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First line of defence: Skin microbiota may protect anurans from infective larval lungworms

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ABSTRACT

Functional roles of the rich microbiota of the skin are not fully understood, but include protection against microbial diseases and other environmental challenges. In experimental studies, we show that reducing the microbiota from cane toad (*Rhinella marina*) skin by gently wiping with absorptive gauze resulted in threefold higher rates of infection by lungworms (*Rhabdias pseudosphaerocephala*) following standardised exposure to infective skin-penetrating larvae. Higher concentrations of microbial DNA were associated with lower rates of lungworm entry. Our data suggest that microbial activity on the anuran skin comprises an important line of defence against attack by macroparasites as well as by fungi and other microbes.

1. Introduction

The outer surfaces of most organisms are covered by a complex array of smaller life forms that may play critical functional roles in maintaining homeostasis and in protecting the organism from environmental challenges (McFall-Ngai et al., 2013; Walke and Belden, 2016; Knutie et al., 2017). Surprisingly, there is little detailed understanding of how, and to what extent, host-associated microbial communities provide benefits for the hosts (Adair and Douglas, 2017; Rebollar et al., 2020) even though such symbiotic relationships are likely the norm (McFall-Ngai et al., 2013).

The moist skin of amphibians supports a diverse array of bacteria and this microbiota is unique to each species, even in cases where different species live in the same environment (Kueneman et al., 2014; Walke et al., 2014; Christian et al., 2018). Correlations between composition of the microbiota and external challenges (e.g., the presence of specific pathogens) suggest that the microbiota may confer protection against pathogenic organisms such as fungi and other microorganisms such as protists (Jani and Briggs, 2014; Federici et al., 2015; Kueneman et al., 2016; Walke and Belden, 2016; Knutie et al., 2017; Ruthsatz et al., 2020). Furthermore, some specific microbes have been identified as conferring protection (Woodhams et al., 2015; Weitzman et al., 2019).

A shielding effect against macroparasites also is plausible and would be advantageous, given that parasites, like microbial pathogens, may massively reduce viability of the host (Kelehear et al., 2011). Several metazoan parasites infect amphibians by penetrating through the skin (e.g. nematodes- *Rhabdias* sp., *Aplectana* sp.; trematodes- *Ribeiroira* sp.) and may also be susceptible to anti-pathogenic effects of the skin microbiota (Knutie et al., 2017; Rebollar et al., 2020). Disruption of the gut and skin microbiota of tadpoles influenced the susceptibility of adult frogs to infection by a skin-penetrating gut parasite, presumably by priming the immune system against parasite exposure later in life (Knutie et al., 2017). However, the adult microbiota offered no protection against the parasites at the time of exposure (Knutie et al., 2017).

The lungworm *Rhabdias pseudosphaerocephala*, brought to Australia with invasive cane toads (*Rhinella marina*) in 1935 (Dubey and Shine, 2008; Pizzatto et al., 2012), can substantially reduce rates of growth and survival of its anuran host at all terrestrial life stages (Kelehear et al., 2011; Brown et al., 2016; Finnerty et al., 2018). Cane toads exhibit a diverse and geographically variable microbiota on the skin (Christian et al., 2018; Weitzman et al., 2018, 2019). To test the hypothesis that the skin microbiota repels attack by infective lungworm larvae, we compared toads exposed to infective lungworm larvae after removing some of the skin microbes (disturbed microbiota) with toads exposed to

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infective lungworm larvae after a sham-removal of microbes (undisturbed microbiota).

2. Material and methods

2.1. Ethical approval

This work was done with approval from the Animal Ethics Committees of Charles Darwin University (A20002) and the University of Sydney (2019/1489).

2.2. Animals

We used captive-reared progeny from three clutches produced by adult cane toads that were wild-caught on the Mitchell Plateau in Western Australia. Toads were induced to spawn using injections of artificial gonadotrophins (Brannelly et al., 2019). 100 tadpoles from each clutch were reared in 70-L pools of water and fed daily on commercial fish flakes. At metamorphosis, toadlets were individually marked and reared separately by clutch in semi-natural outdoor enclosures. At the time of the experiment the toads in two of the clutches were 9 months of age and the toads in the third clutch were 1 month of age. The sample size and mean snout-urostyle length (mm) (\pm SE) of the 34 toads used in the experiment from the three clutches were: $n = 24$, 69.8 ± 1.1 ; $n = 6$, 64.5 ± 6.2 ; and $n = 4$, 35.3 ± 3.4 . Toads were assigned to groups such that each clutch was equally represented in the group with the disturbed microbiota (partially removed from the skin) and the group with the undisturbed microbiota (fully intact at the time the toads were exposed to the lungworms). The mean body sizes of toads assigned to the two groups did not differ significantly (overall range 27.8–95.3 mm snout-urostyle length, $F = 0.44$, $DF = 1,32$, $p = 0.51$).

