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Exploring Differing Host Cutaneous Microbiome and Immune Responses Contributing to Chytridiomycosis Susceptibility in Amphibians

Abstract

Chytridiomycosis is an emerging infectious disease caused by *Batrachochytrium dendrobatidis*, a fungal pathogen affecting the skin of amphibians. Chytridiomycosis is differentially affecting amphibian species and populations across the world, causing severe declines and extinctions. It is spread by contact or zoospores travelling in water sources. It is not yet fully understood why susceptibility is so variable. Understanding differing susceptibility is crucial for realising any successful conservation efforts. Multiple factors appear responsible for the differing susceptibility. The two factors being examined in this literature review are ineffective immune responses and the limitations of the cutaneous microbiome. Relevant papers' significance and limitations are discussed with their provenance and objectivity taken into consideration. The immune system of amphibians comprises innate and acquired defences. The innate immune system has been found to be counterproductive in some cases, much like how an allergic reaction in humans can be detrimental to health. An adaptive immune response has yet to be confirmed in previously exposed individuals. The cutaneous microbiome plays an initial role in the defence against harmful zoospores by making the environment unsuitable or by producing deadly secretions; this consequently prevents colonization by the zoospores. Differing levels of secretions have been measured in response to disease. Most amphibian immune systems and microbiomes are not adapted to deal with chytridiomycosis, and the fungi are adapted to exploit this weakness. There are many difficulties in studying this disease, such as recreating a natural habitat in laboratory conditions, which is vital to get accurate microbiome data. The variety of species and global spread of this disease is incredibly wide ranging with many factors to consider. Many studies are only focused on one aspect of the disease, so a holistic and global approach would be more beneficial.

Introduction

Chytridiomycosis is a skin disease caused by the fungus *Batrachochytrium dendrobatidis (Bd)*. As amphibian skin health is vital for critical functions such as respiration and drinking, chytridiomycosis is a great threat to the amphibian population. The disease was observed in the 1970's and formally described by scientists in 1998. The origins of this disease are still debated, with scientists claiming it originated from Africa[1] or Asia[2]. Chytridiomycosis is now thought to be responsible for the severe decline and extinction of amphibian species such as *Rheobatrachus vitellinus* and *Rheobatrachus silus* (northern and southern gastric brooding frogs) in Australia[1].

Chytridiomycosis has spread globally, now primarily affecting amphibians in Europe, Australia, and the Americas. Some species or populations such as *Litoria verreauxii*, a vulnerable Australian species, are susceptible to the disease whilst others such as *Rhinella marina*[1], a common cane toad, remain unaffected[2]. Amphibians cannot be grouped into affected and non-affected species, as susceptibility variations can occur intra-species. This demonstrates the complexity of establishing susceptibility theories and formulating conservation methods.

A new fungal strain, *Batrachochytrium salamandrivorans (Bs)*, emerged in 2010[3] from the recombination of strains that until now were separated geographically. *Bs* only affects salamanders[4].

It is generally accepted that global geographical movement by humans and environmental change are the primary causes of chytridiomycosis emergence and spread[5]. The introduction of invasive species, such as super spreader *Xenopus laevis*[6], has distributed *Bd* around the globe without they themselves being negatively affected. Wildlife research remains limited with much research conducted on captive species. This can be problematic, especially when considering the microbiome which is highly variable in the same species when the environment is changed. Estimates of *Bd* infection can be greatly influenced by which body part of the frog is swabbed[7].

Emerging infectious disease research looks at the epidemiologic triangle (the components contributing to disease): the host, the pathogen, and the environment. Many factors have the potential to affect amphibian susceptibility to chytridiomycosis; these include environmental conditions, host behaviour, pathogen behaviour, habitat conditions, geographical location, interspecies or intra-species interactions and the wildlife trade. The two factors explored here are host microbiome and immune responses. Research could be used to protect vulnerable species and predict the spread of disease. No effective methods of disease management have been identified.

Methods

A review of papers was conducted by searching on a variety of databases: Science Direct, Web of Science, PubMed and JSTOR. Key words such as chytridiomycosis, susceptibility, microbiome and immune responses were used to narrow down articles relating to the area of interest. Thirty-six papers and websites were read and analysed and chosen for their relevance and objectivity, including those with contrasting results to ensure a fair and unbiased approach was achieved. Instances where methods and sample sizes vary significantly are addressed. There are many key authors on this topic, but this review ensures that conclusions are drawn from a variety of scientists in a variety of locations.

