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Diet- and salinity-induced modifications of the gut microbiota are associated with differential physiological responses to ranavirus infection in *Rana sylvatica*

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Greater knowledge of how host–microbiome interactions vary with anthropogenic environmental change and influence pathogenic infections is needed to better understand stress-mediated disease outcomes. We investigated how increasing salinization in freshwaters (e.g. due to road de-icing salt runoff) and associated increases in growth of nutritional algae influenced gut bacterial assembly, host physiology and responses to ranavirus exposure in larval wood frogs (*Rana sylvatica*). Elevating salinity and supplementing a basic larval diet with algae increased larval growth and also increased ranavirus loads. However, larvae given algae did not exhibit elevated kidney corticosterone levels, accelerated development or weight loss post-infection, whereas larvae fed a basic diet did. Thus, algal supplementation reversed a potentially maladaptive stress response to infection observed in prior studies in this system. Algae supplementation also reduced gut bacterial diversity. Notably, we observed higher relative abundances of Firmicutes in treatments with algae—a pattern consistent with increased growth and fat deposition in mammals—that may contribute to the diminished stress responses to infection via regulation of host metabolism and endocrine function. Our study informs mechanistic hypotheses about the role of microbiome mediation of host responses to infection that can be tested in future experiments in this host–pathogen system.

This article is part of the theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

1. Introduction

Host-associated microbial communities are increasingly recognized as an important factor influencing host–pathogen interactions [1–3]. From very early in development, symbiotic microbes can influence pathogen invasion of hosts via competitive and inhibitory interactions [4,5]. As the host continues to develop, the microbiota contribute substantially to digestion and energy assimilation, and also affect development, growth and the immune system [6,7]. Extensive cross-talk between the gut microbiota and host occurs through metabolite secretion and signalling [8,9], forming a relationship that primes the immune system early on and lasts throughout an organism's life [10–13]. Bacterially produced short-chain fatty acids are integral to this communication, and their influence extends from the gut to the neuroendocrine and immune systems, where they help modulate diverse host processes such as nutrient uptake, fat storage, mucus production and inflammatory responses [14–16]. Host-associated microbial communities, therefore, have the potential to affect

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susceptibility to infectious diseases through direct interactions with pathogens, by increasing resources available for host immune function, and by regulating the neuroendocrine and immune system's responses to infection.

Diet may be one of the most important factors influencing the diversity and function of host-associated gut microbial communities [17,18]. Diets with high food quality improve nutrient acquisition by the host as well as alter the types of metabolites that can be used and produced by both microbes and the host organism [9]. For example, in mammals, diets that are high in fibre result in more diverse microbiota and increased production of short-chain fatty acids that are linked to improved pathogen defence by the host [14,19]. High-fibre diets in mammals also affect growth and fat deposition [20]. While the effects of resource abundance and quality on disease severity have been documented in a variety of taxa [21], we are only just beginning to decipher the contribution of microbial symbionts to these patterns.

Species- and population-level extinctions are occurring at a greater rate in amphibians than any other group of vertebrates [22], with infectious diseases among the important drivers of those declines [23]. Understanding microbiome–disease interactions and how the environment influences these interactions in amphibians is critical and timely. Because of the connection between the fungal disease chytridiomycosis and amphibian decline [24,25], considerable progress has been made in terms of characterizing amphibian skin microbiomes, which are among the first lines of defence against the causative agents of chytridiomycosis: *Batrachochytrium dendrobatidis* [26] and *Batrachochytrium salamandrivorans* [27]. However, interactions between the gut microbiome and disease in amphibians have received far less attention. Recent experimental work has demonstrated that manipulating the microbiota in larval amphibians alters growth and development rates, and dysregulating assembly of the gut microbiota increases susceptibility to fungal infections, viral infections and parasites later in life [7,28,29]. Less understood are the integrated mechanisms by which the microbiota interact with host physiological systems to influence infection severity. In a conservation context, improved understanding of how microbiome–host interactions vary with anthropogenic environmental change is needed to better understand stress-mediated disease outcomes [30].

2. Study system

We investigated how the environment affects infection severity and physiological host responses, and whether these outcomes were associated with altered microbiome assembly within the wood frog : ranavirus host–pathogen system. Ranaviruses (family Iridoviridae) are a leading cause of die-offs of amphibian larvae in North America [31] and post-metamorphic amphibians in Europe [32,33]. Initial ranavirus infection in tadpoles occurs through the gastrointestinal system [34], then spreads through infected immune cells to the body (e.g. liver and kidney) to ultimately cause mortality due to organ necrosis and massive haemorrhaging [35]. Ranavirus infections are particularly severe in larval wood frogs and have been associated with mass mortality events [36,37]. In the northeastern USA, ranavirus is ubiquitous in breeding ponds [38], with prevalence, disease severity and die-offs strongly associated with tadpoles undergoing metamorphosis [37]. Experiments have shown that ranavirus infection in late-stage larvae activates the hypothalmus–pituitary–interrenal (HPI) axis (measured by whole-body or kidney corticosterone concentration), reduces body weight and accelerates development rate [36,39], causing some larvae to die while others successfully metamorphose and survive [40,41]. Although it is not clear what determines an individual tadpole's fate, developmental stage and corticosterone levels at time of infection are important predictors [41,42]. Furthermore, increased salinization of freshwater systems, primarily studied within the context of road de-icing salt run-off, has been linked to higher ranavirus loads and increased probability of ranavirus-related mass mortality events of larval wood frogs [36]. In laboratory experiments, chronic exposure to elevated salinity during the larval period magnified the virus-induced corticosterone response, lowered splenocyte proliferation (an indication of suppressed immune response to the virus) and increased the probability of mortality [36]. These findings show that host responses to ranavirus infection in larval wood frogs depend on complex interactions among environmental conditions and the neuroendocrine, immune and developmental systems of the host.