2.3. Experimental treatments

The steps involved in the experimental treatment of two groups of toads are illustrated in Fig. 1. To control for the effects of handling, the individuals in group 1 (undisturbed microbiota, $n = 17$) were gently rinsed with sterile water and the entire dorsal surface was wiped by a

gloved hand (using a fresh pair of latex gloves for each toad) for 2 min. The latex gloves represent a non-absorptive control for the wiping action that was deemed less likely to disturb (or remove) the microbiota than a swab. The toads were then inoculated with lungworm larvae by adding 200 μ l of water containing infective larvae (50 larvae for toads > 50 mm SVL, $n = 30$ toads; 20 larvae for toads < 50 mm SVL, $n = 4$ toads) to a 2×2 cm square of paper towel placed onto the anuran's back. The *Rhabdias* larvae were obtained from faeces produced by captive toads originating from Kununurra, Western Australia. The skin microbiota of group 1 (undisturbed microbiota, $n = 17$) toads was sampled by swabbing with a sterile synthetic swab (FLOQSwabs 552C, Copan Flock Technologies, Brescia, Italy) 48 h after inoculation. The dorsal surface of each toad was sampled by 30 swipes of the swab (Christian et al., 2018).

The toads in group 2 (disturbed microbiota, $n = 17$) were gently rinsed with sterile water then swabbed (as above) to obtain a pre-removal measure of the microbiota. The microbiota was then partially removed by wiping with sterile cotton gauze (30 swipes, 2 min handling time) and the microbiota was immediately re-swabbed, to determine the extent of removal of microbiota. Toads were then inoculated with lungworm larvae as above.

Toads and their infective larvae were held overnight in individual 1-L plastic containers. We washed out the infection containers the following morning, and counted the number of remaining larvae (i.e., those that had failed to penetrate the toads). Because 19 toads defecated in their chambers during trials and the water was too murky to see the larvae, we could only count the number of larvae remaining for the other 15 toads. Following infection, all toads were housed in individual 15-L containers lined with newspaper and provided with a shelter tube and a water dish. Toads were held post-infection for 18 days in a shaded building exposed to ambient temperature and fed crickets 3 times each week, then humanely euthanized with an overdose of Lethobarb (Virbac (Australia) Pty Limited). They were then dissected, and their lungs were inspected so that the lungworms could be counted.

2.4. Microbial sample analysis

Swabs were stored overnight at -20 °C before the microbial DNA was extracted using a Norgen 64100 Microbiota DNA Isolation Kit

