understory of primary tropical forests. Females are attracted by advertisement calls of the territorial males and deposit clutches of 11-25 eggs in the leaf litter (Fischer et al., 2020; Stückler et al., 2019; Weygoldt, 1980). Fathers care for their offspring by attending clutches and shuttle hatched tadpoles to water bodies where the social tadpoles grow up sharing a pool (Lescure, 1976; Ringler et al., 2013, 2018).

Leptodactylus longirostris (Ll) (Boulenger, 1882; IUCN Conservation status: Least Concern) are medium sized nocturnal leptodactylid frogs (Family *Leptodactylidae*) that inhabit the rock outcrops. They shelter in rock crevices and ground covering vegetation during the day and dwell in seasonal rock pools on the Inselberg plateau during nights. They breed in the rainy season and lay their eggs in ephemeral rock pools. After clutch deposition, they do not care further for their tadpoles, which collectively grow up in the deposition pond and feed on algae and plant debris.

Sampling of frogs in the field

Frogs were caught in fresh plastic bags upon encounter, rinsed with sterile water to remove transient bacteria and soil particles and then swabbed with 20 strokes each on the dorsal and lateral sides as well as on each leg and between the toes using Sterile Polyester Applicators (Puritan Medical Products, 25-206 1PD BT). Due to limitations in the availability of dry ice at the remote study site, applicators were directly transferred into 800 µL of buffer CD1 (a lysis buffer containing chaotropic salts) of the Qiagen PowerSoil Pro extraction kit and frozen until DNA extraction. Previous studies have found no difference in OTU richness and evenness between native frozen and lysis buffer-stored swabs (Hallmaier-Wacker et al., 2018).

Field sampling of tadpoles and their environment

To avoid disturbing microbial communities of tadpoles and their habitats before sampling, free-swimming tadpoles were visually categorized as "small" (with a body length of less than 6 mm, typically Gosner stages 25 and 26), "medium" (with a body length exceeding 6 mm but no visible dorsal pattern, stages 26 - 33), "large" (visible yellow dorsal pattern, stages 34 - 41) or "metamorph" (front limbs visible, stages 42 - 46). Tadpoles of stages 29 – 41 (Gosner) were collected from bromeliad pools for swabbing when they at least doubled the body size of freshly hatched tadpoles, at the earliest four weeks after deposition. The water and sediment of the nursery was sampled prior to tadpole extraction by submerging a sterile applicator tip into the leaf axil pool and moving it over all plant material contained in the water for 10 seconds. *Rv* tadpoles were extracted from bromeliad leaf axils with a custom designed vacuum extractor consisting of an inlet hose wide enough to allow the passage of a late-stage tadpole and a suction hose with air filter, both tightly connected to the lid of a one-liter bottle. The inlet hose was submerged into the bromeliad pool while applying suction to the second hose to create a vacuum that transferred the pool content into the collector. After each use, all parts of the collector were cleaned with soapy water and rinsed, followed by a sodium hypochlorite disinfection for 1 hour and a rinse in 70% Ethanol as described for the dissection tools. After collection, tadpoles were transferred to a petri dish with sterile water twice, using individual sterile transfer pipettes with a cut tip. For swabbing, tadpoles were collected in the bulb of a new sterile pipette. The water was discarded, and the tadpole was swabbed on all body parts with a sterile applicator tip by moving the tip over its body for 10 seconds. We further processed swabs in the same way as adult swabs. *Af* tadpoles of stage 25 - 26 (Gosner) were collected from an artificial pool in the vicinity of camp 'Saut Parare'. *Ll* tadpoles of Goser stage 34 - 41 were collected from three natural pools in the study plot 'Inselberg'. We sampled triplicates of each pool containing multiple tadpoles. All tadpoles were processed for swabbing in a similar way as *Rv* tadpoles.