Interestingly, elevated salinity also alters resources in aquatic systems, causing increased periphyton growth in mesocosm experiments [43] and increased algal growth in culture conditions [44]. Periphyton growth and salinity covary in at least some wood frog breeding ponds [45], raising questions about how pollution-induced changes in host resource availability could alter outcomes of infectious diseases. Wood frog larvae opportunistically consume diverse food items ranging from low-quality pond detritus to higher-quality non-filamentous algae [46]. On the one hand, an increase in diet quantity or quality (e.g. increased dietary algae for tadpoles) could be beneficial for wood frog larvae. Algae are high in protein and antioxidants, and algal supplements are known to enhance immune defence, growth and development rates in larval anurans [47,48], which could potentially counteract ranavirus virulence through supporting host immunity. Alternatively, infections could be more severe in animals with higher-quantity or -quality diets if pathogens are better able to exploit energetic resources and the allocation of resources to immunity in the host is weak [49]. The gut microbiota could play a central but understudied role in mediating interactions between diet, pollution and pathogens, considering that gut microbiota rapidly respond to changes in diet and environmental conditions, including salt [50]. Specifically, through the production of metabolites such as short-chain fatty acids, gut bacteria can provide energetic resources and signal biomolecules that interact with the host immune system and neuroendocrine axes to regulate growth and development [15,51] under benign and stressful conditions.

To characterize associations among realistic environmental variation, gut bacterial assembly and infection severity in an amphibian host–pathogen system, we conducted two experiments. In the first experiment, wood frog tadpoles were raised on a diet with or without an algal supplement to examine the effects of diet on growth, development rates and fat storage. In a second experiment, we used similar dietary treatments crossed with low and high salinity (based on natural variation observed in breeding ponds) and experimental ranavirus infections to assess the influence of these conditions on host physiology (growth, development and

resting interrenal corticosterone concentration), gut bacterial community assembly and ranavirus proliferation.

3. Methods

(a) Algae supplementation experiment

Our first experiment examined the effects of diet supplementation on growth, development, fat storage and timing of metamorphosis. We chose *Chlorella* algae for diet supplementation because they are easily culturable and digestible single-cell, suspended green algae [52], similar to non-filamentous algae that are common in wild larval diets [46]. We obtained wood frog eggs from Alberta, Canada within 1–2 days of fertilization and drove them to Washington State University (WSU) in ice-packed coolers. Upon arrival, eggs were evenly divided into 10-gallon tanks under ambient light cycle (13L : 11D) and 10–12°C at an indoor animal housing facility until they reached independent feeding stages (Gosner stages 25 and 26) [53]. Larvae were then brought to the Airport Garden outdoor amphibian research facility on the WSU campus and haphazardly assigned to 50-gallon mesocosms. Mesocosms were set up with tap water treated with Novaqua (Kordon, Hayward, CA, USA) water conditioner. Water conductivity, an indicator of salinity, was approximately 350–400 µS cm^{-1} in the mesocosms. All mesocosms had 1 gallon of dried maple/poplar leaves submerged in water for at least one week before tadpoles were added. We also seeded each mesocosm with approximately 20 *Daphnia magna* (Carolina Biological Supply, Burlington, NC, no. 142330) to establish a natural grazer population to facilitate stable water conditions. We added 5 g of boiled organic alfalfa pellets once a week to supplement food in all tanks, and later in the experiment, natural opportunistic algal growth occurred along surfaces. This diet has been used in prior mesocosm experiments with wood frog larvae and resulted in steady growth and development rates through to metamorphosis [40,54]. Water temperature and quality (nitrate, nitrite, ammonia and alkalinity) were monitored throughout and did not exceed safe standards.

At Gosner stages 34 and 35, we transferred a subset of these tadpoles to 10-gallon tanks (still at the outdoor facility, $n = 12$–13 tadpoles per tank), which were randomly assigned one of two diet treatments: basic ($n = 3$ tanks) and algae (basic diet plus 5 ml homogenized *Chlorella* culture supplement added twice a week; $n = 3$ tanks). No additional alfalfa was added to these tanks so we could best characterize any effects of algae supplementation. We purchased living stocks of *Chlorella* (Carolina Biological Supply no. 152069), which we cultured in 250 ml Erlenmeyer flasks under full-spectrum lights on an oscillating table at room temperature. At each tadpole feeding, we centrifuged a culture flask and resuspended the pellet with 15 ml aquarium water, briefly vortexed the mixture and then split it into 5 ml aliquots, which were emptied into each tank. After 10 days, 8 or 9 tadpoles from each tank ($n = 25$ or 26 total per diet) were haphazardly sampled (the remainder were used for another experiment) and brought into an indoor animal housing facility (maintaining light cycle and average temperatures from the field site). We weighed and staged 18 tadpoles per diet (6 per tank per treatment), placed them in individual 700 ml containers with de-chlorinated water and gave them their respective diet for up to 14 days. At that time, surviving tadpoles were euthanized, weighed and staged. We also euthanized a smaller sample of larvae per diet ($n = 6$ or 7) that were at Gosner stages 39 and 40 coming out of the outdoor tanks, from which abdominal fat bodies were dissected and weighed.

Statistical analyses were conducted in R v. 4.0.2 [55] here and throughout. To assess diet effects on body weight and development stage, we used linear mixed models (LMMs) with diet as a fixed effect and tank as a random effect nested within diet, and for body weight analyses we used developmental stage as a covariate. To compare development rates, we used a log-rank test to determine differences in time to metamorphic climax (Gosner stage 42) between diets. For the sample of larvae from which abdominal fat bodies were dissected, we compared fat wet mass using Student's *t*-test.

(b) Influence of diet and salinity on host responses to ranavirus infection

Wood frog eggs from 10 different clutches from each of three wetlands (30 clutches total) were collected from the University of Connecticut Forest within days of being fertilized. Eggs were transported to WSU via overnight courier in coolers with ice packs. Upon arrival, eggs from each clutch were evenly divided into two levels of salinity that reflected levels observed in woodland ponds (low: 200–400 µS cm^{-1}) and roadside ponds affected by de-icing salt run-off (high: 1500 µS cm^{-1}) [56]: the low-salinity condition was made from reverse-osmosis water brought up to this conductivity with Instant Ocean (Blacksburg, VA, USA) and the high salinity was made by adding road salt from Connecticut Department of Transportation, consisting mostly of NaCl, to the low-salinity water. Eggs were divided evenly in 51 containers and housed in Panasonic Health Care MIR154PA incubators under 13L : 11D light cycle and 8–16°C diurnal thermocycles. Upon reaching independent feeding stages (Gosner 25 or 26), larvae were brought to the Airport Garden outdoor amphibian research facility at WSU and haphazardly assigned to 50-gallon mesocosms.

To determine how differences in salinity and diet affect gut bacterial community assembly and responses to ranavirus infection, we designed a 2 × 2 factorial experiment in which wood frog tadpoles were reared under the two salinity conditions described above (low and high) and two diets (basic: leaf detritus + alfalfa pellets; algae: basic diet plus live *Chlorella* cultures added four weeks after introductions into mesocosms, midway through larval development). We set up two replicate mesocosms of each salinity–diet treatment combination, 16 tadpoles per mesocosm, arranged in spatial blocks to account for differences in light/temperature across the array.

