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Department of Life and Environmental Sciences

Brandon A. Sheafor — 4/30/18

Director — Date

Brandon A. Sheafor

Print Name

Reader — 4/30/18

Date

Kyle Strode

Print Name

Reader — 4/30/18

Date

Stefanie Otto-Hitt

Print Name

Analysis of Antimicrobial Peptide Efficacy Against Chytridiomycosis from Skin Secretions of Columbia Spotted Frogs (*Lithobates luteiventris*)

Investigator: Emma KJ Esposito

Contact Information:
Emma KJ Esposito
1601 N. Benton Ave
Helena, MT 59625
Phone: (406) 459-5761
eesposito@carroll.edu

Host Institution: Department of Life and Environmental Sciences, Carroll College, Helena, MT

Abstract:

Amphibian populations have been declining in size in recent years. A major contributing factor to this decline is the fungal disease chytridiomycosis. Chytridiomycosis occurs when the zoospores of the fungus *Batrachochytrium dendrobatidis (Bd)* imbed into amphibian skin and disrupt the homeostatic functions the skin provides, leading to death in most amphibians. Amphibian skin can produce antimicrobial peptides (AMPs) that inhibit *Bd* infection. The goal of this experiment was to determine whether or not the amount and type of AMPs a frog produces affect its ability to defend against chytridiomycosis. Columbia spotted frogs were chosen as a model organism because they vary in susceptibility to the disease, yet their populations have not been drastically affected by the fungus. Skin secretion samples were collected from ten Columbia spotted frogs at four different locations in western Montana (40 samples total). The protein concentration for each sample was determined using a micro BCS assay. The minimal inhibitory concentrations (MICs) were determined using 96 well plate growth inhibition assays. In the assay, a set of ten protein dilutions from each frog (ranging from 100-1000 μg/mL) were used. The *Bd* was grown in each dilution and growth was measured after four days as the change in absorbance measured at 492 nm. Infection load of frogs was determined using Quantitative PCR analysis. From the data collected, there was no statistically significant correlation between the MIC and infection load.

Introduction:

Amphibian populations are decreasing at exponential rates (McCallum 2007). When comparing current and natural extinction rates of amphibians over a

505-year period, McCallum found that the number of extinct species since 1500 is more closely approximated to the taxon extinction rate during a million years (McCallum 2007). Therefore, it is important to gain an understanding of the causes of these increased rates, study ways amphibians are combatting the decline, and determine ways to aid in prevention.

The loss of amphibians could have major impacts on ecosystems. Amphibians create links between aquatic and terrestrial habitats (Lips et al. 2005). Due to these linkages, the declines are expected to produce effects that cascade through food webs of both habitats (Lips et al. 2005). Rantala et al. (2014) studied the ecological effects of the loss of amphibian populations due to disease in Panamanian streams and found that suspended particulate organic matter concentrations decreased, biomass of filter-feeding macroinvertebrates decreased, biomass of collector-gatherer macroinvertebrates increased, and macroinvertebrate taxa richness decreased. Rantala et al. (2014) also found that overall community respiration decreased, gross primary production decreased, and ammonium uptake length increased. Collectively these studies show that the loss of amphibians can have a substantial negative effect upon the ecosystem in which they live.

Chytridiomycosis, a disease caused by the fungus *Batracochytrim dendrobatidis,* plays a major role in amphibian population declines (Skerratt et al. 2007). Ryan et al. (2008) studied the invasion of *Bd* into populations of healthy Robber frogs (*Craugastor punctariolus*) in Panama, and the entire population perished within four months of the first detection of *Bd*. Because chytridiomycosis is a major reason for the decline of amphibians, it should be thoroughly studied.

