Charles Darwin University

Protection against anuran lungworm infection may be mediated by innate defenses rather than their microbiome

Weitzman, Chava L.; Brown, Gregory P.; Day, Kimberley; Shilton, Catherine M.; Gibb, Karen; Christian, Keith

Published in:
International Journal for Parasitology

DOI:
10.1016/j.ijpara.2025.01.010

Accepted/In press: 01/01/2025

Document Version
Peer reviewed version

Link to publication

Citation for published version (APA):
Weitzman, C. L., Brown, G. P., Day, K., Shilton, C. M., Gibb, K., & Christian, K. (in press). Protection against anuran lungworm infection may be mediated by innate defenses rather than their microbiome. *International Journal for Parasitology*, 1-7. https://doi.org/10.1016/j.ijpara.2025.01.010

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 23. May. 2025

ARTICLE IN PRESS

International Journal for Parasitology xxx (xxxx) xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Protection against anuran lungworm infection may be mediated by innate defenses rather than their microbiome ☆

Chava L. Weitzman [a,*], Gregory P. Brown [b], Kimberley Day [a], Catherine M. Shilton [c], Karen Gibb [a], Keith Christian [a]

[a] Research Institute for the Environment and Livelihoods, Charles Darwin University, Brinkin, NT 0909, Australia
[b] Department of Biological Sciences, Macquarie University, New South Wales 2109, Australia
[c] Berrimah Veterinary Laboratories, Northern Territory Department of Primary Industries and Fisheries, Berrimah, NT 0828, Australia

ARTICLE INFO

Article history:
Received 18 March 2024
Received in revised form 22 December 2024
Accepted 31 January 2025
Available online xxxx

Keywords:
Host–parasite–microbiome
Rhinella marina
Rhabdias pseudosphaerocephala
Parasite susceptibility
Microbiome protection
Symbiotic interactions

ABSTRACT

Host-associated microbiomes provide protection against disease in diverse systems, through both direct and indirect interactions with invaders, although these interactions are less understood in the context of non-gut helminth infections in wildlife. Here, we used a widespread, invasive host–parasite system to better understand helminth–amphibian–microbiome dynamics. We focus on cane toads and their lungworm parasites, which invade the host through the skin, to study the interactions between lungworm infection abundance and skin and gut (colon) bacterial microbiomes. Through two experiments, first reducing skin bacterial loads, and second reducing bacterial diversity, we found no evidence of protection by skin bacteria against infection. We also did not find divergent gut communities dependent on lungworm infection, signifying little to no immune modulation from infection causing changes to gut communities, at least in the first month after initial parasite exposure. In light of previous work in the system, these results underscore the contribution of toads' innate susceptibility (including possible protection provided by skin secretions) rather than skin microbes in determining the chance of infection by these macroparasites.

© 2025 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

There is growing support for the importance of bacterial communities in the success or failure of parasite and pathogen invasion in hosts (Koskella and Bergelson, 2020). Bacterial microbiomes provide protection when interacting directly with invaders, particularly on mucosal surfaces, but also through indirect interactions via bacteria-stimulated immune pathways (Thomason et al., 2017; Leung et al., 2018; Rebollar et al., 2020). There is a particular emphasis in the literature on the importance of gut bacteria in immune homeostasis, with dysbiotic gut microbiomes associated with increased susceptibility to disease (Ichinohe et al., 2011; Abt et al., 2012; Rosshart et al., 2017; Leung et al., 2018). However, bacteria on skin surfaces, which comprise part of a host's first line of defense, can also interact with hosts and parasites to impact infection outcomes (Rebollar et al., 2020). Furthermore, physiological and behavioral changes due to infection itself can provide feedback, causing changes in a host's microbiome (Leung et al., 2018; Rapin and Harris, 2018; Sencio et al., 2021).

In wildlife, most of our understanding of these interactions is in the context of microparasites (viruses, bacteria, and fungi), with less focus on interactions with a size imbalance, such as between a macroparasite (metazoan) invader and the bacterial microbiome. Again, our understanding of these interactions focuses on gut microbes, particularly in interactions with human or other mammalian hosts, most often in the context of gut helminth infections (Leung et al., 2018; Rapin and Harris, 2018), although an increased interest in non-model, non-gut infections is apparent (DeCandia et al., 2020; Lu et al., 2023). When helminths infect hosts outside of the gut, for example, in the case of urogenital schistosomiasis in human children, infection correlates with dysbiosis of gut microbes (Ajibola et al., 2019; Osakunor et al., 2020). However, there is little evidence of lungworm infection affecting gut microbes in rats, and no indication that lungworms alter skin communities of rats (Rollins, R., 2023. Impact of environment, host microbiome, and stress on *Angiostrongylus cantonensis* (rat lungworm) transmission. PhD Thesis, University of Hawai'i at Manoa, USA). Many wildlife systems are difficult to manipulate, and how

☆ Note: Supplementary data associated with this article.

* Corresponding author at: Research Institute for the Environment and Livelihoods, Charles Darwin University, Ellengowan Drive, Yellow 2, Brinkin, NT 0909, Australia.

E-mail address: chava.weitzman@cdu.edu.au (C.L. Weitzman).

https://doi.org/10.1016/j.ijpara.2025.01.010
0020-7519/© 2025 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Please cite this article as: C.L. Weitzman, G.P. Brown, K. Day et al., Protection against anuran lungworm infection may be mediated by innate defenses rather than their microbiome, International Journal for Parasitology, https://doi.org/10.1016/j.ijpara.2025.01.010

helminth infections interact with microbes in terrestrial ectotherms, i.e., amphibians and reptiles, is an overlooked branch of disease and microbial ecology.

