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Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines

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As habitat range increases with OMZ expansion and intensification, this role will only become more visible and significant. Therefore, the SUP05 metagenome provides a functional template for analysis of gene expression in relation to climatologically relevant biogeochemical transformations within oxygen-deficient oceanic waters. This information should prove useful in the development of monitoring tools to assess microbial community responses to OMZ expansion and intensification.

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Supporting Online Material

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Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines

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The pathogen *Batrachochytrium dendrobatidis* (*Bd*), which causes the skin disease chytridiomycosis, is one of the few highly virulent fungi in vertebrates and has been implicated in worldwide amphibian declines. However, the mechanism by which *Bd* causes death has not been determined. We show that *Bd* infection is associated with pathophysiological changes that lead to mortality in green tree frogs (*Litoria caerulea*). In diseased individuals, electrolyte transport across the epidermis was inhibited by >50%, plasma sodium and potassium concentrations were respectively reduced by ~20% and ~50%, and asystolic cardiac arrest resulted in death. Because the skin is critical in maintaining amphibian homeostasis, disruption to cutaneous function may be the mechanism by which *Bd* produces morbidity and mortality across a wide range of phylogenetically distant amphibian taxa.

Infectious disease can cause population declines (*1*), and potentially extinctions (*2*), if multiple variables create favorable conditions for severe outbreaks. A striking example is the global loss of amphibians due to chytridiomycosis (*1*, *3*, *4*). Despite an initial reluctance to accept disease as a direct cause of declines (*5*), *Batrachochytrium dendrobatidis* (*Bd*) is now recognized for its ability to spread rapidly though amphibian populations (*6*, *7*), infect numerous species (*1*, *6*), cause high rates of mortality (*6*, *8*), and persist even at low host densities (*7*, *9*). These disease characteristics render population recovery from chytridiomycosis especially difficult and provide strong evidence for disease-induced extinctions (*2*, *8*, *10*). However, the mechanism by which *Bd* kills amphibians is unknown.

The pathogenesis of chytridiomycosis has been difficult to determine because cutaneous fungal infections are rarely fatal without other predisposing factors (*11*). Furthermore, *Bd* is in a phylum of fungi not previously known as pathogens of vertebrates (*12*), it is confined to the superficial layers of the epidermis (*2*, *13*) with minimal host reaction to infection (*13*, *14*), and no consistent pathological changes in internal organs of diseased amphibians are detectable with light microscopy (*3*). Differential expression of peptidase genes suggests that *Bd* pathogenicity may have a genetic basis (*15*), but determining the proximate cause of death has been inherently challenging because multiple physiological systems shut down before death.

Amphibian skin is unique among terrestrial vertebrates because it is actively involved in the exchange of respiratory gases, water, and electrolytes (*16*–*19*). Because of the role of amphibian skin in maintaining osmotic balance, other studies have suggested that *Bd* might disrupt cutaneous osmoregulation (*3*, *20*). To test this hypothesis, we tracked the development of *Bd* infections in green tree frogs (*Litoria caerulea*), which are susceptible to chytridiomycosis in

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laboratory experiments (*21*), with polymerase chain reaction (PCR) analysis and histopathology on skin biopsies. Clinical signs of disease and mortality occurred in *L. caerulea* individuals with the highest burdens of *Bd* (Fig. 1) and with greatest histopathological changes in the epidermis (fig. S2). We measured epidermal electrolyte transport in isolated skin preparations, monitored changes in blood and urine biochemical parameters, and monitored cardiac electrical activity with implanted biotransmitters in control, aclinical, and clinically diseased frogs.

To maintain osmotic balance, amphibians must sustain a hyperosmotic internal environment relative to the external environment (*16*–*19*). This is accomplished by tight regulation of electrolyte absorption across the epidermis, involving sodium channels and Na^+/K^+ pumps (*16*–*19*). Basal electrolyte transport across the skin, measured as equivalent short-circuit current in electrophysiological tests, was lower in skin samples from diseased frogs than in those from control frogs (Fig. 2A) and was accompanied by reduced transepithelial resistance (Fig. 2B). We estimated sodium absorption as the component of the short-circuit current that was blocked by amiloride (Fig. 2E), a specific inhibitor of the sodium channel (*22*). The residual short-circuit current in the presence of amiloride did not differ between skin samples from diseased and control frogs (Fig. 2F), indicating that *Bd* infection predominantly inhibits sodium absorption. Additionally, responses to carbachol (Fig. 3G), which activates chloride secretion in frog skin (*23*), and to noradrenaline (Fig. 3H), which stimulates sodium absorption and chloride secretion (*24*), indicated reduced sodium and chloride channel activity in epidermis from clinically diseased frogs. The biochemical mechanisms of epidermal channel disruption are unknown but could be due

