The gut bacteria of an invasive amphibian respond to the dual challenges of range-expansion and parasite attack

Jia Zhou[1,6*], Tiffanie Maree Nelson[2], Carlos Rodriguez Lopez[3], Shao Jia Zhou[1,4], Georgia Ward-Fear[5], Katarina C. Stuart[6], Richard Shine[5], Lee Ann Rollins[6]

[1]School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB 1 Glen Osmond, South Australia, Australia, 5064.

[2]Queensland Facility for Advanced Bioinformatics, School of Medicine, Menzies Health Institute Queensland, Building G40, Level 9, Gold Coast Campus, Griffith University, Southport, QLD, Australia, 4215.

[3]Environmental Epigenetics and Genetics Group, Department of Horticulture, College of Agriculture, Food and Environment, University of Kentucky, Lexington, KY, USA, 40546.

[4]Robinson Research Institute, University of Adelaide, South Australia, Australia, 5005

[5]Department of Biological Sciences, Macquarie University, North Ryde, NSW, Australia, 2109.

[6]Ecology & Evolution Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, Biological Sciences South (E26) UNSW, Kensington, Australia, 2052

*** Correspondence:**
Jia Zhou
E-mail: jiazhou0116@gmail.com

Keywords (Six words): microbiome, biological invasion, Cane toad, 16S rRNA, predicted bacterial functions, host-parasite

Word count: 6329 words including title, abstract, keywords, text, figure/table legends and references.

Abstract

Gut bacterial communities influence, and are influenced by, the behaviour and ecology of their hosts. Those interactions have been studied primarily in humans and laboratory model organisms, but we need field research to understand the relationship between an organism's gut bacteria and its ecological challenges such as those imposed by rapid range expansion (as in invasive species) and the presence of host-manipulating parasites. Cane toads (*Rhinella marina*) provide an excellent model system in this respect, because the species' ongoing colonization of Australia has enforced major changes in phenotypic traits (including behaviour), and lungworm parasites (*Rhabdias pseudosphaerocephala*) modify host gut function in ways that enhance the viability of lungworm larvae. We collected female toads from across the species' invasive range in tropical Australia, and studied toad morphology, behaviour, parasite infection status and gut bacterial community. Range-core *versus* range-edge toads differed in morphology, behaviour, gut bacterial composition and predicted gut bacterial function but did not differ in the occurrence of parasite infection nor in the intensity of infection. Toads infected with lungworms differed from uninfected conspecifics in gut bacterial composition and diversity. Our study demonstrates strong associations between gut bacterial community and host ecology and behaviour.

Introduction

The bacterial community within an organism's intestines can be strongly influenced by host behaviour and ecology, such as habitat selection and diet [1–5]. But that interaction runs both ways because in turn, gut bacteria can influence behaviour and ecology of the host. For example, transferring gut contents can modify the recipient host's behaviour (exploratory behaviour in laboratory mice [6]; emotional reactivity in Japanese quail, *Coturnix japonica* [7]). Similarly, altering gut microbial communities by administering antibiotics or altering dietary composition triggered

aggressive behaviour in leaf-cutting ants (*Acromyrmex echinatior* [8]). Remarkably, changes in only a single bacterial species within the gut can affect behaviour of the host (e.g., in fruit flies, *Drosophila melanogaster* [9]; in zebrafish, *Danio rerio* [10]; in mice, *Mus musculus* [11]). Gut microbiota can also affect mating choices [12] and foraging [13–15].

To date, most evidence for effects of intestinal bacteria on host behaviour comes from studies on humans or "model organisms" in the laboratory. To elucidate the functional significance of this phenomenon, we need to extend such studies to free-ranging animals, and to examine a wider range of taxa [16]. Invasive species offer good models for such research, because novel challenges in the invaded range create an opportunity to compare closely-related organisms exposed to profoundly different environments [17,18]. Host-parasite relationships also may be disrupted during biological invasions, due to processes such as "enemy release" (loss of co-evolved native pathogens from the native range [19]). Parasites can manipulate host behaviour and physiology in ways that enhance parasite fitness but reduce host fitness [20]. Thus, if the gut bacterial community provides a mechanism for such effects, parasite-infected individuals should exhibit different gut bacteria than uninfected conspecifics.

These ideas suggest two predictions: (i) that the gut bacterial community should differ between populations of an invasive species (e.g., between the range-core and the invasion-front); and (ii) that the gut bacterial community should differ between parasitized hosts and non-parasitized conspecifics. The colonization of Australia by cane toads (*Rhinella marina*) provides a robust opportunity to test these predictions. Since their release in north-eastern Australia in 1935, the toads have dispersed into areas that are much hotter and more seasonally arid than in the native range or the initial release sites [21]; and the toads have brought with them a native-range nematode lungworm (*Rhabdias pseudosphaerocephala*) that can have devastating impacts on host viability, and induces behavioural and physiological changes in the host [22]. Notably, infected toads produce copious watery faeces [22]; and hence, we expect lungworm-infected toads to exhibit different gut bacterial communities than uninfected individuals.

Methodology

Study species, sample collection and behavioural assays

Cane toads are native to north-eastern South America, and were introduced into Australia in 1935 as a biocontrol for pests of sugar cane crops [23]. As the toads spread through tropical Australia, they fatally poisoned many native predators that attempted to eat them [23]. Toads from range-core populations (eastern Australia) differ from invasion-front conspecifics (in north-western Australia) in phenotypic traits that confer increased dispersal ability, such as endurance [24], limb morphology [25], boldness and exploratory behaviour [17,26]. Toads from the invasion-front also have lower rates of infection of the co-introduced parasitic lungworm [27]. Drivers of variation in invasion-related behaviours in this species include genetics, morphology, habitat, diet, prior experience and parasites [17,28–31]. However, the possible role of gut bacteria as a potential driver of behavioural shifts across the invasive range has not been studied. Understanding how gut bacterial communities influence their host's rate of invasion may facilitate management of this species.

We hand-captured 60 wild adult female toads from three sites on the invasion-front and three sites in the range-core (for details, see Table S1). We conducted brief behavioural assays upon collection including: (i) struggle score (number of arm or leg kicks after being picked up until toad remains still for 5 seconds; struggle likelihood) and (ii) righting effort (time to right itself, number of limb kicks within two minutes after the toad is placed on its dorsal side; righting effort likelihood) [17]. These measures are predictive of traits such as speed and stamina (K. Stuart, pers. comm.), suggesting that these simple assays may reveal a toad's dispersal potential. We then placed animals into individual, moist, calico bags and weighed, measured (snout urostyle length; SUL) and euthanised them by injecting tricaine methanesulfonate (MS222) buffered with bicarbonate of soda.

We dissected the toads and scored the presence of two types of toad parasite: the gut-encysted physalopterine larvae [32] and adult lungworms [22,33]. Lungworm larvae pass through the gut, but are less easily detected and reliably counted than are adult lungworms. From each toad, we removed 0.3cm of colon near the cloaca (including gut contents) and preserved it in 95% ethanol (See Supplementary Material for justification of using colon contents).