Invasive cane toads (*Rhinella marina*) and their nematode lungworms (*Rhabdias pseudosphaerocephala*) are native to South America, but are now widespread across Australia (Mayer et al., 2021b). Larval (L3) lungworms invade toads by burrowing through the skin to reach the lungs, where they grow and mature. Eggs produced by these hermaphroditic adults migrate to the digestive tract and hatch, and L1s defecated by the toad undergo an adult sexual stage, producing L3s that infect the next toad (Baker, 1979). Infected cane toads can experience reduced growth, performance, and survival (Kelehear et al., 2009, 2011; Finnerty et al., 2018). When *R. pseudosphaerocephala* burrow into toad skin, they interact with host skin secretions alongside microbes on the skin. Unlike other amphibians, toads (Bufonidae) do not appear to produce antimicrobial peptides in their skin (Conlon, 2011), but bufadienolides and other mucosal toxins from toads' skin and parotoid glands have antimicrobial and antiparasitic properties, including against the fungal pathogen *Batrachochytrium dendrobatidis* (Banfi et al., 2016; Schmeda-Hirschmann et al., 2016; Barnhart et al., 2017; de Medeiros et al., 2019). One study revealed that skin secretions from toads in the eastern part of its invaded Australian distribution (range-core, where they first invaded) attract larval lungworms (Mayer et al., 2021a), which may be an adaptive response by the lungworms to those toads that have lower susceptibility to infection than toads farther west in the invaded range (Eyck et al., 2022). In toads from Western Australia, two studies used a similar swabbing or wiping technique of toad skin to demonstrate that reducing skin secretions and microbes increases lungworm establishment (Christian et al., 2021; Mayer et al., 2021a). However, these studies did not distinguish between protection from microbes and skin secretions. In the present study, we aimed to further describe the interactions between lungworm infection and host bacterial communities, both directly on the skin (protective effects) and indirectly with gut communities, by manipulating skin microbes without disrupting natural skin secretions.

We investigated the protective role of skin bacteria in toads' susceptibility to lungworm infection through two experiments, first by reducing skin bacterial loads, and second by reducing bacterial diversity, before exposing toads to lungworms. In the second experiment, we used amplicon sequencing of skin and gut bacterial communities to further explore direct and indirect interactions between infection and host microbiomes. With these experiments, we begin to address the hypotheses that: i) presence and diversity of symbiotic skin bacteria affect infectivity of lungworms to cane toads, and ii) through indirect immune interactions, lungworm infection alters gut bacteria in cane toads. We predicted that lungworms would have greater infection success in toads with reduced skin bacterial load and diversity. Furthermore, we predicted that toads in two rearing treatments (second experiment) would have differing skin and gut bacterial communities, assessed with amplicon sequencing, and that community diversity and composition would correlate with infection status and infection abundance. An association between infection and skin bacterial diversity early in infection may suggest protective effects of skin bacteria. In the toad gut, a correlation between infection and bacterial diversity would suggest an indirect interaction between the two, mediated by the toad immune system.

2. Materials and methods

2.1. Detecting microbiome protection against infection

2.1.1. General methods

Our first aim was to detect the protective capacity of complex skin microbial communities against larval lungworm infection of cane toads. This objective was in light of previous evidence that perturbing cane toad skin by wiping increased their susceptibility to infection (Christian et al., 2021; Mayer et al., 2021a), with our methods here focusing on manipulating the skin microbiome without impacting the toad's mucosal protection. Through two experiments, we compared susceptibility between control toads raised in a semi-natural environment with i) toads with reduced microbiome load (biomass), and ii) toads with reduced skin microbiome diversity through rearing in a comparatively clean environment. We used captive-reared toads of known parentage to reduce the influence of interfamilial variation in susceptibility to infection (Brown and Shine, 2016) and ensure experimental animals were naïve to the lungworm parasite.

Separate clutches of full-sibling toads were used in the two experiments. Toads were mated and hatched at the Macquarie University Middle Point Tropical Research Station, Northern Territory, Australia from locally sourced parental toads. Injections of artificial gonadotropins were used to induce spawning (Brannelly et al., 2019). Eggs and tadpoles were reared in 700 L tanks outdoors and fed twice weekly on commercial fish flakes (VitaPet Ltd., Australia). After emergence, metamorphs were reared in 700 L bins on a flooring of Middle Point soil. Soil was air-dried to prevent infection from lungworms in the environment. After emergence, toads were fed locally sourced termites, then weaned to dry dog food (Mars Petcare, Australia). Each toad was uniquely marked with toe-clipping. Toads were too small to sex prior to treatment assignment, although we do not expect sex to be a factor in toad lungworm infection based on previous research (Pizzatto et al., 2013). At the beginning of each experiment, toads were moved to single-housed enclosures.

Rhabdias pseudosphaerocephala lungworms used in the experiments were larval clones sourced from Middle Point toads (collected from one toad per experiment). Toads were exposed to 30 lungworm larvae suspended in water and pipetted onto the toad's dorsum. Lungworm larvae were given 12 h overnight to invade the toads, at which point toads were returned to their usual housing in a covered shed exposed to ambient temperatures and humidity. Over the following weeks, lungworms matured in the toads, during which time the toad faeces of a subset of randomly chosen individuals were monitored for *R. pseudosphaerocephala* larvae. Each experiment concluded soon after larval detection. *Rhabdias*-exposed toads were humanely euthanised with a lethal dose of Lethabarb (Virbac (Australia) Pty Ltd.) and dissected to count lungworm abundance. Experimental procedures were approved by the Animal Ethics Committees of Charles Darwin University, Australia (permits A21010 and A23016) and Macquarie University, Australia (permit 2021/001-4).