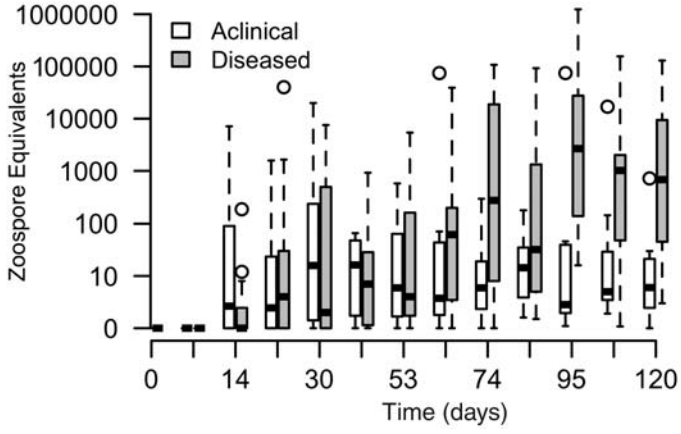

Fig. 1. Load of *Batrachochytrium dendrobatidis* in infected *Litoria caerulea*. Bars show first and third quartiles with medians (black lines) ± SD (whiskers) and outliers (open circles) of zoospore equivalents determined from PCR analysis on skin swabs collected over the course of infection. Results were grouped according to disease status (aclinical, $N = 8$; clinically diseased, $N = 15$) and differed among groups [repeated-measures analysis of variance (ANOVA), $P < 0.001$].