Field experiment to compare transported and non-transported *Rv* tadpoles

To directly compare parental frogs with transported and non-transported tadpoles while reducing predation and variation in the quality of their nurseries, we reared ten tadpoles that were collected from the back of eight caregivers and ten tadpoles that hatched from eggs without experiencing transport in polypropylene cups (USP #77876, 60 ml). Each cup was punctured at the 50 ml water level, covered with a sterilized mosquito net secured with a rubber band to minimize predation, and attached to the stem of a Clusia tree. All tadpoles received water collected from bromeliads as their initial aquatic environment with Clusia leaves as shelter and food. Cups were exposed to rain and during dry periods, rainwater was collected and mixed with leaf litter to refill cups. Triplicates of water samples were obtained using the procedure

described for bromeliad water. We caught transporting frogs upon encounter, transferred tadpoles from their backs to cups, and swabbed the parenting frog as described above. To avoid transport, eggs from 5 clutches were transferred to cups before tadpoles hatched. Tadpoles were sampled after growing for at least 26 days (min: 26, max: 77), when all tadpoles of a group exceeded ~5 mm in body size, as smaller individuals exhibited increased mortality after swabbing in previous lab experiments. After swabbing, tadpoles were measured and photo documented.

DNA extraction and 16S rRNA gene sequencing

The Qiagen PowerSoil Pro Kit was used to extract DNA from all swabs and tissues. The protocol was adapted for use with swabs as described previously (Caty, Alvarez-Buylla, et al., 2024). DNA concentrations were quantified using a Qubit. Samples were pooled by volume and the V4 region of the 16S rRNA gene was amplified using 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) primers (Bletz et al., 2017) and barcoded using standard Illumina unique dual indices (UDIs). We performed two separate sequencing runs: Laboratory collected samples were sequenced in a 2x300nt paired-end configuration on an Illumina MiSeq v3 run, field collected samples in a 2x250nt paired-end configuration on a NovaSeq 6000 SP Flowcell (Roy J Carver Biotechnology Center, University of Illinois).

Sequence processing and bioinformatics

We annotated in-line barcodes based on the first 7 bases of each sequencing read (umi-tools, Smith et al., 2017), split out reads that matched each known barcode combination (grep, GNU Project, 1998), trimmed the remaining primer sequences from the sequencing reads (cutadapt, Martin, 2011) counted the number of sequencing reads in each file and removed files (including negative controls) with low read numbers (<100 reads) from the dataset. We processed the remaining reads with the R Divisive Amplicon Denoising Algorithm package "dada2" (version 1.28.0) (Callahan et al., 2016). Taxonomy was assigned using the Silva 138 database (Ref NR99) (Quast et al., 2012). The count table, taxonomy table, and sample-associated data were integrated into a phyloseq object using the R package "phyloseq" (version 1.44.0) (McMurdie & Holmes, 2013) for further data analysis. Any ASVs detected in control sequencing reactions without DNA as well as ASVs with a phylum designation that was "NA", Eukaryotic, belonging to the family of Mitochondria or the class of Chloroplasts were removed from the datasets. Samples with very low reads (clearly below the first quartile) were excluded from each group.