2.1.2. Experiment 1 – Reducing bacteria with antibiotics

We used an antibiotic bath to reduce bacterial loads on cane toad skin immediately before exposing toads to lungworm larvae to investigate the impact of bacterial presence on lungworm infectivity. Juvenile toads (53–78 mm snout-vent length, SVL) were

fasted for 3 days prior to antibiotic or control treatment to eliminate the chances of defecation during the treatment.

From a pilot experiment (Supplementary Data S1, Supplementary Figs. S1–S3), we determined that a 2 h bath treatment was adequate to reduce skin bacterial loads. Toads were randomly assigned to a control or antibiotic treatment (n = 20 each, similar SVL between the treatments) and placed in individual 15 × 10 × 6.5 cm containers, where 250 mL of amphibian Ringer's solution (per litre: 6.6 g of NaCl, 0.15 g of KCl, 0.15 g of $CaCl_2$, 0.2 g of $NaHCO_3$; Sigma, USA) or enrofloxacin (Baytril, Elanco Australasia Ltd., Australia) diluted to 6.7 mg/L (Wright and Whitaker, 2001) in amphibian Ringer's solution were poured over each toad based on their assigned treatment. Every 30 min, toads were showered in 60 mL of their treatment solution using a 20 mL syringe to ensure dorsal coverage. After 2 h, we rinsed the toads with 200 mL of ultra-pure water to remove excess treatment solution. Toads were then exposed to lungworms (see section 2.1.1) before returning to their long-term individual housing containers. After 7 weeks (51–52 days), toads were euthanised to quantify infection loads in lungs. We compared lungworm abundances between the two treatment groups in R v4.3.1 in RStudio v2023.06.2 (https://www.R-project.org/; https://www.rstudio.com/) with a Kruskal–Wallis test.

2.1.3. Experiment 2 – Modifying communities with rearing conditions

To further detect protective effects of healthy bacterial communities against lungworm infection, we reared cane toads in environments with differing complexities, resulting in divergent skin bacterial diversities at the time of infection. Toads were reared and maintained in two treatments to manipulate the skin bacteria without disturbing the naturally-occurring skin secretions produced by the toads. Alongside the control toads raised under semi-natural conditions (see section 2.1.1; "dirty" treatment), we reared a second, "clean," treatment of toads from a subset of toad eggs. Clean treatment toads were reared in 70 L containers indoors with water changed daily. After metamorphosis, these clean toads were kept indoors on damp paper towels. Every day, faeces was removed from the enclosures and paper towels and water were changed. This clean treatment continued throughout the experiment.

Toads entered the experiment at sizes ranging from 32–52 mm SVL, at which point 20 toads from each rearing treatment (not differing in average SVL) were exposed to *R. pseudosphaerocephala* lungworm larvae. Upon dissection to count lungworm abundance (18–23 days after exposure), we also removed and froze the colons of all *Rhabdias*-exposed toads in this experiment. We compared lungworm abundances between the two treatment groups with a Kruskal–Wallis test.

2.2. Microbiome composition and infection

An aim of this study was to determine whether infection abundance correlated with multiple microbiome features on the skin and in the gut, representing both direct and indirect interactions between microbiomes and infection. We addressed this objective with samples from Experiment 2 to assess patterns in both clean- and dirty-raised toads. We collected samples of the skin microbiome shortly after lungworm exposure, and colon samples upon euthanasia at the end of the experiment.

2.2.1. Microbiome sampling and DNA extraction

For skin microbiome characterisation, we swabbed toads 2 days after lungworm exposure to avoid impacting the toads' skin mucus prior to infection. An additional eight toads from each rearing treatment not exposed to *R. pseudosphaerocephala* were swabbed on this date (co-housed until swabbing) to determine whether day-2 samples represented the starting communities on individuals. Skin swab samples were collected as previously described (Christian et al., 2018; Weitzman et al., 2018). Briefly, toads were rinsed with 75 mL of ultra-pure water and swabbed with a flocked swab (Copan FLOQSwabs, Copan Diagnostics Inc., USA) using 30 strokes to sample microbes from the entire body surface, avoiding the face and cloaca. We preserved swabs in 300 µL of Zymo DNA/RNA Shield (Zymo Research, USA), storing swabs at −20 °C until DNA extraction. Colons removed during necropsy were stored in clean 1.5 mL microcentrifuge tubes at −20 °C.

We extracted DNA from swabs and colons (n = 16 per sample type per rearing treatment of those exposed to lungworms; n = 8 swabs per rearing treatment from unexposed toads) with the Norgen Microbiome DNA Isolation Kit (Norgen, Canada). Samples from lungworm-exposed toads were chosen for extraction based on the total lungworm count per toad, extracting samples representing toads with the full range of lungworm abundance per treatment. DNA was also extracted from two extraction controls and one environmental control (swab sample exposed to the ambient swabbing environment with no toad sample).

2.2.2. Bacterial load and amplicon sequencing

We used quantitative PCR (qPCR) to quantify the relative loads of bacteria in skin swab samples between our rearing treatments, amplifying a portion of the V4 region of the bacterial 16S rRNA gene with the Earth Microbiome Project primers, 515F/806R (Caporaso et al., 2011; Apprill et al., 2015; Parada et al., 2016). The qPCR runs included a serial dilution of toad colon DNA to calculate relative loads in the skin swab samples. We sent 200 ng of DNA per sample to the Australian Centre for Ecogenomics (University of Queensland, Australia) for Illumina MiSeq sequencing (2 × 300 bp chemistry) of amplicons produced with the Earth Microbiome Project primers (Caporaso et al., 2011; Apprill et al., 2015; Parada et al., 2016).

