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Dietary carotenoid supplementation has long-term and community-wide effects on the amphibian skin microbiome

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Abstract

The amphibian skin microbiome plays a crucial role in host immunity and pathogen defence, yet we know little about the environmental drivers of skin microbial variation across host individuals. Inter-individual variation in the availability of micro-nutrients such as dietary carotenoids, which are involved in amphibian immunity, may be one factor that influences skin microbial assembly across different life history stages. We compared the effect of four carotenoid supplementation regimes during different life stages on the adult skin microbiome using a captive population of the critically endangered southern corroboree frog, *Pseudophryne corroboree*. We applied 16S rRNA sequencing paired with joint-species distribution models to examine the effect of supplementation on taxon abundances. We found that carotenoid supplementation had subtle yet taxonomically widespread effects on the skin microbiome, even 4.5 years post supplementation. Supplementation during any life-history stage tended to have a positive effect on the number of bacterial taxa detected, although explanatory power was low. Some genera were sensitive to supplementation pre-metamorphosis, but most demonstrated either additive or dominant effects, whereby supplementation during one life history stage had intermediate or similar effects, respectively, to supplementation across life. Carotenoid supplementation increased abundances of taxa belonging to lactic acid bacteria, including *Lactococcus* and *Enterococcus*, a group of bacteria that have previously been linked to protection against the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (Bd). While the fitness benefits of these microbial shifts require further study, these results suggest a fundamental relationship between nutrition and the amphibian skin microbiome which may be critical to amphibian health and the development of novel conservation strategies.

KEYWORDS
amphibian, cutaneous microbiome, diet, host–microbe interactions, immunity, nutrition, southern corroboree frog, threatened species

1 | INTRODUCTION

The amphibian skin is a permeable organ that is covered with a sugar-rich mucosal layer that acts as a growth substrate for commensal microbes, termed the skin or cutaneous microbiome (Kueneman et al., 2014; Ross et al., 2019). This microbial community is a critical component of host immunity because bacterial symbionts generate antimicrobial and antifungal molecules, while

also stimulating host immune responses that are important for pathogen defence (Knutie et al., 2017; Walke & Belden, 2016). As such, there is growing evidence that inter-individual variation in the amphibian skin microbiome is associated with susceptibility to pathogens (Chen et al., 2022; Harrison et al., 2019; Piovia-Scott et al., 2017). However, we know little about the proximal mechanisms that shape skin microbiome assembly and composition across individuals, which may hinder efforts to promote amphibian health and resilience against pathogens in ex-situ conservation programs.

Widespread loss of amphibian species due to the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, Bd) has driven increased research effort on understanding the ecological drivers of the amphibian skin microbiome and how subsequent variation in microbial communities mediates infection outcomes (Bates et al., 2022; Rebollar et al., 2020). Variation in the amphibian skin microbiome has been associated with species identity (Kueneman et al., 2014), life history stage (Harrison et al., 2019), bioclimate (Kueneman et al., 2019) and microhabitat (Muletz Wolz et al., 2018), yet the mechanisms that underpin these associations remain poorly understood. A potential mediator of these effects is nutrition, which can vary with diet and climatic conditions. For example, diet varies with amphibian life history stage and may in part contribute to differences in the microbiome observed between tadpoles and frogs, while microclimates may differ in their nutrient availability. Recent evidence indicates diet can mediate infection responses via shifts to the amphibian gut microbiome (Hughey et al., 2023), yet studies on the link between diet and the skin microbiome in amphibians are rare and limited to culturing methods (Antwis et al., 2014; Edwards et al., 2017), which underrepresents true microbial diversity.

One group of nutrients that may be important for shaping microbiome-pathogen interactions are dietary carotenoids, a group of essential nutrients that are often involved in immunity and colouration (Szuroczki et al., 2016, 2019; Weaver et al., 2018). Previous research has shown that carotenoid supplementation increased the total abundance and diversity of culturable bacterial isolates on frog skin in the southern corroboree frog *Pseudophryne corroboree* (Edwards et al., 2017) and the red-eyed tree frog *Agalychnis callidryas* (Antwis et al., 2014). Experimental design differed between these two studies, with *P. corroboree* supplemented both pre-metamorphosis and post-metamorphosis, and *A. callidryas* supplemented post- metamorphosis only. Because increased bacterial diversity is one of the most consistent microbial traits linked to lower disease susceptibility (Chen et al., 2022; Harrison et al., 2019), nutrient supplementation may represent an untested approach for promoting disease-resistant microbiomes. However, it is unknown whether the link between carotenoid supplementation and skin bacterial diversity remains when measuring a much broader range of bacteria, including non-culturable species. Moreover, nutrition during host development may be particularly important for shaping adult skin microbial communities via early priority effects (Barnes & Lewis, 2021), yet how nutrition during different life history stages influences the adult skin microbial landscape also remains unknown.

In this study, we experimentally tested the effect of dietary carotenoid supplementation at different life history stages on the adult skin microbiome of a captive population of southern corroboree frogs based on 16S rRNA sequencing, and used joint species distribution models to identify the co-occurring suites of taxa affected by carotenoid treatment. The southern corroboree frog is a critically endangered montane frog from the southeast Australia that is primarily under threat from the amphibian chytrid fungus. Previous studies on this species have demonstrated that carotenoid supplementation has positive yet dose- and carotenoid class-dependent effects on time until metamorphosis (McInerney et al., 2019), skin colour hue and saturation (Umbers et al., 2016), physical performance (Silla et al., 2016), and escape response (McInerney et al., 2020), although it does not affect larval growth or survival (Byrne & Silla, 2017). As such, there is strong evidence that carotenoids influence the colour and physiology of this species in a way that may affect performance and viability.