Analyses of morphological and ecological data

We compared host morphology and host behaviour between regions (range-core *versus* invasion-front toads), and as a function of infection status (lungworm infected *versus* non-infected toads).

Because body length (SUL) and mass were correlated, we only included SUL as our measure of host morphology in further analyses. We used a t-test to compare mean SUL between regions and infected/non-infected toads. For associations between region or infection status with host characteristics or behavioural traits, we used SUL as a covariate in generalized linear models (GLM).

See Supplementary Material and code resources for details of our statistical analyses.

Molecular methodologies and analyses

Laboratory methods and data pre-processing for characterizing gut bacterial community composition and predicted functions are described in Supplementary Material. In summary, we calculated within-individual (alpha) bacterial diversity and between-site (beta) bacterial diversity. For the latter variable, we subset our data to include the Core50 gut community (Amplified Sequence Variants (ASVs) present in a minimum of 50% of toads from each site [2]). We predicted bacterial functions and generated pathway abundance based on Core50 ASVs (Supplementary Material). We compared bacterial composition and predicted function between regions, and between lungworm-infected and non-infected toads. We also identified differences in individual ASVs and predicted bacterial functions between range-core and invasion-front toads and further identified associations between host characteristics (including infection with parasites) with bacterial communities and predicted bacterial functions. Analyses were conducted mainly through QIIME2 [34], PICRUST2 [35], and R packages in R version 4.0.2 [36].

Results

Ecological traits of range-core versus invasion-front toads

Wild-caught invasion-front toads were larger than range-core toads (Tables S2, S3; mean SUL t = 2.54, df = 53.90, p = 0.014). Neither counts nor presence of parasites (lungworm and gut) differed significantly across the range (Table S2). Range-core toads were more likely to struggle (Tables S2, S3; p = 0.008, 95% CI: core [-0.174, 0.005], edge [-0.023, 0.199]) and, in those that did struggle, the number of struggle movements was higher for range-core toads (Tables S2, S3; p = 0.002, 95% CI: core [-0.057, 0.026], edge [0.046, 0.2]). Range-core toads also were more likely to attempt to right themselves when turned over (Tables S2, S3; p = 0.036, 95% CI: core [-0.092, 0.04], edge [-0.006,

0.19]) (Tables S2, S3), but righting effort and righting time did not differ significantly between range-core and invasion-front toads (Tables S2, S3).

Ecological traits of infected versus non-infected toads

Because there were no significant differences in prevalence or intensity of lungworm between the range-core and invasion-front toads (Table S2), we combined samples for an analysis of correlates of lungworm infection. Infected toads were similar in SUL to non-infected toads (Tables S3, 4; $t = 0.86$, $df = 57.19$, $p = 0.393$), with no significant behavioural differences between the two groups (Table S3, 4).

Gut bacterial community composition and predicted bacterial function

Alpha diversity did not differ significantly between regions (Supplementary Material), but beta diversity of bacterial taxonomic communities differed between regions (Figures 1A, S1; $R^2 = 0.050$, $F = 3.050$, $p < 0.001$) and sampling sites (Table S5; all p-values < 0.001). Among 230 ASVs that were assigned to family level, the abundance of 124 ASVs differed between the colons of range-core *versus* invasion-front toads (Table S6). The number of significantly different ASVs in each phylum were: Bacteroidetes (60 ASVs), Firmicutes (55 ASVs), Proteobacteria (7 ASVs), Actinobacteria (1 ASVs), Verrucomicrobia (1 ASV) (Table S6, Figure 2A).

Among the identified 474 predicted bacterial functions, we found significant differences between invasion-front and range-core toads (Figure 1B; $R^2 = 0.064$, $F = 4.110$, p-value= 0.002). Pairwise tests between sampling sites indicated that toads from Kununurra had different bacterial functions to toads from Rossville (p-value= 0.009) and Lucinda (p-value = 0.046), but no other sites differed functionally from each other (Table S7; all other p-values > 0.05). In total, 84 predicted bacterial functions differed between invasion-front and range-core toads (Table S8, Figure 2B). Range-core toads had more abundant bacterial function in the superpathway of pyrimidine ribonucleosides degradation (log2FoldChange = 5.98) and less abundant bacterial function in phosphopantothenate biosynthesis III (log2FoldChange = -4.98), superpathway of sialic acids and CMP-sialic acids biosynthesis (log2FoldChange = -4.89) and factor 420 biosynthesis (log2FoldChange = -4.72) than did invasion-front toads (Table S8, Figure 2B). Among the 30 most abundant functions, range-core toads had lower bacterial function in urate biosynthesis/inosine 5'-phosphate degradation (log2FoldChange = -0.10) than did invasion-front toads (Figure 3, Table S8).

Associations between ecological traits and intestinal bacteria

To assess correlates of gut bacterial composition and function, we compared characteristics of individual hosts to bacterial variation. Only the occurrence of lungworms was significantly associated with the bacterial composition ($R^2 = 0.128$, $p = 0.02$) (Table 1; Figure S2A, B).

In a redundancy analysis combining ecological traits measured here, the model that explained the most variation in the bacterial community assemblage included only the occurrence of lungworms (AIC = 178.58). The best model to explain variation in predicted bacterial functions included the likelihood of righting (AIC = 53.613), the occurrence of lungworms (AIC = 54.297) and righting time (AIC = 56.912). The combination of these three factors explained 17.8% of total variation in predicted bacterial functions (Figure 4).

In explicit tests of whether bacterial community assemblages differed in infected *versus* non-infected toads, we found a significant association with lungworm occurrence (Table 2, p=0.005). Intensity of lungworm infection was not significantly associated with gut bacterial community but did have a significant interaction with region in this analysis (Table 2, p=0.04).

Discussion

Bacteria influence animal behaviour in diverse ways [16,37], but the ecological drivers of variation in gut bacterial composition remain largely unstudied. Our analysis of cane toads from two regions within their invasive range documents substantial variation in community assemblage and function of gut bacteria. Importantly, that variation was associated with two traits that we predicted to influence gut bacterial assemblages: invasion history and parasite infection. Interestingly, toad behaviour differed across the invasive range, and toad righting behaviour was associated with bacterial function but not with parasite infection.

Geographic divergence in gut bacteria

First, we consider the differences in gut bacteria between toads from the invasion-front and the range-core. Although these populations have been separated by less than a century, the toads have diverged remarkably in morphology, physiology and behaviour and much of that divergence is heritable [38,39]. Some of those shifts likely reflect evolutionary pressures for increased rates of dispersal, due to adaptive (natural selection) and non-adaptive (spatial sorting) mechanisms [40,41].