We used the DADA2 package (Callahan et al., 2016) to quality-filter MiSeq reads and QIIME2 v2022.8 (Bolyen et al., 2019) to further filter taxa and calculate diversity metrics. With the filterAndTrim function and otherwise default quality-filtering parameters, we removed primers, trimmed reads (260 bp forward, 180 bp reverse), and allowed maximum expected errors of 2 or 5 in forward and reverse reads, respectively. With merged reads of 250–257 bp length, we removed chimeras and assigned taxonomy to the amplicon sequence variants (ASVs) with the Silva v138.1 database (Quast et al., 2012; Yilmaz et al., 2014). We retained bacterial reads, further removing those assigned to mitochondria or chloroplast. To remove potential spurious taxa remaining in the data set, we removed ASVs that did not occur above 0.05% relative abundance in any one sample, and further removed ASVs present in only one sample (Reitmeier et al., 2021). The three negative controls (extraction and environmental controls) were included in amplicon sequencing, but resulted in fewer reads than the toad samples, with distinct communities from the other sample types. These negative control samples were excluded from further analyses.

After verification of asymptotes in rarefaction curves, signifying that subsetting the number of reads per sample results in representative data, we normalised toad samples to the lowest read depth of 13,566 reads per sample. We extracted α and β diversity metrics for analyses, specifically ASV richness and Bray–Curtis dissimilarity.

2.2.3. Microbiome statistics

Because infected toads were moved from group-housed to single-housed enclosures after lungworm exposure, we first wanted to determine if the skin swab samples represented communities at the time of lungworm exposure. Changes could be explained by either infection itself or being single-housed. To

answer this question, we compared richness (with ANOVA) and beta diversity (with permutational multivariate analysis of variance, PERMANOVA, in the vegan package (https://CRAN.R-project.org/package=vegan)) between lungworm-exposed toads and unexposed toads, which were group-housed until swabbing, assessing the effects of infection treatment, rearing treatment, and their interaction.

Next, we assessed associations between skin microbial communities on lungworm-exposed toads and lungworm abundance to detect correlations between lungworms and microbiome load and community diversity. Although we have limited capacity to predict cause and effect from our sampling scheme, because skin communities were collected after lungworm infection, we use lungworm abundance as a predictor variable in analyses. We tested the effects of rearing treatment and lungworm abundance on relative bacterial load (from qPCR, log_{10}-transformed) and ASV richness with linear models, and tested their effects on beta diversity with a PERMANOVA.

Lastly, we determined whether there were associations between colon bacterial communities and lungworm abundance, the results of which could be affected by both colon bacteria impacting lungworm susceptibility and impacts of infection on colon bacteria. As a consequence of the study design, only toads exposed to lungworms were dissected, and no colons from unexposed toads were available for inclusion in analyses. We tested the effects of rearing treatment and *R. pseudosphaerocephala* abundance on ASV richness and beta diversity with the same methods as those used on skin swab samples above.

2.3. Data accessibility

Amplicon sequence data have been submitted to the NCBI Sequence Read Archive (BioProject accession ID: PRJNA1078995).

3. Results

3.1. Lungworm abundance is not affected by the skin microbiome

After antibiotic treatment to reduce bacterial loads on toads (antibiotic effectiveness verified in a pilot study; Supplementary Fig. S2), 9/20 toads became infected, compared with 7/20 control toads. Total *R. pseudosphaerocephala* abundance did not differ between the two treatments, with control and antibiotic-treated toads housing an average (±S.D.) of 0.7 ± 1.17 and 1.7 ± 2.62 lungworms, respectively (Fig. 1A; Kruskal-Wallis χ^2 = 1.84, *P* = 0.2).

In a second experiment, we modified skin bacterial communities by rearing toads in two different environments, control semi-natural (dirty) and clean, to determine whether more complex communities provide protection against lungworm infection. Although the clean rearing treatment effectively reduced bacterial diversity on the toads (Fig. 2A), infection prevalence from this experiment was similar between the two groups, with 18/20 dirty-raised toads and 17/20 clean-raised toads becoming infected. We again found similar lungworm abundances between the two experimental groups, which had 6.7 ± 6.10 (dirty) and 6.3 ± 4.84 (clean) lungworms at the end of the experiment (Fig. 1B; Kruskal-Wallis χ^2 = 0.003, *P* = 1). In this experiment, larger toads had significantly fewer lungworms than smaller toads (Supplementary Fig. S4).

3.2. Toad microbiome composition and lungworm infection

The filtered dataset from MiSeq amplicon sequencing resulted in 1,201 ASVs in 2,555,104 total reads among toad skin swabs (*n* = 48) and colons (*n* = 32). The normalised dataset used in analyses below included 1,194 ASVs.

3.2.1. Rearing affected microbiome but not infection

Skin swab samples from lungworm-exposed toads had similar bacterial loads (by qPCR) between the two rearing treatments, regardless of infection treatment. Despite similarities in bacterial loads, toads in the two rearing groups had significantly different alpha and beta diversities (Table 1, Fig. 2A, B). Toads in the two infection treatments (lungworm-exposed and unexposed) also differed in diversity. The significant interaction between infection and rearing treatments on beta diversity likely describes the phenomenon in Fig. 2B where uninfected and exposed toads had greater divergence in dirty- compared with clean-raised individuals. Because lungworm-exposed toads were single-housed between the day of infection and swabbing on day 2, compared with unexposed toads that were group-housed until swabbing,