Amphibian and *Batrachochytrium* Biology and Interaction

Amphibians are found on all continents apart from Antarctica. They are vertebrate ectotherms, relying on external factors to control their temperature[8]. Their habitats vary widely. They have a moist skin used for respiration. Most species metamorphose, growing from young to adult form such as tadpole to frog, by changing their body structure[9]. Amphibians are an indicator species, used to assess ecosystem health due to their sensitivity to change[10].

Batrachochytrium dendrobatidis and *Batrachochytrium salamandrivorans* are fungi which are parasitic in amphibians, leading to chytridiomycosis disease. *Bd* fungus anchors itself to the epithelial cells of the amphibian skin and gets pushed deeper via germ tubes, spreading the infection to further cells[4]. *Bd* reproduces by uniflagellate zoospores which possess a tail-like structure that allows locomotion. They are initially contained in a zoosporangium casing. As the infected epithelial cells differentiate, the fungi spores are carried to the skin surface and distributed to the environment.

Zoospores have a membrane and can live for weeks in water. They reproduce asexually and spread by direct contact, through water or moist surfaces. In laboratory conditions, zoospores can travel two centimetres in 24 hours. In fast moving water, they are able to spread faster than this. With moisture, zoospores can live for seven weeks, but when desiccated for three hours, they die. It is unsure whether *Bd* uses keratin, a protein layer of amphibian skin, as a nutrient source or a protected place to live. Ideal conditions for *Bd* are between 17°C to 25°C and a pH between 6 to 7. Outside of these parameters, *Bd* can still live, but either growth is slow or development stops[11].

Tadpoles cannot die from chytridiomycosis because they have only a small amount of keratin present until they are in adult form[12]. They can distribute spores by keratin shedding. The disease presents itself by weight loss, skin shedding, lethargy, red skin, convulsions, mouth discoloration, and feeding changes[4]. Skin diseases are devastating in amphibians as it affects vital functions such as respiration, water uptake, and osmosis. The susceptibility to chytridiomycosis is thought to affect the conservation status of the species[13].

The Amphibian Immune System

The amphibian immune system consists of components and processes similar to humans, having an innate and acquired immune system. Innate refers to an element of the system that an organism is born with, such as post injury tissue inflammation, as a protective mechanism. Acquired or adaptive (used interchangeably) immunity is a specific immunity or response developed after infection or exposure to a disease or virus, such as varicella zoster virus or chickenpox. Acquired immune response includes B and T lymphocytes, which are leukocytes (white blood cells) common in most organisms. They are controlled by specific receptors and determine the body's response to infection. Host immune system suppression is a method of survival for fungal pathogens.

No effective adaptive immune response to chytridiomycosis has been confirmed[3]. The following stages should be occurring in the immune system upon skin infection[14]:

1. Innate & adaptive components are triggered at the site of infection and in organs such as the liver and spleen.
2. Early innate immune responses include increased hepatic gene expression, such as increased CRP (C-reactive protein) productivity, as this is the liver's response to pathogens. This is an inflammatory protective response.
3. Innate responses include activation of macrophages, neutrophils, and Langerhans cells along with inflammatory signalling to produce more leukocytes.
4. Cell surface receptors, like the major histocompatibility complex (MHC) (proteins that assist the adaptive immune system in recognising disease), activate along with cytokines and anti-microbial peptides (chemicals expressed on the skin).
5. Adaptive immune responses, such as lymphocyte maturation (B and T cell receptors); and antibodies, antigens, and cytokine production; activate.

Immune systems develop and adapt over time[15]. One species 20 years ago is not sufficient to make assumptions of that same amphibian immune system now. Repetition of research is vital to stay accurate, adaptive and to observe changes over long periods.

Over-activation of Hormones and Regulatory Gene Responses

Serotonin is a hormone which acts on immune system receptors as an immunoregulator in amphibian skin. Claytor et al. studied levels of serotonin produced by *Litoria verreauxii*, a vulnerable Australian species[16]. Frogs able to maintain stable serotonin production during *Bd* infection were more likely to survive than those whose levels rose. *Bd* excretes tryptophan, an essential amino acid, in susceptible amphibians, which could be the trigger of serotonin level rise. Savage et al. came to a similar inference about excessive immune responses when looking at the gene immune responses of *Rana yavapaienis*[17]. This species is of particular interest as susceptibility is unique to the individual frog, even when living in the same population. A highly reactive immune system was likely to lead to mortality[17]. Over-activation was ineffective; instead, survival improved in amphibians that maintained a non-reactive balance in MHC variants, a T cell adaptive immune response, produced upon infection.

