The Effect of Host, Habitat and Fasting Time on the Gut Microbiota of Amphibian

Qing Tong

Jiamusi University

Li-yong Cui

Jiamusi Branch of Heilongjiang Academy of Forestry Sciences

Zong-fu Hu

Northeast Agricultural University

Xiao-peng Du

Northeast Agricultural University

Hong-bin Wang (✉ hbwang1940@neau.edu.cn)

Northeast Agricultural University

Research Article

Keywords: Amphibian, Fasting, Hibernation, Microbial diversity, Captive, Microbiome

Posted Date: February 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-162992/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Wild animals entering captivity experience radical lifestyle changes resulting in microbiota alterations, in large part due to differences in diet. However, little is known about how external environmental factors influences the gut microbiota and the interaction of the environment-host-microbe interactions in host fasting. The gut microbiota in the early stage (amA and dyA groups) and late stage of hibernation in *Rana amurensis* and *R. dybowskii* of entering captivity (amL and dyL groups) and wild environments (amS and dyS groups) was determined, and the effects of host, environmental factors and fasting time on the gut microbiota were investigated via high-throughput Illumina sequencing. The Shannon index differed significantly between the amL and dyL groups and between the amA and amS groups. The PD index differed significantly between the dyL and dyS groups. Eight core OTUs were widely distributed between species, habitats and fasting times and were dominant in abundance. Captive and wild environments, host species, and fasting time significantly affected the composition and structure of the gut microbiota. Akaike information criterion (AIC)-based model results suggested that the environment and host were the variables that needed to be included in redundancy analysis (RDA) to explain the variance in taxa. The pairwise distances between the early and late stages of hibernation of were greater in *R. amurensis* and *R. dybowskii* entering captivity than in wild. The average of OTUs shared by early and late stages of hibernation of captive frogs was significantly lower than the average of wild frogs. These results can reveal the impact of environmental changes on the gut microbiota, thereby revealing the important interactions between environment-host-microbes, and helping to protect vertebrate hosts.

1 Introduction

Gut microbial diversity is important for the nutrition, physiology, and pathology of host organisms (Napflin & Schmid-Hempel 2018; Strano et al. 2018). Patterns of host and microbial associations result from evolutionary and ecological processes acting simultaneously on both host and microbe (Muletz Wolz et al. 2018). The evolutionary history of the host, environmental conditions and host-microbe interactions are important factors shaping the host-associated microbiota (Muletz Wolz et al. 2018). Compared with terrestrial amniotic vertebrates, amphibians inhabit more complex ecological environments, and their gut microbiota has greater diversity and complexity (Bletz et al. 2016). At present, the gut microbiota of mammals is a popular topic in microbial ecology (Nelson 2015), but little is known about the gut microbiota of amphibians, the intermediate clade between fishes and reptiles (Jiménez & Sommer 2016).

Among the factors that may change the gut microbiota, host genotype has a significant effect on its composition (Napflin & Schmid-Hempel 2018). Genetic factors can determine the physiological structure of the host gut, the function of the gut barrier and host immune function, which interact directly with the gut microbiota and participate in the production and stability of the gut microecological balance (Benson et al. 2010; Goodrich et al. 2016). Different host species have different physiologies and gut structures (Ley et al. 2008), resulting in variations in host-associated gut microbiota composition among host species in different environments (Bletz, Perl & Vences 2017; Perl et al. 2017). These traits can differ

among species because of underlying differences in ecological and evolutionary processes (Muletz Wolz et al. 2018). It is unknown whether host-associated traits that influence microbiomes are dissimilar among all species (Muletz Wolz et al. 2018). For instance, amphibian gut microbiota studies have found that microbiota patterns differ among species from different orders and families, but few studies have been carried out on frogs of the same genus in the same habitat and different habitats.

Genetic factors can determine host dietary preferences. Vertebrates can selectively filter specific microbial members from the external environmental microbiome to recruit them as intestinal residents at different stages of development (Nyholm & McFall-Ngai 2004; Yan et al. 2016). Bacterial communities are assembled in a deterministic manner rather than being randomly ingested from food or the environment. Previous studies focused on the host gut microbiota while the host was eating (Weng, Yang & Wang 2016; Wiebler et al. 2018). The effect of food on the intestinal microbiota is extremely significant (Smith et al. 2015; Martinez-Mota et al. 2020). Interestingly, studying different wildlife (such as *Rana amurensis* and *Rana dybowskii*) that are fasting in the same habitat may help us understand the effects of host factors on the gut microbiota. *Rana amurensis* and *R. dybowskii* use the same physiological mechanisms during hibernation, such as fasting and changes in host nutrition, the structure and function of digestive organs and immunosuppression (Tattersall & Ultsch 2008), which minimize the effects of food, host physiological state, habitat, temperature, and environmental reservoirs and provide extensive information on the host effect on the gut microbiota.

The environment is a strong predictor of amphibian gut microbiota structure (Michaels, Antwis & Preziosi 2014; Rothschild et al. 2018). Wild animals entering captivity experience radical lifestyle changes resulting in microbiome alterations (Martinez-Mota et al. 2020). The changes in the gut microbiota diversity associated with a transition to captivity have been shown to be driven by many different factors (Martinez-Mota et al. 2020). For example, some factors in captive aquatic environments, such as diet, dissolved oxygen, microbial environmental reservoirs and pH, may change the diversity of the gut microbiota in aquatic animals from that observed in the wild environment (Edwards et al. 2017). In particular, shifts in dietary regimes in captivity may drive rapid and significant modifications of gut microbiota composition due to differences in substrate availability (Muletz Wolz et al. 2018; Martinez-Mota et al. 2020). However, no studies have been performed in which the effects of diet are controlled for in exploring the effects of the captive environment on the gut microbiota.

Fasting affects the gastrointestinal (GI) tract and the gut microbiota (Sommer et al. 2016). Previous studies have shown significant differences in the gut microbiota between hibernation fasting frogs and frogs that are eating (Weng, Yang & Wang 2016; Wiebler et al. 2018). Amphibians (such as *R. amurensis* and *R. dybowskii*) living in the temperate zone experience changes in the annual cycle of feeding or fasting and hibernation, and they hibernate and fast for more than 6 months (Tattersall & Ultsch 2008). There may be large differences in the immune system in terms of intestinal nutrients, intestinal structure and host in the early and late stages of hibernation fasting, which may affect the gut microbiota. Although some studies have investigated seasonal and fasting effects on the amphibian gut microbiota

(Kohl et al. 2014), the difference between the effects of long-term fasting and short-term fasting is unclear.

In this study, the gut microbiota in the early and late stages of hibernation in two frog species in two different hibernation habitats (a natural hibernation pool and a captive hibernation pool) was investigated, and the effects of host, environmental factors and hibernation fasting time on the gut microbiota were investigated. We hypothesized that (1) Wild animals entering captivity experience lifestyle changes resulting in microbiome alterations; (2) there would be significant differences in the gut microbiota diversity between short-term fasting and long-term fasting; and (3) there were significant differences between the gut microbiota of the two species in the same habitat, i.e., the host had a significant effect on the gut microbiota.