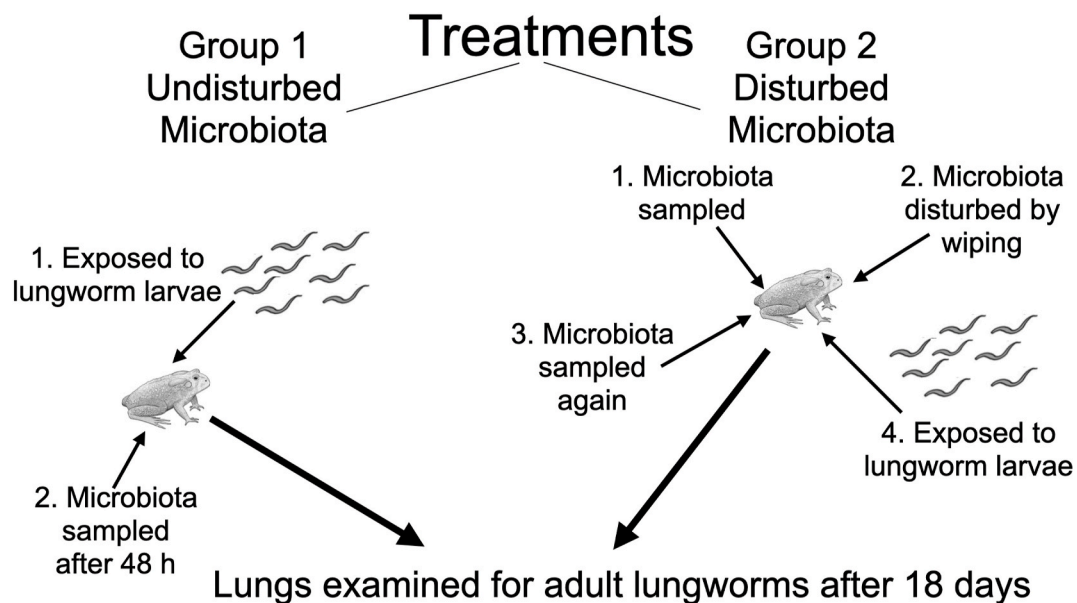


Fig. 1. The steps involved in the two experimental treatments. The undisturbed skin of group 1 toads was swabbed for microbes 48 h after being exposed to lungworm larvae so that the swabbing process did not disturb the microbiota before exposure. The skin of group 2 toads was swabbed before and after being wiped with sterile gauze so that the efficacy of this disturbance or “cleaning” action could be evaluated. Group 2 toads were exposed to lungworm larvae after the second swab.

following the manufacturer's protocol. Primers 515-F (GTGY-CAGCMGCCGCGGTAA) and 806-R (GGACTACNVGGGTWTCTAAT) amplified the 16S rRNA gene (Apprill et al., 2015) using the qPCR Rotor-Gene Q Software 2.3.1.49. DNA concentrations (ng/ μ L) were quantified with a standard curve generated from one of the samples.

2.5. Microscopic skin examination

To determine if wiping the toads with a sterile cotton gauze affected the skin differently from wiping with a gloved hand, we compared the histology of the skin following the two wiping techniques (cotton gauze and gloved hand). Five toads similar in size to the toads used in experimental infections were wiped on their left dorsal surface for 2 min, using the microbiota removal technique described above (using cotton gauze). The right dorsal surface of each toad was wiped for 2 min using the sham wiping technique described above (using a latex gloved hand). Immediately after wiping, toads were euthanized with an intracoelomic injection of Lethobarb. Two 1-cm-square sections of skin, one from the lateral body and one from the dorsal body, were excised from each of the left and right swabbed surfaces, fixed in 10% formalin, processed in standard fashion for histology and stained with haematoxylin and eosin.

Prepared slides of the skin were blindly assessed for alterations in the skin surface, epidermis, dermis and dermal glands. The following characteristics were semi-quantitatively assessed: thickness of the stratum corneum (as a proportion of the epidermis), compactness of the stratum corneum (whether there was delamination or fraying in the keratin), number of cell layers in the epidermis and degree of disorganization of epidermal cells (the latter two parameters were used to assess erosion or other damage to the epidermis).

2.6. Statistical analysis

We used an unpaired t test, accounting for unequal variance (Welch t test), to compare the undisturbed skin microbial DNA concentration (ng/ μ L) in group 1 toads with that of group 2 toads before they were wiped. To quantify the extent of microbiota removal in group 2 toads, we used a paired t test, accounting for unequal variance, to compare the microbial DNA concentration (ng/ μ L) before and after wiping with sterile gauze. We used a quasi-binomial model to test for proportional differences between groups 1 and 2 in the numbers of established worms in their lungs after 18 days, after accounting for total number of larvae to which each toad was exposed. An overdispersion parameter was fitted which was estimated to be above 1.

The relationship between the proportions of worms recovered in the lungs and the DNA concentration of the skin microbiota was examined using nonparametric Spearman's correlation and a quasi-binomial model with lungworm proportional establishment success as outcome and DNA concentration (log-2 transformed) as the predictor variable.

To compare variables scored from histological examinations of skin swabbed by the two techniques on five toads, we used Wilcoxon signed-rank tests on the matched paired data.

3. Results

3.1. Microbial DNA concentrations

The initial, undisturbed skin microbial DNA concentration (ng/ μ L) was similar for group 1 (3.08 ng DNA/ μ L) and group 2 toads (1.52 ng DNA/ μ L, unpaired Welch t test, $t = 1.5$, $p = 0.16$). Wiping with sterile gauze removed much of the microbiota from group 2 toads, creating a fivefold difference in mean microbial DNA concentrations before and after the wiping treatment (1.52 vs 0.30 ng DNA/ μ L, $t = 5.1$, $p < 0.0001$).