Other geographically variable aspects of toad phenotypes likely are responses to different climatic conditions in the newly-invaded regions (hot, seasonally arid) compared to the range-core (cooler, more equable climate) [42]. Similar geographic divergence has been reported for the microbiome on the toad's skin [43]. Our data illustrate that the invasion of Australia by cane toads has been accompanied by substantial divergence in gut bacterial communities. Alpha diversity in gut bacteria was similar in invasion-front and range-core individuals, but there were differences in both the gut bacteria composition and predicted bacterial function between toad populations across the species' Australian invasive range. Predicted bacterial functions better explained cane toad righting behaviour than did gut bacterial community composition. Intriguingly, similarity between gut bacterial communities between individuals within regions in Australia is related to the similarity of their host's epigenome, and this relationship is strengthened in populations where genetic diversity is lowest, such as on the invasion front [44]. Relationships between gut bacterial communities and their hosts are complex, and that a clear understanding of these relationships requires careful consideration of numerous environmental, host and gut bacterial factors.

The diversity and composition of bacterial communities differed between range-core and invasion-front toads, despite an overall similarity in their dominant phyla and alpha diversity. ASVs in the family Veillonellaceae were higher at the invasion-front (Figure 2A). Previous work has shown that the abundance of this bacterial family may influence host metabolic regulation. For example, in Brandt's voles (*Lasiopodomys brandtii*) exposed to colder temperatures, voles which huddled had more Veillonellaceae and more short-chain fatty acids (SCFAs) in their intestines than did non-huddling voles [45]. This family produces SCFAs such as propionic acid [46,47], which can increase locomotor activity [48]. The link to host metabolic regulation suggests that invasion-front toads might fuel their invasion in this way [24]. ASVs from another family of SCFA-producing bacteria, Clostridiaceae [49], were also higher in invasion-front toads than those from the range-core. Furthermore, the family Veillonellaceae may be associated with host sociality. A reduction of Veillonellaceae has been observed in children with Autism Spectrum Disorder, often known for desiring social isolation, when compared to a neurotypical group of children [50]. Higher abundance of Veillonellaceae in invasion-front toads could foster their "bolder" personality, retaining a higher propensity for exploration and risk-taking [26,51].

Several other ASVs that differed across the cane toad's range also may affect behaviour. ASVs from the family Peptococcaceae, more common in invasion-front toads (Figure 2A), are related to host

neurotransmitter levels (noradrenaline linking visual awareness to external world events [52]). For example, Peptococcaceae levels in the caecum are positively correlated with noradrenaline levels in mice [53]. ASVs from family Bacillaceae, lower in invasion-front toads (Figure 2A), might be related to host anxiety (e.g., abundant in methamphetamine-treated rats [54], and in exercised *versus* sedentary mice [55]). Abundant Bacillaceae might induce anxiety-like behaviours, thus intensifying the stress response [54] and decreasing exploratory behaviour in new environments [56]. In summary, invasion-front toads possessed gut bacterial communities that in other studies have been associated with SCFAs production and neurotransmitters. That pattern supports the idea that gut microbes in invasion-front toads may increase locomotor ability, alertness and propensity for exploration and risk-taking. In comparison, range-core toads possessed bacterial taxa that have been associated with anxiety, and a decreased propensity to explore.

Geographic variation was less obvious in the predicted bacterial functional groups than in community composition (Figure 1), consistent with the hypothesis that bacterial function is more conservative than taxonomic composition (e.g. in fire salamanders [2]). Different gut microbiota can have similar bacterial functions, increasing resilience and functional stability [2,3,57]. Despite this broad similarity, bacterial functions differed between range-core and invasion-front toads. These differences included those involved in functional pathways related to food sources and metabolism. Invasion-front toads had less bacterial function in the superpathway of pyrimidine ribonucleosides degradation, which provides a nitrogen source for microbes [58] and plays an important role in perturbations in the uridine monophosphate (UMP) biosynthetic pathways. This pathway allows the bacterial cell to sense signals such as starvation, nucleic acid degradation, and availability of exogenous pyrimidines, and to adapt the production of the extracellular matrix to changing environmental conditions [59]. This function might help to explain the disappearance of Verrucomicrobia as a dominant taxon. As for microbe metabolism, invasion-front toads have higher abundance of bacterial functions in factor 420 biosynthesis, critical to bacterial metabolism and mediating important redox transformations involved in bacterial persistence, antibiotic biosynthesis, pro-drug activation, and methanogenesis [60].

We also detected geographic variation in bacterial functional pathways that contribute to host health. Invasion-front toads exhibited bacterial functions beneficial to general host health and immunity: (i) phosphopantothenate biosynthesis, the first step for the bacterial production of coenzyme A (CoA), an indispensable enzyme cofactor [61]; and (ii) superpathway of sialic acids biosynthesis, generating

sialic acids that play many roles in immunity including acting as host receptors and pathogen decoys for viruses and bacteria [62] and are especially critical for preventing neural tissue damage [63]. Despite this abundance of health-promoting bacterial functions, these toads may also face health challenges. Invasion-front toad bacteria had a higher abundance of urate biosynthesis function (urate biosynthesis/inosine 5'-phosphate degradation, the only significantly different one out of the top 30 abundant functions), which affects serum urate levels [64]. High levels of urate can result in the formation of needle-like crystals of urate in the joints (gout), perhaps related to severe spinal arthritis in invasion-front cane toads [65].

Associations between lungworms and host gut bacteria

Pathogens and parasites impact the composition of the host microbiota and can modify host behaviour in a manner that improves parasite transmission and survival [66–68]. Lungworms can affect cane toad locomotor performance and reduce host endurance, curtailing oxygen supply from infected lungs [69]. Lungworms also can alter a cane toad's thermal preference and manipulate the timing and location of defecation, thereby enhancing lungworm egg production and larval survival [22]. Lungworms are reported to lag behind their host on the invasion-front by 2-3 years [27] and to affect righting behaviour (prolongs righting time [70]). In the current study, although we collected invasion-front toads in recently invaded areas, we found no difference in lungworm presence or intensity between the invasion-front *versus* range-core toads, nor did we find differences in behaviour in lungworm-infected *versus* uninfected toads.

Infection by parasitic nematode lungworms was associated with differences in the gut bacteria. In this case, the direction of causation is less ambiguous than is the case for geographic variation in the gut bacteria (above). We doubt that a toad's bacteria affect its probability of carrying adult lungworms, although bacterial-driven differences in habitat selection might create such a link. Instead, we suggest that the presence of lungworms induces a shift in the gut bacteria. Consistent with that hypothesis, experimental trials have shown that lungworms modify gastric function in their toad hosts, changing the volume and consistency of faeces produced in ways that enhance survival of larval lungworms [22]. Shifts in the microbiome inside the gut might be either causes or consequences of that shift in gastric function. Moreover, *C. elegans* are known to prefer specific bacterial foods [71], suggesting that lungworm larvae may also feed selectively on bacteria in the gut, generating differences in bacterial communities between lungworm-infected toads and non-infected conspecifics. Additionally, gut bacteria may affect lungworms via microbiome-induced shifts in host immunity [72].