Batrachochytrium dendrobatidis is a fungus, first isolated and identified in 1998 from a captive Blue poison dart frog (*Drendrobates azureus*) in the National Zoological Park in Washington, DC (Berger et al. 2005). The discovery required the creation of a montypic genus in the fungus phylum Chytridiomycota, which had not previously contained vertebrate pathogens (Voyles et al. 2011). DNA sequencing also revealed that the fungus was unlike any other known Chytrid (Berger et al. 2005). The life cycle of *Bd* (Fig 1.) consists of two main stages, the zoospore and the zoosporangium (Berger et al. 2005). The zoospores are first mobile and, after a dispersal period, absorb their flagella and form cell walls (Berger et al. 2005). Next, they enter the germling stage that consists of growing rhizoids (Berger et al. 2005). The cytoplasm of the zoospore develops immature sporangia, becoming multinucleated through mitotic divisions, and develops papillae (Berger *et al*. 2005). The cytoplasmic contents form into zoospores, and once fully developed, the mature sporangium releases them to continue the life cycle (Berger et al. 2005). When the zoospores are able to insert their nuclear content into the epidermal cells of amphibians, chytridiomycosis results (Berger et al. 2005).

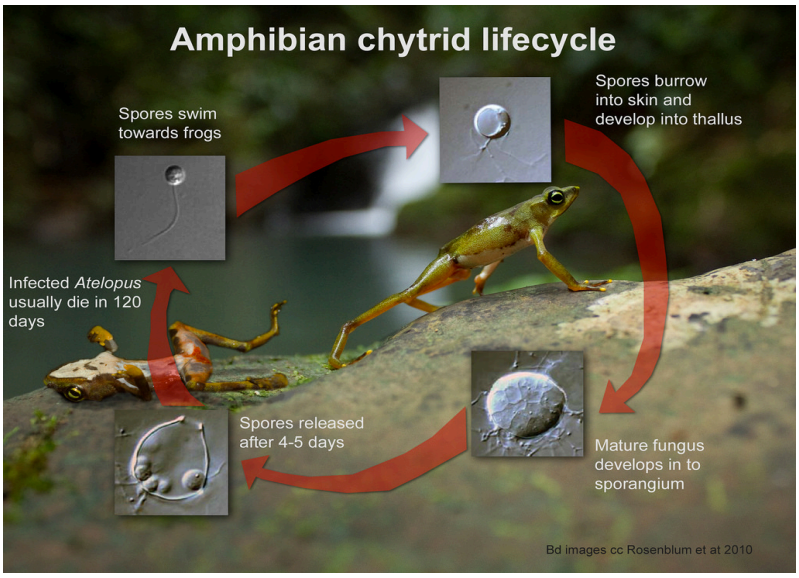


Figure 1. Lifecycle of *Batrachochytrium dendrobatidis* fungus during infection of amphibians.

In order to understand why chytridiomycosis is fatal to amphibians, the anatomical and physiological importance of their epidermis must be understood. The epidermis of amphibians is highly permeable and plays a major role in maintaining homeostasis (Voyles et al. 2011). The skin transports water, respiratory gases, and ions, such as sodium and magnesium (Voyles et al. 2011). These functions are used to maintain intra and extra cellular solute concentrations (Voyles et al. 2011). The epidermis of amphibians contains multiple layers of epithelial cells and specialized cell types (Voyles et al. 2011). Studies concerning chytridiomycosis focus on the stratum corneum whose cells differentiate as keratinocytes (Voyles et al. 2011). Fully developed *Bd* is found mainly in cells with keratin in the cytoskeleton, but has also been found in deeper layers as zoospores (Voyles et al. 2011). Infection causes the stratum corneum to harden and thicken, and causes cytoplasmic degeneration and vacuolation in deeper epidermal cell layers (Voyles et al. 2011). These changes hinder the ability of skin to maintain homeostatic concentrations. Voyles et al. (2011) noted that infected amphibians had reduced

electrolyte levels, deterioration of cardio electrical functioning, and reduced sodium and potassium concentrations. The homeostasis disruption caused abnormal skin sloughing, irregular posture, decreases in activity, and loss of righting reflex, eventually ending in death from cardiac arrest (Voyles et al. 2011).

However, many amphibians appear to have biological defenses against *Bd*. *Silurana (Xenopus) laevis* (African clawed frogs), a species of frog used to study amphibian immunity, has shown a significant increase in susceptibility to *Bd* infections when immuno-suppressed (Ramsey et al. 2010). Ellison et al. (2014) found that *Atelopus zeteki* (Panamanian golden frogs) acquired immune responses, such as increased expression of immunoglobins and major histocompatibility complex genes, when infected with *Bd.* However, in both studies, the innate immune response alone was not enough to fight off the fungus. Becker et al. (2015) found that frog skin accommodates many different bacterial communities, and frogs able to fight *Bd* infections had very different bacterial communities than those that had chytridiomycosis. Frogs also produce antimicrobial peptides (AMPs) from granular glands as another form of defense (Rollins-Smith et al. 2002). These antimicrobial peptides are the main focus of my research.

