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Early-Life Diet Affects Host Microbiota and Later-Life Defenses Against Parasites in Frogs

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Synopsis Food resources can affect the health of organisms by altering their symbiotic microbiota and affecting energy reserves for host defenses against parasites. Different diets can vary in their macronutrient content and therefore they might favor certain bacterial communities of the host and affect the development and maintenance of the immune system, such as the inflammatory or antibody responses. Thus, testing the effect of diet, especially for animals with wide diet breadths, on host-associated microbiota and defenses against parasites might be important in determining infection and disease risk. Here, we test whether the early-life diet of Cuban tree frogs (*Osteopilus septentrionalis*) affects early- and later-life microbiota as well as later-life defenses against skin-penetrating, gut worms (*Aplectana hamatospicula*). We fed tadpoles two ecologically common diets: a diet of conspecifics or a diet of algae (*Arthrospira* sp.). We then: (1) characterized the gut microbiota of tadpoles and adults; and (2) challenged adult frogs with parasitic worms and measured host resistance (including the antibody-mediated immune response) and tolerance of infections. Tadpole diet affected bacterial communities in the guts of tadpoles but did not have enduring effects on the bacterial communities of adults. In contrast, tadpole diet had enduring effects on host resistance and tolerance of infections in adult frogs. Frogs that were fed a conspecific-based diet as tadpoles were more resistant to worm penetration compared with frogs that were fed an alga-based diet as tadpoles, but less resistant to worm establishment, which may be related to their suppressed antibody response during worm establishment. Furthermore, frogs that were fed a conspecific-based diet as tadpoles were more tolerant to the effect of parasite abundance on host mass during worm establishment. Overall, our study demonstrates that the diet of Cuban tree frog tadpoles affects the gut microbiota and defenses against parasitic gut worms of frogs, but these effects depend on the stage of the host and infection, respectively.

Introduction

Resource availability can significantly alter host defense strategies, such as resistance, against parasites (Lee et al. 2006; Sternberg et al. 2012; Howick and Lazzaro 2014; Knutie et al. 2017). Resistance mechanisms, such as immune responses that serve to reduce the damage that parasites cause by reducing parasite fitness, require an energetic investment by the host

(Read et al. 2008; Rohr et al. 2010). Because immune responses can be energetically costly to produce, only hosts in good condition may be physiologically able to invest in these defenses (Sheldon and Verhulst 1996; Svensson et al. 1998; Lochmiller and Deerenberg 2000; Demas 2004). For example, higher food availability (i.e., quantity) can increase host resistance to infections by providing more resources for

immunity (Sternberg et al. 2012; Howick and Lazzaro 2014; Knutie et al. 2017).

The quality (e.g., nutritional content) of food can also affect the maintenance and development of the immune system (Kelly and Coutts 2000; Gil and Rueda 2002; Venesky et al. 2012). Specific macronutrients may be beneficial for different defense mechanisms. Supplemented protein can increase cellular (e.g., eosinophils, globule leukocytes, and mast cells) (reviewed in Coop and Kyriazakis 2001) and humoral immunity (e.g., Ig antibodies) (Datta et al. 1998) to parasites. For example, hosts fed a high protein diet produce more eosinophil cells and IgG antibodies to parasitic worms, which decreased infection risk, compared with hosts fed a low protein diet. Additionally, a high lipid diet can increase leptin hormone production, which in turn, increases cellular immunity, including the inflammatory response (Demas 2004, reviewed in Kau et al. 2011). Thus, food quality, especially for animals with a wide breadth of diet composition, is likely an important factor that determines infection risk.

Host diet composition can also alter the gut microbiota of hosts (David et al. 2014; Carmody et al. 2015; Bletz et al. 2016). For example, hosts that consume a plant-based diet have different bacterial communities than hosts that consume an animal-based diet (David et al. 2014). Such differences in the host-associated gut microbiota may affect the development and maintenance of the immune system of the host. For example, early-life reductions in certain bacterial taxa (e.g., *Bacteroides fragilis*, *Cetobacterium* sp.) in the guts of hosts can adversely affect development of the immune system (reviewed in Round and Mazmanian 2009; Hooper et al. 2012) and can decrease later-life resistance to infection (Knutie et al. 2017). Thus, the host-associated microbiota may play a role in mediating the effect of diet on host health, and specifically predicting infection risk.

Here, we tested whether the early-life diet in Cuban tree frogs (*Osteopilus septentrionalis*) affects host-associated microbiota and later-life infection risk. Cuban tree frog tadpoles have a wide breadth of diet composition. For example, two common resources consumed by tadpoles are algae and conspecifics (Crump 1986; Babbitt and Meshaka 2000; Smith 2005), which vary in their nutritional content (e.g., amount of protein, lipid, and fiber). Because nutritional content of a diet can affect both host-associated microbiota and immunity in other systems, tadpole diet may play an important role in determining gut bacterial community composition and infection risk in Cuban tree frogs.