2 Materials And Methods

2.1 Sample collection

There were two main experimental groups. The gut microbiota in the early and late stages of hibernation (amA, dyA, amS, and dyS groups) in *R. amurensis* and *R. dybowskii* in captive and wild environments (amL, dyL, amS, and dyS groups) were obtained and studied, and the effects of host, environmental factors and hibernation fasting on the gut microbiota were investigated (Table 1).

Table 1
Summary of sampling by species of frog and date

	Habitat	Early stage of hibernation	Late stage of hibernation
<i>Rana amurensis</i>	Wild	amA group: 2016 (<i>n</i> = 5)	amS group: 2017 (<i>n</i> = 7); 2018 (<i>n</i> = 7)
<i>Rana dybowskii</i>	Wild	dyA group: 2016 (<i>n</i> = 5)	dyS group: 2017 (<i>n</i> = 7); 2018 (<i>n</i> = 7)
<i>Rana amurensis</i>	Captive		amL group: 2018 (<i>n</i> = 10)
<i>Rana dybowskii</i>	Captive		dyL group: 2018 (<i>n</i> = 10)

On October 5, 2016, April 5, 2017, and April 1, 2018, 38 individuals of the two frog species were captured in Luobei County, Heilongjiang Province, China (47.6467 N, 130.3436 E, 98 m alt.). Samples from these frogs were labelled amA (5 samples), dyA (5 samples), amS (14 samples), and dyS (14 samples) (Table 1). Nineteen *R. amurensis* samples were collected on October 5, 2016 (amA01-amA05, early stage of hibernation), April 5, 2017 (amS01-amS07, late stage of hibernation), and April 1, 2018 (amS08-amS14, late stage of hibernation) (Table 1). Nineteen *R. dybowskii* samples were collected on October 5, 2016 (dyA01-dyA05, early stage of hibernation), April 5, 2017 (dyS01-amS07, late stage of hibernation), and April 1, 2018 (dyS08-dyS14, late stage of hibernation) (Table 1). The frogs were active and robust and weighed 22.32 ± 4.24 g (*R. dybowskii*) and 21.58 ± 1.93 g (*R. amurensis*), with a female:male ratio of 3:2 in the early stage and 7:7 in the late stage of hibernation. All frog samples were brought to a

laboratory at Northeast Agricultural University for immediate profiling of their intestinal microbial composition.

On October 1, 2017, wild *R. amurensis* and *R. dybowskii* were captured from their natural habitat (47.6467 N, 130.3436 E, 98 m alt.) and hibernated in captive wintering pools. On February 1 and April 1, 20 individuals of the two frog species were captured from the captive hibernating ponds, and samples from these frogs were labelled the amL group (10 samples, amL01-amL10) and the dyL group (10 samples, dyL01-dyL10) (Table 1). The female:male ratio was 1:1. The weight of *R. amurensis* was 20.96 ± 1.63 g, and that of *R. dybowskii* was 25.85 ± 3.09 g. In an aquarium, the conditions were maintained as follows: low temperature (1.22 ± 0.13 °C), pH 7.6, natural photoperiod and oxygen saturation of 90%. The density of frogs was 30/m³.

Rana amurensis and *R. dybowskii* live in similar habitats (Tong et al. 2019). In summer, both species spread into different areas to increase their food resources (Che et al. 2007; Chen & Lu 2011). However, the trophic positions of the two species are similar, and insects are the main food source in their diets (Xu et al. 2010). When the temperature drops with the onset of autumn, both species migrate to wintering ponds. Because of the low temperature, all frogs began fasting on approximately September 20. Both species have a 6-month fasting period from late October to early April. *Rana amurensis* mostly overwinters in quiet waters such as ponds, puddles, marshes, ditches and rice paddies in plains and open areas. *Rana dybowskii* mostly hibernates in freshwater ponds in forests, most of which are connected to streams and continue to flow in winter. However, both species may hibernate in each of these two habitats (She & Liu 2009).

All frog samples were brought to a laboratory at Northeast Agricultural University for immediate profiling of the intestinal microbial composition. Gut content samples were collected from brown frog intestinal contents within 20 min after euthanasia. A mixture of ether and alcohol was used to anaesthetize the frogs, after which cervical dislocation was performed (Tong et al. 2020). Then, the digestive tract was carefully isolated from the body, and lower GI tract contents were collected. To avoid cross-contamination, each sample was collected using a fresh pair of sterile tweezers. The contents of each intestine were emptied into a sterile vial and immediately stored at -80 °C.

2.2 DNA extraction and PCR amplification

DNA was extracted using an E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). DNA quantity and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and DNA quality was examined by 1% agarose gel electrophoresis. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using a PCR thermocycler (GeneAmp 9700, ABI, USA) with the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Each PCR was conducted in triplicate. Each reaction was performed in a 20 µl mixture consisting of FastPfu buffer (5×, 4 µl), dNTPs (2.5 mM, 2 µl), FastPfu polymerase (0.4 µl), template DNA (10 ng) and each primer (5 µM, 0.8 µl). The PCR products were extracted from a 2% agarose gel, further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and quantified via the QuantiFluor™-ST

system (Promega, USA) according to the manufacturer's instructions. The PCR procedure was carried out as follows: denaturation at 95 °C for 3 min; 27 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s; and a final extension at 72 °C for 10 min.

2.3 Illumina MiSeq sequencing

PCR products were purified by using a trans-PCR purification kit and quantified using the QuantiFluor™-ST system. Each sample was mixed in equimolar amounts. Then, sequence libraries were prepared using an NEBNext® Ultra™ DNA library prep kit for Illumina (New England Biolabs Inc., Ipswich, MA, USA) as per relevant instructions. Library quality was assessed spectrophotometrically, and 300-bp paired-end sequences were established on a MiSeq PE300 platform with a 600-cycle MiSeq Reagent Kit v3 (both Illumina Corporation, San Diego, CA, USA).

2.4 Ecological and statistical analysis

The paired-end sequences were merged into a single 434-bp-long sequence using FLASH (Magoč & Salzberg 2011). The low-quality sequences (i.e., ≥ 6 bp homopolymer, primer mismatch, or mean quality score < 25) were eliminated with QIIME 1.17 (Kanehisa et al. 2012), and chimaeric sequences were excluded with UCHIME (Edgar et al. 2011). Operational taxonomic units (OTUs) were then clustered with USEARCH 7.1 (<http://drive5.com/uparse/>) (Edgar et al. 2011) based on a 97% identity threshold.