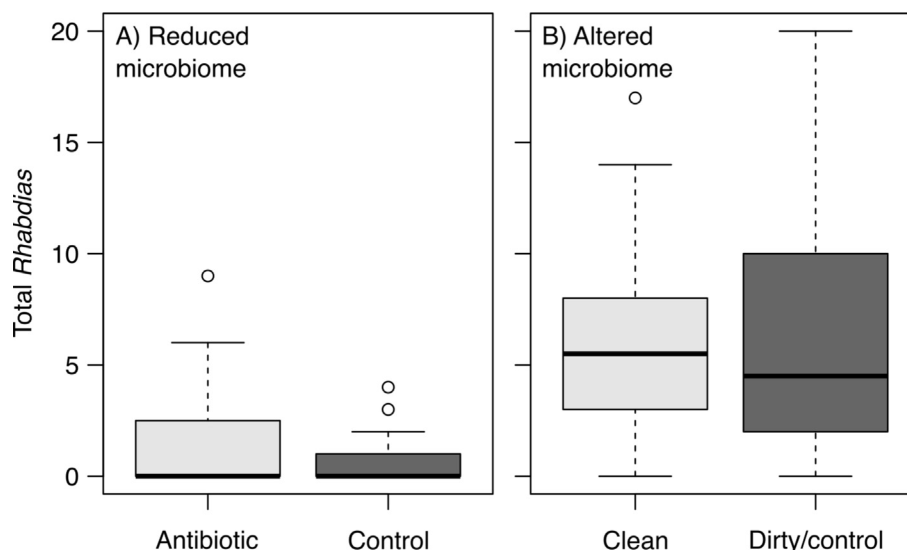

Fig. 1. Skin microbiome did not provide protection against *Rhabdias pseudosphaerocephala* in cane toads. Boxplots depict *R. pseudosphaerocephala* abundance in two experiments, (A) comparing control toads (bathed in Ringer's solution) with antibiotic-treated toads to reduce skin bacterial loads, and (B) comparing control toads (reared in dirty, semi-natural conditions) with toads reared in clean conditions to reduce skin bacterial diversity. *n* = 20 toads per experimental group. Boxplots show the median, interquartile range, reasonable range of the data (whiskers), and outliers (open circles).

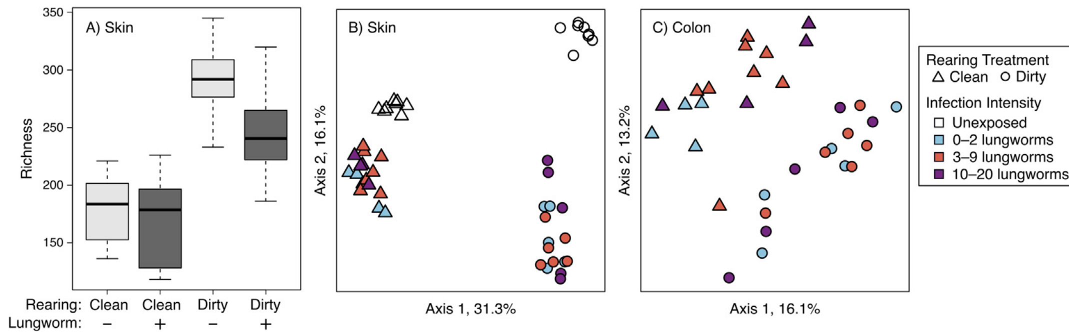

Fig. 2. Skin and colon bacterial microbiomes differed between cane toads raised in dirty and clean conditions, but were not correlated with *Rhabdias pseudosphaerocephala* abundance. (A) Richness of amplicon sequence variants in skin microbiomes on day 2 of infection was lower in clean- compared with dirty-raised toads. Reduced richness in lungworm-exposed toads may be explained by their reduced bacterial reservoirs in single-housed enclosures. Boxplots show the median, interquartile range, and range of the data (whiskers). (B) Beta diversity of skin microbiomes differed between the two rearing treatments and infection treatments, but was not associated with lungworm abundance. (C) Colon microbiome beta diversity was not associated with lungworm abundance. Colon samples were only collected from toads exposed to *R. pseudosphaerocephala* to focus on associations between microbiome diversity and infection outcome. n = 16 per rearing group for samples from lungworm-exposed toads. n = 8 per rearing group for unexposed toads.

Table 1
Statistical results of analyses comparing bacterial microbiomes from toad skin (A,B) and colons (C) based on rearing treatment and infection metrics. Analyses are grouped by samples. Differences were assessed between skin samples from toads exposed to lungworms and unexposed toads to identify if swab samples on day 2 of infection represented starting communities (A). The remaining analyses assess associations of rearing treatment or lungworm abundance on bacterial load and diversity metrics.

	Dependent	Predictor	Statistic	*P*
A) Swab samples, lungworm-exposed and unexposed				
	Bacterial load	Infection Treatment	$F_{1,44}$ = 0.015	0.9
		Rearing Treatment	$F_{1,44}$ = 0.50	0.5
		Infection × rearing	$F_{1,44}$ = 0.016	0.9
	Richness	Infection Treatment	$F_{1,44}$ = 7.65	**0.008**
		Rearing Treatment	$F_{1,44}$ = 77.79	**< 0.0001**
		Infection × rearing	$F_{1,44}$ = 2.95	0.09
	Bray–Curtis	Infection Treatment	$F_{1,44}$ = 17.66, R^2 = 0.15	**0.001**
		Rearing Treatment	$F_{1,44}$ = 39.58, R^2 = 0.35	**0.001**
		Infection × rearing	$F_{1,44}$ = 12.73, R^2 = 0.11	**0.001**
B) Swab samples, lungworm-exposed				
	Bacterial load	Rearing Treatment	t = 0.70	0.5
		Total *Rhabdias*	t = 0.28	0.8
	Richness	Rearing Treatment	t = 5.86	**<0.0001**
		Total *Rhabdias*	t = 0.04	1
	Bray–Curtis	Rearing Treatment	$F_{1,29}$ = 24.54, R^2 = 0.45	**0.001**
		Total *Rhabdias*	$F_{1,29}$ = 1.29, R^2 = 0.02	0.2
C) Colon samples, lungworm-exposed				
	Richness	Rearing Treatment	t = 2.15	**0.04**
		Total *Rhabdias*	t = −1.70	0.1
	Bray–Curtis	Rearing Treatment	$F_{1,29}$ = 5.21, R^2 = 0.15	**0.001**
		Total *Rhabdias*	$F_{1,29}$ = 0.85, R^2 = 0.02	0.6

