

RESPONSE

Innate immune defenses of amphibian skin: antimicrobial peptides and more

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Chytridiomycosis is an emerging disease of amphibians with global impact (Skerratt *et al.*, 2007). Innate immune defenses may contribute to the ability of some species to coexist with endemic *Batrachochytrium dendrobatidis* (*Bd*) (Retallick, McCallum & Speare, 2004; Woodhams *et al.*, 2007b). We appreciate the thoughtful commentaries by Fisher (2007), Garner (2007) and Kurtz & Scharsack (2007) to the paper 'Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses' (Woodhams *et al.*, 2007a). The commentators endorsed the ecological immunology approach of the work and its utility for informing conservation. They included excellent suggestions for future studies including the following:

- (1) Examining population, family and individual level variation in amphibian immune defenses.
- (2) Testing for mucosal antibodies, immune memory and induction of innate defenses in addition to constitutive innate defenses.
- (3) Determining how behavioral differences among species affect the rate of encountering the pathogen and response to encounter.
- (4) Understanding how environmental conditions affect innate immunity.

The commentators pointed out that immunity can vary among lineages of frogs (Pearman & Garner, 2005) and stickleback fish (Rauch, Kalbe & Reusch, 2006), suggesting that some of the among-species differences we detected might be due to differences among lineages within species. This is true, however, as the work cited by the commentators illustrates, fully exploring variation in disease susceptibility within even a single species requires very large, complex experiments. For our initial exploration of variation among species, we chose to use single sibships to minimize within-species variation, maximizing the power of our comparisons among species. This also minimized the probability of chance differences in peptide expression between infected and control frogs. This was advantageous because we could collect peptides only from uninfected control frogs, because

depletion caused by norepinephrine induction could have affected the survival of infected frogs.

We agree that much remains to be investigated in terms of variation in innate immunity among sibships and populations; however, it seems likely that variation of skin peptide defenses among species is greater than within-species variation. For example, Apponyi *et al.* (2004) found substantial variation on a large geographic scale in the composition of skin peptides profiled within two species of Australian treefrogs, and also showed that most species produced profiles containing distinctive families of peptides, and that on smaller geographical scales profiles did not differ among individuals within species. Our own studies of peptide variation among individuals of two populations of *Rana muscosa* in the Sierra Nevada Mountains of California suggest very limited variation in skin peptides (Woodhams *et al.*, 2007b). This suggests that sibship-level differences in skin peptide defenses are not likely to have affected the results of this study in which four species from the same region were compared.

Some antimicrobial peptides are constitutive and others may be induced in response to infection (Boman, Nilsson & Rasmuson, 1972; Zasloff, 2002; Cunliffe & Mahida, 2004; Izadpanah & Gallo, 2005). Here, peptide induction has two meanings: transcription and storage in glands or secretion from glands onto the skin. We did not test for induction of skin peptides in response to *Bd* exposure in this study. However, the constitutive peptide defenses may be a good measure of immune condition before *Bd* exposure. Any induction of new skin peptides that may occur in response to *Bd* infection may not be effective because chytridiomycosis leads to mortality of susceptible species between 18 and 48 days post-infection (Berger *et al.*, 2004; Woodhams *et al.*, 2007a), and production of peptides in granular glands after discharge can take as long as 6 to >21 days (Flucher *et al.*, 1986; Giovannini *et al.*, 1987; Rollins-Smith & Conlon, 2005). Of course the quantity of peptides released from the granular glands after administration of norepinephrine is

not the same as that which infectious *Bd* zoospores are likely to encounter on the skin of a frog in nature. Additional studies are needed to determine the mechanisms of peptide release onto the skin and the degree to which constitutive peptides are effective against *Bd in vivo*. Therefore, although the natural mixtures of skin peptides from all four species were capable of inhibiting *Bd in vitro*, other factors probably contribute to induction of skin defense and species-specific survival rates.

In addition to skin peptides, we found that populations of circulating neutrophilic and eosinophilic granulocytes decreased after exposure to *Bd* in *Litoria chloris*, and suggested that they may migrate toward foci of degeneration in the epidermis. Although inflammation appears to occur in approximately one-third of cases in species that are susceptible to chytridiomycosis (Nichols *et al.*, 2001; Berger, Speare & Skerratt, 2005), perhaps it is more common in resistant species. We did not histologically examine infected *L. chloris* skin for inflammation.