The amphibian immune system is reacting to *Bd*, but these reactions are counterproductive[16,17]. Amphibian survival rate decreased when these immune responses were upregulated. This is seemingly counterintuitive and not what was initially predicted. The adaptive immune response is producing more B and T lymphocytes, as it should, but *Bd* can easily destroy these. The reaction is futile and energy consuming. Savage et al. did not attempt to explain why some *Rana yavapaienis* reacted differently to others, which would be a valuable study[17]. Research relating to gene responses can be applied to a wide variety of amphibian species due to strong similarities in their genes.

Leukocytes and Lymphocyte Responses

Ellison et al. and Grogan et al. discovered susceptible amphibians have weak and late leukocyte responses which are insufficient to protect against chytridiomycosis[18,19]. Key components of the tricarboxylic acid cycle, the second stage of cellular respiration, were depleted upon *Bd* infection[19]. These components are a nutrient pathway for the immune system, required for effective function.

Ellison et al. compared the transcriptome, all coding and non-coding genes, of *Atelopus zeteki* in three different tissues, including the spleen, that are important to immune function. Comparisons were performed on naïve frogs (previously uninfected) and on frogs which previously survived *Bd* infection[18]. Interleukins, which are immune inflammatory proteins, were disproportionately represented in infected naïve frogs but were minor in previously infected frogs. The spleen showed the greatest difference in immune responses of naïve and previously infected frogs. Three hundred differentially expressed genes were found between the naïve and previously infected frogs, showing potential acquired immunity. It must be noted that the sample size of previously infected exposed frogs (n=2) was small due to the susceptibility of death from chytridiomycosis in this species. This same study should be repeated on different species.

Evidence of late-stage infection (white blood cell response) was found in the form of neutrophil-associated genes in *Xenopus tropicalis*[14]. This reaction was too late to have any significant effect on survival rates. Some immune responses were detected early in the spleen, such as interleukins; however, these responses were too weak to impact survival. The most

significant finding of this experiment concerned cytochrome p450 in the liver of infected frogs. 51 out of the 70 p450 genes showed decreased expression in infected frogs. Rosenblum et al. commented that very few of the expected immune responses occurred on *Bd* infection of *Xenopus tropicalis*[14]. There was no significant difference between naïve and previously infected frogs, contrary to the conclusion made by Ellison et al.[18] This could be a result of the small sample size or a difference in response of the species studied. Contrary findings are important in deciphering factors of differing susceptibility between species.

Lymphocytes can be inhibited by metabolites produced by *Bd*. *Bd* was shown to adapt its metabolism to alter the skin environment of the host, inhibiting immune responses[20]. Researchers compared metabolites produced by *Bd* to a closely related pathogen, *Homolaphlyctis polyrhiza*, which did not produce these metabolites. They were trying to establish why this form of chytrid fungi was so effective in inhibiting the host's immune defences. The metabolites were methylthioadenosine (MTA), tryptophan, kynurenine (Kyn). MT, and Kyn, which independently inhibit lymphocytes; when acting together, they have a combined greater impact on immune inhibition, even at low concentrations. The exact mechanism MTA uses to inhibit lymphocytes in amphibians is unknown. Much of these conclusions are assumptions that mechanisms are akin to those exhibited by mammalian MTA. Further study on amphibians would be required for confirmation. This study also found that tryptophan was converted to Kyn which, when released, can interrupt the effector lymphocytes, inhibiting natural immune responses[20].

Amphibian Cutaneous Microbiome

The cutaneous microbiome is the community of bacteria and fungi that lives on the skin of most organisms. The symbiotic relationship between host and microbiome is believed to be an important factor in disease prevention and susceptibility in amphibians. Cutaneous microbiomes vary significantly between species and environments. They are subject to changes, especially during transition from juvenile to adult. The topography of the bacterial communities on the amphibians also varies greatly. *Bombina orientalis* in the wild has higher skin bacterial diversity in the dorsal regions; in captivity, however, the richness is higher in the ventral regions[21]. *Bombina orientalis* in the wild is resistant to decline from *Bd*[22]. These observations could be factors in susceptibility variation and important to consider during research.

Given that *Bd* fungi lives on amphibian skin, the cutaneous microbiome plays an important protective and defensive role. A study highlighted three preventative interactions by the cutaneous microbial community, reworded here[3]:

1. Zoospore colonization ability is reduced as the skin is already colonised by bacteria which can block adhesion sites for the pathogen.
2. The microbiome can produce secretions, changing the skin pH, making the environment unsuitable for zoospore survival.
3. Some of the bacteria species can produce enzymes and secondary metabolites which directly kill the invading microorganisms.