ITS sequence processing and bioinformatics

Amplification targeted the second internal transcribed spacer (ITS2) region of the fungal ribosomal cistron using primers ITS3 and ITS4 (White et al., 1990). Full primer sequences included Illumina Nextera transposase adapters (underlined) and were as follows: ITS3-5'-TCG TCG GCA GCG TCA GAT GTG TAT AA GAG ACA GGC ATC GAT GAA GAA CGC AGC-3' and ITS4-5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA TGC-3'. Reaction mixes contained 1x AccuStart II PCR SuperMix (Quantabio), 0.4 µM forward and reverse primers, and 2 µl of template DNA. The thermal cycling regime consisted of an initial denaturation and enzyme activation step at 94°C for 3 min, followed by 30 cycles of 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec, with a final extension step at 72° for 10 min. Ten additional PCR cycles were used to tag diluted amplicons with unique dual indices (Illumina; cycling regime as above but with annealing at 54°C). Amplicons were pooled, cleaned using AMPure XP beads (Beckman Coulter), and sequenced on an Illumina MiSeq instrument (2 x 300 nt) at the DNA Services Lab, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Reads processing was consistent with the DADA2 ITS workflow (https://benjjneb.github.io/dada2/ITS_workflow.html). Raw reads were trimmed of non-biological sequence using cutadapt (Martin, 2011). DADA2 was used to filter and truncate the reads, infer amplicon sequence variants (ASVs), and remove chimeras (Callahan et al., 2016). Taxonomy was assigned using DADA2's implementation of the RDP naïve Bayesian classifier (Q. Wang et al., 2007) against a UNITE reference database (version 9.0; Abarenkov et al., 2023). The ASV count table, taxonomy table, and sample-associated data were integrated into a single object in R using the phyloseq package (McMurdie & Holmes, 2013).

Microbiota composition analyses

All statistical analyses were performed in R (v 4.3.2) (R Core Team, 2023), and "ggplot2" (v 3.4.4) (Wickham, 2016) was used for visualizations if not stated otherwise. Illustrations of all species were created in Adobe Illustrator (v 28.3) (Adobe Inc., 2024) and all figures were compiled in InkScape (v 1.1) (Inkscape Project, 2021).

Microbiota composition analyses of captive frogs and source tracking

First, we calculated beta diversity between skin communities of adult frogs that served as transporting frogs to determine if communities are distinct enough to distinguish them as microbial source communities. We applied Principal Coordinates Analysis (PCoA) using the package "phyloseq" to visualize variation in the genus-agglomerated dataset based on two dissimilarity indices: Bray-Curtis, a widely used measure considering relative abundances and unweighted Unifrac, a presence-absence measure of ASVs based on

phylogenetic distances. Data were converted to relative abundances before calculating Bray-Curtis or rarefied using a rarefaction over 300 iterations (function phyloseq_mult_raref_avg in the package 'metagmisc' (Mikryukov, 2023) before calculating Unifrac distances. We confirmed equal dispersion between compared groups using the betadisper function of the package "vegan" (v2.6.4) (Oksanen et al., 2022) followed by a Tukey post-hoc test. Variation was analyzed with a PERMANOVA (function 'adonis' within the package "vegan") using Bray-Curtis and unweighted Unifrac dissimilarities as responses and species or transporting species as predictors. Significant PERMANOVAs were followed by pairwise multilevel post-hoc comparisons between groups with the function pairwise.adonis (package "pairwiseAdonis", (Arbizu, 2017). We applied the same method to compare if communities between tadpoles that had been transported by a homospecific frog (*Rv*) or a heterospecific frog (*Os*) differed after six hours of tadpole transport. We further used 'Sourcetracker' (Knights et al., 2011) to determine whether transporting frogs served as a source for bacteria detected on tadpole skin after six hours of transport. This Bayesian approach was developed to estimate proportion of contaminants in a given community that come from possible source environments and is commonly used to determine if parental communities serve as source for offspring communities (Kouete et al., 2023; Murphy et al., 2023; Switzer et al., 2023). We performed two sets of analysis to determine the source proportions of adult *Rv* and *Os* in tadpole communities. First, we defined transporting frogs of both species as possible microbial sources and all tadpoles including non-transported controls as sinks. Then, we defined transporting frogs and non-transported control tadpoles as possible sources to visualize the proportion of bacteria acquired in the clutch prior to transport. For both approaches, we rarefied the source dataset of transporting frogs to decrease the influence of high-coverage source samples on the analysis. For the first approach, differences in community proportions shared with the transporting species ('transporting frog') or the non-transporting species ('non-transporting frog') were determined for non-transported and transported tadpoles and compared using a Kruskal Wallis test, with p-values adjusted for multiple comparisons using Benjamini Hochberg correction (R Core Team, 2023). Additionally, we plotted the source proportion of *Rv* and *Os* communities for non-transported tadpoles, tadpoles transported by *Rv*, and tadpoles transported by *Os*, and we performed the same analysis between the experimental conditions for each source (*Rv* or *Os*) separately. For the second approach, we determined the source proportions that transported tadpoles had acquired from the clutch, from *Rv* adults, and from *Os* adults, and we determined differences between tadpoles transported by *Rv* or by *Os* using a paired Wilcoxon test with Benjamini Hochberg correction (R Core Team, 2023).