In our study, we experimentally fed Cuban tree frog tadpoles either a diet consisting of spirulina algae (*Arthrospira* sp.) or conspecifics. We then: (1) characterized the gut microbiota of tadpoles and adults; and (2) measured host defenses against an environmentally common, skin-penetrating, gut worm *Aplectana hamatospicula* (Ascaridida: Cosmocercidae). Juvenile worm larvae penetrate the skin of frogs and then, in approximately three weeks, establish, mature, and reproduce in the gastrointestinal tract (Ortega et al. 2015; Knutie et al. 2017). Worm eggs and larvae (they are ovoviparous) are defecated by frogs, and after approximately a week of development, juvenile worms can infect the next host. We experimentally exposed adult frogs, that were fed different diets as tadpoles, to juvenile *A. hamatospicula* and then quantified host resistance of frogs during the skin penetrating and gut establishment stages of the parasite. We also measured host tolerance, which was quantified as the reaction norm between parasite abundance and change in host body mass (Simms 2000), while worms established in the gut.

We predicted that the diet treatment of tadpoles would alter bacterial communities in tadpoles but that these effects would not be enduring into adulthood, as found in other studies (Knutie et al. 2017; Vences et al. 2016). We also predicted that the early-life diet treatment would affect later-life infection risk; if a conspecific-based diet in tadpoles alters the microbiota that affect the development of resistance mechanisms (e.g., bacterial diversity and relative abundance of phylum Fusobacteria and genus *Cetobacterium*; Knutie et al. 2017), then frogs that were fed a conspecific-based diet as tadpoles would be differentially resistant to worm infections compared with frogs that were fed an alga-based diet as tadpoles. Additionally, the nutritional content (e.g., protein vs. lipids) of either diet could favor the development of resistance mechanisms, such as the inflammatory or antibody response. Because the inflammatory response of the skin is likely important during the penetration stage of the worm and studies have shown that a lipid-rich diet can favor the development of the inflammatory response (Kau et al. 2011), we predicted that an alga-based diet (higher lipid content) would favor resistance to worm penetration. Additionally, because the antibody response is important during the establishment stage of the worm (Knutie et al. 2017) and studies have shown that a protein-rich diet can favor the antibody response (Datta et al. 1998), we predicted that an alga-based diet (higher protein content) would favor resistance to worm establishment. Finally, we

predicted that tadpole diet treatment would not affect later-life host tolerance of infection because adult frogs would have access to *ad libitum* food resources (Knutie et al. 2017).

Methods

Experimental protocol

Osteopilus septentrionalis tadpoles were collected from the University of South Florida Botanical Gardens. In the laboratory, tadpoles (all at Gosner stages ~25; Gosner 1960) were divided randomly among 60 tanks with 3 L of pond water and housed at a density of three tadpoles per tank. Tanks were assigned to one of two experiments to test the effect of tadpole diet on either: 1) early- and later-life host-associated microbiota ($n=30$ tanks), or 2) later-life infection risk ($n=30$ tanks). Tanks were then assigned to receive either *ad libitum* diets of conspecifics or algae ($n=15$ tanks per treatment per experiment), which were suspended in agarose to prevent spoiling. To create the agarose mixture, 4 g of agarose powder were added to 200 mL of deionized (DI) water and then heated for 1 min in the microwave until it reached approximately 100 °C. The boiling agarose mixture was then added to each of the two diet mixtures (100 mL each) and refrigerated for 4 h until solidified. For the alga-based diet preparation, three tablespoons of spirulina powder (NOW foods, Bloomington, IL) were added to 100 mL of DI water. The powder contained approximately 60% protein, 0% lipids, and 0% fiber. For the conspecific-based diet preparation, 10 conspecific tadpoles (approximately three tablespoons; presumably non-kin, fed an alga-based diet, and *Aplectana*-free) were euthanized and then macerated with a sterile mortar and pestle and added to 100 mL of DI water. Tadpoles contain approximately 40% protein, 25% lipids, and 15% fiber (Nagai et al. 1971; Sogbesan and Ugwumba 2007, 2008; Sogbesan et al. 2007; Hindatu and Solomon 2017). Food was prepared twice per week for the duration of the tadpole diet experiment (approximately 8 weeks). Tadpoles were maintained under laboratory conditions (12 h light cycle, air temperature: 22 °C, water temperature: 22.6 °C, pH 7.7, dissolved oxygen: 59, nitrates: 0.96). Water was changed weekly and survival was checked daily.