3.2. Lungworm infection

More larvae crossed the skin barrier and entered the toads whose skin had been wiped prior to infection (10.1% remaining in the container, SE = 2.8) compared to conspecifics with an intact microbiota (20% remaining, SE = 2.9; $F_{1,13} = 5.3$, $p = 0.038$). When dissected 18 days post-infection, two toads with an intact microbiota did not have adult *Rhabdias* in their lungs, and the other 32 toads each had 1 to 23 worms. Thus, infection success ranged from 0% to 95% (0/50 larvae to 19/20 larvae). More larvae established in the lungs of toads whose skin had been wiped prior to infection (28.9%, SE = 5.6) compared to conspecifics with an intact microbiota (9.9%, SE = 2.1; $p = 0.003$; Fig. 2). This result is unchanged if the younger toads from the third clutch are excluded from the analysis ($p = 0.003$). The odds of a larva establishing in the lungs averaged 3.3 times higher following disturbance to the skin microbiota (95% CI 1.6 to 7.0). The proportion of lungworms that established in lungs was negatively correlated with concentration of skin microbial DNA (Spearman's rho = -0.40, $p = 0.021$). For every doubling of the DNA concentration of the skin microbiota, the odds that a larva would successfully establish in the lungs decreased by 12% ($p = 0.049$).

3.3. Microscopic skin examination

The two wiping techniques (gauze versus a latex glove) did not result in histologically detectable differences in the structure of the skin, with no overt alterations in the surface stratum corneum, and no evidence of epidermal structural alteration or necrosis (Fig. 3). Corneum thickness (as a proportion of epidermal thickness) of all sections examined was identical between the two techniques (0.25, Wilcoxon signed rank S = 0.00, $p = 1.00$). The epidermis was, on average 3.35 cell layers thick both after microbiota-removal wiping and after sham wiping with the gloved hand (Wilcoxon signed rank S = 1.00, $p = 1.00$). Our scores of keratin compactness and epithelial cell jumbling were also indistinguishable between the wiping methods (both Wilcoxon signed rank S > 7.00, $p > 0.13$). Thus, there was no evidence of physical disruption of

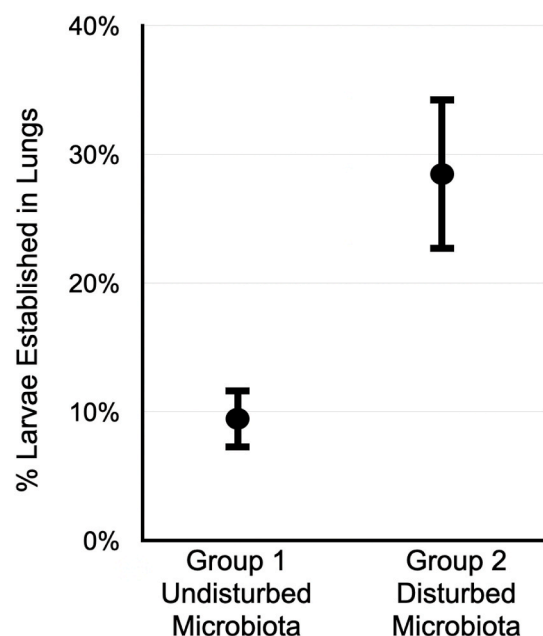


Fig. 2. The consequences of inoculation of cane toads with larval lungworms, as a function of whether the toads had an undisturbed skin microbiota (group 1) or a disturbed (partially removed) skin microbiota (group 2). Establishment success of the lungworms was measured by the mean percentage \pm SE of the larva that established themselves as adults in the lungs after 18 days.