Associations between behaviours and host gut bacteria

Interestingly, behaviours including righting effort likelihood and righting time were associated more closely with predicted bacterial functions than with gut bacterial taxonomic composition. Multiple identified taxa may share the same bacterial function, or one taxon may contribute to multiple bacterial functions, obscuring the relationship between host behaviour and taxonomic composition of the gut bacteria. Nonetheless, these relationships we found between gut bacterial function and righting behaviours may be related to toad health and/or rearing conditions. A dampened stress response (lower level of corticosterone) in invasion-front toads [73] could result from higher abundance of bacterial functions beneficial to host health and immunity, especially the superpathway of sialic acids biosynthesis [63]. Further, invasion-front toads are more reluctant to flee in simulated predation trials [74]. Dampened stress responses can be related to more exploratory behaviour [56], and to greater dispersal ability [26]. Rearing conditions also affect righting behaviour [17]. Although manipulative studies are needed to clarify causal relationships between stress responses, proactive behaviours, and gut bacterial functions, our results indicate that behaviour and toad colon bacterial functions are related, suggesting that gut bacteria may be an important driver of invasion.

Our study is preliminary, and has identified patterns rather than testing alternative hypotheses about underlying causal processes. To clarify causal mechanisms underlying the geographic divergence in gut bacteria across the toads' Australian range, future studies could transfer range-core toads to the range-edge, and *vice versa*, and examine if (and how) their gut bacteria respond to novel environmental conditions. Breeding these animals, and raising their offspring under common-garden conditions, could further reveal the degree to which a toad's gut bacteria is driven by the host's genetics *versus* by rearing conditions [75,76]. To clarify the hypothesis that changes in the gut bacteria mediate the ability of lungworm parasites to modify gut function of the host, we could transplant colon contents from infected to uninfected toads. In short, our discovery of strong associations between the gut bacteria and important facets of toad ecology provides the opportunity to move to hypothesis-testing experimental studies.

Our research illustrates that during invasion, as a species expands across a novel and variable landscape, a complex relationship between a host's behaviour, its parasite community, and its microbiome may unfold. A clearer understanding of these relationships and how they influence the rate of expansion are key to understanding the role of the holobiont during invasion [77]. Such

studies also will advance our understanding of co-evolution and may facilitate innovative approaches to invasive species management.

Ethics

All work with animals has been approved by the University of Adelaide Animal Ethics Committee (approval number: S-2018-056).

Author Contributions

1. Designed research: JZ, TMN, CRL, SJZ, LAR
2. Performed Research: JZ, CRL, GWF, KS, LAR
3. Analyzed data: JZ, TMN, CRL, SJZ, KS, LAR
4. Wrote the paper: JZ, RS, TMN, CRL, SJZ, GWF, KS, LAR

Funding

This project was supported by the Holsworth Wildlife Research Endowment (Ecological Society of Australia) and Adelaide Graduate Research Scholarship (University of Adelaide) to JZ, and a Scientia Fellowship to LAR (UNSW). CMRL is partially supported by the National Institute of Food and Agriculture, AFRI Competitive Grant Program Accession number 1018617 and National Institute of Food and Agriculture, United States Department of Agriculture, Hatch Program accession number 1020852.

Acknowledgments

We thank Dr. Alice Russo, and Ms. Rita Kurpiewski contributed to field sample collections. Dr. Eve Slavich from Stats Central in UNSW provided statistical advice.

Data Availability Statement

All the data and supporting information will be made available online.

1. Supplementary methods
2. Supplementary results
3. Supplementary Figures S1-2.
4. Supplementary Tables S1-8.
5. Codes: https://github.com/jiazhou0116/gut-microbiome-analyses-2

The sequence data that support the findings of this study are openly available in NCBI Sequence Read Archive (accession number: PRJNA670039). The raw data of host, environmental and behavioural measurements, and correlation tests of all factors are available on Dryad (doi:10.5061/dryad.v15dv41tw).

Bibliography

[1] Hopkins WA. Amphibians as models for studying environmental change. ILAR J 2007;48:270–7. doi:10.1093/ilar.48.3.270.

[2] Bletz MC, Goedbloed DJ, Sanchez E, Reinhardt T, Tebbe CC, Bhuju S, et al. Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. Nat Commun 2016;7:13699. doi:10.1038/ncomms13699.

[3] Huang B-H, Chang C-W, Huang C-W, Gao J, Liao P-C. Composition and functional specialists of the gut microbiota of frogs reflect habitat differences and agricultural activity. Front Microbiol 2017;8:2670. doi:10.3389/fmicb.2017.02670.

[4] Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Pharmacol Res 2013;69:52–60. doi:10.1016/j.phrs.2012.10.020.

[5] Zmora N, Suez J, Elinav E. You are what you eat: diet, health and the gut microbiota. Nat Rev Gastroenterol Hepatol 2019;16:35–56. doi:10.1038/s41575-018-0061-2.

[6] Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 2011;141:599–609, 609.e1. doi:10.1053/j.gastro.2011.04.052.

[7] Kraimi N, Calandreau L, Zemb O, Germain K, Dupont C, Velge P, et al. Effects of gut microbiota transfer on emotional reactivity in Japanese quails (Coturnix japonica). J Exp Biol 2019;222. doi:10.1242/jeb.202879.

[8] Teseo S, van Zweden JS, Pontieri L, Kooij PW, Sørensen SJ, Wenseleers T, et al. The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants. Anim Behav 2019;150:239–54. doi:10.1016/j.anbehav.2018.12.017.

[9] Schretter CE, Vielmetter J, Bartos I, Marka Z, Marka S, Argade S, et al. A gut microbial factor modulates locomotor behaviour in Drosophila. Nature 2018;563:402–6. doi:10.1038/s41586-018-0634-9.

[10] Borrelli L, Aceto S, Agnisola C, De Paolo S, Dipineto L, Stilling RM, et al. Probiotic modulation of the microbiota-gut-brain axis and behaviour in zebrafish. Sci Rep 2016;6:30046. doi:10.1038/srep30046.

[11] Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterol Motil 2014;26:1615–27. doi:10.1111/nmo.12427.

[12] Sharon G, Segal D, Ringo JM, Hefetz A, Zilber-Rosenberg I, Rosenberg E. Commensal bacteria play a role in mating preference of Drosophila melanogaster. Proc Natl Acad Sci USA 2010;107:20051–6. doi:10.1073/pnas.1009906107.

[13] Vogel H, Shukla SP, Engl T, Weiss B, Fischer R, Steiger S, et al. The digestive and defensive

basis of carcass utilization by the burying beetle and its microbiota. Nat Commun 2017;8:15186. doi:10.1038/ncomms15186.

