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The Effects of Chytridiomycosis on Myocardial Oxygenation in Columbian Spotted Frogs (Rana luteiventris)

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Abstract

Chytridiomycosis is an infection in amphibians caused by the fungus *Batrachochytrium dendrobatidis* that has been linked to world-wide amphibian decline. However, there is currently no clear explanation of how this infection is killing amphibians. I hypothesize that the fungus is preventing effective cutaneous gas exchange, causing hypoxia in the heart which could lead to myocardial ischemia. The objectives of this study are to find an organism that is susceptible to Chytridiomycosis which can be used as a model for detecting myocardial ischemia in frogs and to establish a protocol for ischemia detection.

Rana luteiventris (Columbian Spotted Frogs) were collected and swabbed from two Montana lakes. Of the 23 total frogs sampled two from Park Lake and one from Krohn Lake tested positive for *B. dendrobatidis* according to a Taqman quantitative PCR assay. Captive *R. luteiventris* inoculated with the fungus showed signs of infection. A spleen sample was collected from a frog with artificially compromised cutaneous gas exchange. This sample was run out on a 10% polyacrylamide native gel and stained for LDH activity. When compared to a control spleen sample the frog with the compromised cutaneous gas exchange showed a 6.4% increase in Heart-type LDH indicating possible myocardial damage. In conclusion, the detection of Chytridiomycosis in wild-caught *R. luteiventris* and the results of the inoculations indicate that this species is an acceptable model organism. The established protocol using LDH isoenzyme native gels shows promise of being an effective method of assessing myocardial damage in amphibians.

Introduction

The fungus *B. dendrobatidis* causes an often fatal disease in amphibians known as Chytridiomycosis (Berger et al., 1998). This disease was first implicated in the rapid decline of amphibian populations in Costa Rica and Panama (Lips, 1999) as well as Australia (Daszak et al., 1999). It has now been identified on amphibians in Europe (Mutschmann et al., 2000), North America (Bradley et al., 2002), and New Zealand (Waldman et al., 2001). Evidence is being complied that indicates Chytridiomycosis is linked to a world-wide decline of amphibians (Daszak et al., 2003).

Batrachochytrium dendrobatidis infects the keratinized cells of the epidermis of amphibians (Daszak et al., 2001). This causes hyperplasia (excessive cell production) and hyperkeratosis (excessive thickening of skin) resulting in excessive skin sloughing (Pessier et al., 1999). However, none of these symptoms is severe enough to be fatal. To date there is no clear explanation of how this skin infection is killing amphibians. There are two current hypotheses. The first is that the fungus is releasing a toxin; however, *B. dendrobatidis* has not yet been shown to produce any such toxins. The second hypothesis is that it is interrupting the osmoregulation across the skin. This hypothesis is more convincing as it is supported by research that showed a reduction of blood plasma osmolality and electrolyte concentrations as the disease progressed (Voyles et al., 2007).

Amphibian skin not only acts as a protective barrier, but can function as the site for gas exchange and osmoregulation (Heatwole & Barthalmus, 1994). The heart relies on cutaneous gas exchange to oxygenate the heart because amphibian hearts are composed of spongy myocardium that lacks coronary vessels (Pough et al., 2009). Therefore, the oxygen demand of the myocardium is met by the blood that fills the

chambers (Pough et al., 2009). In mammals, the right side of the heart receives blood from the systemic loop which would be relatively low in oxygen; however in an amphibian the blood returning from the skin is oxygen rich and also feeds into the venous return, therefore supplying oxygen to that side of the heart. If *B. dendrobatidis* is interfering with cutaneous gas exchange then hypoxia in the heart may result.

While there is evidence to support that amphibians that are infected by *B. dendrobatidis* show a disruption in osmoregulation, there is no clear explanation of what causes this disruption. For my research project I proposed that the lack of osmoregulation is a result of a larger problem. It is possible that the fungus is interrupting cutaneous gas exchange which leads to hypoxia in the heart. This can lead to poor oxygenation of all tissues resulting in a shortage of cellular energy. ATP is essential for the active transport required to maintain proper ion balance. My hypothesis was that poor cutaneous gas exchange and hypoxia could cause myocardial ischemia resulting in death of the animal. The objectives of this study were to find an acceptable model organism for the study of the effects of *B. dendrobatidis* on amphibian physiology and to establish a protocol for detecting myocardial ischemia in frogs. Hopefully later research will be able to use these protocols to determine if there is a correlation between chytridiomycosis and myocardial damage.