Rearing treatment = dirty- or clean-housed throughout development. Infection treatment = lungworm-exposed (n = 16 per rearing treatment) or unexposed (n = 8 per rearing treatment). Total *Rhabdias* = abundance of *Rhabdias pseudosphaerocephala* from the 30 lungworms each toad was exposed to. Bold values indicate significance.

differences between these two groups could be an effect of either the infection itself or a change in housing conditions. Regardless, this difference indicates that swab data from lungworm-exposed toads do not represent pre-exposure starting communities.

Among lungworm-exposed toads, we found no association between *R. pseudosphaerocephala* abundance and skin bacterial load or diversity (Table 1, Fig. 2A, B). Similarly, in the colon samples, we found no correlations between lungworm abundance and diversity metrics. Diversity was only significantly affected by rearing treatment (Table 1, Fig. 2C). On average, dirty-housed toads had 51% greater bacterial richness on their skin and 11% greater bacterial richness in their colons than clean-reared toads.

4. Discussion

Bacterial microbiomes provide protection against pathogenic invaders in diverse wildlife systems (Thomason et al., 2017; Rebollar et al., 2020), but there is less evidence for such protection against macroparasitic infections. A previous study in this cane toad–lungworm system found possible evidence of protection by skin bacteria (Christian et al., 2021), but in our two experiments presented here, we found similar susceptibility to lungworms regardless of skin microbiome treatment (control, reduced bacterial biomass, reduced bacterial diversity). With amplicon sequencing, we found no evidence of bacterial community composition,

either on the skin or in the gut, associated with infection abundance after lungworm exposure. These results suggest that skin bacteria (directly) and gut bacteria (indirectly) provide little to no protection against lungworm invasion, and infection with lungworms does not influence gut communities through immune modulation (Rapin and Harris, 2018), at least in the first weeks of infection.

In a previous study (Christian et al., 2021), a wiping treatment was used to reduce bacterial load on toads before exposure to lungworm larvae. While the treatment did not impact the toads' skin anatomy, it may have reduced skin secretions alongside skin bacteria, and reduction in skin secretions may have led to the result of increased lungworm abundance in treated individuals. Another study also found that swabbing to reduce skin secretions, which likely reduced symbiotic skin bacteria as well, revealed defense provided by one or both of these intertwined skin features (Mayer et al., 2021a). The role of skin secretions in this infection is complex, however, as lungworm larvae appear to be attracted to secretions from toads in northern Queensland, Australia (Mayer et al., 2021a). Alternatively, there may be a protective effect of skin microbes against lungworm invasion, but it could depend on specific community members, as has been the case in another wildlife disease system (Thomason et al., 2017; Weitzman et al., 2021). If that were the case, skin microbes on toads in the present study were not protective despite protection provided in the past (Christian et al., 2021). Furthermore, toads and lungworms in the present study were sourced from the Northern Territory, while those used by Christian et al. (2021) and Mayer et al. (2021a) were sourced from Western Australia. As outcomes of toad–lungworm interactions depend on location of origin (Mayer et al. 2021a), our results may not be generalisable across the toad distribution.

Our amplicon sequence data highlight how influential the environment is on host microbiomes. Skin samples collected 2 days after toads were single-housed and exposed to lungworms had different communities from those of toads group-housed and unexposed to the parasite. Because we found no other support for lungworm infection correlating with skin microbiomes, we suspect this difference was due to a change in housing. A sudden lack of conspecifics as a reservoir for skin taxa could cause a decline in diversity. Indeed, others have found that within 1 day, amphibians moved from a complex (wild) to a simple (captive) environment experience significant reductions in skin microbial diversity (Jones et al., 2021). While our sampling scheme was chosen to avoid perturbing natural skin secretions, those samples did not completely represent the bacterial communities lungworms interacted with.

Expectedly, our two rearing treatments resulted in differing skin and gut communities in toads. Rearing toads in a clean environment reduced both skin and gut community richness with consequent differences in beta diversity. Others have similarly manipulated amphibian microbiomes to demonstrate their role in disease resistance (Knutie et al., 2017).

In our study, we found no evidence of bacterial communities providing protection against lungworm macroparasites in cane toads, despite the amphibian skin microbiome's role in creating an unwelcoming environment to invading microparasites (e.g., *Batrachochytrium dendrobatidis*, (Rebollar et al., 2020)). We also did not detect an impact of lungworm infection on gut communities in the first month after parasite exposure, signifying minimal immune modulation. Cane toads' innate susceptibility, including their skin secretions, may play a larger role in determining the chance of lungworm invasion and establishment than the toad's microbial communities (Mayer et al., 2021a; Eyck et al., 2022).