[14] Wong AC-N, Wang Q-P, Morimoto J, Senior AM, Lihoreau M, Neely GG, et al. Gut Microbiota Modifies Olfactory-Guided Microbial Preferences and Foraging Decisions in Drosophila. Curr Biol 2017;27:2397–2404.e4. doi:10.1016/j.cub.2017.07.022.

[15] Jones JC, Fruciano C, Marchant J, Hildebrand F, Forslund S, Bork P, et al. The gut microbiome is associated with behavioural task in honey bees. Insectes Soc 2018;65:419–29. doi:10.1007/s00040-018-0624-9.

[16] Davidson GL, Raulo A, Knowles SCL. Identifying Microbiome-Mediated Behaviour in Wild Vertebrates. Trends Ecol Evol (Amst) 2020. doi:10.1016/j.tree.2020.06.014.

[17] Stuart KC, Shine R, Brown GP. Proximate mechanisms underlying the rapid modification of phenotypic traits in cane toads (*Rhinella marina*) across their invasive range within Australia. Biological Journal of the Linnean Society 2019;126:68–79. doi:10.1093/biolinnean/bly150.

[18] Wagener C, Mohanty NP, Measey J. The gut microbiome facilitates ecological adaptation in an invasive vertebrate. BioRxiv 2020. doi:10.1101/2020.12.10.418954.

[19] Colautti RI, Ricciardi A, Grigorovich IA, MacIsaac HJ. Is invasion success explained by the enemy release hypothesis? Ecol Lett 2004;7:721–33. doi:10.1111/j.1461-0248.2004.00616.x.

[20] Poulin R. Parasite manipulation of host behavior: an updateand frequently asked questions. Advances In The Study Of Behavior, vol. 41, Elsevier; 2010, p. 151–86. doi:10.1016/S0065-3454(10)41005-0.

[21] Tingley R, Ward-Fear G, Schwarzkopf L, Greenlees MJ, Phillips BL, Brown G, et al. New weapons in the toad toolkit: A review of methods to control and mitigate the biodiversity impacts of invasive cane toads (*rhinella marina*). Q Rev Biol 2017;92:123–49. doi:10.1086/692167.

[22] Finnerty PB, Shine R, Brown GP. Survival of the feces: Does a nematode lungworm adaptively manipulate the behavior of its cane toad host? Ecol Evol 2018;8:4606–18. doi:10.1002/ece3.3870.

[23] Shine R. The ecological impact of invasive cane toads (Bufo marinus) in Australia. Q Rev Biol 2010;85:253–91. doi:10.1086/655116.

[24] Llewelyn J, Phillips BL, Alford RA, Schwarzkopf L, Shine R. Locomotor performance in an invasive species: cane toads from the invasion front have greater endurance, but not speed, compared to conspecifics from a long-colonised area. Oecologia 2010;162:343–8. doi:10.1007/s00442-009-1471-1.

[25] Hudson CM, Vidal-García M, Murray TG, Shine R. The accelerating anuran: evolution of locomotor performance in cane toads (Rhinella marina, Bufonidae) at an invasion front. Proc R Soc Lond, B, Biol Sci 2020;287:20201964. doi:10.1098/rspb.2020.1964.

[26] Gruber J, Brown G, Whiting MJ, Shine R. Geographic divergence in dispersal-related

behaviour in cane toads from range-front versus range-core populations in Australia. Behavioral Ecology And 2017.

[27] Phillips BL, Kelehear C, Pizzatto L, Brown GP, Barton D, Shine R. Parasites and pathogens lag behind their host during periods of host range advance. Ecology 2010;91:872–81. doi:10.1890/09-0530.1.

[28] Child T, Phillips BL, Shine R. Abiotic and biotic influences on the dispersal behavior of metamorph cane toads (Bufo marinus) in tropical Australia. J Exp Zool A Ecol Genet Physiol 2008;309:215–24. doi:10.1002/jez.450.

[29] Selechnik D, Richardson MF, Shine R, DeVore JL, Ducatez S, Rollins LA. Increased adaptive variation despite reduced overall genetic diversity in a rapidly adapting invader. Front Genet 2019;10:1221. doi:10.3389/fgene.2019.01221.

[30] Brown GP, Kelehear C, Pizzatto L, Shine R. The impact of lungworm parasites on rates of dispersal of their anuran host, the invasive cane toad. Biol Invasions 2016;18:103–14. doi:10.1007/s10530-015-0993-1.

[31] Hudson CM, Brown GP, Shine R. Evolutionary shifts in anti-predator responses of invasive cane toads (Rhinella marina). Behav Ecol Sociobiol (Print) 2017;71:134. doi:10.1007/s00265-017-2367-4.

[32] Kelehear C, Jones HI. Nematode larvae (order Spirurida) in gastric tissues of Australian anurans: a comparison between the introduced cane toad and sympatric native frogs. J Wildl Dis 2010;46:1126–40. doi:10.7589/0090-3558-46.4.1126.

[33] Baker MR. The free-living and parasitic development of *Rhabdias* spp. (Nematoda: Rhabdiasidae) in amphibians. Can J Zool 1979;57:161–78. doi:10.1139/z79-014.

[34] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science 2018. doi:10.7287/peerj.preprints.27295v1.

[35] Douglas GM, Maffei VJ, Zaneveld J, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2: An improved and extensible approach for metagenome inference. BioRxiv 2019. doi:10.1101/672295.

[36] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; 2020.

[37] Davidson GL, Cooke AC, Johnson CN, Quinn JL. The gut microbiome as a driver of individual variation in cognition and functional behaviour. Philos Trans R Soc Lond B, Biol Sci 2018;373. doi:10.1098/rstb.2017.0286.

[38] Rollins LA, Richardson MF, Shine R. A genetic perspective on rapid evolution in cane toads (Rhinella marina). Mol Ecol 2015;24:2264–76. doi:10.1111/mec.13184.

[39] Shine R. The impact of an invasive amphibian: the cane toad Rhinella marina. In: Heatwole H, Rowley J, editors. Status of Conservation and Decline of Amphibians: Australia, New Zealand,

and Pacific Islands, Victoria, Australia: CSIRO Publishing; 2018, p. 107–24.

[40] Shine R, Brown GP, Phillips BL. An evolutionary process that assembles phenotypes through space rather than through time. Proc Natl Acad Sci USA 2011;108:5708–11. doi:10.1073/pnas.1018989108.

[41] Brown GP, Kelehear C, Shine R. The early toad gets the worm: cane toads at an invasion front benefit from higher prey availability. J Anim Ecol 2013;82:854–62. doi:10.1111/1365-2656.12048.

[42] Kosmala GK, Brown GP, Shine R. Thin-skinned invaders: geographic variation in the structure of the skin among populations of cane toads (Rhinella marina). Biological Journal of the Linnean Society 2020;131:611–21. doi:10.1093/biolinnean/blaa128.

[43] Weitzman CL, Kaestli M, Gibb K, Brown GP, Shine R, Christian K. Disease exposure and antifungal bacteria on skin of invasive cane toads, australia. Emerging Infect Dis 2019;25:1770–1. doi:10.3201/eid2509.190386.