We implemented four carotenoid supplement regimes whereby individuals were not supplemented at all (UU), supplemented pre-metamorphosis only (as tadpoles; CU), post-metamorphosis only (as juveniles and adults; UC), or during both stages (CC; Figure 1a). Skin swabs were then taken as 4.5-year old adults and the microbiome quantified (Figure 1b). We generated a hypothesis framework to visually define four types of effects we could expect based on how supplementation might interact with life history stage to influence microbial traits (Figure 1c). These are categorized as pre-metamorphic effects, whereby a microbial trait is disproportionally affected by supplementation regime as a tadpole; post-metamorphic effects, whereby a microbial trait is disproportionally affected by supplementation regime post metamorphosis; additive effects, whereby supplementation either pre- or post-metamorphosis have intermediate effects on a microbial trait compared to supplementation across life; and dominant effects, whereby supplementation during any life history stage have similar effects. Because nutrition during early development may be particularly important for shaping long-term microbial assembly (Indrio et al., 2017; Lavoie et al., 2021) and microbiome-mediated effects on adult physiology (Knutie et al., 2017), we predicted that pre-metamorphic effects would be the most common type of effect for microbial traits that are significantly influenced by carotenoid treatment.

2 | METHODS

All procedures outlined in the present study were approved by the University of Wollongong Animal Ethics Committee (Protocol Number: AE17/14).

2.1 | Study species and cohort

The southern corroboree frog *P. corroboree* is a critically endangered terrestrial frog restricted to the sub-alpine regions of Kosciuszko

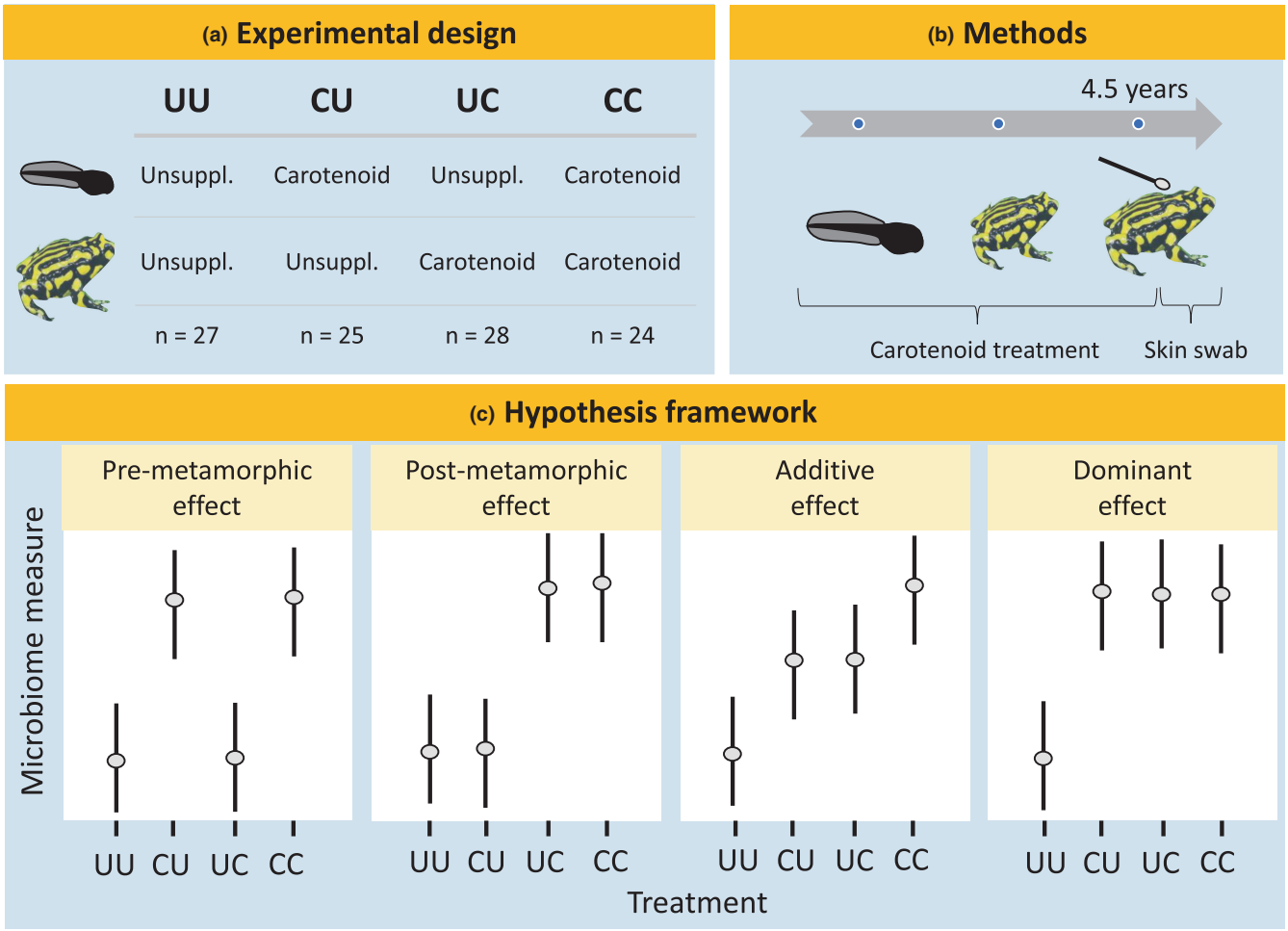

FIGURE 1 Conceptual outline of (a) experimental design, (b) sampling methodology and (c) our hypothesis framework with some of the expected effects of carotenoid supplementation on microbial phenotypes. Note that other types of effects or intermediate effects are also possible.

National Park, New South Wales. The species is nationally listed as Critically Endangered, having been driven to the brink of extinction by the amphibian chytrid fungus (McFadden et al., 2018). A multi-institutional conservation breeding program was established for the species in the late 1990s, successfully generating an increasing captive population which annually supplies offspring for wild reintroductions and conservation research (McFadden et al., 2018). Native *P. corroboree* typically feed on algae and organic matter as tadpoles, and ants and other small invertebrates post-metamorphosis as juveniles and adults (Osborne, 1991). These prey items all contain carotenoids, so it is assumed that carotenoids form a natural component of the diet of *P. corroboree*.