Alpha diversity (phylogenetic diversity (PD), Shannon, and Sobs indices) was analysed using mothur (Hadizadeh et al. 2017). Relative abundance differences between groups of bacterial taxa were compared using the Wilcoxon rank sum test. Only differences with corrected P values < 0.05 are presented. Ordination plots were constructed with Bray-Curtis distances and unweighted UniFrac distances using the R vegan package. These matrices were further analysed in the R vegan package with nonmetric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) (Mcardle & Anderson 2001) to statistically analyse the comparisons outlined above. Furthermore, host species, habitat, and fasting time effects on the gut microbiota were evaluated using two-way PERMANOVA (Zhang & Li 2018).

A member of the core gut microbiota of the frogs was assigned if it was found in 90% of the groups and represented $> 0.1\%$ of the reads. Differential microbial taxa between the two frog species in two different hibernating habitats and fasting times were identified using the linear discriminant analysis (LDA) effect size (LEfSe) technique (Zhong, Yan & Shangguan 2015), which considers both statistical significance and biological relevance. Differences between populations were analysed using one-way analysis of variance (ANOVA) at a significance level of $P < 0.05$.

The Venn analysis method performs pairwise analysis on the shared OTUs of each sample in the early hibernation (amA and dyA) and each sample in the late hibernation (amL, dyL, amS and dyS groups). The chi-square test was used to assess differences in the proportions of shared OTUs and total OTUs of the groups. $P < 0.05$ was considered as a significant difference. As a measure of similarities between the gut and skin bacterial communities over time, the pair-wise distance of Bray-Curtis dissimilarities and

weighted UniFrac distances of the gut microbiota between the early and late stages of hibernation of captive and wild were compared via the Wilcoxon rank-sum test. Only differences with p values of < 0.05 are presented.

Since the longest gradient of all axes determined by detrended correspondence analysis (DCA) was < 4 , redundancy analysis (RDA) was used to link the changes in the microbial community with environmental variables (Janczyk et al. 2010). All variables were standardized by the “scale” function before analysis. Since there may be multicollinearity among the three impact factors, the variance inflation factor ($VIF < 3$) for each variable was estimated using the R package. Akaike’s information criterion (AIC) was applied to select useful impact factors in an AIC-based model for vector fitting. Permutation tests with marginal effects and 1,000 permutations were applied to estimate the significance of the AIC-based model and the VIF-based model. The smaller the AIC value was, the better the model fit. All of the analyses were performed using R software (version 3.5.3), and the vegan package was used for RDA and nonparametric multivariate ANOVA.

3 Results

3.1 Overview of alpha diversity and the core microbiota

Rana amurensis and *R. dybowskii* were sampled for sequencing, resulting in 2,496,447 high-quality sequences with a mean of 43,042 sequences per sample. A total of 2,607 OTUs were obtained with an average length of 442.74 bp per read. The average number of OTUs per sample was 325.78, with a range of 72 (sample dyL02) to 1,140 (sample dyS02). Rarefaction analysis showed that the sequenced samples mostly reached the plateau phase, particularly the amL and amA samples (Fig. S1).

There were no significant differences in the Sob index between groups (Wilcoxon rank sum test, $P > 0.05$, Fig. 1a). The Shannon index differed significantly between the amL and dyL groups and between the amA and amS groups (Wilcoxon rank sum test, $P < 0.05$; Fig. 1b). The PD index differed significantly between the dyL and dyS groups (Wilcoxon rank sum test, $P < 0.05$) (Fig. 1c).

With respect to the core microbiota of all frogs, there were 8 core OTUs (found in $> 90\%$ of all frogs) (Fig. 1d and 2). Twenty and 7 species-specific core OTUs were identified in *R. amurensis* and *R. dybowskii*, respectively (Figs. 2). Most notable were *Arthrobacter*, *Chryseobacterium*, *Erysipelatoclostridium*, *Hafnia-Obesumbacterium* and *Pseudomonas*, as they were widely distributed between species, habitats and fasting times and were dominant in abundance (Fig. 1d and 2).

3.2 Effects of captive and wild environments and host species on the gut microbiota

The effects of host and habitat on the gut microbiota were assessed using two frog species that hibernated in different habitats, and the results showed that the overall composition and structure of the gut microbiota were strongly associated with the effects of host species (two-way PERMANOVA: Bray-

Curtis, $P = 0.001$; unweighted UniFrac, $P = 0.001$) and habitat (two-way PERMANOVA: Bray-Curtis, $P = 0.003$; unweighted UniFrac, $P = 0.003$), yet no significant effects were detected for their interactions (two-way PERMANOVA: Bray-Curtis, $P = 0.079$; unweighted UniFrac, $P = 0.130$) (Table 2; Fig. 3a and b).

Table 2
Results of PERMANOVA examining the effects of host, habitat, and host
× habitat interaction on two beta diversity metrics (Bray-Curtis and
unweighted UniFrac distance matrices)

	Bray-Curtis			Unweighted UniFrac		
	F	R^2	P	F	R^2	P
Host	3.933	0.075	0.001	4.253	0.080	0.001
Habitat	3.055	0.058	0.003	3.388	0.064	0.003
Host × habitat	1.546	0.029	0.079	1.431	0.027	0.130

The gut microbiota differed between host species. The composition and structure of the gut microbiota of the same host (*R. amurensis* and *R. dybowskii*) differed significantly between the captive and wild environments (*R. amurensis*, Adonis: Bray-Curtis, $R^2 = 0.091$, $P = 0.022$; unweighted UniFrac, $R^2 = 0.088$, $P = 0.037$; *R. dybowskii*, Adonis: Bray-Curtis, $R^2 = 0.094$, $P = 0.014$; unweighted UniFrac, $R^2 = 0.109$, $P = 0.015$) (Fig. 3a and b; Table S1).

The gut microbiota differed between captive and wild environments. In natural habitat, the composition and structure of the gut microbiota differed significantly between the two species (Adonis: Bray-Curtis, $R^2 = 0.121$, $P = 0.001$, unweighted UniFrac, $R^2 = 0.091$, $P = 0.011$; Table S1). In captive habitat, there were significant differences in the composition and structure of the gut microbiota based on unweighted UniFrac distances (Adonis: unweighted UniFrac, $R^2 = 0.142$, $P = 0.004$), but there were no significant differences based on Bray-Curtis distances (Adonis: Bray-Curtis, $R^2 = 0.081$, $P = 0.058$) (Fig. 3a and b; Table S1).

The dominant phyla in the gut microbiota of both species in both habitats ranked by abundance (> 5%) were Bacteroidetes, Proteobacteria, Firmicutes, Actinobacteria and Spirochaetes, accounting for $96.34 \pm 0.65\%$ (amL), $96.59 \pm 0.52\%$ (amS), $92.81 \pm 0.52\%$ (dyL) and $98.06 \pm 0.67\%$ (dyS) of the total phyla, respectively (Figs. 4a and S2). The Wilcoxon rank sum test showed that host had a significant effect on Actinobacteria (amL vs. dyL, $P < 0.05$; Table S2) and Spirochaetes (amS vs. dyS, $P < 0.05$) and that habitat had a significant effect on Actinobacteria (dyL vs. dyS, $P < 0.05$; Table S2). *Pseudomonas*, *Bacteroides*, *Chryseobacterium*, *Erysipelatoclostridium* and *unclassified_f_Erysipelotrichaceae* were the most abundant genera among the amL, dyL, amS, and dyS groups (Figs. 4b and S3). Of the 745 genera, 116 (e.g., *Bacteroides*, *Chryseobacterium* and *Deefgea*) were significantly different (Kruskal-Wallis H test, $P < 0.05$; Table S3).