Microbiota composition analyses of wild populations

We calculated mean and standard deviation across all samples per group for the number of phyla and families from a rarefied dataset to compare groups. The total number of unique phyla and families for each

group was calculated from the unrarefied dataset to include low abundance taxa. We plotted the relative abundance of taxa agglomerated at the phylum level with an abundance greater than 2%. In addition, we identified the most abundant genera (N = 10) for each group based on overall mean relative abundances. The number of *Batrachochytrium*-inhibiting taxa was calculated by blasting the rarefied dataset against the AmphiBac-Database-2023.2 (Woodhams et al., 2015, updated by Bletz 2023); Antifungal database: AmphibBac_InhibitoryStrict_2023.2; https://github.com/AmphiBac/AmphiBac-Database) and calculating the average across all samples of a group.

All alpha diversity analyses were conducted with datasets rarefied to 90% of the read number of the sample with the fewest reads in each comparison and visualized with boxplots. We compared richness, Shannon index and evenness between different life stages of the same species, as well as between tadpoles from different species and their environments. Differences in alpha diversity measures between groups were determined using an ANOVA (for normally distributed, homoscedastic data) followed by a Tukey post-hoc test or Kruskal-Wallis (for non-normally distributed data) followed by a Dunn test. P-values were adjusted for multiple comparisons using Benjamini-Hochberg corrections.

We determined beta diversity across frog species, life stages, and aquatic environments using two commonly used dissimilarity measurements as described for the captive-bred dataset. ASVs found in only one frog were removed from the full, non-rarefied dataset. Variation was analyzed with a PERMANOVA (function adonis within the package "vegan"), using Bray-Curtis and unweighted Unifrac dissimilarities as responses and species, life stage, species- life stage interaction and parenting behavior as predictors. Significant PERMANOVAs were followed by pairwise multilevel post-hoc comparisons between groups with the function pairwise.adonis (package "pairwiseAdonis", Martinez Arbizu, 2020).

As previously suggested (Neu et al., 2021), we determined genus level agglomerated taxa between adults, tadpoles and aquatic environments from unrarefied data converted to relative abundances across different prevalence (prev) and abundance (abd) cutoffs. This approach avoids using arbitrary thresholds to define a taxonomic 'core'. The following cutoffs were evaluated using the function "core_members" of the package "microbiome" (v1.22.0, Lahti & Shetty, 2012, 2023): (1) prev 100% without abd cutoff, (2) prev >75% with abd >1%, (3) prev >75% with abd >0.1% and (4) prev >75% without abd cutoff. We listed the most abundant genera for each species (*Rv*, *Af* and *Ll*) and life stage (adult or tadpoles) with respect to their presence or absence in the core of the aquatic environment of the respective species and used the package "eulerr" (Larson, 2022) to visualize overlaps of genera with prevalence over 75% and relative abundance over 0.1% with a Venn diagram.

To test whether community distances between tadpoles and adults are smaller in poison frogs than in a species without parental care, we performed a beta regression with an underlying logit function (R package "betareg", Zeileis et al., 2021) to analyze unweighted Unifrac distances of a rarefied dataset. This approach is suited to model predictions on our bounded distance dataset with high variance. ASVs were agglomerated at the genus level prior to calculating distances between tadpoles and adults of different species, while distances between related and unrelated pairs were calculated on a ASV level. To account for differences in samples size between groups, we performed iterated analyses with random subsamples of equal sample size (N = 8 for distances between adults and tadpoles between species and N = 10 for related vs. not-related pairs of *Rv*) and report averaged p-values (following the approach of Hughey et al., 2017).