Our treatment regime for studies of leukocytes included housing frogs in groups to ensure constant exposure to *Bd* from contact with other infected individuals. It is possible that common environmental factors may have affected groups of frogs housed together. However, given that our results did not show any effects of our deliberate environmental manipulation of temperature on leukocyte populations, it seems unlikely that the much smaller differences between containers produced the differences we observed. It also seems unlikely that any direct influences of frogs on one another, other than reinforcement of infection by transmission of zoospores, produced correlations among individuals within containers. Garner (2007) suggested sham-infecting frogs with filtered media in which *Bd* was previously grown for a negative control. This might be an interesting experimental treatment, but it is not clear whether it would be a better control treatment. If *Bd* produced toxins or other compounds that could elicit an immune response, then 'control' frogs so treated would not be true controls. In any case, it now appears that *Bd* causes death via electrolyte depletion, resulting in osmotic imbalance (Voyles *et al.*, 2007), rather than by dehydration or toxicity.

Disease ecology is still a young field. Defense mechanisms of amphibians against both resident and invading pathogens are not well understood. As a first indication of adaptive immunity to *Bd*, Kurtz & Scharsack (2007) suggest testing whether survivors of infection are less susceptible upon repeat exposure, which may indicate immune memory. It may also indicate that the innate immune mechanisms that originally prevented mortality are still in effect. Indeed, Kurtz & Scharsack (2007) suggest that additional mechanisms or immune factors might contribute to extreme differences in survival among species. We certainly agree. For example, behavioral differences among species may affect rates of transmission (Rowley & Alford, 2007a,b) or survival (e.g. Lips, Reeve & Witters, 2003). Ongoing experiments in the Rollins-Smith laboratory have demonstrated the development of a robust antibody response to *Bd* in *Xenopus laevis* following immunization. On the list of questions to be

addressed are whether antibodies to *Bd* can be detected in the mucus, and whether this response inhibits chytridiomycosis.

Recent evidence suggests that natural microbiota may also be important in preventing disease in amphibians (Austin, 2000; Harris *et al.*, 2006; Lauer *et al.*, 2007; Woodhams *et al.*, 2007b). Some species or assemblages of microbiota may act as mutualists by preventing infection by pathogenic organisms or by preventing other members of the microbiota from proliferating and causing disease (Belden & Harris, 2007). If mutualistic microbiota are an extension of the host innate immune system, rather than representing chance colonization from the host's environment, the following conditions are predicted to apply:

- (1) Individual hosts that are resistant to a disease (i.e. chytridiomycosis) should have a greater abundance or diversity of symbiotic microbes that benefit the host by resisting pathogen colonization or inhibiting growth. For example, within a *Bd*-infected host population, individuals with low-intensity infection or no infection should carry *Bd*-inhibiting symbionts or communities.
- (2) Populations of hosts that are resistant to chytridiomycosis should have a higher proportion of individuals with *Bd*-inhibiting microbiota than susceptible populations (Woodhams *et al.*, 2007b).
- (3) After removal of symbiotic microbiota, hosts should be more susceptible to pathogen infection and disease development.
- (4) Addition of mutualistic microbiota to 'clean' or microbiota-reduced amphibians should reduce their susceptibility to pathogen colonization and disease.
- (5) The host immune defenses should interact with the microbiota such as to reduce potential pathogens but not eliminate the mutualistic symbionts. For example, antimicrobial skin peptides may be upregulated and selectively reduce the microbiota. (In a preliminary experiment, we found skin-associated anti-*Bd* microbiota that could resist high concentrations of antimicrobial peptides, see Fig. 1.)