For the microbiota experiment, a subset of tadpoles ($n=10$) was euthanized and necropsied to collect the gut contents after six weeks on their respective diet. For the remaining tadpoles, individuals with all four limbs (metamorphs) were removed from the tanks daily, weighed (g), and placed

individually in cups (6 cm high $\times$ 12 cm diameter) with sterilized organic *Sphagnum* moss (autoclaved for 30 min at 121 °C). Adults were maintained in the laboratory (12 h light cycle, 22 °C) on non-sterile vitamin- and mineral-dusted crickets (fed *ad libitum*) and survival was checked daily. Approximately three months after metamorphosis, adult frogs were euthanized and their guts were collected and stored at –80 °C until DNA extractions. Adults were also weighed (g) and their snout–vent length (SVL) was measured (mm).

For the infection experiment, metamorphs were also removed from the tanks daily, placed individually in cups, and maintained under laboratory conditions, as described above. Adult frogs were then exposed or sham-exposed to *A. hamatospicula* worms. Frogs were weighed (g) and then placed individually in parafilm-sealed petri dishes (100 mm diameter) with an air hole at the top of the lid. Frogs were exposed to worms by pipetting 20 infectious larval *A. hamatospicula* worms (collected from naturally-parasitized adult frogs) in 3 mL of autoclaved pond water or sham-exposed by pipetting 3 mL of autoclaved pond water without worms through the hole in the lid. After 24 h in the petri dish, frogs were returned to their individual cups with sterile *Sphagnum* moss and a dissecting microscope was used to count the worms remaining in the petri dish to determine the number of worms that penetrated each frog. All frogs survived parasite exposure in the petri dishes. Three weeks after exposure to *A. hamatospicula*, frogs were weighed (g) and their SVL was measured (mm) and then they were euthanized. Blood samples were collected from the heart with heparinized microcapillary tubes and stored in 0.6 mL centrifugation tubes on wet ice. Within 2 h of collection, samples were spun for 5 min at 13,000 rpm to separate the plasma from red blood cells. Samples were stored in a freezer at –20 °C until the plasma was used in the immunoassay. Frogs were then necropsied under a dissecting microscope to count the number of worms that had established in the colon.

Quantifying the antibody-mediated immune response

An enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of IgY antibodies in frog plasma following the protocol from Knutie et al. (2017). Each well of a 96-well plate was coated with 100 μ L of individual frog serum diluted 1:100 in carbonate coating buffer (0.05 M, pH 9.60). Each sample was applied in triplicate (i.e., across three

wells) on the plate. The plate was incubated overnight at 4°C and then washed and coated with 200 µL/well of bovine serum albumin (BSA) blocking buffer and incubated for 30 min at room temperature on an orbital table. Between each of the following steps, the plate was washed five times with a Tris-buffered saline wash solution, loaded as described, and incubated for 1 h on an orbital table at room temperature. The plate was then loaded with 100 µL/well of primary detection antibody (Goat- α -Alligator-IgG, diluted 1:1000; Bethyl Laboratories) for 1 h, washed, then loaded with 100 µL/well of a conjugate detection antibody (Rabbit- α -Goat-IgG, diluted 1:5000; Bethyl Laboratories) for 1 h. Finally, the plate was loaded with 100 µL/well of peroxidase substrate (tetramethylbenzidine; Bethyl Laboratories) and incubated for exactly 30 min. The reaction was halted using 100 µL/well of stop solution (Bethyl Laboratories). Optical density (OD) was measured with a spectrophotometer (BioTek, PowerWave HT, 450-nanometer filter).

Bacterial DNA extraction and sequencing

We isolated total DNA from frog guts using a MoBio PowerFecal DNA Isolation Kit; DNA extracts were then sent to Argonne National Laboratory for sequencing. We also extracted and sequenced “blank” samples, which were collected using sham-necropsies and sham-extractions (i.e., without an experimental sample) to control for methodological contamination (Salter et al. 2014). Bacterial DNA was also sequenced from the tank water ($n = 2$ per treatment; 50 mL of water from each tank) and bacterial communities (diversity, structure, membership) were similar between treatments.

Bacterial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R and paired end sequencing with v2 chemistry (2×150 bp) on an Illumina MiSeq platform using a single barcode approach (Caporaso et al. 2012). Sequences were analyzed using QIIME version 1.9.1 (Caporaso et al. 2010b). We applied standard quality control settings and split sequences into libraries using default parameters in QIIME. Sequences were grouped into operational taxonomic units (OTUs) using `pick_open_reference_otus.py` with a minimum sequence identity of 97%. The most abundant sequences within each OTU were designated as a “representative sequence” and aligned against the Greengenes core set (DeSantis et al. 2006) using PyNAST (Caporaso et al. 2010a) with default parameters set by QIIME.

A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. A phylogenetic tree of representative sequences was built using FastTree (Price et al. 2009). OTUs were classified taxonomically using UCLUST (Edgar 2010) with the reference Greengenes database (DeSantis et al. 2006). Singleton OTUs and sequences identified as chloroplasts or mitochondria were removed from the analysis. Additionally, any OTUs present in the “blank samples” were considered contaminants and were removed from all other samples (Salter et al. 2014). Contaminant OTUs were largely similar to those presented in Salter et al. (2014).