Frogs used in the present study ($n = 104$) were reared to maturity and maintained in a biosecurity facility within the Ecological Research Centre, University of Wollongong, between 2013 and 2018. Individuals were obtained on the 19th of July 2013, as captive-bred F1 embryos generated by an ex-situ population located at Melbourne Zoo, Australia. Immediately following hatching, animals were housed in isolation throughout all life-stages in order to eliminate the transfer of microbiota between individuals. All animals were subjected to the same standardized husbandry conditions (described below), apart from the provision of dietary carotenoid supplementation. Individuals were randomly assigned to one of four dietary treatments, consisting of groups that: (1) received no carotenoid supplementation (unsupplemented—UU; $n = 27$), (2) were carotenoid supplemented as tadpoles but unsupplemented post metamorphosis (CU; $n = 25$), (3) were carotenoid supplemented post metamorphosis but unsupplemented as tadpoles (UC; $n = 28$) or (4) supplemented with dietary carotenoids across both life-stages (CC; $n = 24$).

2.2 | Developmental (tadpole) diet and husbandry

Tadpoles were housed in individual plastic containers (10 cm D × 10.5 cm H) filled with 600 mL of reverse-osmosis (RO) water according to methods described previously (Silla et al., 2016). All containers received a partial water change (ca. 50% volume, using a RO water system) three times a week and excess food and faeces were siphoned from each container weekly. Tadpoles were held in an artificially illuminated constant-temperature room maintained at 12°C on an 11.5:12.5 h light: dark cycle. In addition to overhead room

lighting, UV-B light was provided for 1 h/day using fluorescent strip bulbs to ensure vitamin D3 synthesis (Michaels et al., 2015).

The unsupplemented larval diet (UU and UC treatments) consisted of 2 g of ground fish flake (75:25 mixture of Sera Flora/Sera Sans) suspended in 20 mL of RO water. The carotenoid-supplemented diet (CC and CU treatments) consisted of 2 g of ground fish flake (75:25 mixture of Sera Flora/Sera Sans) and 0.04 g of carotenoid mixture (Superpig, Repashy Ventures, Inc., Oceanside, CA, USA) suspended in 20 mL of RO water. Food suspensions were prepared in batches and frozen at −20°C until required. Individual tadpoles received two droplets of thawed homogenized food suspension (0.0585–0.0685 g wet mass, 0.015–0.018 g dry mass) thrice weekly for the first 8 weeks of development. The quantity of food provided was then increased to four droplets of thawed homogenized food suspension (0.117–0.137 g wet mass, 0.030–0.036 g dry mass) thrice weekly until forelimb emergence (Gosner stage 42). During the transition from forelimb emergence (Gosner stage 42) to full tail absorption (Gosner stage 46), individuals satisfy nutritional needs through the absorption of the tail tissue; consequently, food was not provided during this period (mean ± SEM duration = 25.24 ± 0.65 days).

2.3 | Post-metamorphic (frog) diet and husbandry

Once metamorphosis was complete (full tail absorption; Gosner stage 46), frogs continued to be housed individually throughout post-metamorphic life. Frogs were housed in cylindrical plastic containers (10 cm D × 10.5 cm H) for the first 3-years of post-metamorphic life, then in April 2017 the frogs were transferred into larger plastic containers (21 cm L × 14 cm W × 12 cm H). All containers contained a layer of aquarium gravel covered by a layer of sphagnum moss. Twice weekly, containers were flushed with approximately 600 mL of reverse-osmosis water to remove nitrogenous waste, and sphagnum moss was replaced every 4 weeks. Artificial lighting was provided by fluorescent strip bulbs controlled by timers which were cycled annually to reflect natural seasonal conditions (daylight hours ranged from 10 to 14 h/day). The facility was temperature-controlled, and ambient air temperature inside the facility was also cycled annually, to reflect natural seasonal changes, which included an eight-week overwintering period. Temperature throughout the year ranged from a minimum of 5°C during the winter brumation period, to a maximum of 20°C during austral summer. During the winter brumation period, feeding ceased and resumed once the temperature was above 15°C.

Post-metamorphic frogs were fed hatchling crickets, *Acheta domestica*, twice weekly. The unsupplemented post- metamorphic diet (UU and CU treatments) consisted of crickets that had been fed 48 h earlier with apple. The carotenoid supplemented diet (CC and UC treatments) consisted of crickets that had been fed 48 h earlier with carrots and dusted with 1.0 g of carotenoid mixture (Superpig) immediately prior to feeding. Once per week, crickets in all treatments were dusted with an additional 0.2 g of calcium powder (Repti-Cal) to prevent frogs developing disorders associated with calcium deficiencies.

2.4 | Sample collection

At approximately 4.5 years post-hatching, frogs were swabbed for skin microbiome quantification. Frogs were swabbed (1 swab per frog) across each of the following surfaces five times: dorsal (anterior to posterior), ventral (anterior to posterior), lateral (left and right sides), front legs from armpit to wrist (left and right sides) and back legs from groin to ankle (left and right sides). Skin swabs were collected over two sampling days; 10/04/2018 ($n = 14$) and a week later on 17/04/2018 ($n = 90$). At the time of sample collection, frogs were maintained at a constant temperature of 20°C and a 12:12 h day: night lighting cycle. During swab collection, frogs were removed from their individual containers and handled with separate sterile gloves to prevent cross contamination of skin bacteria. Immediately prior to sampling, frogs were rinsed once with 20-mL sterile water to remove transient bacteria, ensuring minimal handling. Skin swabs were collected using sterile rayon-tip swabs. Swabs were placed in individual Eppendorf tubes and stored dry in a cooler box for <6 hours (storage temp = 5.5 ± 0.6°C) before being transferred to a −80°C freezer until further processing. Immediately following swab collection, frogs were weighed to the nearest 0.01 g. The sex of each frog was confirmed at the end of their life span via dissection and inspection of the gonads.

2.4.1 | Sample processing

DNA extraction, amplification and sequencing was outsourced to the Australian Genome Research Facility (AGRF). Briefly, DNA was extracted using DNeasy power soil kit following manufacturer's instructions. The V3–V4 region of the bacterial 16S rRNA gene was amplified using 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers. PCR thermocycling was completed with an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 (Life Technologies, Australia) for the primary PCR. The first stage PCR was cleaned using magnetic beads, and samples were visualized on 2% Sybr Egel (Thermo-Fisher). A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were cleaned again using magnetic beads, quantified by fluorometry (Promega Quantifluor) and normalized. Amplicons were sequenced on an Illumina MiSeq (San Diego, CA, USA) with a V3, 600 cycle kit (2 × 300 base pairs paired-end).