Defence Abilities of the Cutaneous Microbiome

Susceptible species are thought to have a less rich cutaneous microbiome than those less susceptible. Researchers tested frog species with varying susceptibilities from Panama[23]: *Atelopus certus, Colosthetus panamansis, Espadarana prosoblepon, Craugastor fitzingeri , and Strabomantis bufoniformis*. They found that 11 amplicon sequence variants (groups of bacteria) made up the core microbiome. Four were affected by *Bd*, suggesting that the presence of certain bacteria could be a good indication of *Bd* susceptibility[23]. Research found microbiomes of *Rana muscosa* and *Rana sierrae* did not recover from previous forms after *Bd* infection, likely due to some of the bacteria being affected and unable to recolonise[24]. Amphibians were only tested for recovery over 48 days. A useful follow-up study would have been examining if these amphibians are now more susceptible to mortality from chytridiomycosis with degraded microbiomes. Captive frogs were used in both these studies[23,24]. Microbiomes of wild species may have differing microbial communities which presents an experimental obstacle when studying this defence mechanism. Natural interactions would influence the bacteria able to colonise the skin. A study of a variety of species and their cutaneous microbiome community would be necessary to gather enough data to perform a test for significance of certain bacteria present against susceptibility.

The unsusceptible salamander species, *Plethodon cinereus*, was found to have *Janthinobacterium lividum* bacteria on its skin[25]. This provides an antifungal layer able to produce secondary metabolites, such as violacein, which inhibit the growth of *Bd*. When the microbiome of *P. cinereus* is altered, symptoms of chytridiomycosis worsen[3]. Researchers reduced the cutaneous bacteria on *P. cinereus* and compared this to the same species with an undisturbed microbiome[3]. They found that salamanders with removed bacteria lost significantly more body weight. Supporting results on the same species show a reduction of *Bd* prevalence where more *J. lividum* was present[26]. Treating susceptible species Rana muscosa with *J. lividum* increased their survival rate by 40%[27]. Studies on other species have found contrasting results. When *Atelopis zeteki* was treated with *J. lividum*, the survival rate did not improve. *J. lividum* may be a species-specific protective mechanism, or it may be part of a group of bacteria required[28].

When amphibians are in their optimal environment, their microbiome is most effective in dealing with *Bd* infection. Optimal temperature varies between species and populations depending on their natural environment. *Eleutherodactylus coqui*, susceptible frogs endemic to Puerto Rico, were infected with *Bd* during their optimal conditions (warm and wet) and during less ideal conditions (cool and dry)[29]. In their prime season, they were able to recruit skin bacteria that helped to fight off infection and return to the same pre-infection levels of bacterial richness and diversity. In the cool dry season, they were unable to do this, and infections rose as a result.

The microbiome of frogs freshly caught in the wild changes upon placement in a laboratory setting. Recreating wild conditions, including seasonal changes, adds value to research. The researchers used mesocosms, setting their experiment up in the natural environment, then used rRNA amplicon sequencing to detect changes in frog condition. The finding highlights that seasons have a direct effect on the cutaneous microbiomes' ability to protect against *Bd*. In the warm wet season, the frogs were significantly more able to fight infection and return to pre-infection condition[29].

Roback and Richards-Zawacki demonstrated the complexity of reaching conclusions about the microbiome[30]. They tested antifungal bacterium *Stenotrophomonas maltophilia* on *Acris crepitans*. The individual frogs with more antifungal bacteria survived longer when infected with *Bd* but only at temperatures of 14°C and not at 26°C. All amphibian species have specific and greatly varied optimum temperatures, indicating this would be a useful study to repeat on a wide variety of species for comparison against *Bd* susceptibility. This study again provides indication of the multifactorial nature of the disease.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are part of the early innate immune response in most mammals and amphibians. The immune system produces antimicrobial peptides, which are chemicals expressed on the skin to protect from infection. They can interact with the cutaneous microbiome.

Antimicrobial peptides are important in the development of host immunity and susceptibility. They are effective in decreasing the survival of zoospores on the skin of *Speleomantes*, a European salamander genus with 8 species. AMPs, although part of the innate immune system, have been found to adapt further after *Bd* exposure, making them more effective[31]. The combination of AMPs and their interactions with skin microbes affects the response to *Bd*. Both *Dendropsophus labialis* and *Rheobates palmatus*, frog species living symbiotically (beneficially interacting) in the Columbian Andes, both host *Bd* without succumbing to chytridiomycosis[32]. By testing 158 bacterial isolates (populations of microbes) found on these amphibians, 80% displayed antifungal properties. Some of the iso

lates, however, promoted the growth of *Bd*. The combination and ratio of AMPs could be key in determining the susceptibility of certain amphibians. Susceptibility differences could be due to AMPs working in synergy with bacteria from the cutaneous microbiome[33]. The metabolite 2,4-diacetylphloroglucinol is produced by *Pseudomonas fluorescens*, a cutaneous bacterial species found on *Rana muscosa*. This bacteria worked with AMPs to inhibit *Bd* growth at a concentration 4-fold lower than if either acted alone. This demonstrates the importance of observing multiple aspects of host defence when considering susceptibility as evidence points to a multi-factorial explanation.