(i) Mesocosm set-up

Mesocosms were set up with tap water treated with Novaqua water conditioner (plus dissolved road salt for high-salt treatments). All mesocosms had 1 gallon of dried maple/poplar leaves, which were submerged in water for at least one week before tadpoles were added. We again seeded each mesocosm with approximately 20 *D. magna* (Carolina no. 142330) and allowed natural growth of opportunistic algae. Throughout development, we added 5 g of boiled organic alfalfa pellets once a week. For the algae supplementation treatment, after four weeks in mesocosms, we added 10 ml of *Chlorella* suspended culture, which was homogenized and split evenly across all tanks (see above methods). Water temperature and quality (nitrate, nitrite, ammonia and alkalinity) were monitored throughout and did not exceed safe standards. Survival was high (0–3 mortalities) in each of the mesocosms. Tadpoles were collected from mesocosms at Gosner stage 33–38, and transferred to individual 700 ml containers (maintaining the same water and diet treatments) in an indoor animal housing facility for ranavirus exposures.

(ii) Ranavirus exposure

We followed similar methods to Hall *et al*. [36] for ranavirus exposures. Briefly, we haphazardly assigned tadpoles from each rearing treatment into two groups: ranavirus exposed (10^3 pfu FV-3 ranavirus cultured from an Adirondack, NY,

population, provided by J. Brunner) or not exposed (equal volume of virus culture medium, 10% fetal bovin serum : Hank's minimum essential medium FBS : HMEM). Tadpoles were weighed and staged, and placed in individual containers with 700 ml water at the same salinity and diets as experienced in mesocosms for 24 h. Then, tadpoles were transferred to a new container with 200 ml of aquarium water pre-mixed with ranavirus culture at the desired concentration, or an equal volume of water inoculated with culture medium, at the same salinities in which they were reared. After 24 h, all tadpoles were transferred to clean aquarium water (at same salinities), maintaining their respective diets. Tadpoles were euthanized after 6 days, a time when systemic infections had established but prior to death [36]; indeed, no mortality was observed in this experiment. Upon euthanasia, tadpoles were again weighed and staged, and then the gut, liver and kidneys were dissected. All tissues and the remaining carcass were placed in individual tubes that were snap frozen with dry ice slurry and kept at −80°C until analysis. Ranavirus titres were assayed from DNA extracted from liver tissues (Qiagen DNeasy kit, following the manufacturer's instructions) and quantified by qPCR following methods in Hall *et al*. [36]. Measuring ranavirus loads in liver indicates the severity of infection after it has spread systemically throughout the body.

(iii) Corticosterone analysis

Resting corticosterone concentrations in kidneys dissected from the ranavirus-exposed larvae were determined following methods validated in Hall *et al*. [36]. (Note, corticosterone is synthesized on demand within interrenal cells of the kidney.) Briefly, we weighed tissue and used a double diethyl ether procedure to extract lipids. Samples were dried under a nitrogen stream and resuspended in 250 µl ELISA buffer (Cayman Scientific, Ann Arbor, MI, USA). We assayed each sample in duplicate using Cayman Corticosterone ELISA kits (no. 501320) according to the manufacturer's instructions. We adjusted corticosterone amounts by the weight of the kidney tissue (reported as pg mg^{-1}). Intra-assay CV was 7.9% and interassay CV was 13.6%; serial dilutions of samples were parallel to standard curves (see electronic supplementary material, figure S1).

(iv) Statistical analyses

To determine the effects of salinity and diet on growth, body weight upon collection from mesocosms (prior to ranavirus exposure) was compared using LMMs, with diet and salinity as fixed effects, spatial block as a random factor, and Gosner stage as a covariate. To determine the effects of diet and salinity on liver ranavirus titres (in ranavirus-exposed treatments only), we conducted LMMs with diet and salinity as fixed effects, spatial block as a random effect, and body weight, Gosner stage, and resting kidney corticosterone concentration (log_{10}-transformed for normality) as covariates. To determine if salinity influenced the effects of ranavirus infection on host physiology across diets, we compared per cent body weight change, change in Gosner stage, and resting kidney corticosterone concentrations (log_{10}-transformed) using LMMs, with salinity, diet and ranavirus exposure as fixed effects, and spatial block as a random effect. We initially included interaction terms in our models but removed them if not significant. When an interaction term was significant, we conducted *post hoc* analyses to explore the nature of the interaction using the emmeans package in R.

(c) Influence of diet and salinity and ranavirus infection on gut bacterial assembly

We extracted whole-genome DNA from gut tissues using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). We followed the manufacturer's protocol, including the pretreatment for Gram-negative bacteria with 180 µl enzymatic lysis buffer at 37°C for 1 h. Prior to the start of the pretreatment, samples were mechanically ground for 5 s with a sterile pestle (Fisher Scientific, Waltham, MA). We modified the final elution volume to 100 µl. To characterize the bacterial communities in the gut, we followed Illumina's 16S metagenomic sequencing library preparation protocol, which consists of two PCR reactions. The first generated amplicons targeting the V4 region of the 16S rRNA gene using primers 515F and 806R [57] with overhang adapter sequences, and the second added Illumina sequencing adapters and dual-index barcodes to the amplicon target using Nextera XT Index primers (Illumina, San Diego, CA, USA). Amplicons were quantified using a Qubit fluorometer (Invitrogen, Waltham, MA, USA), and 4 nM of each amplicon was combined into one of two libraries consisting of 36 and 32 randomly selected samples, respectively. The libraries were sequenced using a 150 bp forward-end strategy on an Illumina iSeq 100 at Vassar College (Poughkeepsie, NY, USA). Reads were processed using the default settings in QIIME 2 [58]. DADA2 [59] was used for denoising and dereplication. Scikit-learn v1.1 was used to assign taxonomy to representative sequences. We pre-trained a naive Bayes classifier on a dataset representing 99% operational taxonomic units (OTUs) from the 515F/806R region from the SILVA 138 database. The following filtering steps were performed: all reads representing less than 0.01% of the total reads; reads not identifiable to phylum; reads assigned to chloroplast, mitochondria or Eukaryota; reads present in only a single sample. Lastly, all samples in the final dataset were rarefied to 4500 reads. Our final table contained a total of 563 unique amplicon sequence variants (ASVs) across 66 samples (range 59–287 ASVs per sample).