Materials and Methods

Collection and care of specimens

Wild-caught frogs from the species *Rana luteiventris* (Columbian Spotted Frog) were used for this study. Columbian Spotted frogs were collected in Montana from Park Lake in the Helena National Forest and Krohn Lake outside of Lincoln. They were

initially swabbed and tested for *B. dendrobatidis* using quantitative PCR (see protocol below). If the test results came back negative the frogs were put in a large tank with recirculating water. Infected frogs were isolated and placed in individual containers containing 150 mL of artificial pond water (Provasoli solution) which was changed twice a week. Containers were autoclaved before and after use. All frogs were fed approximately five large crickets per week.

Detection of the fungus

In order to detect *B. dendrobatidis* the underside of each frog was swabbed with disposable cotton swabs (10 strokes on left side of the abdomen, five strokes on the left fore limb, and five strokes on the left hind limb). The DNA was extracted from the swab using a Qiagen DNeasy Blood & Tissue Kit and following the directed protocol. Real-time Taqman PCR was performed on the samples according to the protocol established by Boyle (2004). This allowed for the determination of the relative number of *B. dendrobatidis* zoospores on each swab.

Inoculation

Frogs were inoculated in 10 mL of artificial pond water and approximately 1 million zoospores for eight hours. Inoculations were done once a week for two weeks. Frogs were monitored for signs of infection such as unusual skin sloughing, posturing, and lack of appetite.

Collection of Samples

To collect tissue samples, frogs were placed in a solution of MS222 (0.5g/L, pH=7.0) to anesthetize them. Once the frogs were unresponsive, they were removed from the solution and placed on a dissecting tray. The frogs were cut open to expose the

spleen. One frog that was clear of infection had both cutaneous arteries clamped to prevent effective gas exchange across the skin for 90 minutes before the samples were collected. This was done to model the compromised cutaneous gas exchange that may exist in amphibians with Chytridiomycosis. Samples were collected immediately for the control frog. The spleen was removed and placed in a glass homogenization tube along with 19 volumes (w/v) of homogenate buffer (175mM KCl, 2mM EDTA, 10mM Tris, pH=7.0). The tissue was ground using a glass pestle until fully suspended in the buffer. The homogenized tissue was placed in a microcentrifuge tube and centrifuged at 13,000 rpm for two minutes and the supernatant was removed and placed in the freezer for future use.

LDH native gel electrophoresis and staining

LDH native gels were used to detect myocardial damage. The protocol of Sheafor (1999) was used for both gel electrophoresis and staining with some modifications. Ten percent polyacrylamide gels with a six percent stacking gel were poured and allowed to polymerize. Electrophoresis lasted approximately 8 hours at a current of 15 milliamps. Gels were stained for 1 hour to allow the bands to appear.

Densitometry and gel interpretation

Gels were imaged using the Kodak Gel Logic 1500 Imaging System and were further examined using ROI Analysis. This program allows the user to select a region of interest (ROI) and calculate the background-subtracted pixel values in that region. Each band was selected and the net intensity was recorded. The intensities were compared within each lane to determine the relative intensity of each LDH isoform. That data was then used to determine the ratio of the Heart-type (H-type) to Muscle-type (M-type)

isozymes. Because LDH forms as a teramer, there are five possible isoforms. Below is a table that shows the compositions of the five LDH isoforms (Table 1). The composition of the isoform was used to determine the percent intensity that is contributed by each isozyme.

Table 1

Isoform	Composition	% M-type	% H-type
LDH-5	MMMM	100	0
LDH-4	MMMH	75	25
LDH-3	MMHH	50	50
LDH-2	MHHH	25	75
LDH-1	HHHH	0	100

To calculate the relative amount of H-type in a sample the intensity of each band was multiplied by the percent composition of H-type. The sum was then divided by the total intensity of all the bands to determine the percent H-type in the sample.