All amphibian species and populations have varying abilities within their immune processes due to their varying habitats and life cycles. This makes it difficult to draw any overarching conclusions about the ability to adapt AMPs. Age, life history, and environmental habitat would need to be considered for repeatable experiments to be valuable.

Differential Recovery Impacts

Some species or populations have made recoveries after a decline from chytridiomycosis while others have not. Understanding differential recovery could lead to a greater understanding of susceptibility.

Australia has the longest spanning history of scientifically recorded chytridiomycosis data. Scheele et al. looked at these data to establish how the epidemic has affected species long term from 1978 to 2017[24]. This type of study is vital in tracking disease trajectory to identify if the biological data supports the numbers in terms of extinctions and recoveries. Out of 238 Australian species, chytridiomycosis was believed to be responsible for 43 species extinctions or declines. However, 11 of these species are showing signs of recovery[24]. None of the research can empirically confirm whether host genetic adaptation or a decrease in *Bd* virulence is the contributing factor to recovery. Research certainly suggests host resistance is developing in the species studied in Panama[15].

Decades have passed since some studies were conducted, and species' immune systems and genetic alterations are now more readily examined. There are signs of potential adaptations and resistance. Researchers revisited an area in Panama where 12 species of frog had previously been recorded at critical levels due to chytridiomycosis[15]. The populations of *Atelopus varius* and *Colostethus panamansis* had recovered to pre-epidemic levels. *Bd* was still present in the current populations at a lower level. When previously uninfected captive frogs were exposed to *Bd*, both historic and contemporary isolates, they reacted the same as they originally did upon *Bd* emergence. However, wild samples previously exposed to *Bd* exhibited greater inhibitory effects when tested through skin secretions samples. This could indicate a developed host resistance.

Heritability of *Bd* resistant genes was demonstrated in *Bufo spinosus*[35]. Trait changes do not develop in isolation so researchers looked at the fungal burden of *Bd* and development time of *Bufo spinosus*. An associated trait was found to be to be significantly heritable, suggesting that adaptation to *Bd* could occur in this species. To conduct their research, a semi-natural environment was used in this study, and amphibians were exposed to temperature and varied environmental conditions. These findings are supported by the genetic adaptations found in the loci of *Litoria dayi*[36]. Selection was occurring for resistance to *Bd* infection. Loci are specific locations on a chromosome where a gene is located. This adaptation is occurring by heritable Bd resistant genes being selected for. This study is an unrepeated, novel finding. The researchers acknowledge that the conclusion assumes that the microbiome does not affect the outcome, which is a big assumption given the role the microbiome plays in disease defence. This study requires repetition with microbiome analysis to confirm conclusions with more certainty.

This research indicates that developments are occurring in some species to prevent or stimulate recovery from chytridiomycosis, whether through acquired immunity or genetic adaptation toward *Bd* resistance. These developments must be compared to environmental changes and other factors to determine the predominant susceptibility factors.

Conclusions and Recommendations

Although most often considered individually, this review demonstrates the importance of linking and understanding multiple factors which can contribute simultaneously to the differing susceptibility of amphibians to chytridiomycosis. Meeting the objectives of the review, the emergence, major theories, and limitations of previous theories and studies have been discussed. Combining and reviewing research reveals different factors work together to affect susceptibility such as immune responses, including AMPs interacting with the cutaneous microbiome.

Some amphibian species are still declining, whilst others are showing signs of recovery. Analysis on species recovery has revealed possible evidence of innate and acquired immunity along with *Bd* resistant heritable genes. These recent studies require corroboration and repetition. Knowledge on recovery can reveal information on susceptibility.

Limits and uncertainties discussed reveal wildlife experiments are difficult to replicate in a laboratory. This is particularly relevant in cutaneous microbiome research which is highly susceptible to change and variability. Some studies evaluated reveal particular bacteria with strong species specific *Bd* resistance. This review proposes further research in this area may produce significant findings.

The global nature of chytridiomycosis and the variety of species involved require collaboration from researchers. The weakness in current research lies in the lack of connections to other areas of biology, even in review papers where the opportunity is more practical. The sharing of information in a database would establish possible susceptibility associations not yet linked.

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