Several measurements of alpha diversity were calculated. We calculated the number of observed OTUs (species richness), equitability (species evenness), the Shannon index, and Faith’s phylogenetic diversity (Faith 1992), the latter of which measures the cumulative branch lengths from randomly sampling 1000 sequences from each sample (the minimum number of sequences returned from each sample). For each sample, we calculated the mean of 20 iterations of random sampling. We calculated unweighted and weighted UniFrac distances between samples in QIIME using 1000 sequences for bacterial community composition analyses.

All sequences were deposited in the Sequence Read Archive under accession PRJNA383840.

Statistical analyses

We determined the effect of tadpole diet on bacterial diversity metrics using generalized linear models (GLMs) with Gaussian errors because we only characterized the microbiota of one tadpole per tank. To determine the effect of tadpole diet on bacterial diversity metrics of adults, health metrics of tadpoles and adults, host resistance and tolerance of infection, as well as the effect of health metrics on infection risk, we used generalized linear mixed models (GLMMs) with Gaussian errors and tank as a random effect because we often had more than one individual per tank. Analyses were conducted using the `glm` (GLM) and `lmer` (GLMM) functions with the `lme4` package. We determined the effect of diet on tadpole and adult survival using a censored Cox mixed effects model (Coxme) with the `coxme` function. Probability values were calculated using log-likelihood ratio tests using the `Anova` function in the `car` package. GLM, GLMM, and survival analyses were conducted in RStudio (2013, version 0.98.1062). All figures were made in Prism (2008, version 5b).

Table 1 Effect of tadpole diet treatment on the health of tadpoles and adult frogs

Variable	Algae	Conspecifics
Time to metamorphosis	34.54 $\pm$ 2.59 days (28)	37.15 $\pm$ 4.06 days (26)
Mass at metamorphosis	0.38 $\pm$ 0.03 g (13)	0.33 $\pm$ 0.03 g (13)
Tadpole survival		
By individual	51/80 individuals	50/80 individuals
By replicate	28/30 tanks	27/30 tanks
Adult mass	0.46 $\pm$ 0.02 g (26)	0.44 $\pm$ 0.01 g (23)
Adult snout–vent length	17.74 $\pm$ 0.24 mm (26)	17.62 $\pm$ 0.25 mm (23)
Adult survival		
By individual	42/51 individuals	39/50 individuals
By replicate	28/28 tanks	26/27 tanks

Notes: Time, mass, and length are displayed as mean $\pm$ SE. Numbers in parentheses represent the number of tanks. Survival by replicate is displayed as the number of tanks for which at least one individual survived, out of the total number of tanks. The tadpoles that were necropsied ($n=10$ per treatment), and therefore did not complete the experiment, were removed from the survival analyses.

We determined the effect of tadpole diet on bacterial community membership (unweighted UniFrac distances) and structure (weighted) using PERMANOVA+ (2008, version 1.0.1; with 999 permutations) in PRIMER (2008, version 6.1.11). Bonferroni post-hoc multiple comparison tests were used to compare bacterial communities among treatment levels. For adults, tank of origin was included as a random effect. We used Principal Coordinate Analyses (PCoA) on unweighted UniFrac distances to visualize similarities of bacterial community membership across water treatments. Unweighted scores represent bacterial community membership, which is based on the presence or absence of bacterial taxa, whereas weighted scores represent bacterial community structure, which also takes into account relative abundance of bacterial taxa.

To compare relative abundances of bacterial taxa across groups, we first removed any phyla that were present in less than 25% of samples. Given that the gut bacterial community is largely restructured over the course of metamorphosis (Kohl et al. 2013), we compared relative abundances of bacteria in tadpoles and adult frogs separately. Relative abundances (arcsine square root transformed; Shchipkova et al. 2010; Kumar et al. 2012) of bacterial phyla in tadpoles and adults were analyzed in JMP (2016, version 13) using one-way ANOVAs with water treatment as an independent variable and, for adults, with tank as a random effect. For all analyses, P -values were corrected using the false discovery rate correction for multiple comparisons.

Results

Effect of diet on host health

Tadpole diet treatment did not affect time to metamorphosis (GLMM, $\chi^2=0.11$, $df=1$, $P=0.74$), mass

at metamorphosis ($\chi^2=2.04$, $df=1$, $P=0.15$), tadpole survival (Coxme, $\chi^2=0.17$, $df=1$, $P=0.68$), adult mass (GLMM, $\chi^2=1.18$, $df=1$, $P=0.28$), adult SVL ($\chi^2=0.21$, $df=1$, $P=0.65$), or adult survival (Coxme, $\chi^2=0.28$, $df=1$, $P=0.60$) (Table 1).