2.4.2 | Sequence processing

All sequence reads were processed using QIIME2 version 2018.8 (Bolyen et al., 2019). Sequences were merged, quality filtered, and chimera filtered using the DADA2 pipeline to generate amplicon sequence variants (ASVs; Callahan et al., 2016). Primers were trimmed and reads were truncated at 277 (forward) and 220 (reverse) base pairs. ASVs were assigned a taxonomy using SILVA version 132

(Pruesse et al., 2007). A microbial phylogenetic tree was built using FastTree (Price et al., 2009). The phylogenetic tree was rooted with an Archaea sequence that was subsequently removed. ASVs were filtered if they were not bacteria, not assigned to a phylum (as these are assumed to be spurious), or if they were classified as mitochondria or chloroplasts. This filtering step removed 0.2% of reads and 5% of ASVs. Fourteen replicate samples were sequenced twice on different sequencing runs and demonstrated high repeatability in microbial composition (Figure S1).

2.4.3 | Statistics

Alpha diversity: We applied linear models to quantify the effect of diet treatment on three measures of alpha diversity (within individual diversity): observed ASV richness, inverse Simpsons index and Shannon index. These three indices were chosen because they showed limited correlation with each other and because they demonstrated distributions close to normal. Both inverse Simpson and Shannon indices attempt to measure the evenness of the community, although Shannon is more sensitive to sequencing depth and is not easily biologically interpretable. We rarefied data for alpha diversity analyses to 6700 reads, because this reduced the strong effect of sequencing depth on alpha diversity measures. To choose the best model structure, each alpha diversity metric was modelled using three probability distributions (gaussian, gamma with a log link, and gamma with no link) and the best model was chosen based on AIC and distribution of residuals. This step indicated that the best models for observed ASV richness and Shannon diversity were general linear models with a gaussian distribution, while the best model for the inverse Simpson was a generalized linear model assuming a gamma distribution with a log link. Treatment, sex, and sequencing depth were included as predictors in all models. Date of swab collection was not significantly associated with any measure and was excluded from models. Mass was also excluded because mass and sex were highly correlated (females are heavier than males with a largely non-overlapping distribution), and model comparison indicated variation in the microbiome was better attributed to sex than to mass. The importance of individual predictors was measured using partial R^2.

Beta diversity: We analysed community composition based on two distance matrices: weighted Unifrac, which accounts for abundance and microbial phylogeny, and Bray-Curtis, which accounts for abundance but not phylogeny. Prior to analysis the data were normalized by rarefaction to 6700 reads. We modelled associations between distances and sample variables using a distance-based redundancy analysis (dbRDA; a type of constrained ordination) and applied using vegan::capscale (Jari Oksanen et al., 2018). The dbRDA model included treatment, sex and sequencing depth as co-variates. The model was summarized using vegan::anova.cca. As with alpha diversity, mass was excluded because sex provided a better model fit and fitting models that included mass as a predictor separately to males and females did not show a convincing effect of mass.

Joint modelling with generalized linear latent variable models (GLLVM): Genus- and ASV-level abundances were modelled using a joint modelling approach using the function gllvm::gllvm (Niku et al., 2019), specifying three latent variables, a negative binomial distribution, and including treatment, sex and sequencing depth as predictor variables. Model selection was based on AIC, which indicated five latent variables and excluding mass was the most parsimonious model. GLLVMs are superior to methods such as differential abundance analysis because they model joint responses of species to explanatory variables and aim to tease apart the causes of species co-occurrence. For the model at the genus level, microbial data were agglomerated to genus level and only genera that were present in 70% of samples and had over 20 reads included, which consisted of 62 genera that made up 89% of all reads. For the model at the ASV level, 67 of the most common ASVs were included (prevalence above 70% and above 20 reads). These 67 ASVs accounted for 61% of all reads. Counts were not normalized but were modelled using the negative binomial distribution and controlling for sequencing depth, as recommended by model developers for positively skewed microbial count data. Quality checks on model fit were performed to ensure model assumptions were met. The same model structure was used as for the model at genus level.

3 | RESULTS

The skin microbiome of the southern corroboree frog was represented by 11,151,387 sequence reads that were assigned to 5776 ASVs from 104 samples, with a mean read count of 106,204 per sample. Sixteen per cent of all ASVs were shared across all treatment groups (Figure S2a) and this increased to 70% when excluding rare ASVs that occurred in only three individuals or fewer (Figure S2b). The most abundant phylum was Proteobacteria (67% of reads) followed by Bacteroidetes (22%), with rarer Actinobacteria and Firmicutes making up approximately 6% (Figure 2a). The four most abundant taxonomic orders (Pseudomonadales, Enterobacteriales, Flavobacteriales and Betaproteobacteriales) made up between 10 and 19% relative abundance each (Figure 2b).

3.1 | Carotenoid treatment increased alpha diversity

We modelled the effect of carotenoid treatment on three measures of alpha diversity, controlling for sequencing depth. For ASV diversity and Inverse Simpson, but not Shannon, alpha diversity was significantly higher for frogs that were supplemented post-metamorphosis compared to unsupplemented frogs (Figure 3a–c; Table 1), with carotenoid supplemented frogs having 20% (95% CI: 5–37%, $p = .01$) higher ASV diversity than control frogs. Carotenoid supplementation pre-metamorphosis (during the tadpole phase) did not significantly increase any alpha diversity measure (Figure 3; Table 1). However, treatment independently explained only 6% of observed variation (measured using partial R^2), suggesting that despite an overall increase in alpha diversity with supplementation,