Many of the above predictions have been tested in coral reef systems in which microbial symbionts are thought to confer benefits that are particularly important because corals do not have vertebrate-type immune systems (Reshef *et al.*, 2006). This was used as support for the 'hologenome theory of evolution' (Rosenberg *et al.*, 2007). The hologenome (the combined genomes of host and symbionts) is thought to change with environmental conditions more quickly than the host genome alone and allows the combined holobiont organism greater adaptive potential. Changing environmental conditions are often cited as possible causes of amphibian population declines (Kiesecker, Blaustein & Belden, 2001; Pounds *et al.*, 2006; Fisher, 2007). Belden & Harris (2007) suggest that abiotic and biotic environmental changes may disrupt the natural microbiota and lead to disease outbreaks. The hologenome theory suggests that evolutionary responses to such disruptions may also be rapid. Therefore, knowledge of microbial interactions with host immune defenses, mechanisms of skin peptide induction and conditions under which stable or even host-protective associations occur are important to our

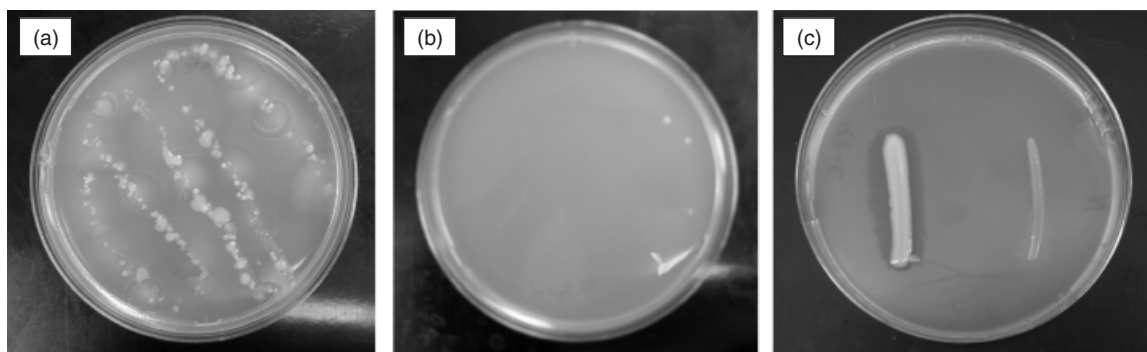


Figure 1 A preliminary analysis of *Rana pipiens* skin bacteria swabbed before (a) and after (b) secretion of AMPs demonstrates that AMPs inhibit many, but not all, culturable bacteria. The skin of 16 *R. pipiens* (BioCorporation, Alexandria, MN, USA) were rinsed to remove transient bacteria (Lauer *et al.*, 2007), and swabbed using a sterile technique before and after inducing skin peptides with a subcutaneous injection of 40 nmol per gram body weight (gbw) norepinephrine-bitartrate (Sigma-Aldrich Co., St. Louis, MO, USA). After inducing skin peptides ($1\text{--}2\text{ mg gbw}^{-1}$), few bacteria remained. These remaining bacteria were isolated and co-cultured with *Batrachochytrium dendrobatidis* (*Bd*) as described in Harris *et al.* (2006). (c) A co-culture assay testing the ability of *Serratia marcescens* (left streak) to inhibit the growth of *Bd* (zoospores plated as lawn). The control streak on the right side of the plate is a non-inhibitory bacterium. Photo credit: J. L. Banning. The microbiota of 69% (11 of 16 frogs) contained bacteria that could inhibit growth of *Bd* in the co-culture assays. The diversity of microbiota on frogs in their natural environment may be higher than after a period of time in the lab. However, one frog hosted six different isolates that inhibited *Bd*. These isolates were identified by extracting DNA (UltraClean Microbial DNA isolation kit, MoBio Laboratories Inc., Carlsbad, CA, USA), PCR amplification with the universal eubacterial primers 8F and 1492R (Lane, 1991) and sequencing of ~ 1400 bases of the 16S rRNA gene. Identifications were based on sequence similarity to GenBank database sequences (<http://www.ncbi.nlm.nih.gov>). The organisms identified were closely related to (accession numbers): *Pseudomonas fluorescens* (AY447046), *Chryseobacterium meningosepticum* (AF207077), *Chryseobacterium ginsengisoli* (AB245373), two isolates of *S. marcescens* (AY514431, AY514435) and Bacterium 2–4 (DQ163943). Thus, we found skin-associated anti-*Bd* microbiota that could resist high concentrations of antimicrobial peptides.

understanding of disease resistance and the evolution of disease resistance, and may offer avenues for manipulations that could allow management of resistance in natural populations of hosts. We suggest that increasing this understanding should be one of the major goals of future research in disease ecology.

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