(i) Statistical analyses

Microbiome assembly analyses were conducted primarily using the vegan package [60]. Prior to analysis, raw sequence counts were converted to relative abundances. We tested for main and interactive effects of salinity, diet and ranavirus exposure; interaction terms were removed if not significant. To compare overall levels of ASV diversity across treatments, we used a LMM with the effective number of species [61] as the response variable, including block as a random effect. To compare variation in the kinds of ASVs present and their relative abundances (i.e. differences in community composition and structure), we used a permutational multi-variate analysis of variance (PERMANOVA) [62] on a Bray–Curtis dissimilarity index, including block as strata in the model. We also tested for differences in dispersion among treatments using multi-variate homogeneity of group dispersions (function betadisper in the vegan package). To better understand which taxa were changing in response to our experimental treatments, we first compared the relative abundance of each phylum across treatments using Kruskal–Wallis tests. We also conducted an analysis of composition of microbiomes (ANCOM) [63] to identify genera that were differentially abundant among treatments.

4. Results

(a) Algae supplementation experiment

Survival to metamorphosis of larvae in the algae-supplemented treatment was 94.4% versus 83.3% for the basic diet treatment. After accounting for the random effect of tank, and the covariance between Gosner stage and weight ($\chi^2_1 = 9.04$, $p = 0.003$), tadpoles fed a diet supplemented with algae were significantly heavier ($\chi^2_1 = 7.66$, $p = 0.006$) and were at a more advanced developmental stage after 14 days than tadpoles fed a basic diet (electronic supplementary

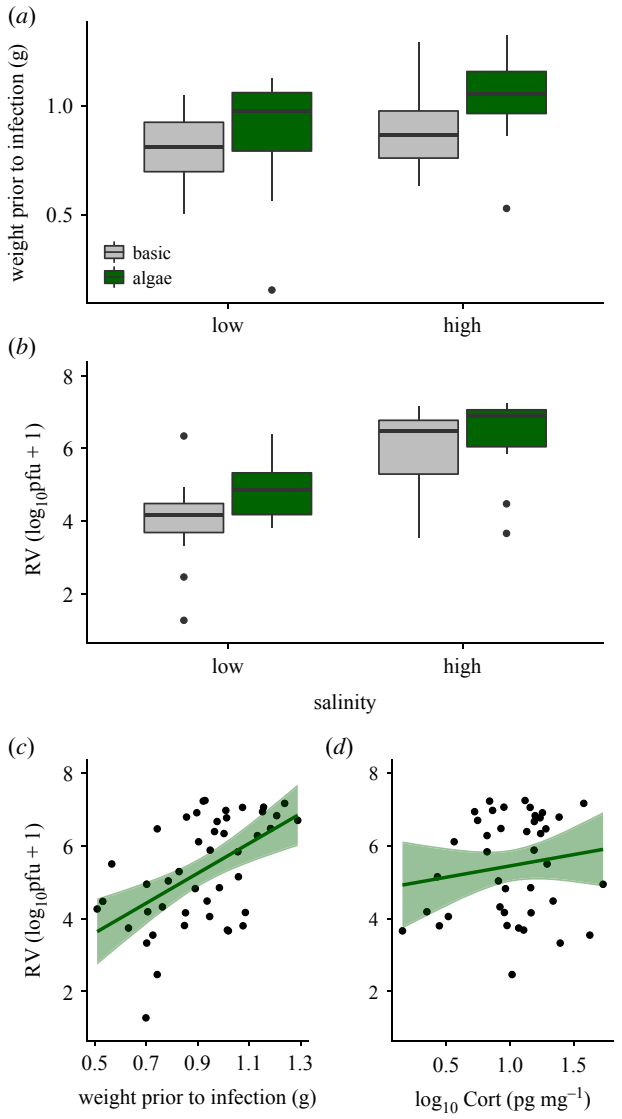

Figure 1. Effects of diet and salinity on (*a*) body weight (measured prior to infection with ranavirus) and (*b*) ranavirus titres (RV) in liver tissue (log_{10}pfu +1) at 6 days post-infection in prometamorphic wood frog larvae (Gosner stages 33–38). Medians (lines), 1–3 interquartiles (boxes), and maximum and minimum (error bars) shown per group; dots indicate outliers. Log_{10} ranavirus load (pfu +1) plotted against (*c*) weight prior to infection, and (*d*) kidney corticosterone (Cort) concentrations (both were significant covariates in models, see Results). Sample sizes were as follows: weight: $n = 25$ or 26, except low salt, algae $n = 17$; ranavirus: $n = 12$–13, except low salt, algae $n = 7$. See electronic supplementary material, table S2 for results of LMMs. (Online version in colour.)

material, figure S2*a*,*b* and table S1; $\chi^2_1 = 8.49$, $p = 0.004$). Time to metamorphic climax was significantly accelerated in the algae-supplemented group relative to larvae that were fed a basic diet (electronic supplementary material, figure S2*c*; log-rank test $\chi^2_1 = 4.16$, $p = 0.04$). Fat mass (wet) was also greater in larvae given the algae-supplemented diet (electronic supplementary material, figure S2*d*, table S3 and fig. S4; $t = 5.95$, $p = 0.0009$, $n = 6$ basic, $n = 7$ algae).

(b) Influence of diet and salinity on host response to ranavirus infection

Both algal supplementation and elevated salinity resulted in higher body weights of prometamorphic tadpoles, as measured prior to ranavirus exposure. Diet had a more pronounced effect on body weight than salinity (figure 1*a*; electronic supplementary material, table S2; diet: $\chi^2_1 = 12.83$, $p = 0.0003$; salinity: $\chi^2_1 = 5.88$, $p = 0.02$, Gosner stage: $\chi^2_1 = 48.75$, $p < 0.0001$). All tadpoles exposed to ranavirus developed infections, as indicated by detectable viral load in livers. Algal supplementation and elevated salinity additively increased liver ranavirus loads, with chronic exposure to high salinity increasing viral loads by approximately two orders of magnitude, and diet adding to a lesser degree (figure 1*b*; electronic supplementary material, figure S3 and table S2; diet: $\chi^2_1 = 5.36$, $p = 0.02$; salinity: $\chi^2_1 = 14.25$, $p = 0.0002$). Body weight prior to infection ($\chi^2_1 = 10.53$, $p = 0.001$) and resting kidney corticosterone concentrations after exposure to ranavirus ($\chi^2_1 = 12.75$, $p = 0.0004$) were significant covariates in the model and were positively related to ranavirus loads (figure 1*c*,*d*; electronic supplementary material, figure S3 and table S2). Gosner stage prior to exposure, which ranged from 33 to 38 because we restricted the range to the prometamorphic stages, did not influence subsequent ranavirus load ($\chi^2_1 = 1.60$, $p = 0.2$, electronic supplementary material, figure S3).