The LEfSe results revealed significant differences in the gut bacterial taxa, mainly in the phyla Actinobacteria (dyL group), Bacteroidetes (amS group) and Spirochaetes (amL group) (LDA > 4, $p < 0.05$; Fig. 5a). The LEfSe results at the genus level indicated that 5 of the 745 genera present in the dataset were differentially abundant in the two species between different habitats. *Flavobacterium*, *unclassified_f__Comamonadaceae* and *unclassified_f__Flavobacteriaceae* were most abundant in the dyL group, *Acinetobacter* was most abundant in the amS group, and *Deefgea* was most abundant in the dyS group (LDA > 4, $P < 0.05$; Fig. S4).

3.3 The gut microbiota of frog entering captivity environments occurred more significant changes

The gut microbiota differed with fasting time in wild environments. In *R. amurensis*, the composition and structure of the gut microbiota differed significantly between the early and late stages of hibernation (Adonis: Bray-Curtis, $R^2 = 0.179$, $P = 0.001$; unweighted UniFrac, $R^2 = 0.145$, $P = 0.014$; Fig. 3a and b). In *R. dybowskii*, the composition of the gut microbiota differed significantly between the early and late stages of hibernation according to Bray-Curtis distances (Adonis: Bray-Curtis, $R^2 = 0.101$, $P = 0.032$; Fig. 3a) but not unweighted UniFrac distances (Adonis: unweighted UniFrac, $R^2 = 0.090$, $P = 0.084$; Fig. 3b).

The gut microbiota differed with fasting time in captive environments. In *R. amurensis*, the composition and structure of the gut microbiota differed significantly between the early and late stages of hibernation (Adonis: Bray-Curtis, $R^2 = 0.198$, $P = 0.005$; unweighted UniFrac, $R^2 = 0.147$, $P = 0.022$; Fig. 3a and b). In *R. dybowskii*, the composition of the gut microbiota differed significantly between the early and late stages of hibernation (Adonis: Bray-Curtis, $R^2 = 0.273$, $P = 0.001$; unweighted UniFrac, $R^2 = 0.267$, $P = 0.016$) (Table S4; Fig. 3a and b).

The Bray-Curtis dissimilarities and unweighted UniFrac distances between the early and late stages of hibernation were greater in captive environments than in wild environments (Fig. 3a and b). Based on Bray-Curtis and unweighted UniFrac distance and comparison of the pairwise distance of the gut microbiota between the early and late stages of hibernation of captive and wild, the pairwise distances of the early and late stages of hibernation between captive and wild were significant (Wilcoxon rank-sum test, $P < 0.05$; Fig. 5a).

We used Venn analysis to pairwise analysed the shared OTUs in each sample in the early of hibernation (amA and dyA) and each sample in the late stages of hibernation (amL, dyL, amS, and dyS groups). The average of the shared OTUs in amA and amL groups was 78.64, and that of the shared OTUs in amA and amS groups was 116.90. There were significant differences between the two groups in the average of the shared OTUs (ANOVA, $F = 119.425$, $df = 1$, $P < 0.01$; Fig. 5b). The average of the shared OTUs in dyA and dyL groups was 90.16, and that of the shared OTUs in dyA and dyS groups was 115.10. There were significant differences between the two groups in the average of the shared OTUs (ANOVA, $F = 10.818$, $df = 1$, $P < 0.01$; Fig. 5b).

3.4 The best variables for predicting microbiota structure as selected by the RDA model

Constrained ordination (RDA) was used to explore the potential relationships between microbial communities and the environment, fasting time, and host species. Three impact factors with VIF values below 3 were included in the RDA model, including host (VIF: 1.001), fasting (VIF: 2.5385), and habitat (VIF: 2.386). The variation partitioning analysis (VPA) results showed that 5.98% of the variance in gut microbiota structure could be explained by the host (3.94%), fasting (1.43%), and habitat (1.33%) (Fig. 6a). However, the AIC-based model results suggested that host and habitat were the variables that needed to be included in the RDA to explain the variance in taxa (AIC: -25.778, $P < 0.05$; Fig. 6b).

4 Discussion

4.1 Captive and wild environments

In the present study, the intestinal microbiota of fasting amphibians entering captivity environments occurred more significant changes. Vertebrates may selectively filter particular microbial members from the exogenous species pool to function as gut residents. However, rearing environments may have no significant effects on the composition of the animal gut microbiota (Seedorf et al. 2014; Yan et al. 2016). Similar results were also found for the gut microbiota assembly processes in mammals, where exogenous selection always generates more Firmicutes regardless of the original inputs involved (Seedorf et al. 2014; Yan et al. 2016). The biological, physical chemistry and anthropogenic factors under captive conditions differ from those in natural habitats, which may affect animal welfare, health and the gut microbiota.

Genetic factors can determine the host's dietary preferences, and diet and feeding pattern affect the animal gut microbiome (Martinez-Mota et al. 2020). However, frogs can choose only a portion of all animals provided in the habitat as their food. Providing the same diet to different species of animals in breeding may not truly reflect the host's dietary preferences, and this may lead to errors in the experiment. The present study selected animals during the fasting period, which better reflected the effect of the host's environment (captive or natural habitat) on the gut microbiota.

In this study, the density of frogs was 30/m³, while in natural habitats, the density of frogs is much lower than that in cultured habitats (She & Liu 2009). During high-density farming, farmed animal excreta can lead to water quality deterioration (Tovar et al. 2000). For example, studies have found that the pH of cultured water decreases with an increase in culture density and time, and the amounts of ammonia, nitrite and nitrate nitrogen increase. The concentrations of ammonia, nitrite and nitrate nitrogen in high-density cultures are also higher than those in low-density cultures. When the aquatic environment changes, the intestinal microenvironment of aquatic animals changes (Rudi et al. 2018). This shows that a high culture density affects animal growth performance and immunity but also significantly changes

culture water quality, and water quality changes may also affect the intestinal microbiota of cultured aquatic animals.

Space is not a single factor, and its internal complexity (shelter or cover provision) constitutes a spatial element, such that excessive exposure can increase the rigid, tense and fearful behaviours of animals (Michaels, Antwis & Preziosi 2014). Environmental enrichment can limit fighting and cannibalism and can optimize the general health, fecundity, and welfare of captive amphibians (Chum et al. 2013). Both species studied here hibernate mostly in the soil, under rocks, and around roots at the bottom of ponds (She & Liu 2009). In this study, no hiding places were set up for the hibernating frogs, *R. dybowskii* individuals did not gather together, and both species hibernated in dimly lit areas. Both species are sensitive to exotic stimuli in the summer, and during hibernation, low temperatures make them unresponsive to outside stimuli (Vo & Gridi-Papp 2017). Therefore, due to low temperatures, the effects of space on the physiology, behaviour and intestinal flora of frogs may be limited.