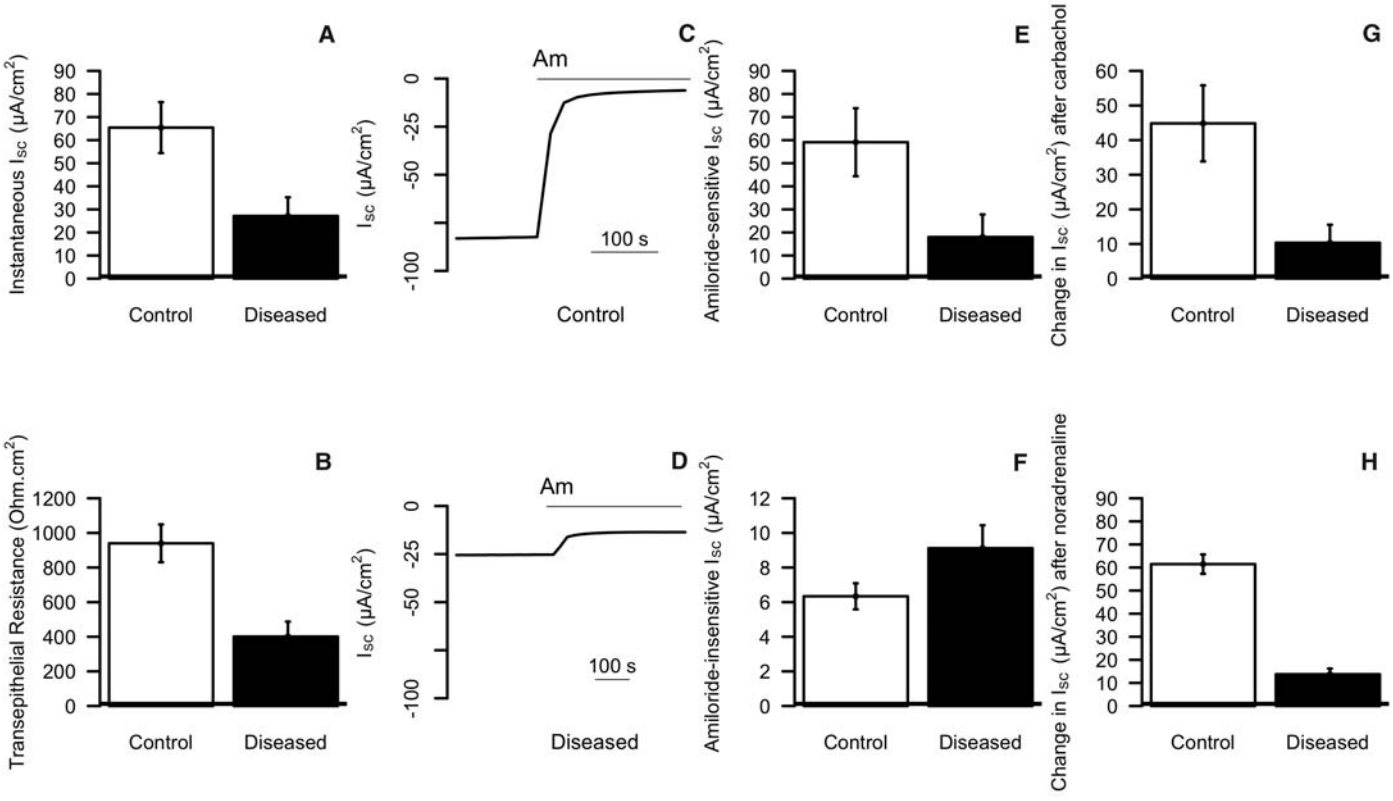

Fig. 2. Electrophysiological measurements of electrolyte transport across ventral skin samples from *L. caerulea* infected with *B. dendrobatidis*. Data in bar graphs show means (±SEM) from control ($N = 7$) and clinically diseased ($N = 7$) *L. caerulea*. (**A** and **B**) Instantaneous short-circuit current and transepithelial resistance were reduced in skin samples from clinically diseased *L. caerulea* [(A), Student's *t* test, $P = 0.009$; (B), $P = 0.006$]. (**C** and **D**) Original tracings show short-circuit current before and after blocking of sodium absorption with amiloride in skin samples from a control (C) and a clinically diseased *L. caerulea* (D). (**E** and **F**) The component of the amiloride-sensitive current (E) differed (Student's *t* test, $P = 0.038$) between the two groups, but amiloride-insensitive (residual) current (F) did not (Student's *t* test, $P = 0.79$), indicating inhibition of sodium absorption in clinically diseased *L. caerulea*. (**G** and **H**) The change in transepithelial short-circuit current was reduced in clinically diseased *L. caerulea* after treatment with carbachol [(G), Student's *t* test, $P = 0.015$] or noradrenaline [(H), Student's *t* test, $P = 0.001$].

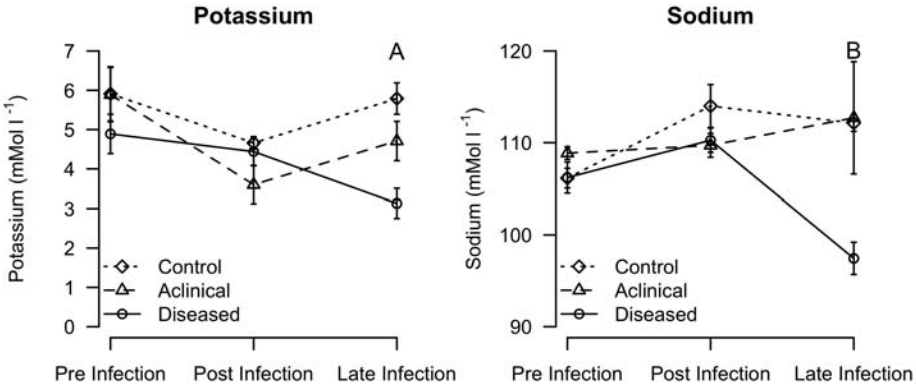

Fig. 3. Blood plasma potassium and sodium concentrations in *L. caerulea*. Blood samples were collected from infected *L. caerulea* (aclinical, $N = 7$; clinically diseased, $N = 11$; uninfected control, $N = 7$) on three sample occasions: 20 days before exposure (Pre Infection), 30 days after exposure (Post Infection), and 60 to 123 days after exposure when clinical signs of disease were obvious (Late Infection). (**A** and **B**) Data show mean (±SEM) concentrations of plasma potassium [(A), repeated-measures ANOVA, $P = 0.028$] and plasma sodium [(B), repeated-measures ANOVA, $P = 0.002$].