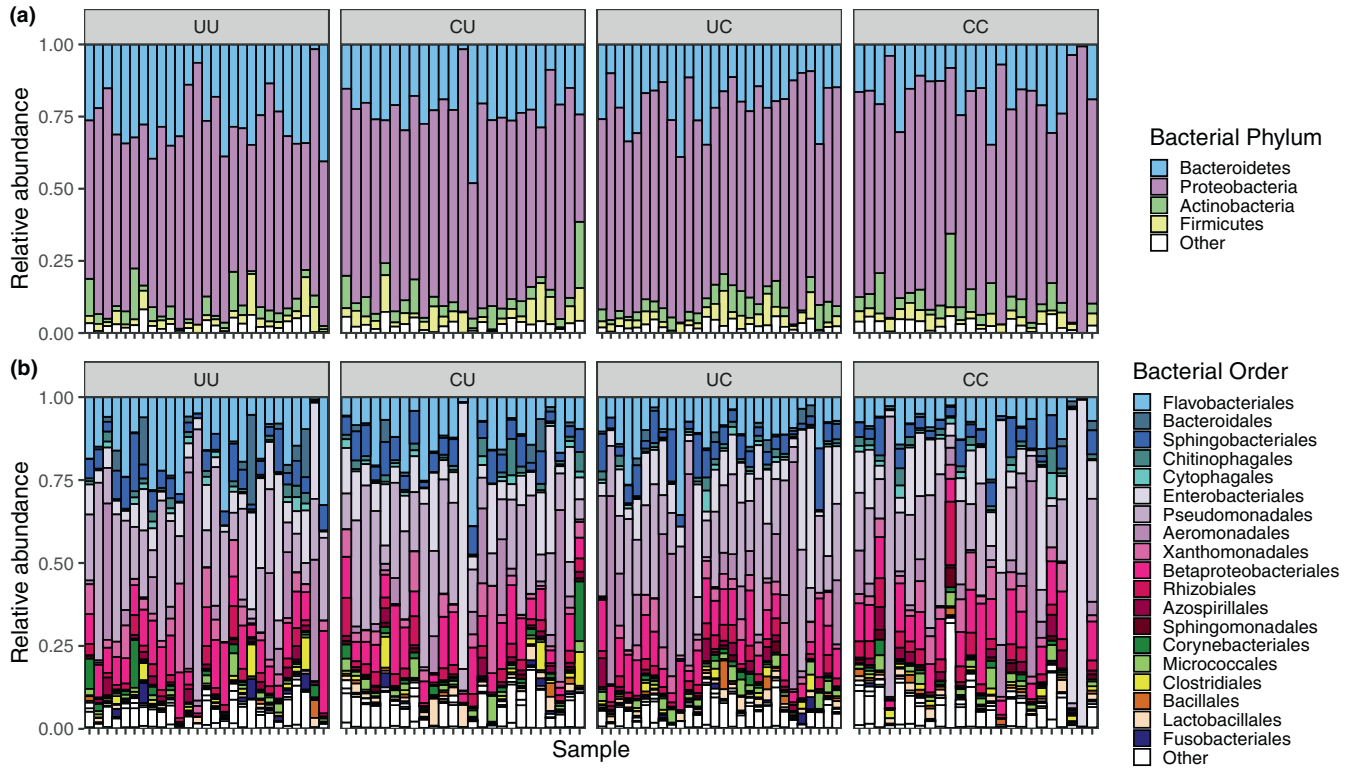

FIGURE 2 Taxonomic composition per sample, grouped by carotenoid treatment, and coloured by (a) phylum and (b) order. Bacterial orders are ordered and coloured according to the Phylum they belong to (Bacteroidetes—blues; Proteobacteria—purples/reds; Actinobacteria—greens; Firmicutes—yellow/brown).

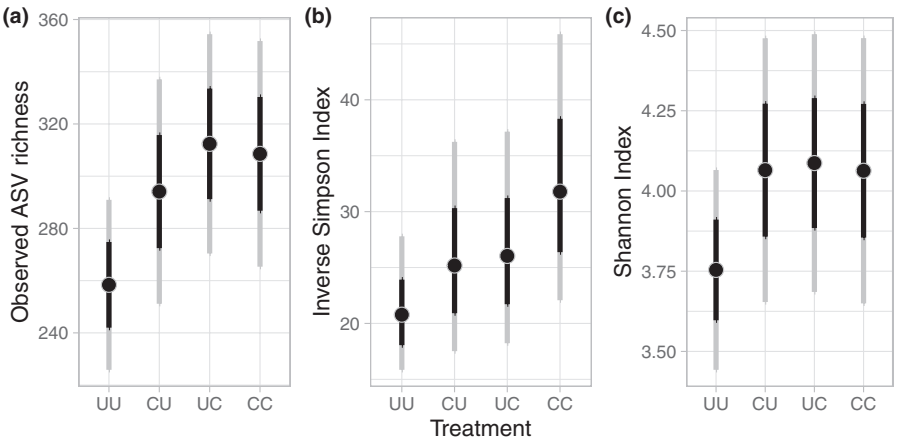

FIGURE 3 Model estimates for the effect of carotenoid treatment on three measures of alpha diversity; (a) observed ASV richness, (b) inverse Simpson index and (c) Shannon index. Black bars represent standard errors while grey bars represent 95% confidence intervals. See Table 1 for statistics.

individual variation across treatment groups was still very high. Sex was also weakly associated with Inverse Simpson ($t = -2.3$, $p = .02$) and Shannon diversity ($t = -2.1$, $p = .03$), but not observed ASV richness ($t = -1.7$, $p = .1$), with males having lower alpha diversity compared to females across measures.

3.2 | Carotenoid treatment had additive effects on beta diversity

To identify how carotenoid treatment affected overall microbial community composition, we applied constrained ordinations on two community distance metrics, weighted Unifrac (Figure 4a) and Bray-Curtis (Figure 4b). Carotenoid treatment was significantly associated with community composition for both distance metrics (Table 2), with the skin microbiome of unsupplemented frogs (UU) being most different from frogs that were supplemented with carotenoids across life (CC; Figure 4a,b). Frogs that were treated only pre- or post-metamorphosis (UC and CU) had skin microbial compositions that were broadly intermediate in comparison to unsupplemented (UU) and fully supplemented frogs (CC). Sex was also a significant predictor of beta diversity (Figures 4c,d), in particular when applying Bray Curtis (Figure 4d), which accounts for ASV identity but not phylogeny.

TABLE 1 Model statistics for three models predicting (a) observed ASV richness, (b) inverse Simpson and (c) Shannon index, visualized in Figure 3.