Results

Detection of the Fungus

Twelve Columbian Spotted frogs were caught and swabbed at Krohn Lake and eleven at Park Lake. Two individual frogs from Park Lake and one from Krohn Lake tested positive for *B. dendrobatidis* according to the Taqman PCR assay (Table 2). All other frogs tested were negative for *B. dendrobatidis* meaning their genomic equivalent was less than one.

Table 2

Individual	Genomic equivalent per swab
Rl #3 KL	8.42E+07
Rl #1 PL	4.72E+02
Rl #9 PL	2.02E+07

Inoculation

Three frogs were inoculated to determine susceptibility of the species. All three frogs that were inoculated with *B. dendrobatidis* showed signs of infection including unusual skin sloughing, loss of appetite, and abnormal posturing. Subject two died within the first week before the second inoculation. Subjects one and three died at approximately three weeks and four weeks respectively.

LDH native gel electrophoresis and staining

Two gels were run with the same spleen samples from both the clamped and unclamped frogs (Figure 1). Samples were run at full strength as well a 50% dilution to control for oversaturation of the imaging software. The average percent H-type for the clamped frog spleen sample was 53.4%. The average percent H-type for the control frog spleen sample was 47.0%.

Figure 1

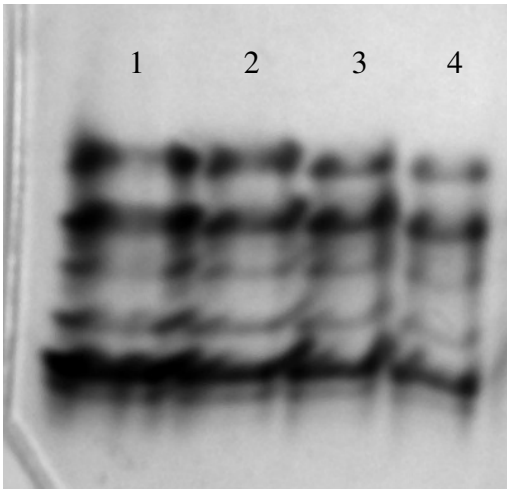

Figure 1: LDH isoform gel. Lane 1 is spleen tissue clamped frog, Lane 2 is diluted spleen tissue clamped frog, Lane 3 is spleen tissue unclamped frog, Lane 4 is diluted spleen tissue unclamped frog.

Discussion

One objective of this study was to determine if the Columbian Spotted Frog was an acceptable model organism for examining the effects of *B. dendrobatidis* on amphibian physiology. According to the results of this study they appear to be adequate. Detecting the fungus in wild-caught frogs suggests that this species can be infected with and carry *B. dendrobatidis*. The inoculation of the frogs and the subsequent deaths of all the subjects suggest that this species is susceptible to *B. dendrobatidis* and will succumb to the disease.

Another objective of this study was to determine an effective protocol for detecting if there is heart damage in frogs infected with *B. dendrobatidis*. Based on the results of this study, the protocol used for visualizing LDH isoforms from spleen tissue is a possible candidate. The sample from the frog with clamped arteries was collected to simulate a frog with compromised cutaneous respiration which I hypothesize could be a deadly consequence of a *B. dendrobatidis* infection. The increase in the H-type isozyme

in the spleen tissue of the clamped frog as compared to the control frog can be an indication of damage to the heart. It appears that the procedures outlined above are sensitive enough to detect this damage and may be used in future studies.

Conclusion

The protocol established in this study shows promise in being able to determine if myocardial damage is in fact a part of the physiological consequence of a *B. dendrobatidis* infection. More trials are needed to ensure these results are consistent and reproducible and not the result of chance variation among individual frogs. While I was unable to obtain clean blood samples from the frogs I used, in the future it may be beneficial to find a way to collect blood samples rather than spleen tissue. This could produce more accurate results by ensuring that the spleen tissue is not interfering with the LDH level readings in the blood . Further research is needed to establish a trial where blood samples are taken from individuals infected with *B. dendrobatidis* and analyzed for signs of a myocardial ischemia in order to determine if there is a correlation between the fungal infection and myocardial damage.

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