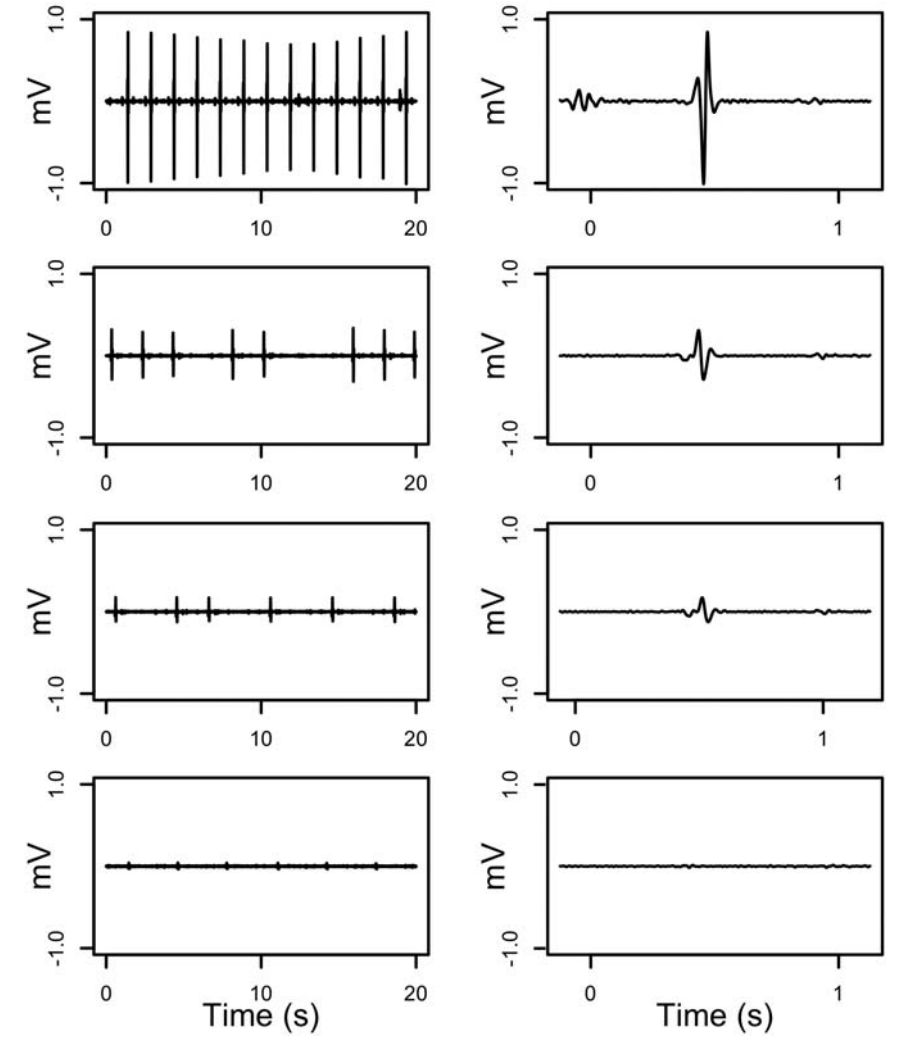

Fig. 4. Original cardiac electrogram tracings from one *L. caerulea* with clinical chytridiomycosis. Heart rate and signal amplitude were determined using 20-s sweeps (left column). Cardiac electrical signal morphology was assessed using 1-s sweeps (right column). Electrograms show cardiac electrical patterns consistent with asystolic cardiac arrest: slowing heart rate, decreasing ventricular depolarization amplitude, and increasing time of ventricular depolarization from (in order from row 1 to row 4) 18, 3, 2, and 0.5 hours before death.

to release of a fungal toxin or direct damage to infected host cells. Nonetheless, these results show that *Bd* compromises electrolyte transport, and thus osmoregulatory function, in the skin of infected *L. caerulea*.

We tested multiple blood and urine parameters as markers of organ function and general health (tables S1 and S2) and observed the greatest and most consistent changes in plasma electrolyte concentrations. Plasma sodium and potassium concentrations were significantly reduced as a result of disease when assessed according to clinical status (Fig. 3). Significant negative correlations existed between intensity of infection (*Bd* load) and the change in plasma sodium, potassium, and calcium concentrations (Pearson correlations: sodium, $r = –0.64$, $P = 0.001$, $N = 23$; potassium, $r = –0.47$, $P = 0.03$, $N = 22$; calcium, $r = –0.44$, $P = 0.04$, $N = 22$). None of the additional parameters changed significantly (tables S1 and S2). We observed no significant decrease in body masses or increases in albumin and total protein concentrations in frogs with chytridiomycosis (table S1), which suggests that there was no change in water volume. Because body mass did not change in diseased frogs, the observed osmotic imbalance most likely resulted from electrolyte loss rather than dilution caused by water uptake.