	(a) Observed ASV richness			(b) Inverse Simpson			(c) Shannon		
Predictors	**Estimates**	**CI**	***p***	**Estimates**	**CI**	***p***	**Estimates**	**CI**	***p***
Intercept (UU)	258.4	225.85 to 290.94	**<.001**	20.78	15.85 to 27.79	**<.001**	3.75	3.44 to 4.07	**<.001**
Treatment (CU)	35.71	−7.25 to 78.67	.102	1.21	0.84 to 1.74	.301	0.31	−0.10 to 0.72	.136
Treatment (UC)	53.99	12.00 to 95.97	**.012**	1.25	0.88 to 1.79	.214	0.33	−0.07 to 0.73	.103
Treatment (CC)	50.15	6.94 to 93.36	**.023**	1.53	1.06 to 2.21	**.023**	0.31	−0.10 to 0.72	.141
Sex (Male)	−25.68	−56.30 to 4.95	.099	0.73	0.56 to 0.95	**.019**	−0.32	−0.61 to −0.02	**.035**
Sequencing depth	40.25	25.03 to 55.47	**<.001**	1.1	0.97 to 1.26	.142	0.2	0.05 to 0.34	**.008**
Observations	104			104			104		
R^2/R^2 adjusted	.297/.261			.135			.143/.099		

P values in bold are significant to .05.

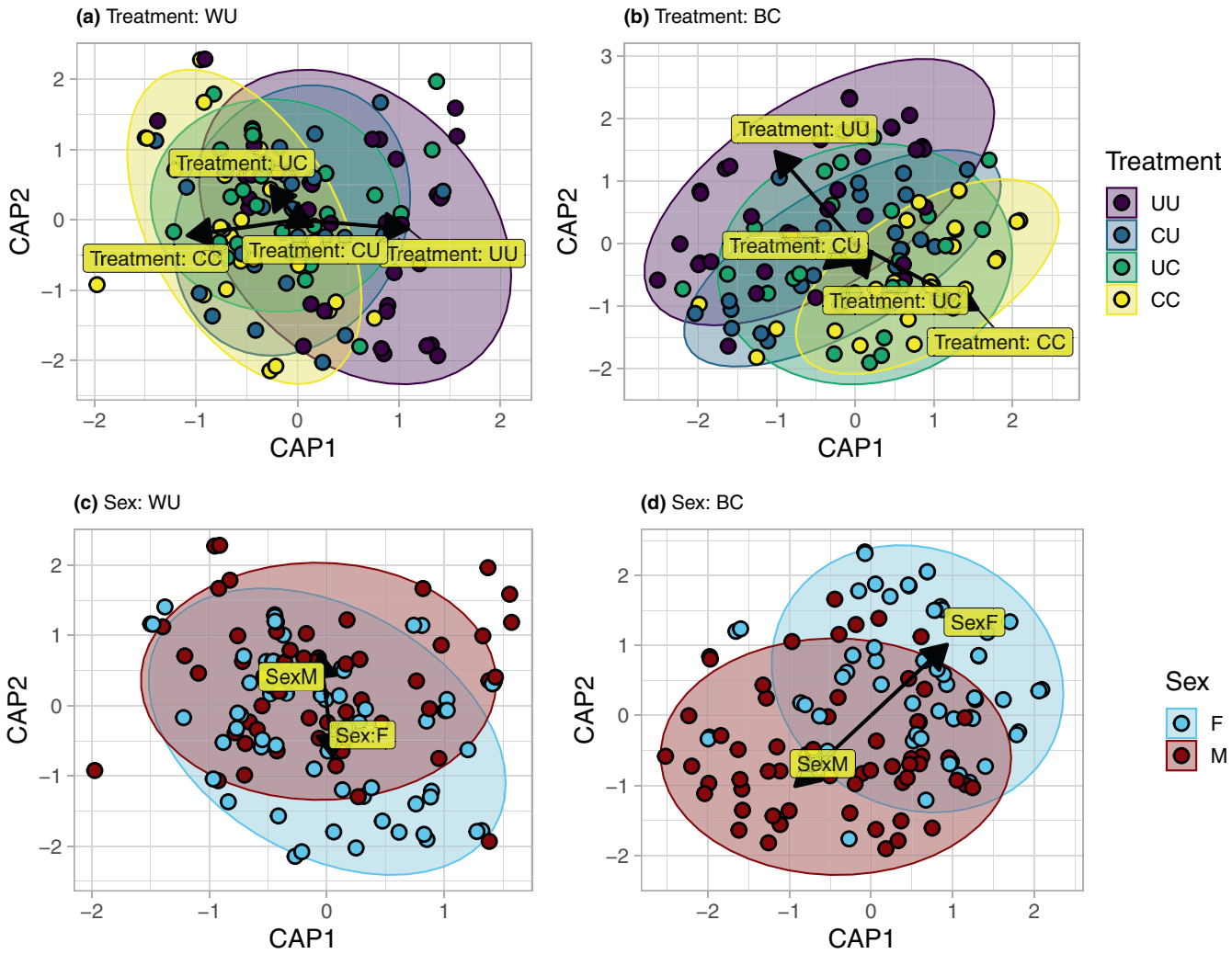

FIGURE 4 Constrained ordinations visualizing associations between microbial composition and both carotenoid treatment (a and b) and sex (c and d), applying weighted Unifrac (a and c) and Bray-Curtis (b and d) distance matrices. Ellipses show 90% confidence intervals around the group centroids. Arrows point towards group centroids.

3.3 | Carotenoid treatment had additive and dominant effects on taxon abundances

We next examined how carotenoid treatment affected co-occurring suites of taxa at the genus level by applying a generalized linear latent variable model, a type of joint model, controlling for sex and sequencing depth. Out of 51 genera, 12 (19%) significantly varied with carotenoid treatment (Figure 5; see Figure S3 for all genera). We predicted that many taxa would be sensitive to pre-metamorphic effects, yet most significant effects were either additive or dominant, or a mix of dominant and both pre- and post-metamorphic effects. Carotenoid supplementation pre-metamorphosis (during the tadpole stage) influenced to a certain degree the adult microbial abundances of *Lactococcus* and *Methylobacterium*. Overall, the joint model indicated that carotenoid supplementation at any life history stage increased abundances of *Enterococcus*, *Lactococcus*, *Enterobacter and Kluyvera*, and decreased abundances of *Comomonas*, *Empedobacter* and *Flavobacterium*. Repeating the joint model at the ASV level found similar results, with 25% of common ASVs significantly affected by carotenoid supplementation (Figure S4). We ran significant ASVs through a nucleotide BLAST, which indicated carotenoid-mediated increases to taxa with good matches to *Lactococcus garvae*, *Enterococcus faecalis*, *Vagococcus fluvialis* and *Enterobacter cloacae*.