[44] Zhou J, Tesfamicael K, Zhou SJ, Rollins LA, Rodriguez Lopez CM. Genetic similarity enhances the strength of the relationship between gut bacteria and host DNA methylation. BioRxiv 2021. doi:10.1101/2021.07.10.451923.

[45] Zhang X-Y, Sukhchuluun G, Bo T-B, Chi Q-S, Yang J-J, Chen B, et al. Huddling remodels gut microbiota to reduce energy requirements in a small mammal species during cold exposure. Microbiome 2018;6:103. doi:10.1186/s40168-018-0473-9.

[46] Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol 2017;19:29–41. doi:10.1111/1462-2920.13589.

[47] Gamage HKAH, Tetu SG, Chong RWW, Ashton J, Packer NH, Paulsen IT. Cereal products derived from wheat, sorghum, rice and oats alter the infant gut microbiota in vitro. Sci Rep 2017;7:14312. doi:10.1038/s41598-017-14707-z.

[48] Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu S, et al. The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. J Neuroinflammation 2012;9:153. doi:10.1186/1742-2094-9-153.

[49] Hugenholtz F, Davids M, Schwarz J, Müller M, Tomé D, Schaap P, et al. Metatranscriptome analysis of the microbial fermentation of dietary milk proteins in the murine gut. PLoS One 2018;13:e0194066. doi:10.1371/journal.pone.0194066.

[50] Kang D-W, Park JG, Ilhan ZE, Wallstrom G, Labaer J, Adams JB, et al. Reduced incidence of Prevotella and other fermenters in intestinal microflora of autistic children. PLoS One 2013;8:e68322. doi:10.1371/journal.pone.0068322.

[51] González-Bernal E, Brown GP, Shine R. Invasive cane toads: social facilitation depends upon an individual's personality. PLoS One 2014;9:e102880. doi:10.1371/journal.pone.0102880.

[52] Gelbard-Sagiv H, Magidov E, Sharon H, Hendler T, Nir Y. Noradrenaline modulates visual

perception and late visually evoked activity. Curr Biol 2018;28:2239–2249.e6. doi:10.1016/j.cub.2018.05.051.

[53] Houlden A, Goldrick M, Brough D, Vizi ES, Lénárt N, Martinecz B, et al. Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production. Brain Behav Immun 2016;57:10–20. doi:10.1016/j.bbi.2016.04.003.

[54] Ning T, Gong X, Xie L, Ma B. Gut Microbiota Analysis in Rats with Methamphetamine-Induced Conditioned Place Preference. Front Microbiol 2017;8:1620. doi:10.3389/fmicb.2017.01620.

[55] Choi JJ, Eum SY, Rampersaud E, Daunert S, Abreu MT, Toborek M. Exercise attenuates PCB-induced changes in the mouse gut microbiome. Environ Health Perspect 2013;121:725–30. doi:10.1289/ehp.1306534.

[56] Golla A, Østby H, Kermen F. Chronic unpredictable stress induces anxiety-like behaviors in young zebrafish. Sci Rep 2020;10:10339. doi:10.1038/s41598-020-67182-4.

[57] Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012;489:220–30. doi:10.1038/nature11550.

[58] West TP. Degradation of pyrimidine ribonucleosides by Pseudomonas aeruginosa. Antonie Van Leeuwenhoek 1996;69:331–5. doi:10.1007/bf00399622.

[59] Garavaglia M, Rossi E, Landini P. The pyrimidine nucleotide biosynthetic pathway modulates production of biofilm determinants in Escherichia coli. PLoS One 2012;7:e31252. doi:10.1371/journal.pone.0031252.

[60] Bashiri G, Antoney J, Jirgis ENM, Shah MV, Ney B, Copp J, et al. A revised biosynthetic pathway for the cofactor F420 in prokaryotes. Nat Commun 2019;10:1558. doi:10.1038/s41467-019-09534-x.

[61] Sibon OCM, Strauss E. Coenzyme A: to make it or uptake it? Nat Rev Mol Cell Biol 2016;17:605–6. doi:10.1038/nrm.2016.110.

[62] Varki A, Gagneux P. Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci 2012;1253:16–36. doi:10.1111/j.1749-6632.2012.06517.x.

[63] Liao H, Klaus C, Neumann H. Control of innate immunity by sialic acids in the nervous tissue. Int J Mol Sci 2020;21. doi:10.3390/ijms21155494.

[64] Sinnott-Armstrong N, Naqvi S, Rivas MA, Pritchard JK. GWAS of three molecular traits highlights core genes and pathways alongside a highly polygenic background. BioRxiv 2020. doi:10.1101/2020.04.20.051631.

[65] Brown GP, Shilton C, Phillips BL, Shine R. Invasion, stress, and spinal arthritis in cane toads. Proc Natl Acad Sci USA 2007;104:17698–700. doi:10.1073/pnas.0705057104.

[66] House PK, Vyas A, Sapolsky R. Predator cat odors activate sexual arousal pathways in brains of Toxoplasma gondii infected rats. PLoS One 2011;6:e23277.

doi:10.1371/journal.pone.0023277.

[67] Poulin R. Parasite manipulation of host behavior: an updateand frequently asked questions. Advances In The Study Of Behavior, vol. 41, Elsevier; 2010, p. 151–86. doi:10.1016/S0065-3454(10)41005-0.

[68] Gegear RJ, Otterstatter MC, Thomson JD. Bumble-bee foragers infected by a gut parasite have an impaired ability to utilize floral information. Proc Biol Sci 2006;273:1073–8. doi:10.1098/rspb.2005.3423.

[69] Pizzatto L, Shine R. Lungworm infection modifies cardiac response to exercise in cane toads. J Zool 2012;287:150–5. doi:10.1111/j.1469-7998.2012.00898.x.

[70] Finnerty PB. The physiological and ecological impacts of lungworm infection on invasive cane toads. Undergraduate thesis. University of Sydney, 2017.

[71] Abada EA, Sung H, Dwivedi M, Park B-J, Lee S-K, Ahnn J. C. elegans behavior of preference choice on bacterial food. Mol Cells 2009;28:209–13. doi:10.1007/s10059-009-0124-x.

[72] Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. Cell Res 2020;30:492–506. doi:10.1038/s41422-020-0332-7.

[73] Brown GP, Kelehear C, Shilton CM, Phillips BL, Shine R. Stress and immunity at the invasion front: a comparison across cane toad (*Rhinella marina*) populations. Biological Journal of the Linnean Society 2015;116:748–60. doi:10.1111/bij.12623.

[74] Hudson CM, Brown GP, Shine R. Evolutionary shifts in anti-predator responses of invasive cane toads (Rhinella marina). Behav Ecol Sociobiol (Print) 2017;71:134. doi:10.1007/s00265-017-2367-4.

[75] Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J 2010;4:232–41. doi:10.1038/ismej.2009.112.