Effect of diet on host-associated microbiota

Tadpole diet treatment did not affect bacterial phylogenetic diversity (GLM, $\chi^2=0.17$, $df=1$, $P=0.68$) or species richness ($\chi^2=0.03$, $df=1$, $P=0.85$) (Table 2). Tadpoles that were fed a conspecific-based diet tended to have bacterial communities with a higher Shannon index ($\chi^2=3.20$, $df=1$, $P=0.06$) and species evenness ($\chi^2=4.02$, $df=1$, $P=0.045$) compared with tadpoles that were fed an alga-based diet (Table 2). Tadpole diet treatment also altered bacterial community membership (PERMANOVA, $F_{1,15}=1.40$, $P=0.03$) and structure (Fig. 1; $F_{1,15}=2.71$, $P=0.058$) of tadpoles.

Tadpole diet treatment did not affect relative abundance of bacterial phyla (Supplementary Fig. S1). However, tadpoles that were fed a conspecific-based diet had higher relative abundances of genera *Microvirgula* (one-way ANOVA, $F=10.73$, $P=0.049$), *Rathayibacter* ($F=11.55$, $P=0.049$), *Ruminococcus* ($F=10.04$, $P=0.049$), and *Corynebacterium* ($F=8.93$, $P=0.05$), but lower relative abundance of *Nodularia* ($F=8.92$, $P=0.05$) compared with tadpoles that were fed an alga-based diet (Supplementary Table S1). Relative abundances of phylum Fusobacteria and genus *Cetobacterium*, which have previously been shown to relate to later-life host resistance to worm establishment (Knutie et al. 2017), were on average higher in tadpoles that were fed an alga-based diet (Fusobacteria: $13.87 \pm 6.25\%$, 9/9 individuals;

Table 2 Grand mean $\pm$ SE of bacterial diversity metrics from the guts of tadpoles and adults in response to tadpole diet treatment

Diversity metric	Tadpole		Adult	
	Algae	Conspecifics	Algae	Conspecifics
Phylogenetic diversity	85.31 $\pm$ 4.85 (9)	88.45 $\pm$ 5.94 (7)	74.40 $\pm$ 4.57 (11)	79.26 $\pm$ 4.76 (9)
Shannon index	4.63 $\pm$ 0.38 (9)	5.48 $\pm$ 0.24 (7)	5.56 $\pm$ 0.22 (11)	5.73 $\pm$ 0.18 (9)
Species richness	1437.79 $\pm$ 119.22 (9)	1469.40 $\pm$ 116.29 (7)	1511.85 $\pm$ 103.12 (11)	1626.46 $\pm$ 127.35 (9)
Species evenness	0.44 $\pm$ 0.03 (9)	0.52 $\pm$ 0.02 (7)	0.53 $\pm$ 0.02 (11)	0.54 $\pm$ 0.01 (9)

Note: Numbers in parentheses represent the number of tanks.

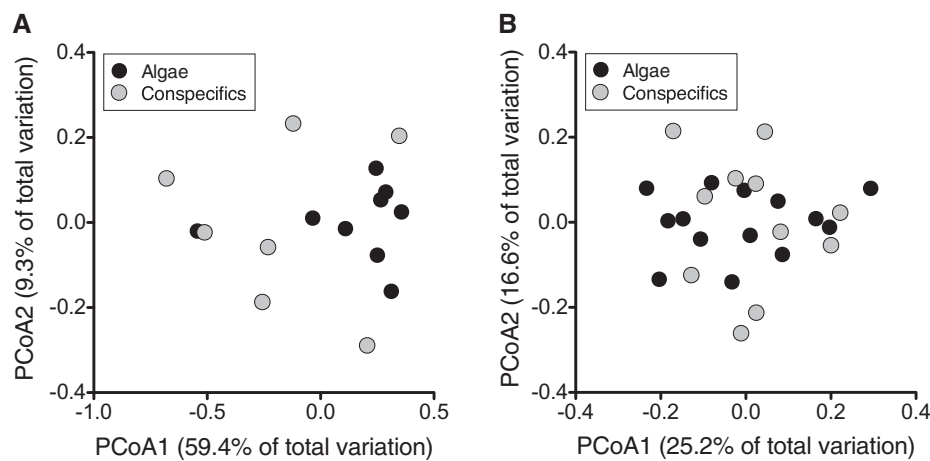


Fig. 1 PCoA of gut bacterial community structure from tadpoles (A) and adults (B) that were fed either an alga- or conspecific-based diet as tadpoles. Tadpole diet treatment affected the bacterial community structure of tadpoles but not adults. Community structure uses weighted Unifrac distances, which considers relative abundances of bacterial lineages.

Cetobacterium: 1.40 $\pm$ 1.28%, 9/9 individuals) compared with tadpoles that were fed a conspecific-based diet (*Fusobacteria*: 5.59 $\pm$ 2.14%, 7/7 individuals; *Cetobacterium*: 0.09 $\pm$ 0.08%, 3/7 individuals), but these differences were non-significant (Supplementary Fig. S1; *Fusobacteria*: $F = 0.94$, $P = 0.33$, *Cetobacterium*: $F = 4.42$, $P = 0.056$).