CRediT authorship contribution statement

Chava L. Weitzman: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Gregory P. Brown:** Writing – review & editing, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Kimberley Day:** Writing – review & editing, Investigation, Conceptualization. **Catherine M. Shilton:** Writing – review & editing, Resources, Investigation. **Karen Gibb:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Keith Christian:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Acknowledgements

This study was supported by a grant from the Australian Research Council (ARC-DP210102176). Thanks to Mirjam Kaestli for her helpful comments. We thank two anonymous reviewers for their time and helpful comments to improve the quality of this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.01.010.

References

Abt, M.C., Osborne, L.C., Monticelli, L.A., Doering, T.A., Alenghat, T., Sonnenberg, G.F., Paley, M.A., Antenus, M., Williams, K.L., Erikson, J., 2012. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 37, 158–170. https://doi.org/10.1016/j.immuni.2012.04.011.

Ajibola, O., Rowan, A.D., Ogedengbe, C.O., Mshelia, M.B., Cabral, D.J., Eze, A.A., Obaro, S., Belenky, P., 2019. Urogenital schistosomiasis is associated with signatures of microbiome dysbiosis in Nigerian adolescents. Sci. Rep. 9, 829. https://doi.org/10.1038/s41598-018-36709-1.

Apprill, A., McNally, S., Parsons, R., Weber, L., 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat. Microb. Ecol. 75, 129–137. https://doi.org/10.3354/ame01753.

Baker, M.R., 1979. The free-living and parasitic development of *Rhabdias* spp. (Nematoda: Rhabdiasidae) in amphibians. Can. J. Zool. 57, 161–178. https://doi.org/10.1139/z79-014.

Banfi, F.F., de Sena Guedes, K., Andrighetti, C.R., Aguiar, A.C., Debiasi, B.W., da Costa Noronha, J., de Jesus Rodrigues, D., Júnior, G.M.V., Sanchez, B.A.M., 2016. Antiplasmodial and cytotoxic activities of toad venoms from Southern Amazon, Brazil. Korean J. Parasitol. 54, 415. https://doi.org/10.3347/kjp.2016.54.4.415.

Barnhart, K., Forman, M.E., Umile, T.P., Kueneman, J., McKenzie, V., Salinas, I., Minbiole, K.P., Woodhams, D.C., 2017. Identification of bufadienolides from the boreal toad, *Anaxyrus boreas*, active against a fungal pathogen. Microb. Ecol. 74, 990–1000. https://doi.org/10.1007/s00248-017-0997-8.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A. D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Brannelly, L.A., Ohmer, M.E., Richards-Zawacki, C.L., 2019. Artificial reproduction using leuprolide acetate in the frog *Rana pipiens*. Herpetol. J. 29, 125–130. https://doi.org/10.33256/hj29.2.125130.

Brown, G.P., Shine, R., 2016. Maternal body size influences offspring immune configuration in an oviparous snake. R. Soc. Open Sci. 3, 160041. https://doi.org/10.1098/rsos.160041.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U.S.A. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107.

Christian, K., Weitzman, C., Rose, A., Kaestli, M., Gibb, K., 2018. Ecological patterns in the skin microbiota of frogs from tropical Australia. Ecol. Evol. 8, 10510–10519. https://doi.org/10.1002/ece3.4518.

Christian, K., Shine, R., Day, K.A., Kaestli, M., Gibb, K., Shilton, C.M., Brown, G.P., 2021. First line of defence: skin microbiota may protect anurans from infective larval lungworms. Int. J. Parasitol. 14, 185–189. https://doi.org/10.1016/j.ijppaw.2021.02.014.

Conlon, J.M., 2011. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res. 343, 201–212. https://doi.org/10.1007/s00441-010-1014-4.

de Medeiros, D.S., Rego, T.B., dos Santos, A.P.D.A., Pontes, A.S., Moreira-Dill, L.S., Matos, N.B., Zuliani, J.P., Stábeli, R.G., Teles, C.B., Soares, A.M., Sperotto, A.R.D.M., Moura, D.J., Saffi, J., Caldeira, C.A.D.S., Pimenta, D.C., Calderon, L.A., 2019. Biochemical and biological profile of parotoid secretion of the Amazonian *Rhinella marina* (Anura: Bufonidae). Biomed Res. Int. 2019, 2492315. https://doi.org/10.1155/2019/2492315.

DeCandia, A.L., Brenner, L.J., King, J.L., vonHoldt, B.M., 2020. Ear mite infection is associated with altered microbial communities in genetically depauperate Santa Catalina Island foxes (*Urocyon littoralis catalinae*). Mol. Ecol. 29, 1463–1475. https://doi.org/10.1111/mec.15325.

Eyck, H.J.F., Brown, G.P., Rollins, L.A., Shine, R., 2022. In an arms race between host and parasite, a lungworm's ability to infect a toad is determined by host susceptibility not parasite preference. Biol. Lett. 18, 20210552. https://doi.org/10.1098/rsbl.2021.0552.

Finnerty, P.B., Shine, R., Brown, G.P., 2018. The costs of parasite infection: effects of removing lungworms on performance, growth and survival of free-ranging cane toads. Funct. Ecol. 32, 402–415. https://doi.org/10.1111/1365-2435.12992.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., Iwasaki, A., 2011. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. U.S.A. 108, 5354–5359. https://doi.org/10.1073/pnas.1019378108.

Jones, K.R., Walke, J.B., Becker, M.H., Belden, L.K., Hughey, M.C., 2021. Time in the laboratory, but not exposure to a chytrid fungus, results in rapid change in spring peeper (*Pseudacris crucifer*) skin bacterial communities. Ichthyol. Herpetol. 109, 75–83. https://doi.org/10.1643/h2020077.