Several hours before death, the cardiac electrical activity of severely diseased frogs resembled patterns associated with cardiac standstill, also known as asystolic or bradyasystolic cardiac arrest, in other organisms including humans (Fig. 4) (*25*). Asystolic cardiac arrest occurs when cardiac electrical abnormalities cause contractile failure, reduced blood flow, and ultimately circulatory collapse and death. Although several conditions may initiate this cycle (*25*), most can be ruled out as factors in this study. Ambient temperatures remained constant, eliminating the possibility of hypothermia. Stable body mass and stable plasma protein and albumin concentrations were evidence against dehydration and hypovolemia (low blood volume). Hypoxia (low blood oxygen) was not completely ruled out, but measurements of peripheral blood oxygen saturation indicated a 20% drop in oxygen only after changes in electrical activity were observed in one individual (*26*). Furthermore, no changes in blood carbon dioxide were detected in a previous study (*20*). Thus, shifts in electrolytes and/or acidosis appear to be the most likely cause of cardiac asystolic death.

Although electrolyte imbalance, hypokalemia (low plasma potassium), and hyponatremia (low plasma sodium) could result from depletion via the epidermis or the kidney (*17*, *18*), plasma biochemistry showed no indication of renal damage (table S1). In contrast, the skin, which regulates the bidirectional flux and overall balance of sodium and potassium (*17*), demonstrated inhibited sodium absorption in the ventral epidermis (Fig. 2), and histology showed degenerative epidermal changes (fig. S2). Thus,

the pathophysiology of chytridiomycosis appears to be disruption to the osmoregulatory functioning of the skin and consequent osmotic imbalance that leads to cardiac standstill.

To test whether treating electrolyte abnormalities would reduce the clinical signs of disease, we administered an oral electrolyte supplement to *L. caerulea* in the terminal stages of infection, when they lost the righting reflex and could no longer correct their body positions (*26*). Frogs under treatment recovered a normal posture and became more active; one individual recovered sufficiently to climb out of the water onto the container walls, and two individuals were able to jump to avoid capture. These signs of recovery were not observed in any untreated frogs. In addition, treated frogs lived >20 hours longer than untreated frogs [mean time after treatment ± SEM: treated frogs (N = 9), 32 ± 2.8 hours; control frogs (N = 6), 10.7 ± 2.2 hours; Student's t test, $P < 0.001$]. All treated frogs continued to shed skin and ultimately died from the infection, as expected. It is unlikely that electrolyte treatment could prevent death unless the epidermal damage caused by *Bd* is reversed. Although amphibians can generally tolerate greater electrolyte fluctuations than other terrestrial vertebrates (*18*), we suggest that depletion of electrolytes, especially potassium, is important in the pathophysiology of chytridiomycosis. Amphibian plasma potassium concentrations are maintained at constant levels across seasons (*27*), and even moderate hypokalemia is dangerous in humans (*28*).

Our results support the epidermal dysfunction hypothesis, which suggests that *Bd* disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance and death. The ability of *Bd* to compromise the epidermis explains how a superficial skin fungus can be fatal to many species of amphibians; their existence depends on the physiological interactions of the skin with the external environment (*16*–*19*). Disease outbreaks capable of causing population declines require the alignment of multiple variables, including a life-compromising pathophysiology (*1*). Resolving the pathogenesis of chytridiomycosis is a key step in understanding this unparalleled pandemic.

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Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome

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Chronic fatigue syndrome (CFS) is a debilitating disease of unknown etiology that is estimated to affect 17 million people worldwide. Studying peripheral blood mononuclear cells (PBMCs) from CFS patients, we identified DNA from a human gammaretrovirus, xenotropic murine leukemia virus–related virus (XMRV), in 68 of 101 patients (67%) as compared to 8 of 218 (3.7%) healthy controls. Cell culture experiments revealed that patient-derived XMRV is infectious and that both cell-associated and cell-free transmission of the virus are possible. Secondary viral infections were established in uninfected primary lymphocytes and indicator cell lines after their exposure to activated PBMCs, B cells, T cells, or plasma derived from CFS patients. These findings raise the possibility that XMRV may be a contributing factor in the pathogenesis of CFS.

Chronic fatigue syndrome (CFS) is a disorder of unknown etiology that affects multiple organ systems in the body. Patients with CFS display abnormalities in immune system function, often including chronic activation of the innate immune system and a deficiency in natural killer cell activity (*1*, *2*). A number of viruses, including ubiquitous herpesviruses and enteroviruses, have been implicated as possible environmental triggers of CFS (*1*). Patients with CFS often have active β herpesvirus infections, suggesting an underlying immune deficiency.

The recent discovery of a gammaretrovirus, xenotropic murine leukemia virus–related virus (XMRV), in the tumor tissue of a subset of prostate cancer patients prompted us to test whether XMRV might be associated with CFS. Both of these disorders, XMRV-positive prostate cancer and CFS, have been linked to alterations in the antiviral enzyme RNase L (*3*–*5*). Using the Whittemore Peterson Institute's (WPI's) national

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