Tadpole diet treatment did not have an enduring effect on bacterial diversity of adult frogs (Table 2; GLMM, phylogenetic diversity: $\chi^2 = 0.47$, $df = 1$, $P = 0.49$; Shannon index: $\chi^2 = 0.41$, $df = 1$, $P = 0.52$; species richness: $\chi^2 = 0.43$, $df = 1$, $P = 0.51$; species evenness: $\chi^2 = 0.37$, $df = 1$, $P = 0.54$), community membership (PERMANOVA, $F_{1,20} = 1.14$, $P = 0.15$) and structure (Fig. 1; $F_{1,20} = 1.10$, $P = 0.35$), or relative abundance of bacterial taxa (Supplementary Fig. S1; $P > 0.05$ for all phyla and genera) of adults.

Effect of diet on host resistance and tolerance of infection

Tadpole diet treatment affected host resistance to worm penetration (Fig. 2A; GLMM, $\chi^2 = 7.14$, $df = 1$, $P = 0.008$); adult frogs that were fed an alga-based diet as tadpoles were penetrated by three

more worms, on average, and were therefore less resistant than frogs that were fed a conspecific-based diet as tadpoles. However once infected, diet treatment did not affect worm establishment (Fig. 2B; $\chi^2 = 0.72$, $df = 1$, $P = 0.40$).

Antibody levels in parasitized frogs that were fed an alga-based diet as tadpoles (0.25 $\pm$ 0.08) were approximately twice as high as parasitized frogs that were fed a conspecific-based diet as tadpoles (0.10 $\pm$ 0.02) and non-parasitized frogs (Fig. 2C; alga-based diet: 0.13 $\pm$ 0.02, conspecific-based diet: 0.15 $\pm$ 0.03; interaction controlling for age: $\chi^2 = 2.96$, $df = 1$, $P = 0.08$). We controlled for adult age in this analysis because age was positively related to antibody levels ($\chi^2 = 6.89$, $df = 1$, $P = 0.009$). Additionally, there was a marginally non-significant effect of the interaction between diet treatment and gut worm infections on antibody levels among parasitized frogs (interaction: $\chi^2 = 3.09$, $df = 1$, $P = 0.08$); antibody levels were negatively related to the proportion of worms that established in the gut for frogs that were fed an alga-based diet as tadpoles, whereas frogs that were fed a conspecific-based diet as tadpoles showed no relationship between antibody

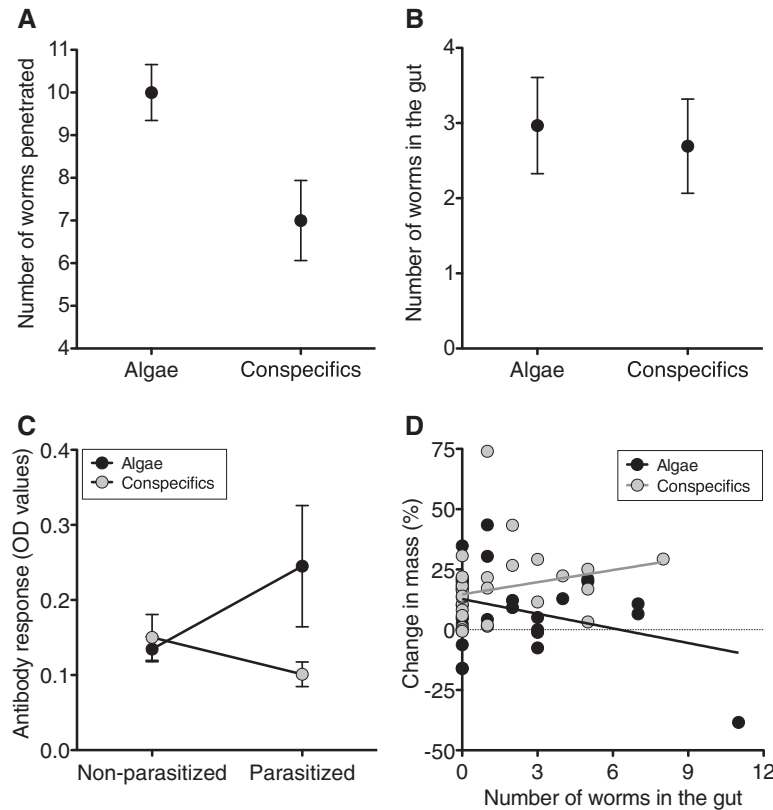


Fig. 2 Effect of early-life diet treatment on host resistance and tolerance of parasitism later in life. Adult frogs that were fed a conspecific-based diet as tadpoles were more resistant to worm penetration than adults fed an alga-based diet as tadpoles (A), however, were less resistant to worm establishment because the number of worms that established in the gut did not differ between treatments (B). Parasitized adult frogs that were fed an alga-based diet as tadpoles had a higher antibody-mediated immune response compared with non-parasitized frogs and parasitized frogs fed a conspecific-based diet (C). In contrast, frogs that were fed a conspecific-based diet as tadpoles were more tolerant to the effect of parasite load on mass compared with frogs fed an alga-based diet as tadpoles (D).

levels and worm establishment. The number of worms that penetrated the host did not affect antibody levels when controlling for diet treatment ($\chi^2 = 0.16$, $df = 1$, $P = 0.69$).