[76] Fan P, Bian B, Teng L, Nelson CD, Driver J, Elzo MA, et al. Host genetic effects upon the early gut microbiota in a bovine model with graduated spectrum of genetic variation. ISME J 2020;14:302–17. doi:10.1038/s41396-019-0529-2.

[77] Arnaud-Haond S, Aires T, Candeias R, Teixeira SJL, Duarte CM, Valero M, et al. Entangled fates of holobiont genomes during invasion: nested bacterial and host diversities in Caulerpa taxifolia. Mol Ecol 2017;26:2379–91. doi:10.1111/mec.14030.

Tables and Figures

Table 1a-d. The association between a) single host factors and microbial community, b) single host factors and predicted microbial function, c) single behavioural trait and microbial community and d) single behavioural trait and predicted microbial function. Significant p-values denoted by an asterisk.

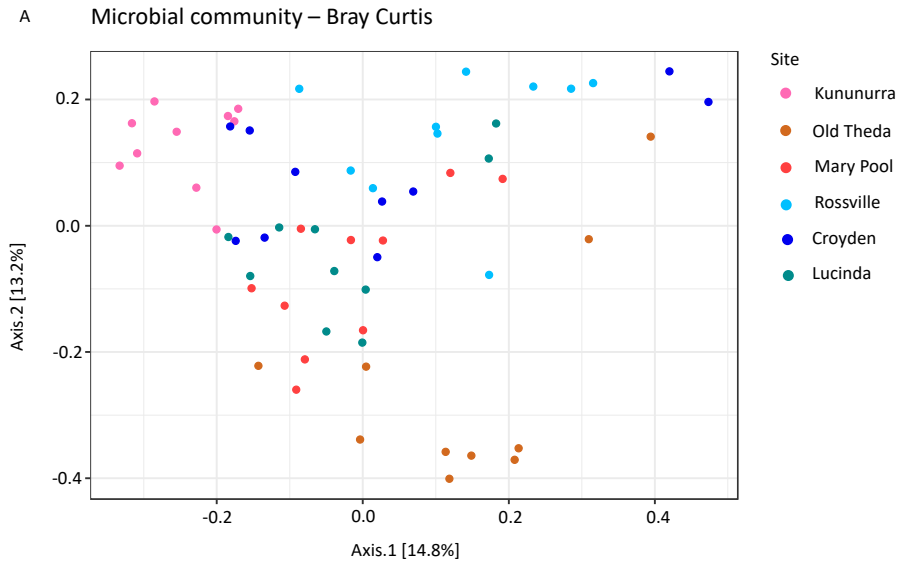
	MDS1	MDS2	r2	Pr(>r)
a. Host factor/microbial community				
SUL	0.170	0.985	0.071	0.119
BodyWeight	-0.009	1.000	0.051	0.231
Lungworms	0.254	-0.967	0.020	0.585
Occurrence of lungworms	0.453	-0.892	0.128	0.023*
b. Host factor/microbial function				
SUL	0.680	0.733	0.021	0.556
BodyWeight	0.610	0.793	0.041	0.311
Lungworms	-0.791	0.612	0.007	0.839
Occurrence of lungworms	-0.827	-0.562	0.059	0.187
c. Behavioural trait/microbial community				
Struggle score	-0.814	0.580	0.044	0.268
Struggle likelihood	-0.880	-0.474	0.021	0.555
Righting effort	-0.124	0.992	0.075	0.121
Righting effort likelihood	0.499	0.867	0.0255	0.474
Righting time	0.933	0.360	0.0615	0.174
d. Behavioural trait/microbial function				

Struggle score	0.376	0.927	0.051	0.221
Struggle likelihood	0.183	0.983	0.017	0.606
Righting effort	0.571	0.821	0.070	0.141
Righting effort likelihood	0.399	0.917	0.035	0.362
Righting time	-0.956	-0.294	0.004	0.894

Table 2. Association between gut microbiota variation and lungworm occurrence and intensity, based on Bray Curtis dissimilarity values for microbial community assemblages.

	Df	SumOfSqs	R2	F	Pr(>F)
Location	1	1.314	0.091	6.026	<0.001***
Lungworm_occurrence	1	0.482	0.033	2.213	0.005**
Lungworms_intensity	1	0.150	0.010	0.688	0.861
Location:Lungworm_occurrence	1	0.331	0.023	1.520	0.075
Location:Lungworm_intensity	1	0.357	0.025	1.638	0.043*
Residual	54	11.774	0.817		
Total	59	14.409	1.000		

Significance codes: 0 '***' ≤0.001 '**' ≤0.01 '*' ≤0.05.



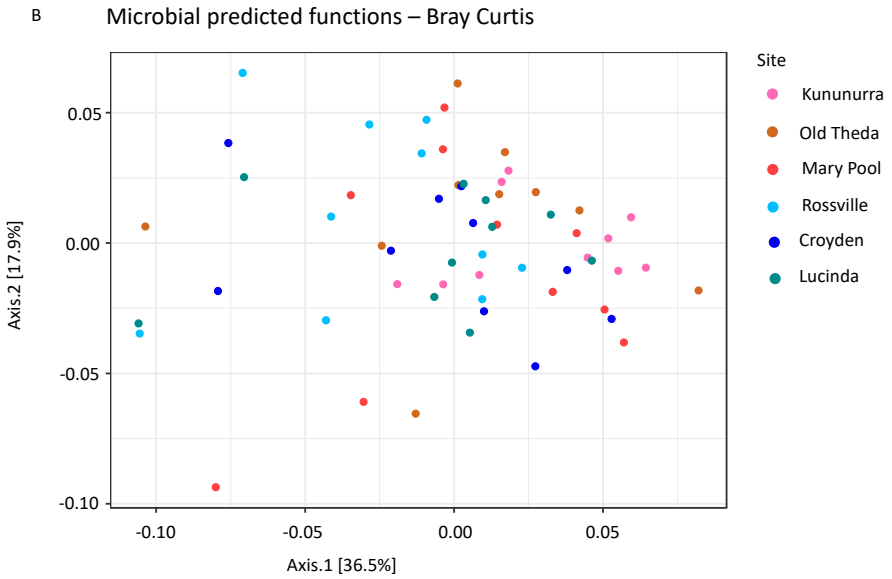


Figure 1. Beta diversity by location. Principle coordinate analysis plot of Bray Curtis distance of microbial community (A) and Bray Curtis distance of predicted functional groups (B) from 60 cane toad individuals of the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda).