Kelehear, C., Webb, J.K., Shine, R., 2009. *Rhabdias pseudosphaerocephala* infection in *Bufo marinus*: lung nematodes reduce viability of metamorph cane toads. Parasitology 136, 919–927. https://doi.org/10.1017/S0031182009006325.

Kelehear, C., Brown, G.P., Shine, R., 2011. Influence of lung parasites on the growth rates of free-ranging and captive adult cane toads. Oecologia 165, 585–592. https://doi.org/10.1007/s00442-010-1836-5.

Knutie, S.A., Wilkinson, C.L., Kohl, K.D., Rohr, J.R., 2017. Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. Nat. Commun. 8, 86. https://doi.org/10.1038/s41467-017-00119-0.

Koskella, B., Bergelson, J., 2020. The study of host–microbiome (co)evolution across levels of selection. Philos. Trans. R. Soc. Lond. B Biol. Sci. 375, 20190604. https://doi.org/10.1098/rstb.2019.0604.

Leung, J.M., Graham, A.L., Knowles, S.C., 2018. Parasite-microbiota interactions with the vertebrate gut: synthesis through an ecological lens. Front. Microbiol. 9, 843. https://doi.org/10.3389/fmicb.2018.00843.

Lu, J., Hamblen, E.E., Brenner, L.J., King, J.L., VonHoldt, B.M., DeCandia, A.L., 2023. Ear mite infection restructures otic microbial networks in conservation–reliant Santa Catalina Island foxes (*Urocyon littoralis catalinae*). Mol. Ecol. 32, 892–903. https://doi.org/10.1111/mec.16795.

Mayer, M., Schlippe Justicia, L., Shine, R., Brown, G.P., 2021a. Host defense or parasite cue: skin secretions mediate interactions between amphibians and their parasites. Ecol. Lett. 24, 1955–1965. https://doi.org/10.1111/ele.13832.

Mayer, M., Shine, R., Brown, G.P., 2021b. Rapid divergence of parasite infectivity and host resistance during a biological invasion. Biol. J. Linn. Soc. 132, 861–871. https://doi.org/10.1093/biolinnean/blaa229.

Osakunor, D.N., Munk, P., Mduluza, T., Petersen, T.N., Brinch, C., Ivens, A., Chimponda, T., Amanfo, S.A., Murray, J., Woolhouse, M.E., 2020. The gut microbiome but not the resistome is associated with urogenital schistosomiasis in preschool-aged children. Commun. Biol. 3, 155. https://doi.org/10.1038/s42003-020-0859-7.

Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ. Microbiol. 18, 1403–1414. https://doi.org/10.1111/1462-2920.13023.

Pizzatto, L., Kelehear, C., Shine, R., 2013. Seasonal dynamics of the lungworm, *Rhabdias pseudosphaerocephala*, in recently colonised cane toad (*Rhinella marina*) populations in tropical Australia. Int. J. Parasitol. 43, 753–761. https://doi.org/10.1016/j.ijpara.2013.05.002.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

Rapin, A., Harris, N.L., 2018. Helminth–bacterial interactions: cause and consequence. Trends Immunol. 39, 724–733. https://doi.org/10.1016/j.it.2018.06.002.

Rebollar, E.A., Martínez-Ugalde, E., Orta, A.H., 2020. The amphibian skin microbiome and its protective role against chytridiomycosis. Herpetologica 76, 167–177.

Reitmeier, S., Hitch, T.C.A., Treichel, N., Fikas, N., Hausmann, B., Ramer-Tait, A.E., Neuhaus, K., Berry, D., Haller, D., Lagkouvardos, I., Clavel, T., 2021. Handling of spurious sequences affects the outcome of high-throughput 16S rRNA gene amplicon profiling. ISME Commun. 1, 31. https://doi.org/10.1038/s43705-021-00033-z.

Rosshart, S.P., Vassallo, B.G., Angeletti, D., Hutchinson, D.S., Morgan, A.P., Takeda, K., Hickman, H.D., McCulloch, J.A., Badger, J.H., Ajami, N.J., 2017. Wild mouse gut microbiota promotes host fitness and improves disease resistance. Cell 171, 1015–1028. https://doi.org/10.1016/j.cell.2017.09.016.

Schmeda-Hirschmann, G., Quispe, C., Arana, G.V., Theoduloz, C., Urra, F.A., Cárdenas, C., 2016. Antiproliferative activity and chemical composition of the venom from the Amazonian toad *Rhinella marina* (Anura: Bufonidae). Toxicon 121, 119–129. https://doi.org/10.1016/j.toxicon.2016.09.004.

Sencio, V., Machado, M.G., Trottein, F., 2021. The lung–gut axis during viral respiratory infections: the impact of gut dysbiosis on secondary disease outcomes. Mucosal Immunol. 14, 296–304. https://doi.org/10.1038/s41385-020-00361-8.

Thomason, C.A., Mullen, N., Belden, L.K., May, M., Hawley, D.M., 2017. Resident microbiome disruption with antibiotics enhances virulence of a colonizing pathogen. Sci. Rep. 7, 16177. https://doi.org/10.1038/s41598-017-16393-3.

Weitzman, C.L., Gibb, K., Christian, K., 2018. Skin bacterial diversity is higher on lizards than sympatric frogs in tropical Australia. PeerJ 6, e5960.

Weitzman, C.L., Rostama, B., Thomason, C.A., May, M., Belden, L.K., Hawley, D.M., 2021. Experimental test of microbiome protection across pathogen doses reveals importance of resident microbiome composition. FEMS Microbiol. Ecol. 97, fiab141. https://doi.org/10.1093/femsec/fiab141.

Wright, K.M., Whitaker, B.R., 2001. Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malibar, Florida.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, D643–D648. https://doi.org/10.1093/nar/gkt1209.