Temperature directly and indirectly affects almost all life activities, such as growth, behaviour and distribution (Vo & Gridi-Papp 2017). Studies have shown that the frog intestinal microbiota varies depending on temperature (Fontaine, Novarro & Kohl 2018). Both study species hibernate under ice and can adjust their position to adjust the temperature of hibernation. However, the maximum temperature of both species for hibernation is 4 °C, and even lower temperatures are conducive to their hibernation, such as temperatures between 0 and 2 °C (She & Liu 2009). In the present study, the temperature of the captive environment was within this range (1.22 °C). However, the response of the gut microbiota to small temperature changes remains unknown.

Captive management may act as a stressor (Michaels, Antwis & Preziosi 2014); for example, the corticosterone levels of red-sided garter snakes significantly increased upon capture, while their testosterone levels decreased significantly (Moore, Lemaster & Mason 2000). In the present study, we managed the environment of the hibernating frogs, including regular water changes, oxygenation, and cleaning, which may hinder the breeding of hibernating frogs and affect the gut microbiota (Michaels, Antwis & Preziosi 2014).

4.2 Amphibian host species

In this study, we selected two frog species with a close genetic relationship that were distributed in the same region to clearly study differences in the gut microbiota of two closely related species. The effect of food on gut microbiota is extremely significant (Jiménez & Sommer 2016; Rowland et al. 2018). In this study, the two frog species were hibernating and fasting, so the effect of food on the gut microbiota was excluded. In addition, the presence of the same conditions, i.e., low temperature, similar water qualities, the same nutritional status among hosts and similar physiological mechanisms of hibernation, minimized the effects of other factors, such as food, temperature, and physiology (Tong et al. 2019). Therefore, our study isolated the effects of the endogenous intestinal environment determined by the genetic differences and immune characteristics of host species.

Our study shows that the main inhabitants of the two frog species are Proteobacteria, Bacteroidetes, and Firmicutes, and these frogs have the same core gut microbiota compositions, which indicates that the intestinal habitats of closely related species are highly similar. This is consistent with results from previous studies (Weng, Yang & Wang 2016; Wiebler et al. 2018). In addition, similarities in the gut microbiota often reflect the phylogeny of the hosts, indicating the coevolution of each host and their gut microbiota (Hale et al. 2018; Muletz Wolz et al. 2018). However, even for these hosts with similar genetic relationships, there were significant differences in their gut microbial diversity in the same hibernation habitat. The beta diversity of the gut microbiota of the two frog species indicated that their microbial communities were significantly different, and the proportion of the core microflora present in *R. dybowskii* (7/2,211) was smaller than that in *R. amurensis* (20/1,752). Thus, the host genotype has a significant effect on the composition of the gut microbiota (Napflin & Schmid-Hempel 2018). Previous studies on the gut microbiota in different mammals or in domesticated and wild animals have demonstrated this effect (Goodrich et al. 2016; Hale et al. 2018). This study shows that there is no exception for species with a close genetic relationship. Although these species live in the same habitat and are exposed to the same environmental conditions, gut microbial diversity varies from species to species (Tong et al. 2019). From the view of host biology, the host can selectively filter specific microbial members from pools of exogenous species and recruit them as intestinal residents at different developmental stages (Yan et al. 2016). The composition and structure of the guts of different hosts may be different, or the genes that interact with specific microorganisms may be different (Ley et al. 2008), resulting in "directed selection" of a specific gut microbiota by the host. Low-abundance species will be at a competitive disadvantage or even eliminated. Host selection of microbial groups can be achieved through a variety of mechanisms that, because they show different behaviours or requirements, are not necessarily "active" (Yan et al. 2016). However, when the microflora is beneficial to the host, a change in the host-selected microbiota is particularly interesting, usually because the host can coordinate the assembly of the microflora, thereby maximizing its benefits (Smith et al. 2015). From the point of view of community ecology, the composition of the gut microbiota is not a random combination, and some microbial species have relationships with some hosts, indicating that the host may be "actively" involved in the community assembly process by selecting from all available microbial species libraries (filtering) (Smith et al. 2015). In other words, members of microbial communities are recruited together rather than being randomly received from food or the environment (Napflin & Schmid-Hempel 2018). The success of specific microorganisms in colonizing the gut seems to depend on several factors, such as transmission restrictions, host filtration and competition among microorganisms (Sabino-Pinto et al. 2017).

4.3 Hibernation fasting

This study investigated the gut microbiota of two frog species at different fasting times and the effects of fasting time and host on the gut microbiota, and the results of two-way PERMANOVA showed that hibernation fasting time had a significant effect on the composition and structure of the gut microbiota. The microbiota in the late stage of hibernation had more time to become permanently established in the gut because frogs in the early and late stages of hibernation were sampled 20 and 180 days after they started to fast, respectively. Previous studies have also shown that hibernation is associated with

changes in the diversity of the gut microbiota; however, in those studies, the gut microbiotas of frogs that were hibernation fasting and frogs that were eating and not hibernating were compared (Weng, Yang & Wang 2016; Wiebler et al. 2018).

There were significant differences in the gut microbiota between the early and late stages of hibernation, which may also be related to host nutrition, the structure and function of the digestive organs and immunosuppression in addition to fasting time. Hibernating frogs must acquire adequate energy in the early stages of hibernation and live for an extended period of time at low temperatures, usually under low-dissolved oxygen conditions (Stewart, Reese & Ultsch 2004; Tattersall & Ultsch 2008). During hibernation, frogs become increasingly sensitive to low temperatures, probably because the weakening of physiological functions intensifies the risk of death (Tattersall & Ultsch 2008).

Gut microbiota changes caused by fasting may promote hibernation-induced alterations in the gut immune system, epithelial barrier function, and other host characteristics, which may alter gut microbiota composition (Carey & Assadi-Porter 2017). Brown frogs usually feed on insects and gradually transition from a feeding state to a fasting state and then to a hibernating state. Thus, their gut microbiota facilitates the gradual transition from a protein-rich food consumption state to a fasting state, and this period coincides with early hibernation. Fasting affects the GI tract (which is affected by undigested nutrients and microbial activity) and the gut microbiota (which depends strongly on the host diet for metabolic substrates) (Sommer et al. 2016). The gut microbiota during the feeding stage utilizes complex substrates (e.g., amino acids and glycoproteins) rather than simple carbohydrates (e.g., galactose, fucose, and glucose) (Dill-McFarland et al. 2014). Fasting during hibernation results in the selection of taxa in the microbiota that can decompose and use a host-derived diet and the loss of taxa that rely on complex plant polysaccharides (Sommer et al. 2016; Carey & Assadi-Porter 2017).