Antimicrobial peptides are 12-46 amino acid chain peptides that are hydrophobic, cationic, and adopt an α-helical structure when binding to cell membranes (Woodhams et al. 2006). The α-helical structure causes cell membranes to become disrupted, which induces cell death (Woodhams et al. 2006). Woodhams et al. (2006) studied the AMPs of Australian frog species whose populations were not declining, such as *Litoria chloris* and *L. lesueuri*, and found that AMPs inhibited

the growth of the *Bd*. Rollins-Smith, et al. (2002) studied similar effects of specific AMPs collected from several frog species, including *Lithobates luteiventris* (Columbia spotted frog) and *Rana catesbeiana* (American Bullfrog). Rollins-Smith et al. (2002) used a growth inhibition assay that analyzed a set of diluted skin peptide solutions collected from the frogs to determine the concentration necessary to inhibit the growth of *Bd*. The minimal inhibitory concentration (MIC), or the lowest concentration of peptides needed to inhibit *Bd* growth, determined the relative strength of the AMPs (Rollins-Smith et al. 2002). However, there have been no studies focused on the Columbia spotted frog (*Lithobates luteiventris*) populations in Montana. The objective of my research was to determine the MICs of Colombia spotted frog samples in Montana and determine if they correlate with *Bd* infection load. I hypothesized that the frogs infected with *Bd* will have higher MICs than those not infected.

This research focused on Columbia spotted frogs in several locations around Helena, Montana. The main goal of this research is to determine if a correlation between MICs and disease status is present. The overall objective of this project is to be able to use the MICs, and other factors, to create a model to predict the susceptibility of amphibians to chytridiomycosis. The other factors that will be taken into account when building the predictive model will be the cutaneous bacterial colony structure, the infection status for each frog, and the water nutrient composition (Kurtz 2017). Once the relationship between these factors is understood, it may be possible to use the model to predict susceptibility in future groups of amphibians.

Materials and Methods:

Sampling locations and amphibian collection: Sites were selected based on *Bd* having been positively identified there previously. These sites were also within a reasonable distance from the laboratory at Carroll College in Helena, Montana (Fig 2). The chosen sites also supported populations of Columbia spotted frog. This species was chosen due to its variability in susceptibility to chytridiomycosis, and it is not considered a species of concern at this time (ICUN Red List). Ten frogs were captured by hand at each location, for a total of 40 frogs. Samples were collected from all 40 frogs.