Ranavirus infection caused significant weight loss across all treatments, compared with unexposed larvae (ranavirus: $\chi^2_1 = 13.64$, $p = 0.0002$), but there was a significant ranavirus × diet effect ($\chi^2_1 = 4.89$, $p = 0.03$), where weight loss was primarily observed in the basic diet treatment (figure 2*a*; electronic supplementary material, table S3 and figure S4*a*). Comparing the relationship between weight change and viral load across diet treatments, we observed that weight loss increased considerably for individuals with increasing viral loads in the basic diet treatment, but not in the algae-supplemented diet (electronic supplementary material, figure S5). Weight loss was exacerbated by high-salinity conditions (salt: $\chi^2_1 = 4.8$, $p = 0.03$), with the greatest weight loss experienced in ranavirus-exposed larvae in the high-salinity–basic diet treatment group (figure 2*a*). Ranavirus infection also caused acceleration of development rates (ranavirus $\chi^2_1 = 6.36$, $p = 0.01$), and again, this effect was largely observed in the basic diet treatment (figure 2*b*; electronic supplementary material, figure S4*b*; ranavirus × diet: $\chi^2_1 = 4.36$, $p = 0.04$). Unlike weight loss, the change in development stage did not vary with ranavirus load in either diet (electronic supplementary material, figure S5). Kidney concentrations of resting corticosterone did not vary between exposure or salinity treatments, but algae supplementation significantly reduced corticosterone concentrations compared with that of larvae fed the basic diet across all treatments ($\chi^2_1 = 11.23$, $p = 0.0008$, figure 2*c*).

(c) Influence of diet and salinity and ranavirus infection on gut bacterial assembly

Algae supplementation and ranavirus exposure, but not salinity, impacted overall ASV diversity in the gut (electronic supplementary material, table S4; LMM: salinity $\chi^2_1 = 0.40$, $p = 0.52$; diet $\chi^2_1 = 9.12$, $p = 0.002$; ranavirus $\chi^2_1 = 8.68$, $p = 0.003$). Specifically, the effective number of species in the gut bacterial communities was lower in animals fed a diet supplemented with algae and in animals exposed to ranavirus (figure 3*a*). Salinity, diet and ranavirus exposure all altered bacterial community structure in the gut (electronic supplementary material, table S5 and figure S6; PERMANOVA: salinity pseudo-$F_{1,61} = 2.83$, $p = 0.001$; diet pseudo-$F_{1,61} = 3.65$, $p = 0.001$; ranavirus pseudo-$F_{1,61} = 2.04$, $p = 0.01$), with a significant interaction between diet and salinity (diet × salinity pseudo-$F_{1,61} = 1.90$, $p = 0.03$). None of these factors led to

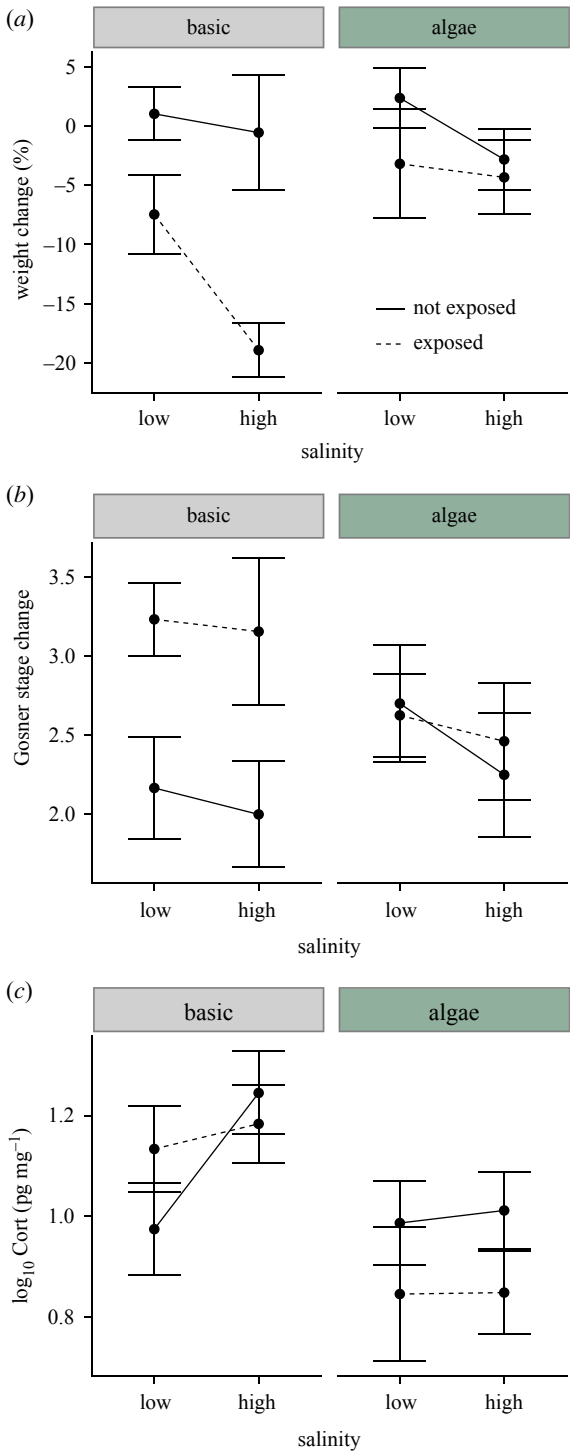

Figure 2. Mean ± s.e.m. of (*a*) percentage weight change, (*b*) Gosner stage change and (*c*) $\log_{10}$-transformed kidney corticosterone (Cort) concentrations 6 days after exposure to ranavirus (exposed) or viral culture medium (not exposed) by salinity treatment (low = 200–400 µS cm^{-1}, high = 1500 µS cm^{-1}) and diet treatment. See electronic supplementary material, table S3 for results of LMMs and electronic supplementary material, figure S4 for results of *post hoc* analyses of significant interaction terms. (Online version in colour.)

differences in dispersion (betadisper: $F_{7,57} = 0.46$, $p = 0.86$). Two phyla, Bacteroidota and Proteobacteria, exhibited the most pronounced responses to these treatments as changes in their relative abundances (figure 3*b*; Kruskal–Wallis: Bacteroidota $p = 0.003$ and Proteobacteria $p = 0.02$). Supplementing the diet with algae dramatically reduced the relative abundance of Bacteroidota—and the prevalence of this phylum as well: nearly all individuals fed a basic diet harboured Bacteroidota in the gut (32/35), but only about half of individuals fed the algae-supplemented diet did (16/28). By contrast, the combined effects of salt and a basic diet increased the relative abundance of Proteobacteria, although supplementing the diet with algae ameliorated the salt effect and suppressed proliferation of Proteobacteria (figure 3*b*).