The joint model also revealed specific microbial associations with sex (Figure S3). In total, 19 genera (37%) were significantly associated with sex. Male microbiomes were enriched in taxa belonging to *Empedobacter*, *Marmoricota* and *Janthinobacterium*, while females were enriched in *Niveispirillum*, *Runella*, *Bacteroides* and *Citrobacter*. There was also some evidence that members of *Proteus* were also enriched in females (Figure S3).

TABLE 2 Model statistics for constrained ordination models represented in Figure 4, using (a) weighted Unifrac and (b) Bray Curtis distance measures.

	(a) Weighted Unifrac			(b) Bray Curtis		
Variable	Sum of squares	*F*	*p* value	Sum of squares	*F*	*p* value
Treatment	0.16	1.7	**.01**	1.06	1.4	**.02**
Sex	0.06	2.1	**.03**	0.81	3.3	**.001**
Sequencing depth	0.04	1.4	.16	0.36	1.4	.09
Observations	104			104		
R^2/R^2 adjusted	.09/.04			.09/.04		

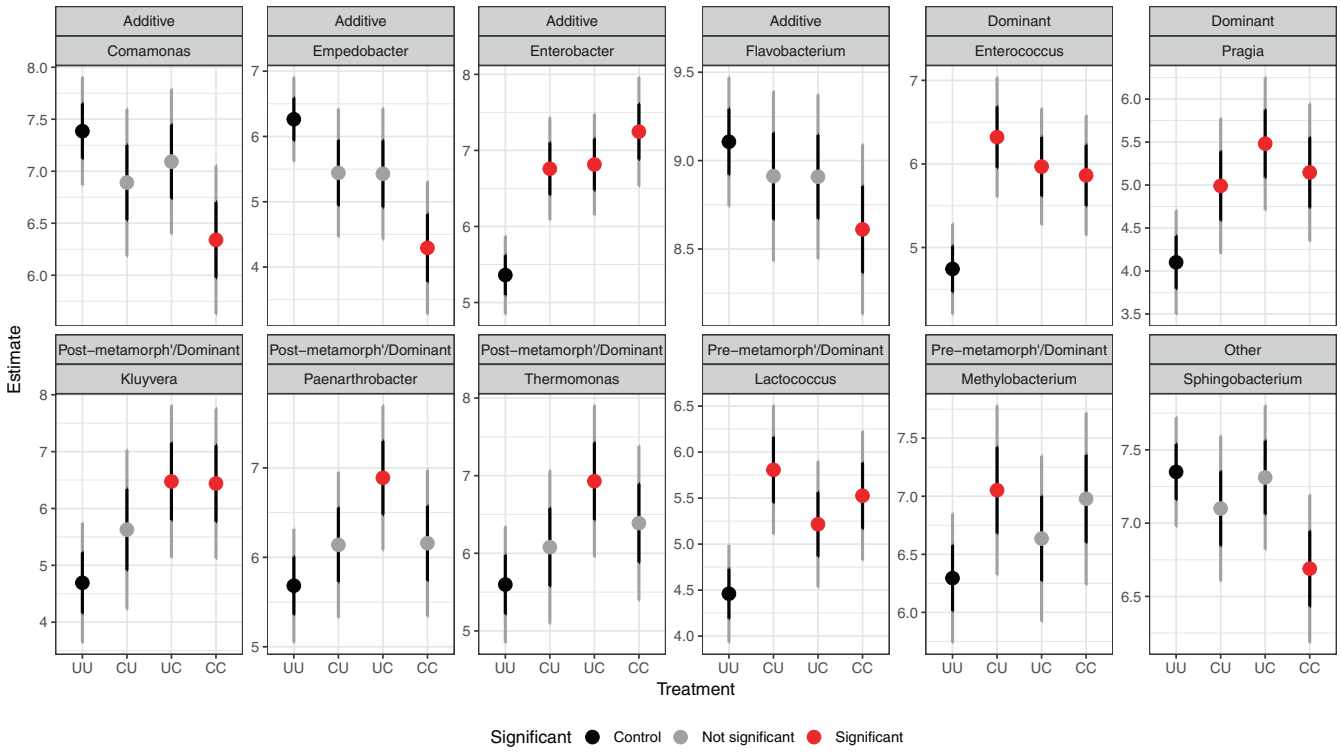

FIGURE 5 Model estimates for key genera that significantly differ by carotenoid treatment group, ordered by effect type. Red points indicate significant difference from the reference control group (UU). Black lines indicate standard errors and grey lines indicate 95% confidence intervals.

4 | DISCUSSION

Dietary carotenoids underpin multiple functions in vertebrates, yet the extent to which carotenoids, and nutrient availability more generally, affect skin microbiome assembly and structure has not been previously tested using culture-independent methods. This study examined the long-term effect of carotenoid supplementation during different life history stages on the skin microbiome diversity and composition of a cohort of captive southern corroboree frogs. The overall effect of carotenoid supplementation on diversity and composition was rather subtle, with some of the most abundant genera, such as *Pseudomonas* and *Aeromonas*, not being affected. Nevertheless, carotenoid supplementation influenced the abundances of 19% of frequently-occurring genera and was associated with increased ASV diversity. While we predicted that many microbial phenotypes would demonstrate strong pre-metamorphic effects, whereby they would be disproportionately influenced by nutrition during the larval stage, this was largely not the case. We found that the strongest effects of carotenoid supplementation were on ASVs belonging to *Enterococcus*, *Enterobacter*, and *Kluyvera*, all of which demonstrated either additive or dominant increases with supplementation, while taxa belonging to *Empedobacter* showed additive declines. Members of *Lactococcus* and the *Methylobacterium* were the only genera that demonstrated specific sensitivity to pre-metamorphic effects. Overall, our results suggest that dietary carotenoids have long term and sometimes accumulating effects on a suite of low-abundance bacterial taxa, yet provide little evidence that the presence of these nutritional conditions during early development is particularly important for the long-term structuring of adult skin microbiomes.