Tadpole diet treatment affected host tolerance to worm establishment (Fig. 2D; interaction: $\chi^2 = 4.61$, $df = 1$, $P = 0.03$). Frogs that were fed a conspecific-based diet as tadpoles did not lose mass in response to increasing parasite abundance, whereas the mass of frogs that were fed an alga-based diet as tadpoles decreased with increasing parasite loads.

Worm penetration and establishment were not affected by days to metamorphosis (penetration: $\chi^2 = 0.09$, $df = 1$, $P = 0.77$; establishment: $\chi^2 = 0.24$, $df = 1$, $P = 0.62$), adult mass (penetration: $\chi^2 = 0.07$, $df = 1$, $P = 0.79$; establishment: $\chi^2 = 0.41$, $df = 1$, $P = 0.52$), or adult age at infection (penetration: $\chi^2 = 0.09$, $df = 1$, $P = 0.77$; establishment: $\chi^2 = 0.24$, $df = 1$, $P = 0.62$).

Discussion

Our study demonstrated that the diet of Cuban tree frog tadpoles affected their gut microbiota and

defenses against parasitic gut worms, but these effects depended on the stage of the host and infection, respectively. Specifically, tadpole diet affected bacterial communities in the guts of tadpoles (Fig. 1A), but did not have an enduring effect on bacterial communities in adults (Fig. 1B). In contrast, tadpole diet had an enduring effect on host resistance and tolerance of infections in adults (Fig. 2). Frogs that were fed a conspecific-based diet as tadpoles were more resistant to worm penetration (Fig. 2A) but less resistant to worm establishment compared with frogs that were fed an alga-based diet as tadpoles, which resulted in no overall effect of the diet on resistance (Fig. 2B). Reduced resistance to worm establishment in frogs fed a conspecific-based diet as tadpoles may have been related to their suppressed antibody-mediated immune response during this stage of infection compared with frogs that were fed an alga-based diet as tadpoles (Fig. 2C). In contrast, frogs that were fed an alga-based diet as tadpoles were less tolerant to the effects of parasite abundance during worm establishment, which may

have been related to the cost of producing an antibody response (Fig. 2D). Our results suggest that the diet of Cuban tree frog tadpoles has enduring effects on infection risk, which could be related, in part, to shifts in the early-life microbiota because a disruption of host-associated microbiota can affect immune system development (reviewed in Round and Mazmanian 2009; Hooper et al. 2012) and later-life resistance to infections (Knutie et al. 2017).

The diet of Cuban tree frog tadpoles affected their gut bacterial communities (Fig. 1A). Tadpoles that were fed a conspecific-based diet had higher species evenness compared with tadpoles fed an alga-based diet (Table 2), suggesting that diet selected for the relative abundance of certain taxa (Supplementary Table S1). Other studies have found an effect of diet on host-associated microbiota (Kau et al. 2011; Gordon et al. 2012; David et al. 2014). David et al. (2014) found that humans who consume almost an exclusively animal-based diet had high abundances of bile-tolerant bacteria (genera *Alistipes*, *Bilophila*, and *Bacteroides*) and low abundances of bacteria that metabolize dietary plant polysaccharides (genera *Roseburia*, *Eubacterium*, and *Ruminococcus*) when compared with humans who consume a plant-based diet. In contrast, tadpoles that were fed a conspecific-based diet in our experiment had a higher relative abundance of *Ruminococcus* compared with tadpoles fed an alga-based diet, which could have been because the conspecific-based diet contained more fiber than the alga-based diet (~15% in conspecifics vs. 0% in algae). Bacteria from the genus *Ruminococcus* can increase butyrate levels in the gut of hosts, which can increase the digestibility of resistant starches (i.e., complex carbohydrates found in high fiber foods) (Tremaroli and Bäckhed 2012; Ze et al. 2012) and also increase the transcription of genes related to the inflammatory response (reviewed in Gourbeyre et al. 2011; Rosenbaum et al. 2015). The potential immune-related function of *Ruminococcus* sp. in other systems could possibly explain the differential resistance to worm penetration observed between treatments, but this idea requires further investigation.