Five genera were identified as differing in abundance across our treatments (figure 4; electronic supplementary material, table S6). Algal supplementation was associated with increases in the relative abundance of *Staphylococcus* (phylum Firmicutes), whereas a basic diet was associated with increases in *Aeromonas* and *Dysgonomonas* (phyla Proteobacteria and Bacteroidota, respectively). Increases in the relative abundance of *Shewanella* (phylum Proteobacteria) were also observed in individuals that were fed a basic diet, but only under high-salt conditions. Ranavirus exposure was associated with decreases in the relative abundance of an uncultured bacterium in the family Alcaligenaceae (phylum Proteobacteria).

5. Discussion

The factors influencing host responses to infection are complex but critical to understand in order to reduce the impacts of infectious disease in wildlife. Our study shows that exposure to salt pollution and diets of differing resources influence the severity of ranavirus infection in larval amphibians. Algal supplementation and elevated salinity were both associated with increased ranavirus proliferation in liver, indicating more severe systemic viral infections. However, responses to infection were mitigated by the resources available to the host. Larvae that were fed a basic diet accelerated development at the expense of body mass, whereas larvae provided with algae did not alter developmental rates or lose weight despite more severe infections. Our characterization of the host neuroendocrine response and gut bacterial communities offers clues as to why these environmental conditions may have led to these contrasting responses to infection (discussed below).

In our experiment, we saw an increase in ranavirus load with elevated salinity, which is expected from prior research [36], but we also showed that algal supplementation increased ranavirus infections in an additive fashion. This finding is counter to what would be expected if increased resources or particular nutrients found in algae (e.g. antioxidants) were enhancing immune function [47,48]. In the case of wood frogs, body mass was positively associated with viral loads across all treatments, and algal supplementation had a positive effect after mass was statistically accounted for in our model. These effects suggest that either mechanisms to allocate resources to immune function are weak in this species, ranaviruses have the ability to efficiently exploit resources, or both—thus, resulting in greater replication rates [49]. How shifts in gut bacteria with algae supplementation relate to this finding is not clear. Larval amphibians mount relatively weak adaptive immune responses and rely primarily on innate immunity, and ranavirus infection stimulates an inflammatory response in amphibians [64]. Gut microbiomes with high Firmicutes : Bacteriodota ratios, as shown in the algae-supplemented diet group, produce abundant short-chain fatty acids, which could be beneficial or detrimental to viral replication within the host [11,65–67]. A limitation of our study is the lack of measurement of inflammation