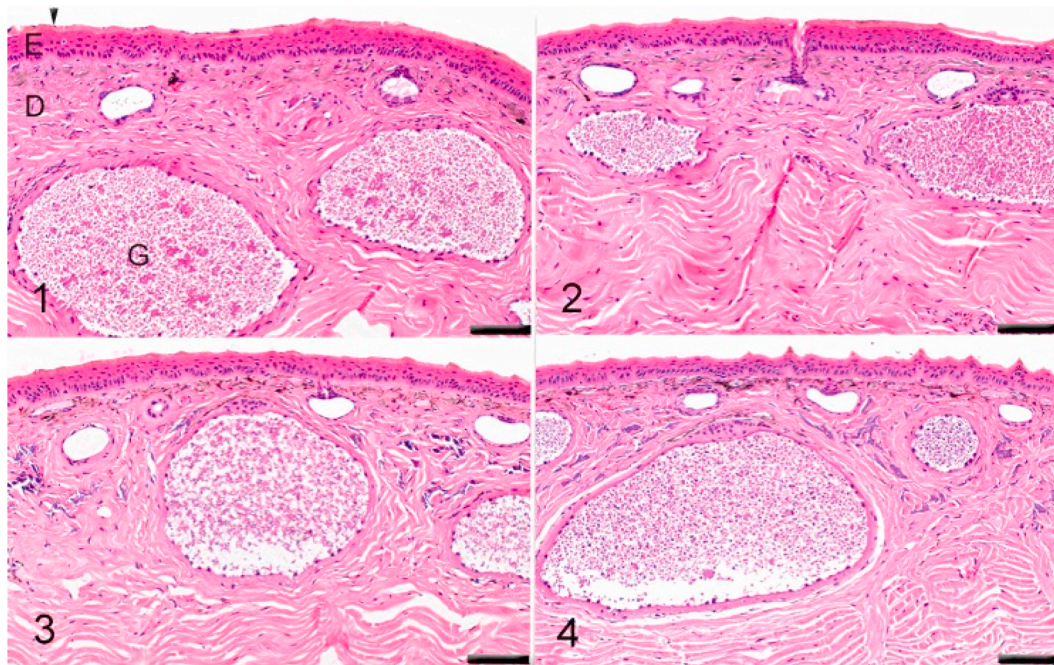


Fig. 3. Histological photomicrographs of toad skin. Skin sections from the lateral body of toad A are depicted in images 1 (sham wiping with a gloved hand) and 2 (microbiota removal technique using sterile cotton gauze). Skin sections from the dorsal body of toad B are depicted in images 3 (sham) and 4 (microbiota removal technique). Notations identifying major skin structures are shown in image 1: E, epidermis, D, dermis, G, large granular gland and stratum corneum (arrowhead). Haematoxylin and eosin stain. Bar = 100 μ m in all images.

the skin, such as architecture changes, erosion, ulceration, or haemorrhage, to indicate that the rubbing procedure damaged the skin.

4. Discussion

Although previous studies have demonstrated that the skin microbiota of adult anurans can protect the host from infection by pathogens such as fungi and other microorganisms (McFall-Ngai et al., 2013; Jani and Briggs, 2014; Federici et al., 2015; Kueneman et al., 2016; Walke and Belden, 2016; Knutie et al., 2017; Weitzman et al., 2019), our data provide the first evidence that the microbiota can also provide protection against macroparasites (lungworms). Our experimental design did not allow us to distinguish if characteristics of the undisturbed skin impair the ability of parasite larvae to penetrate the host's body or affect the viability of larvae that succeed in penetrating. For example, components of the skin microbiota might either attack the lungworm larvae directly, or affect them in a way (e.g., by coating them with specific markers) that makes larvae more easily detectable by the host's immune system as the larva migrates towards the lungs.

Our experimental design manipulated the abundance of microbiota on the host's skin, without causing any overt change to the underlying epidermis (Fig. 3). It would be of interest to know how quickly an anuran can rebuild its skin microbiota after disturbance. Future work could also identify the roles of specific skin microbes and associated gene functions in conferring resistance to macroparasite attack.

Our simple experimental design does not allow us to completely exclude the possibility that the wiping technique disrupted other skin characteristics in addition to the microbiota, such as peptides produced by the toad skin (Rollins-Smith et al., 2011). However, the negative correlation between microbiota concentration (as quantified by DNA assays) and lungworm infection is consistent with the notion that the microbiota acts as a shield against macroparasites. Nevertheless, if skin wiping affected other skin characteristics (e.g., peptides from skin glands) proportionately to the disruption of the microbiota, then a similar result would be expected. More complex experimental designs, involving inoculations of toads from different regions with different

microbial communities or matching specific metabolites with resistance to infection would shed light on the relative roles of the microbiota and the toad skin.

Although previous work found that the skin microbiota on adult frogs did not protect them from a skin-penetrating gut parasite (Knutie et al., 2017), our results suggest that the skin microbiota of cane toads may confer protection against a skin-penetrating lungworm parasite. If so, the skin microbiota would represent an effective external component of the immune system against a wide range of pathogenic organisms.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2021.02.014>.

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