The gut is the first organ system to be directly affected by dietary changes and is very accommodating of variable dietary conditions (Dou et al. 2001; Cramp & Franklin 2005). During hibernation, the morphology of the animal gut markedly changes, exhibiting alterations such as decreases in mass, villus height and enterocyte turnover (Cramp & Franklin 2005; Sonoyama et al. 2009). The intestinal villi, which exhibits the most significant contact area between the animal and the environment, may alter the diversity of the gut microbiota (Purchiaroni et al. 2013).

5 Conclusions

In summary, wild frogs entering captivity experience radical lifestyle changes resulting in microbiome alterations. Eight core OTUs were widely distributed between species, habitats and fasting times and were dominant in abundance. Captive and wild environments, host species, and fasting time significantly affected the composition and structure of the gut microbiota. The host and habitat factors were the best variables for predicting microbiome composition. Studies of the gut microbiota of frogs that hibernate in two habitats can shed light on the effects of environmental changes on the gut microbiota, which may

reveal important environment-host-microbe interactions and help inform the conservation of vertebrate hosts.

Declarations

Acknowledgements

We thank Wei Zhang and Feng-lin Gao for assistance with collecting frogs. Yu Lin advised on the experimental design and statistical analysis.

Funding

This study was supported by the National Natural Science Foundation of China, grant numbers 31772807 and 31972757.

Declarations of interest

The authors declare that they have no conflicts of interest.

Author contributions

QT and LYC: data collection, data analysis and interpretation, and drafting of the article. HBW and QT: conception or design of the work. QT, LYC, and XPD: sample collection. QT, XPD and ZFH: writing and critical revision of the article. HBW: final approval of the version submitted.

Data accession numbers

The obtained raw sequences were deposited in the NCBI database (Accession numbers: PRJNA422735, PRJNA423108, PRJNA626375, PRJNA626393 and PRJNA626389).

Ethical approval

All animal protocols were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (IACUC#2015-035). All experiments were performed in accordance with the approved guidelines and regulations. All experiments involving animals followed the principles of the 3 Rs (replacement, reduction, and refinement) to prevent excessive and unnecessary sacrifice.

References

1. Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M., Oh, P.L., Nehrenberg, D., Hua, K., Kachman, S.D., Moriyama, E.N., Walter, J., Peterson, D.A. & Pomp, D. (2010) Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl. Acad. Sci. U. S. A.*, **107**, 18933–18938.

2. Bletz, M.C., Goedbloed, D.J., Sanchez, E., Reinhardt, T., Tebbe, C.C., Bhuj, S., Geffers, R., Jarek, M., Vences, M. & Steinfartz, S. (2016) Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nat. Commun.*, **7**, 13699.
3. Bletz, M.C., Perl, R.G.B. & Vences, M. (2017) Skin microbiota differs drastically between co-occurring frogs and newts. *R. Soc. Open Sci.*, **4**, 170107.
4. Carey, H.V. & Assadi-Porter, F.M. (2017) The hibernator microbiome: host-bacterial interactions in an extreme nutritional symbiosis. *Annu Rev Nutr*, **37**, 477–500.
5. Che, J., Pang, J., Zhao, E.M., Matsui, M. & Zhang, Y.P. (2007) Phylogenetic relationships of the Chinese brown frogs (genus *Rana*) inferred from partial mitochondrial 12S and 16S rRNA gene sequences. *Zoolog. Sci.*, **24**, 71–80.
6. Chen, W. & Lu, X. (2011) Age and body size of *Rana amurensis* from northeastern China. *Curr. Zool.*, **57**, 781–784.
7. Chum, H., Felt, S., Garner, J. & Green, S. (2013) Biology, behavior, and environmental enrichment for the captive African clawed frog (*Xenopus spp*). *Appl. Anim. Behav. Sci.*, **143**, 150–156.
8. Cramp, R.L. & Franklin, C.E. (2005) Arousal and re-feeding rapidly restores digestive tract morphology following aestivation in green-striped burrowing frogs. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, **142**, 451–460.
9. Dill-McFarland, K.A., Neil, K.L., Zeng, A., Sprenger, R.J., Kurtz, C.C., Suen, G. & Carey, H.V. (2014) Hibernation alters the diversity and composition of mucosa-associated bacteria while enhancing antimicrobial defence in the gut of 13-lined ground squirrels. *Microb. Ecol.*, **23**, 4658–4669.
10. Dou, Y., Gregersen, S., Zhao, J., Zhuang, F. & Gregersen, H. (2001) Effect of re-feeding after starvation on biomechanical properties in rat small intestine. *Med. Eng. Phys.*, **23**, 557–566.
11. Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, **27**, 2194–2200.
12. Edwards, C.L., Byrne, P.G., Harlow, P. & Silla, A.J. (2017) Dietary carotenoid supplementation enhances the cutaneous bacterial communities of the critically endangered southern corroboree frog (*Pseudophryne corroboree*). *Microb. Ecol.*, **73**, 435–444.
13. Fontaine, S.S., Novarro, A.J. & Kohl, K.D. (2018) Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *J. Exp. Biol.*, **221**, jeb187559.
14. Goodrich, J.K., Davenport, E.R., Waters, J.L., Clark, A.G. & Ley, R.E. (2016) Cross-species comparisons of host genetic associations with the microbiome. *Science*, **352**, 532–535.
15. Hadizadeh, F., Walter, S., Belheouane, M., Bonfiglio, F., Heinsen, F.-A., Andreasson, A., Agreus, L., Engstrand, L., Baines, J.F. & Rafter, J. (2017) Stool frequency is associated with gut microbiota composition. *Gut*, **66**, 559–560.
16. Hale, V.L., Tan, C.L., Niu, K., Yang, Y., Knight, R., Zhang, Q., Cui, D. & Amato, K.R. (2018) Diet versus phylogeny: a comparison of gut microbiota in captive colobine monkey species. *Microb. Ecol.*, **75**, 515–527.