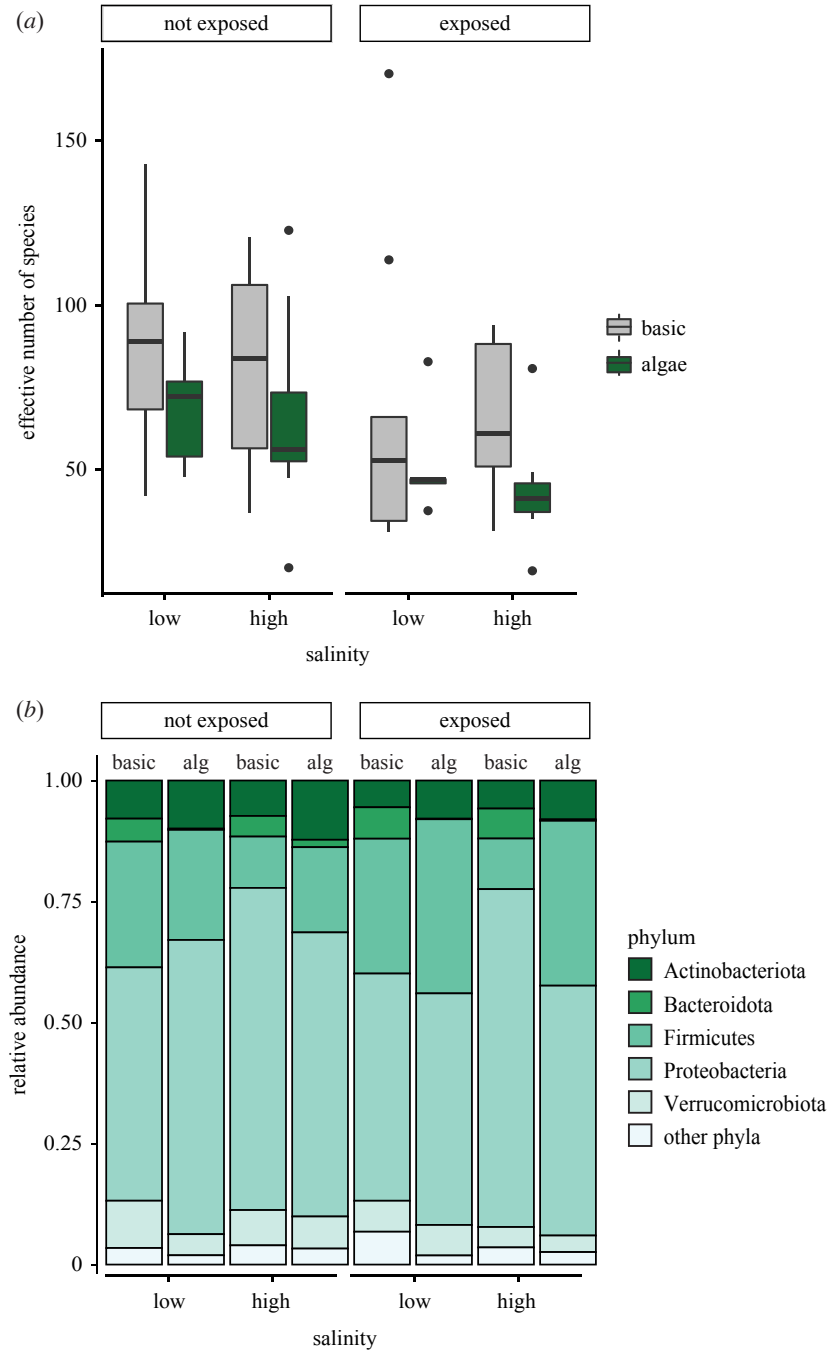

Figure 3. (*a*) Changes in the overall diversity of the gut bacterial communities, measured as the effective number of species, in an experiment testing for effects of algal supplementation and elevated salinity on responses to ranavirus exposure in wood frog tadpoles. Medians (lines), 1–3 interquartiles (boxes) and max and min (error bars) shown per group; dots indicate outliers. (*b*) Changes in the mean relative abundance of the main phyla in the gut bacterial communities across treatments. 'Basic' refers to treatments that were fed a basic diet. 'Alg' refers to treatments in which basic diets were supplemented with *Chlorella* algae. Sample sizes were as follows: basic diet, all treatments $n = 9$–10; algae diet, high-salinity treatments not and exposed to ranavirus $n = 10$ and 8, respectively; algae diet, low-salinity treatments not and exposed to ranavirus $n = 6$ and 5, respectively. See electronic supplementary material, tables S4 and S5 for results of statistical models. (Online version in colour.)

markers or immune response, which is important to many of the microbiome–host–pathogen dynamics we hypothesize.

Nonetheless, the diet-induced shifts in bacterial community structure and HPI axis activity we observed may relate to diet-specific host responses to ranavirus infection. Algae supplementation in all treatment combinations shifted gut bacterial assembly to a greater abundance of Firmicutes, and *Staphylococcus* spp. in particular, which are generally considered to be very effective at extracting energy from foods [68]. Firmicutes are thought to mediate resource allocation of hosts by increasing the production of insulin-like growth factor in the liver via increased short-chain fatty acid mobilization, which promotes growth and fat deposition in diverse animals [15,20]. Consistent with these effects of greater Firmicutes abundance, we show increased growth and fat storage with algal supplementation in wood frog larvae. In addition to the neuroendocrine growth axis, it is possible that the shift in the gut bacteria of algae-fed larvae explains the reduction in HPI axis activity we observed (inferred from lower kidney corticosterone content), since there is a growing body of work in mammals showing that short-chain fatty acids produced by gut bacteria can modulate the HPI axis at the level of the hypothalamus [69] or by strengthening negative feedback loops by activating glucocorticoid receptors in

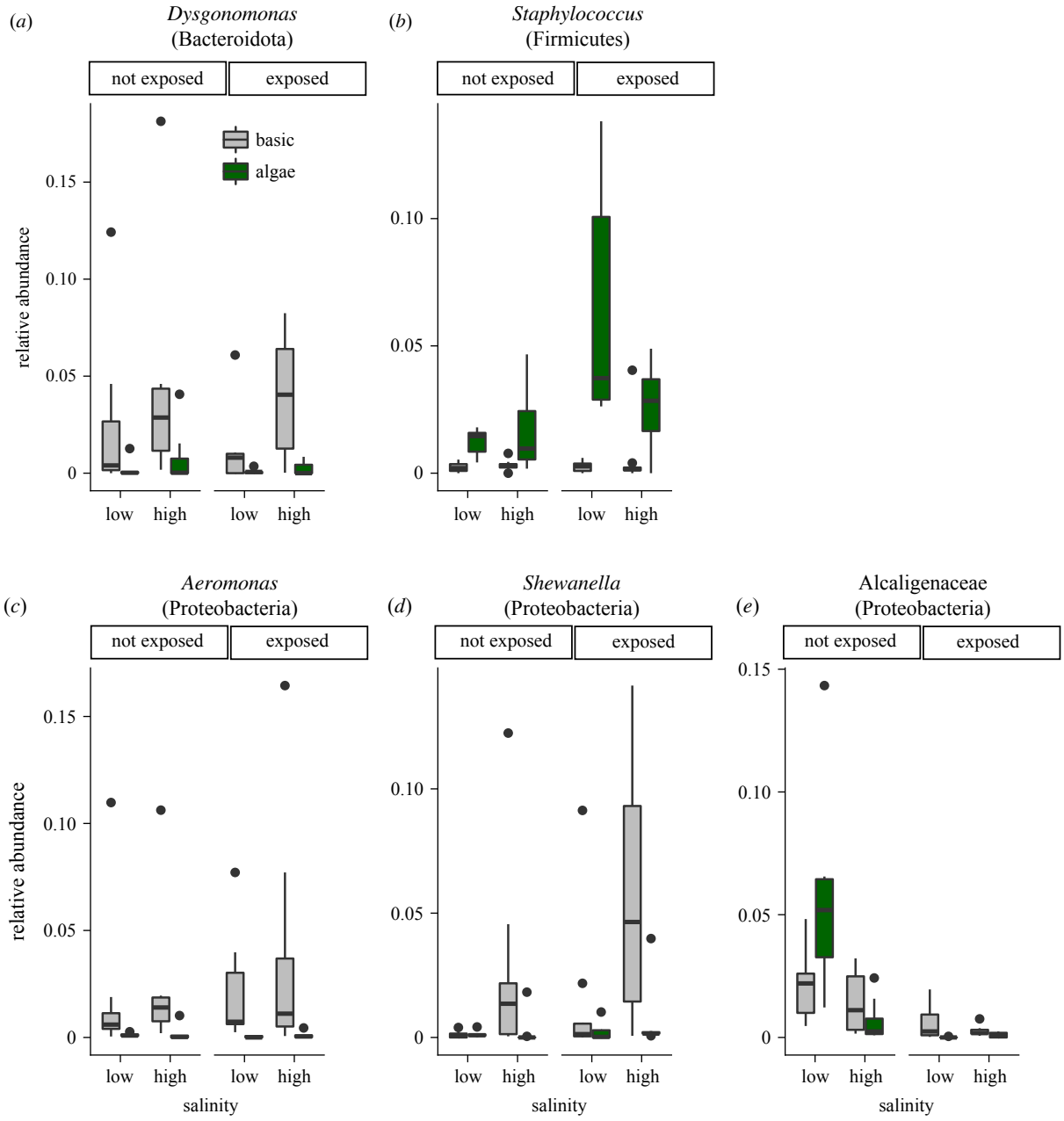

Figure 4. Changes in the relative abundance of selected genera (*a–e*) within the gut bacterial communities in an experiment testing the effects of algal supplementation and elevated salinity on responses to ranavirus exposure in wood frog tadpoles. Genera were identified by ANCOM as differentially abundant across treatments. Phylum classification for each genus is provided in parentheses. One group—Alcaligenaceae—was only identified to family level. Full taxonomic classification is available in electronic supplementary material, table S6. (Online version in colour.)