Our findings that dietary carotenoids influence the skin microbiome for many years post supplemententation have profound implications for the role of diet more broadly in structuring the skin microbiome in amphibians. Our study tests one facet of nutrition; yet individual variation in nutrient availability and foraging strategy are likely to have cumulative effects on host–microbe interactions that might reasonably be expected to have consequences for amphibian health and immunity. However, the molecular mechanisms underpinning this link are poorly understood, in part because the impact of nutrition on amphibian physiology is understudied. Carotenoids are converted into vitamin A, which is involved in multiple physiological processes, including immunity, reproduction, development, and metabolism (Clugston & Blaner, 2014). One possible explanation for our findings is that limitations in dietary carotenoids may affect the normal secretion of innate immune compounds, such as defensins and lysozymes, that likely control the skin microbiota and promote homeostatisis (Colombo et al., 2015; Woodhams et al., 2023). Future studies that link diet treatments to assays on skin immune compounds such as antimicrobial peptides may elucidate the intermediary role of immune function in linking diet to skin microbial communities.

While the extent to which these carotenoid-mediated effects might influence fitness and specifically *Bd* infection outcomes remains to be tested, previous studies have shown that overall bacterial diversity (Chen et al., 2022; Harrison et al., 2019; Piovia-Scott et al., 2017), and the presence of lactic acid bacteria such as *Lactococcus garvae* (Niederle et al., 2019), may provide protective effects against *Bd*. Our findings that carotenoid supplementation increases ASV diversity and abundances of lactic acid bacteria suggest that carotenoid supplementation may be a promising approach to increase amphibian resilience to this disease in ex-situ conservation programs. However, the relationship between the skin microbiota diversity and pathogen susceptibility is not well understood and requires further research. This relationship is generally assumed to be underpinned by the concept that high diversity communities are more resilient to pathogen invasion, because complex networks of interactions promote a stable coexistence between otherwise competing strains (Chang et al., 2023). As such, diet-mediated microbial diversity may shape host susceptibility to pathogens through its influence on microbial network stability and resilience (Banerjee et al., 2018). It should be noted, though, that the importance of high community diversity per se remains unclear and may not hold for all types of host-associated microbiome (e.g. the vaginal microbiome; Baud et al., 2023), and the presence of key functional strains and symbionts embedded within networks may be critical. Experiments that validate downstream consequences of diet-mediated microbiomes on *Bd* infection susceptibility, as well as the role of microbial diversity versus identity, are a promising avenue for future research.

We additionally found that sex was a relatively strong predictor of skin microbiome structure, suggesting that other developmental phases such as sexual maturation may influence skin microbial communities. Sex may be expected to be associated with skin microbial composition in amphibian species such as the southern corroboree frog that use chemical communication for mate choice, because skin microbes can produce chemical compounds involved in chemical signalling (Brunetti et al., 2019; Henneken et al., 2017). However, studies to date have generally found sex to be a poor predictor of amphibian skin microbiomes (Campbell et al., 2019; Douglas et al., 2021; Prado-Irwin et al., 2017). In this captive cohort, sex was a significant predictor of beta diversity, with male microbiomes being enriched in *Empedobacter*, *Marmoricota*, and *Janthinobacterium*, while females were enriched in *Niveispirillum*, *Runella*, *Bacteroides* and *Citrobacter*. As such, this represents one of the first studies to report associations between sex and the amphibian skin microbiome. Two potential explanations for this association are, firstly, that skin microbiomes in captive cohorts may better reflect genetically-based host effects that may be diluted in the wild; and secondly, that the southern corroboree frog uses sex-specific chemical signalling that may in part be mediated by the skin microbiome. The role of host-associated microbiomes in social communication has been largely limited to insects and mammals (Sarkar et al., 2020), yet the importance of the amphibian skin for both social communication and pathogen defence suggest the skin microbiome may be a nexus for these functions.

Our study has two notable limitations that might influence the interpretations of our results. Firstly, we quantify the skin microbiome

4.5 years post metamorphosis and using a cross-sectional study design. The effect of carotenoid treatment pre-metamorphosis on skin microbial communities may decline over time since treatment, and its effect may be much stronger in the months immediately following metamorphosis. The weak effect sizes presented here may therefore underestimate the true influence of carotenoid supplementation on the microbial skin microbiome at earlier time points, and may not have the power to detect short-term effects. Secondly, captive animals have highly altered and lower diversity host-associated microbiomes than their wild counterparts (Schmidt et al., 2019), including amphibian skin microbial communities (Kueneman et al., 2022). Whether carotenoids would influence the skin microbiomes of wild frogs with more complex microbial communities is unknown, yet this would be a valuable comparison in order to understand the ecological relevance of in vitro experiments on host–microbe interactions in natural populations. Longitudinal sampling across captive and rewilded counterparts during experimental diet regimes would further elucidate the extent to which diet influences amphibian skin microbial dynamics over time.

5 | CONCLUSION

We provide the first evidence that carotenoid supplementation causes subtle, yet widespread shifts in the amphibian skin microbiome composition that can last years into adulthood. Our results shed light on the mechanisms driving skin microbial assembly in amphibians and point towards nutrition being a potentially important tool for indirectly shaping amphibian health and pathogen defence via effects on host-associated microbes. Such a mechanism can be exploited for the development of novel conservation strategies that increase the resilience of amphibians to ongoing global threats.

AUTHOR CONTRIBUTIONS

Aimee J. Silla and Phillip G. Byrne conceived the study, acquired funding, were responsible for frog husbandry and collected the data. A. Risely analysed the data and wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

16S sequences are deposited on NCBI under BioProject PRJNA1008088 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1008088). Processed data and R code are available to download at https://zenodo.org/record/8279381 (Risely, 2023).

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SUPPORTING INFORMATION

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