17. Janczyk, P., Pieper, R., Smidt, H. & Souffrant, W.B. (2010) Effect of alginate and inulin on intestinal microbial ecology of weanling pigs reared under different husbandry conditions. *FEMS Microbiol. Ecol.*, **72**, 132–142.
18. Jiménez, R.R. & Sommer, S. (2016) The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.*, **26**, 763–786.
19. Kanehisa, M., Goto, S., Sato, Y., Furumichi, M. & Tanabe, M. (2012) KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.*, **40**, D109–D114.
20. Kohl, K.D., Amaya, J., Passement, C.A., Dearing, M.D. & McCue, M.D. (2014) Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. *FEMS Microbiol. Ecol.*, **90**, 883–894.
21. Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R. & Gordon, J.I. (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.*, **6**, 776–788.
22. Magoč, T. & Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**, 2957–2963.
23. Martinez-Mota, R., Kohl, K.D., Orr, T.J. & Denise Dearing, M. (2020) Natural diets promote retention of the native gut microbiota in captive rodents. *ISME J.*, **14**, 67–78.
24. Mcardle, B.H. & Anderson, M.J. (2001) Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis. *Ecology*, **82**, 290–297.
25. Michaels, C.J., Antwis, R.E. & Preziosi, R.F. (2014) Impact of plant cover on fitness and behavioural traits of captive red-eyed tree frogs (*Agalychnis callidryas*). *PloS one*, **9**, e95207.
26. Moore, I.T., Lemaster, M.P. & Mason, R.T. (2000) Behavioural and hormonal responses to capture stress in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. *Anim Behav*, **59**, 529–534.
27. Muletz Wolz, C.R., Yarwood, S.A., Campbell Grant, E.H., Fleischer, R.C. & Lips, K.R. (2018) Effects of host species and environment on the skin microbiome of *Plethodontid salamanders*. *J. Anim. Ecol.*, **87**, 341–353.
28. Napflin, K. & Schmid-Hempel, P. (2018) Host effects on microbiota community assembly. *J. Anim. Ecol.*, **87**, 331–340.
29. Nelson, K.E. (2015) An update on the status of current research on the mammalian microbiome. *ILAR J.*, **56**, 163–168.
30. Nyholm, S.V. & McFall-Ngai, M.J. (2004) The winnowing: establishing the squid-vibrio symbiosis. *Nat. Rev. Microbiol.*, **2**, 632–642.
31. Perl, R.B., Gafny, S., Malka, Y., Renan, S., Woodhams, D.C., Rollins-Smith, L., Pask, J.D., Bletz, M.C., Geffen, E. & Vences, M. (2017) Natural history and conservation of the rediscovered Hula painted frog, *Latonia nigriventer*. *Contrib. Zool.*, **86**, 11–37.
32. Purchiaroni, F., Tortora, A., Gabrielli, M., Bertucci, F., Gigante, G., Ianiro, G., Ojetti, V., Scarpellini, E. & Gasbarrini, A. (2013) The role of intestinal microbiota and the immune system. *Eur. Rev. Med. Pharmacol. Sci.*, **17**, 323–333.

33. Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P.I., Godneva, A., Kalka, I.N., Bar, N., Shilo, S., Lador, D., Vila, A.V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B.C., Avnit-Sagi, T., Lotan-Pompan, M., Weinberger, A., Halpern, Z., Carmi, S., Fu, J., Wijmenga, C., Zhernakova, A., Elinav, E. & Segal, E. (2018) Environment dominates over host genetics in shaping human gut microbiota. *Nature*, **555**, 210–215.
34. Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I. & Tuohy, K. (2018) Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.*, **57**, 1–24.
35. Rudi, K., Angell, I.L., Pope, P.B., Vik, J.O., Sandve, S.R. & Snipen, L.G. (2018) Stable core gut microbiota across the freshwater-to-saltwater transition for farmed Atlantic Salmon. *Appl Environ Microbiol*, **84**, e01974-01917.
36. Sabino-Pinto, J., Galan, P., Rodriguez, S., Bletz, M.C., Bhujju, S., Geffers, R., Jarek, M. & Vences, M. (2017) Temporal changes in cutaneous bacterial communities of terrestrial- and aquatic-phase newts (Amphibia). *Environ. Microbiol.*, **19**, 3025–3038.
37. Seedorf, H., Griffin, N.W., Ridaura, V.K., Reyes, A., Cheng, J., Rey, F.E., Smith, M.I., Simon, G.M., Scheffrahn, R.H., Woebken, D., Spormann, A.M., Van Treuren, W., Ursell, L.K., Pirrung, M., Robbins-Pianka, A., Cantarel, B.L., Lombard, V., Henrissat, B., Knight, R. & Gordon, J.I. (2014) Bacteria from diverse habitats colonize and compete in the mouse gut. *Cell*, **159**, 253–266.
38. She, K. & Liu, P. (2009) Distribution and protection status of two kinds of brown frog in heilongjiang province. *Nat. Sci. J. Harbin Norm. Univ.*, **25**, 101–103.
39. Smith, C.C., Snowberg, L.K., Gregory Caporaso, J., Knight, R. & Bolnick, D.I. (2015) Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. *ISME J.*, **9**, 2515–2526.
40. Sommer, F., Stahlman, M., Ilkayeva, O., Arnemo, J.M., Kindberg, J., Josefsson, J., Newgard, C.B., Frobert, O. & Backhed, F. (2016) The gut microbiota modulates energy metabolism in the hibernating brown bear ursus arctos. *Cell Rep.*, **14**, 1655–1661.
41. Sonoyama, K., Fujiwara, R., Takemura, N., Ogasawara, T., Watanabe, J., Ito, H. & Morita, T. (2009) Response of gut microbiota to fasting and hibernation in Syrian hamsters. *Appl Environ Microbiol*, **75**, 6451-6456.
42. Stewart, E.R., Reese, S.A. & Ultsch, G.R. (2004) The physiology of hibernation in Canadian leopard frogs (*Rana pipiens*) and bullfrogs (*Rana catesbeiana*). *Physiol. Biochem. Zool.*, **77**, 65–73.
43. Strano, C.P., Malacrino, A., Campolo, O. & Palmeri, V. (2018) Influence of host plant on thaumetopoea pityocampa gut bacterial community. *Microb. Ecol.*, **75**, 487–494.
44. Tattersall, G.J. & Ultsch, G.R. (2008) Physiological ecology of aquatic overwintering in ranid frogs. *Biol. Rev. Camb. Philos. Soc.*, **83**, 119–140.
45. Tong, Q., Du, X.P., Hu, Z.F., Cui, L.Y., Bie, J., Zhang, Q.Z., Xiao, J.H., Lin, Y. & Wang, H.B. (2019) Comparison of the gut microbiota of *Rana amurensis* and *Rana dybowskii* under natural winter fasting conditions. *FEMS Microbiol. Lett.*, **366**, fnz241.