the hippocampus [70], which can alter responses to stressful conditions [13,71–73]. Regardless of microbiome interactions, the reduction in corticoserone in circulation, as suggested by kidney concentrations, is associated with increased growth rate in larval amphibians [74]. Furthermore, lower HPI axis activity of larvae provided supplemental algae may contribute to the absence of the acceleration of development and weight loss responses to ranavirus infections observed in prior experiments in wood frog larvae that were fed diets similar to our basic diet [39]. This response fits theory for complex life histories, whereby larvae in good body condition should prolong the larval period and be less sensitive to stressors (e.g. pathogen infection or pollutants) when growth conditions are optimal [54,75].

We also saw shifts in gut microbiome assembly under chronic exposure to elevated salinity, but in this case the change in composition involved the proliferation of bacteria that are often associated with disrupted metabolic function and disease. In larvae fed basic diets, gut bacteria were enriched in Proteobacteria at the cost of Firmicutes; Proteobacteria are often associated with community instability and disease states in hosts [76]. Furthermore, the presumed reduction in short-chain fatty acid availability from the loss of fermenting Firmicutes could have had downstream effects on energy production in larvae [77,78], making it more difficult to fight infections. High-salinity conditions could also lead to increased inflammation by supporting the proliferation of Gram-negative bacteria. Gram-negative bacteria have cell walls that contain lipopolysaccharides, which are antigens in vertebrate hosts that elicit local and systemic inflammatory responses in diverse taxa [79], including amphibians [80]. At the genus level, *Dysgonomonas* and *Shewanella* increased in abundance in high-salinity–basic diet conditions, but not in algae-supplemented larvae. While

this is the first report, to our knowledge, of *Dysgonomonas* and *Shewanella* in amphibians, these Gram-negative bacterial genera have been shown to promote inflammation in other hosts [81–83]. Furthermore, high-salinity diets in rodents cause degradation of tight junctions in the gut epithelium, thus allowing translocation of pathogenic microbes through the gut epithelium, resulting in systemic infections [84]. Thus, larvae fed a basic diet and exposed to elevated salinity may have experienced systemic inflammation from chronic exposure to Gram-negative bacteria, and this inflammation could have made them more susceptible to subsequent infection by ranavirus. *Aeromonas*, another group that increased in abundance in high-salinity conditions, can become pathogenic under stressful conditions in amphibians [85,86] and has been linked to mass mortality events in wood frog larvae (although the causative agent was unclear) [87]. An important future direction would be isolating specific bacterial taxa and experimentally investigating their impacts on amphibian hosts in the context of disease.

Viruses are also part of the gut microbiome, and the introduction of ranavirus infections had significant effects on bacterial community assembly. We found that the diversity of gut bacteria was diminished 6 days after ranavirus exposure across all salinity and diet treatments. This is consistent with the effects of ranavirus infections on the skin bacteria of post-metamorphic frogs [88,89]. Microbial community composition was altered by ranavirus infection in our study as well. In the wood frog : ranavirus system, the gut is the first site of invasion in the host [34], but by 6 days post-exposure, infections had translocated throughout the body, as shown by measurements of viral load in the liver. Thus, the shifts in microbial assembly we observed likely represent direct viral interactions with microbiota, as well as the host's systemic inflammatory immune responses to the virus, and reduced food intake by infected hosts. At the genus level, we saw that *Alcaligenaceae* was specifically diminished in ranavirus-infected larvae, whereas *Dysgonomonas* and *Shewanella* tended to be most abundant in larvae exposed to high salinity that were infected with ranavirus. Viruses can exploit specific bacterial surface proteins to enhance infectivity of host cells, replication and stability, or to evade immune responses to viral infections [90,91]. The increase in abundance of these latter groups could have enhanced ranavirus infectivity or replication particularly in high-salt conditions, but more research is needed to understand how viruses interact with the gut bacteria to affect viral infection success [90].

6. Conclusion

Alterations to the gut microbiota early in development due to environmental conditions, diet or disease can have long-lasting influences on host health [92,93]. This study highlights how early-life exposure to pollutants and resource availability can influence the gut microbiota, which are integrators of both host and pathogen responses to infection on multiple fronts [11,51]. Our findings give rise to numerous mechanistic hypotheses that can be tested in future experiments to ultimately understand how the gut microbiota interact with the host in response to infection, and how environmentally induced alterations in the gut microbiota can mediate changes in host susceptibility to infection. For larval wood frogs, the question of how the within-host disease dynamics we elucidated could impact the outcomes of ranavirus epidemics and post-metamorphic survival remains open [94,95]. Road de-icing salt run-off has been linked to a higher probability of ranavirus-induced larval die-offs of wood frogs [36], and our findings suggest these die-offs may be less likely in ponds with higher productivity if larvae have the resources to tolerate infection long enough to metamorphose and reach the maturation of a more competent immune system. In this case, we would predict that greater nutrition could allow more individuals to survive as ranavirus carriers, thus increasing ranavirus prevalence among adults in the population. However, ranavirus-infected larvae in high-productivity ponds may simply delay mortality until metamorphosis or soon after. Future work is needed to understand the population-level consequences of variation in these environmental conditions.

Ethics. This research was approved under Washington State University IACUC protocols 6083 and 6936, and collection permits from the Alberta Environment and Parks Policy and Planning Division Fish and Wildlife, Lower Athabasca Region (no. 18-506) and the Connecticut Department of Energy and Environmental Protection (no. 1224001).

Data accessibility. Data are available from Figshare repository: https://figshare.com/s/4c9382177d65dffc0b87.

Data also provided in the electronic supplementary material [96].

Authors' contributions. M.C.H.: data curation, formal analysis, funding acquisition, investigation, methodology, visualization, writing—original draft, writing—review and editing; R.W.: formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; A.D.: data curation, investigation, methodology, writing—review and editing; R.E.R.: data curation, investigation, methodology, writing—review and editing; G.H.C.: data curation, investigation, methodology, writing—review and editing; K.W.: data curation, investigation, methodology, writing—review and editing; D.M.S.: conceptualization, data curation, investigation, methodology, writing—review and editing; E.C.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing.

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

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

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