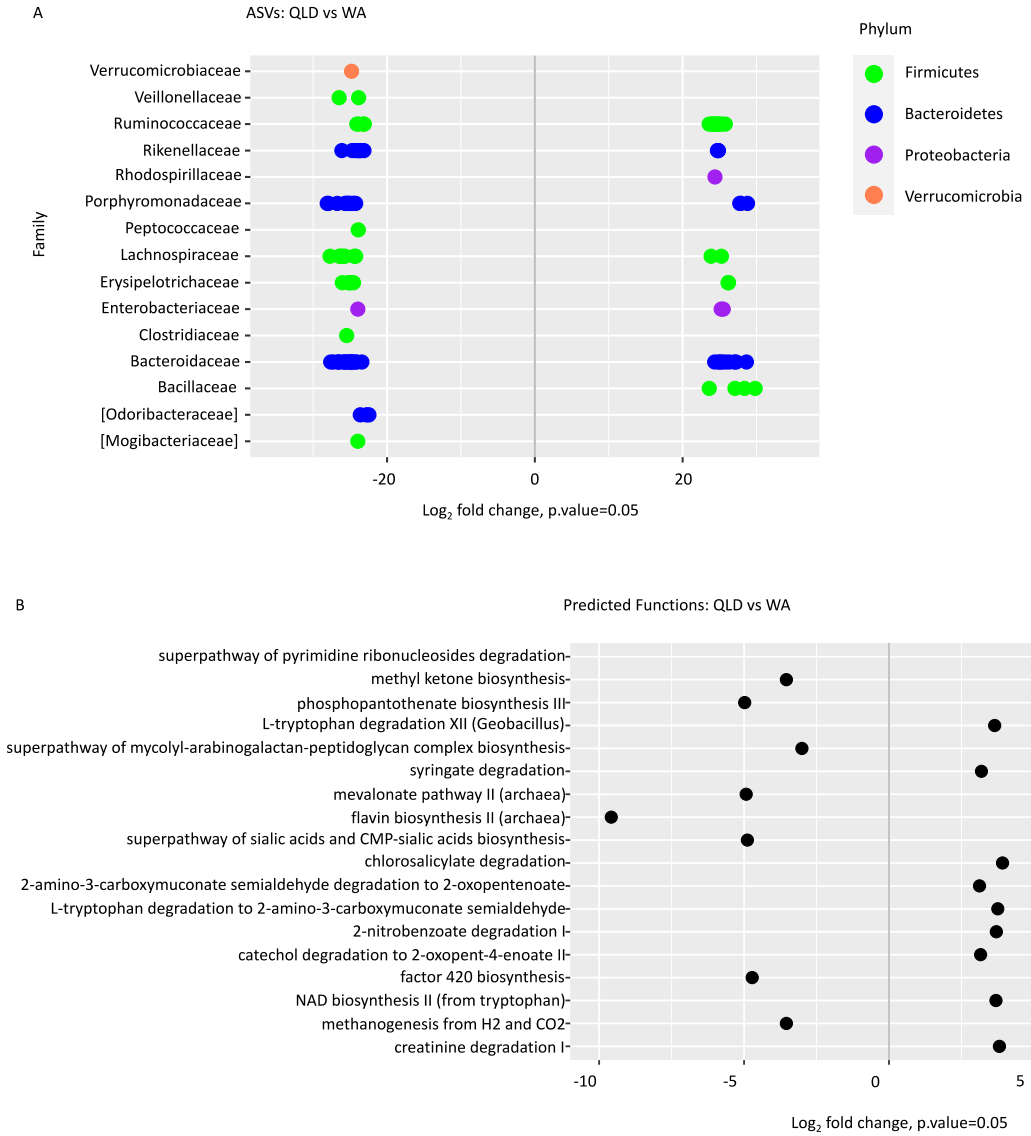


Figure 2. Significantly different bacterial taxa and predicted functions between range-core (QLD) and invasion-front (WA) toads' colon. Significant differences were identified between locations via differential abundance testing based on a negative binomial distribution. The dots represent the average log-2 fold change (x axis) abundance and positive log_2 fold changes signify increased abundance in range-core, and negative log_2 fold changes display increased abundance in invasion-front. Bacterial taxa (A) were classified to the taxonomic level of family (y axis) and coloured by taxonomic level of phylum. Family name in bracket is proposed taxonomy by Greengenes. Only ASVs that could be matched to a known bacterial family and with a log2FoldChange value higher than 20 or lower than -20 are presented. Predicted functions (B) with a log2FoldChange value higher than 3 or lower than -3 are presented.

Figure 3. Heatmap for top 30 functional group abundance.

Heatmap indicates the top 30 functional groups in the intestinal samples from range-core and invasion-front toads. Abundance indicates the raw count of functional groups inferred from taxonomic 16S sequences using PICRUSt where light blue is high abundance and dark blue is lower abundance. Functional pathways that differ significantly between range-core and invasion-front toads are highlighted in bold. Range-core includes Rossville, Croyden, and Lucinda; invasion-front includes Kununurra, Old Theda, and Mary Pool.

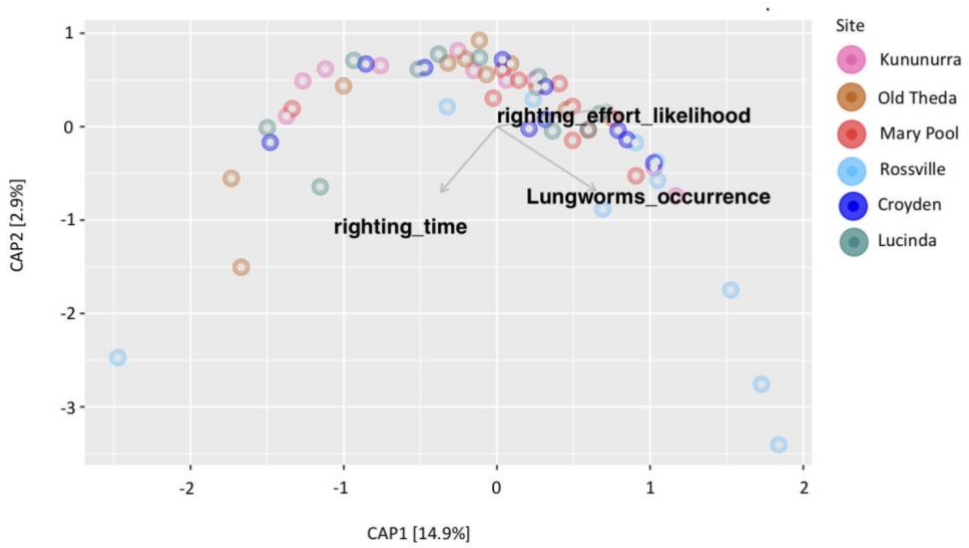


Figure 4. Main variables that affect predicted function differentiation among individuals. CAP (*capscale*) plot displays the combination of variables that explained the greatest variation in the predicted functions through model selection, using 60 cane toad individuals from the invasion-front (Kununurra, Old Theda, and Mary Pool) and the range-core (Rossville, Croydon, and Lucinda). The final model explained 17.8% of variation in the microbial predicted functions, which includes righting effort likelihood (AIC = 53.613), occurrence of lung worms (AIC = 54.297) and righting time (AIC = 56.912) explained the greatest variation.