46. Tong, Q., Hu, Z.F., Du, X.P., Bie, J. & Wang, H.B. (2020) Effects of Seasonal Hibernation on the Similarities Between the Skin Microbiota and Gut Microbiota of an Amphibian (*Rana dybowskii*). *Microbial Ecology*, **79**, 898-909.
47. Tovar, A., Moreno, C., Manuvelz, M.P. & Garciavargas, M.J.W.R. (2000) Environmental impacts of intensive aquaculture in marine waters. *Water Res.*, **34**, 334–342.
48. Vo, P. & Gridi-Papp, M. (2017) Low temperature tolerance, cold hardening and acclimation in tadpoles of the neotropical tungara frog (*Engystomops pustulosus*). *J. Therm. Biol.*, **66**, 49–55.
49. Weng, F.C., Yang, Y.J. & Wang, D. (2016) Functional analysis for gut microbes of the brown tree frog (*Polypedates megacephalus*) in artificial hibernation. *BMC Genom.*, **17**, 1024.
50. Wiebler, J.M., Kohl, K.D., Lee, R.E., Jr. & Costanzo, J.P. (2018) Urea hydrolysis by gut bacteria in a hibernating frog: evidence for urea-nitrogen recycling in Amphibia. *Proc. Biol. Sci.*, **285**, 20180241.
51. Xu, K., Zhu, D.Z., Wei, Y., Schloegel, L.M., Chen, X.F. & Wang, X.L. (2010) Broad distribution of Ranavirus in free-ranging *Rana dybowskii* in Heilongjiang, China. *EcoHealth*, **7**, 18-23.
52. Yan, Q., Li, J., Yu, Y., Wang, J., He, Z., Van Nostrand, J.D., Kempfer, M.L., Wu, L., Wang, Y., Liao, L., Li, X., Wu, S., Ni, J., Wang, C. & Zhou, J. (2016) Environmental filtering decreases with fish development for the assembly of gut microbiota. *Environ. Microbiol.*, **18**, 4739–4754.
53. Zhang, Z. & Li, D. (2018) Thermal processing of food reduces gut microbiota diversity of the host and triggers adaptation of the microbiota: evidence from two vertebrates. *Microbiome*, **6**, 99.
54. Zhong, Y., Yan, W. & Shangguan, Z. (2015) Impact of long-term N additions upon coupling between soil microbial community structure and activity, and nutrient-use efficiencies. *Soil Biol. Biochem.*, **91**, 151–159.

Figures

Figure 1

Comparison of the gut microbiota alpha diversity and the core microbiota. Comparison of Sobs (a), Shannon (b), and phylogenetic diversity (c) index values of the gut microbiota of *R. dybowskii* at different host developmental stages (*, $p < 0.05$). Venn diagram showing the core OTUs in all frog samples, *R. amurensis* samples and *R. dybowskii* samples (d).

Phylum	Genus	OUT	amA	amL	amS	dyA	dyL	dyS	P value
Proteobacteria	Pseudomonas	OTU176	12.71	7.25	6.38	6.33	11.67	8.12	0.10
Proteobacteria	Pseudomonas	OTU188	1.26	8.26	1.23	0.49	0.37	1.84	0.01
Proteobacteria	Pseudomonas	OTU669	2.74	2.34	2.56	0.62	0.34	0.75	0.01
Firmicutes	Erysipelatoclostridium	OTU1087	5.22	4.11	1.99	3.67	2.39	1.10	0.50
Proteobacteria	Hafnia-Obesumbacterium	OTU2529	1.64	1.53	1.49	1.18	0.15	6.97	0.12
Actinobacteria	Arthrobacter	OTU594	2.74	0.85	0.41	6.66	2.10	0.38	0.00
Firmicutes	g__unclassified_Erysipelotrichaceae	OTU2050	0.39	0.53	2.62	1.97	3.19	5.14	0.14
Bacteroidetes	Chryseobacterium	OTU1280	2.07	18.92	21.09	0.06	8.77	1.27	0.00
Bacteroidetes	Flavobacterium	OTU20	3.53	0.65	1.49	0.18	0.02	0.06	0.00
Proteobacteria	Ralstonia	OTU21	0.62	0.45	0.46	0.05	1.65	0.52	0.12
Cyanobacteria	norank_FamilyI	OTU1781	0.01	0.68	0.02	0.00	0.02	0.04	0.00
Firmicutes	Faecalitalea	OTU199	1.55	1.72	3.38	0.28	0.75	0.08	0.01
Bacteroidetes	Flavobacterium	OTU233	1.67	0.14	0.03	0.49	3.80	0.00	0.00
Bacteroidetes	Flavobacterium	OTU240	0.37	0.77	0.37	0.01	0.07	0.01	0.00
Actinobacteria	Arthrobacter	OTU594	2.74	0.85	0.41	6.66	2.10	0.38	0.00
Bacteroidetes	Pedobacter	OTU606	0.47	0.09	0.21	0.11	0.03	0.01	0.00
Bacteroidetes	Bacteroides	OTU838	1.19	0.53	3.07	2.20	0.30	2.18	0.03
Proteobacteria	Janthinobacterium	OTU854	0.22	0.11	0.72	0.04	0.02	0.15	0.00
Proteobacteria	g__unclassified_Comamonadaceae	OTU949	1.51	0.04	0.50	0.03	0.01	0.02	0.00
Bacteroidetes	g__unclassified_Flavobacteriaceae	OTU952	0.28	0.98	1.38	0.07	3.05	0.54	0.09
Proteobacteria	Yersinia	OTU1198	1.68	1.83	0.52	0.14	0.76	0.65	0.02
Bacteroidetes	Bacteroides	OTU2402	0.98	1.14	7.84	1.98	0.85	4.64	0.03

Figure 2

Changes in the relative abundances of core microbial OTUs associated with the hosts, habitats and fasting times. Each column represents a group. Red indicates high abundances, and green represents low abundances.

Figure 3

Microbial community shifts associated with the hosts, captive and fasting times. Nonmetric multidimensional scaling (NMDS) analysis shows patterns of separation by hosts, captive and fasting times (red indicates amA; blue, amA; amL, green; yellow, dyL; purple, dyS; and pink, amS) based on Bray-Curtis (a) and unweighted UniFrac (b) distances. NMDS was based on all OTUs. Each point represents the gut microbial community of a brown frog on a given day.

Figure 4

Comparison of differences in relative abundances of different phyla and genera between *Rana amurensis* and *R. dybowskii*, captive and wild environments, and the early and late stages of hibernation fasting. Community bar plot of bacteria at the phylum level (a) and genus level (b).

Figure 5

Similarities between the early and late stages of hibernation in captive environments and in wild environments. (a) The pair-wise distance of Bray-Curtis dissimilarities (amB or dyB) and weighted UniFrac (amW or dyW) distances of the gut microbiota between the early and late stages of hibernation of captive and wild were compared via the Wilcoxon rank-sum test ($0.01 < p \leq 0.05$ marked as *, $0.001 < p \leq 0.01$ marked as **). *Rana amurensis* is represented by am, *R. dybowskii* is represented by dy. W-W means that the frog is hibernating in the wild habitat, and W-C means that the hibernating frog in the wild is transferred to the breeding habitat to continue hibernation at the initial stage of hibernation. (b) The Venn analysis method performs pairwise analysis on the shared OTUs of each sample in the early hibernation (amA and dyA) and each sample in the late hibernation (amL, dyL, amS and dyS groups).

Figure 6

The best variables for predicting microbiota structure as selected by the RDA model. (a) Variation partitioning analysis (VPA) of the effects of fasting time, habitat, and host on microbial community structure. (b) Redundancy analysis (RDA) showing significant effects of habitat and host on gut microbiota structure.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [C7QX16D8SUPFIGURE.docx](#)
- [C7QX16D8TableS13.docx](#)