Microbiota composition analyses of wild experimental tadpoles

We determined and illustrated alpha and beta diversities of microbial communities on transported and non-transported tadpoles and significant differences between the two groups as described for wild populations. Variation was analyzed with a PERMANOVA (function adonis within the package "vegan"), using unweighted Unifrac dissimilarity measures calculated from a rarefied dataset. Due to the limited sample size and low biomass of some tadpoles, we chose to work with relative ASV abundances rather than rarefaction to determine disparities and overlaps between the groups and their aquatic environment, following previous examples (McMurdie & Holmes, 2013; Prest et al., 2018). Venn diagrams were created without prevalence or abundance cutoffs and include all low abundance taxa. Differentially abundant genera between transported and non-transported tadpoles were determined with the package "ANCOMBC2" (version 2.2.2) (Lin, 2023).

We used 'Sourcetracker' (Knights et al., 2011) on unrarefied ASVs to determine proportions of the communities of adult caregivers (“source”) that can be detected on tadpoles that were collected from their back and then reared in artificial cups for one month (“sink”). For this approach, we defined the communities of each caregiver as source and transported tadpoles as sinks and reported minimum, maximum and median source proportions detected among transported tadpoles.

ASVs shared between the transported tadpole and its transporting caregiver were identified using the function "common_taxa" of the package "phylosmith" (Smith, 2023). We then evaluated the presence and relative abundance of these shared taxa in transported and non-transported tadpoles, in their respective aquatic cup environments, and in the water used to refill their cups. We converted relative abundances to binary presence-absence information for illustration in a bubble chart and determined the ten taxa with the highest relative abundance for each transporting caregiver individually from the unrarefied dataset. To visualize the microbial interconnectedness of generationally shared genera within adult communities, we

used the function "netConstruct" from the package NetCoMi (v1.1.0; Peschel et al., 2021) to construct genus-level microbial networks of *Rv* adults (N = 44) using SPRING associations with a lambda index of 200 over 100 repetitions.

Permits

Approval of the field monitoring by the scientific institution of the authors:

The monitoring project in the Nouragues was reviewed by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University and approved under the APLAC-protocol 33691 (Protocol Director LAO). The APLAC committee is Stanford's Institutional Animal Care and Use Committee (IACUC). It is appointed by the University Vice Provost and Dean of Research.

Approval from local field site:

Non-invasive field experiments included in this publication were approved by the scientific committee of the Nouragues Reserve. The field station in the Nouragues Reserve is managed by the CNRS (Centre National de la Recherche Scientifique), is part of the USR 3456 LEEISA (Laboratoire Ecologie, Evolution Interactions des Systèmes Amazoniens, founded in 2016) and located in the nature reserve 'Les Nouragues', which is operated by the Office national des forêts (ONF) Guyane. Additional tissue collections were approved by the Direction Générale des Territoires et de la Mer (DGTM approval number # R03-2022-08-10-00001).

Data availability

Data files and data analysis scripts used to generate the results are available on DataDryad (pending acceptance).

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Nouragues' in French Guiana, that was founded on land ancestrally inhabited by the Amerindien Nouragues ('Norak') tribe.

Declaration of Interests

The authors declare no competing interests.

Author Contributions

Conceptualization: MTF, SC

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Formal Analysis: MTF, KSX, EKC, MD

Investigation: MTF

Visualization: MTF, LAO

Methodology: MTF

Writing: MTF

Review and Editing: KSX, EKC, DAR, LAO

Project Administration: MTF, LAO

Supervision: KSX, EKC, SC, DAR, LAO

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