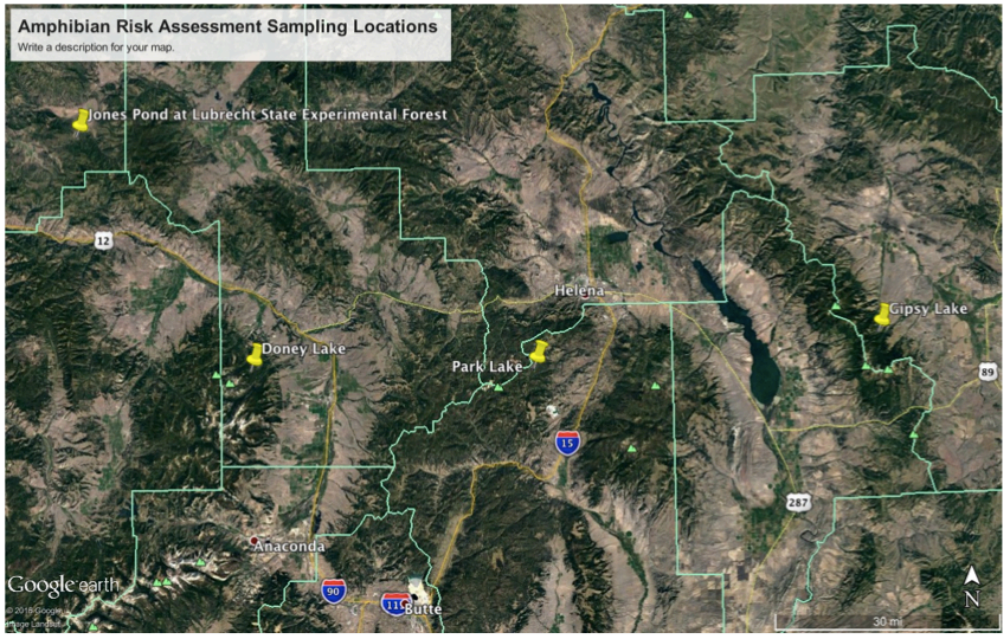


Figure 2. Map showing locations of sample collecting. Locations included Doney Lake, Park Lake, Gipsy Lake, and Jones Pond (Kurtz 2017).

Sampling: Each frog was swabbed on the ventral surface, randomly choosing the right or left side. The surface was swabbed 10 times with sterile foam swabs. This procedure was used to collect bacterial metabolites. After rinsing with sterile

Provasoli medium, the other side was swabbed to collect DNA samples for *Bd* and bacterial community analysis. The metabolic rate of frogs was also assessed using Sable Systems hardware and software. The data collected using these methods was not used further for this research project, but will be used for creating the prediction model.

AMP collection: After swab samples were collected, each frog was placed in 50 mL of sterile collection buffer (50mM NaCl, 25 mM CH_3COONa, pH=7.0) for 15 minutes. The buffer also contained 500 µL of 20 mM norepinephrine hydrochloride (Sheafor et al. 2008). The solution was then extracted from the container and acidified with 500 µL of trifluoroacetic acid (TFA). It was then passed through a C-18 Sep-Pak cartridge, which is a filter that binds to proteins (Waters Corporation, Milford, Massachusetts, USA). The solution and Sep-Paks were stored on ice and all test subjects were released at the site of capture. Peptides were eluted from the Sep-Paks and concentrated using a Spin-Vac centrifuge system (Sheafor et al. 2008).

Bd community analyses: Batrachochytrium dendrobatidis DNA was extracted from swabs using MoBio Powersoil DNA isolation kits (MoBio Labs, Carlsbad, California) (Kurtz 2017). The extracted DNA samples were sent to University of British Columbia where Andrew Loudon carried out Quantitative PCR protocol to quantify the amount of *Bd* present on a frog (Kurtz 2017). The DNA samples were also used to determine the cutaneous bacterial communities of each frog. Communities were

determined by amplifying the 16S rRNA sequence for each sample (Loudon et. al. 2014).

AMP assays: Protein concentrations for each skin secretion sample were determined by Kurtz (2017) using a micro BCS assay using bradykinin as a standard. Those concentrations were used to produce a series of peptide dilutions. The peptide dilutions were between 100 and 1000 μg/mL, in 100 μg/mL increments. The protocol used for the growth inhibition assays was similar to that used by Sheafor et al. (2008) with some alterations. Zoospores from a cultured plate of *Bd* (strain PTH02 from Dr. Joyce Longcore, University of Maine) were collected in a 2% tryptone broth. The concentration of zoospores was determined via hemacytometer counting in a 45 μL volume and diluted to $5x10^5$ zoospores per μL. Each sample assay was carried out on a 96-well plate and dilutions were replicated three times. To each well, 50 μL of one AMP dilution and 50 μL of zoospore broth were added. The plates were analyzed for growth over four days by measuring optical density at 492 nm. Heat killed *Bd* was used as a positive control and live *Bd* was used as a negative control. Each control had 50 μL of water added to ensure a volume equal to the rest of the samples.