In contrast, we found that the diet of tadpoles did not have an enduring effect on gut bacterial communities of adult Cuban tree frogs. We found that bacterial communities across diet treatments homogenized after metamorphosis (Fig. 1B, Table 2, Supplementary Fig. S1), which was likely the result of either the community reconstructing during frog metamorphosis (Kohl et al. 2013; Vences et al. 2016) or the adult diet consisting of non-sterile crickets reducing the treatment-induced variation in the

microbiota that existed in tadpoles. However, wild adult Cuban tree frogs have a variable animal-based diet, which can include con- and heterospecifics (Glorioso et al. 2010), and thus variation in the diet of adults could affect their gut microbiota.

The diet of Cuban tree frog tadpoles affected later-life host resistance to parasitic gut worms, which depended on the stage of infection. A conspecific-based diet favored adult resistance at the worm penetration stage, whereas an alga-based diet favored resistance at the worm establishment stage, which reduced the net effect of resistance on worm survival to zero. Such differences in resistance might be related to the effect of nutrient content on the development of the immune response (Kelly and Coutts 2000; Venesky et al. 2012). That is, the higher lipid content of the conspecific-based diet versus the higher protein content of the alga-based diet might have affected the development of the inflammatory response of the skin and antibody response in the gut, respectively (Demas 2004; Lee et al. 2006). For example, studies suggest that a high lipid diet can increase leptin hormone production, which in turn, can increase cytokine levels to promote the inflammatory response (reviewed in Kau et al. 2011). We did not quantify the inflammatory response in adult frogs but we did quantify the IgY antibody response during worm establishment in the gut. Adult frogs that were fed an alga-based diet (higher protein) as tadpoles produced a higher antibody response to parasitism than parasitized adults fed a conspecific-based diet (lower protein) as tadpoles. Similarly, Datta et al. (1998) found that hosts fed a high protein diet at the time of infection had a higher antibody response to parasitic gut worms compared with hosts on a low protein diet. These results suggest that protein may affect the priming and/or production of the antibody response. Our study found that frogs on an alga-based diet as tadpoles did not produce more baseline antibodies as adults compared with frogs on a conspecific-based diet as tadpoles because antibody levels did not differ between diet treatments in non-parasitized frogs. Instead, the protein-rich, alga-based diet in tadpoles may have favored the development of either: (1) hematopoietic stem cells from the bone marrow where B cells (the Ig antibody precursor) form; or (2) immune organs, such as the spleen, where activation and class switching of B cells to Ig antibodies occur. We did not measure B cell concentration in tadpoles or adults but this hypothesis should be tested in the future.

Observed differences in resistance at the worm establishment stage might also be, in part, related to the early-life microbiota because changes in the

host-associated microbiota can influence the development of the immune system (reviewed in Round and Mazmanian 2009; Hooper et al. 2012), which in turn, may affect infection risk. Knutie et al. (2017) found that an early-life disruption in the microbiota of Cuban tree frog tadpoles can decrease later-life resistance to worm establishment in the gut; the relative abundance of phylum Fusobacteria and more specifically genus *Cetobacterium* in tadpoles negatively predicted worm establishment in adults. In our study, we found that the relative abundance of *Cetobacterium* was higher in tadpoles that were fed an alga-based diet than tadpoles fed a conspecific-based diet, which could, in part, be related to higher later-life resistance to worm establishment in adults.

Adult Cuban tree frogs that were fed a conspecific-based diet as tadpoles were more tolerant to the effects of parasitism compared with adults reared on an alga-based diet. This result was surprising given that frogs were fed the same *ad libitum* diet as adults and a high resource diet favors tolerance to parasitism in the Cuban tree frog-*Aplectana* system (Knutie et al. 2017), as well as other systems (Vale et al. 2011; Sternberg et al. 2012; Howick and Lazzaro 2014; Knutie et al. 2016). However, the difference in tolerance between treatments might be related to the cost of producing an immune response. Adult frogs that were fed an alga-based diet as tadpoles produced a higher antibody response in response to parasitism compared with adults fed a conspecific-based diet, which helped hosts resist worm establishment. Thus, frogs that were fed an alga-based diet as tadpoles may lose mass in response to parasitism due to the cost of producing antibodies (Sheldon and Verhulst 1996; Svensson et al. 1998; Lochmiller and Deerenberg 2000; Demas 2004) or collateral damage related to the immune response (Sears et al. 2011; Cornet et al. 2014).

Our study contributes to the growing body of evidence that environmental factors, such as diet, can affect host-associated microbiota and host-parasite dynamics. Other factors, such as pollutants (Koch and Schmid-Hempel 2011; Shehata et al. 2013; Kohl et al. 2015) and climate (Kohl and Yahn 2016), can disrupt the microbiota of hosts, and as a consequence, could impact infectious disease risk (Rohr et al. 2013; Alberdi et al. 2016). Our study suggests that identifying the environmental factors that affect early-life factors of the host could help predict host-parasite interactions later in life.

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Supplementary data

Supplementary data available at *ICB* online.

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