Calculations: Minimal inhibition concentrations were obtained by creating a logistic curve of the optical density values at the different AMP concentrations using Microsoft Excel. The MIC was calculated to be where the 95% confidence limit above the lower asymptote crossed the regression line (Sheafor et al. 2008) (See Appendix

for graphs of all samples). Mean MICs of infected frogs and frogs that tested negative for the presence of *Bd* were compared. Statistical f-tests were used to determine whether a t-test assuming equal or unequal variances should be performed. This was repeated with MICs normalized for surface area and body weight. The MICs were also normalized for total peptide concentration by dividing the total peptide concentration by the MIC. The normalized MIC averages were compared for *Bd* positive and negative frogs using the same statistical methods as mentioned before (Fig 3). The MICs were then compared to the infection load of individual frogs to infer whether or not a correlation could be observed (Fig 4). A linear regression analysis was performed using the Excel Data Analysis ToolPak in order to determine the statistical significance of that correlation. In order to normalize MICs based on body mass and surface area, the weight and skin surface area of each specimen were observed and calculated (Table 1). Surface area was calculated using the equation Hutchinson et al. (1968) formulated, $SA=1.107W^{0.606}$, relating frog body mass (W) to surface area (SA). This equation was derived for the species *Lithobates pipiens*, and was used in this study based on the similar body size and shape of *L. pipiens* to *L. luteiventris*. The MICs per gram body weight (gbw) and per cm^2 were calculated by taking the measured MIC and dividing by the total peptides per gbw or per cm^2 (Table 1). The MIC equivalents per gbw and per cm^2 were graphed against *Bd* total copy number, and linear regression analysis was used to calculate statistical significance (Fig 5, Fig 6).

Table 1. Summary of data collected from Columbia spotted frogs (*Lithobates luteiventris*). Frogs were labeled based on the lake at which they were captured. DL corresponds to Doney Lake, GR corresponds to Jones Pond, PL corresponds to Park Lake, and GL corresponds to Gipsy Lake.

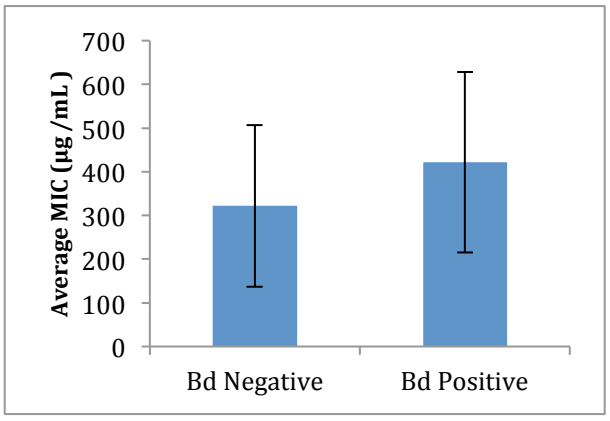
Frog Specimen	Weight (g)	Surface Area (cm^2)	Total peptide concentration (ug/mL)	Total peptides per mass (µg/gbw)	Total peptides per surface area (µg/cm^2)	MIC (µg/mL)	MIC equivalents per gbw	MIC equivalents per cm^2	*Bd* total copy number
DL-1	29.1	8.20	5011	172.20	611.01	518.07	0.33	0.85	0
DL-9	1.90	1.57	1131	595.26	720.75	34.69	17.16	0.05	0
DL-10	19.1	6.35	13471	705.29	2120.02	606.32	1.16	0.29	0
GR-1	15.8	5.66	1821	115.25	321.49	163.69	0.70	0.51	69.34
GR-4	14.1	5.29	2741	194.40	518.48	240.64	0.81	0.46	0
GR-5	10.2	4.34	1541	151.08	354.68	216.74	0.70	0.61	0
GR-6	11	4.55	5621	511.00	1235.88	465.76	1.10	0.38	46.78
GR-7	30.9	8.50	1736	56.18	204.12	177.11	0.32	0.87	0
PL-1	52.2	11.69	4036	77.32	345.37	292.48	0.26	0.85	142.36
PL-2	8.2	3.81	6166	751.95	1619.87	615.23	1.22	0.38	0
PL-4	21.3	6.79	4981	233.85	733.78	341.23	0.69	0.47	0
PL-5	7.2	3.52	2946	409.17	837.41	244.16	1.68	0.29	0
PL-6	12.2	4.84	3356	275.08	693.00	224.64	1.22	0.32	0
PL-7	6.5	3.31	8456	1300.92	2557.33	739.32	1.76	0.29	257.03
GL-1	32	8.69	4326	135.19	497.98	297.73	0.45	0.60	813.49
GL-2	27.5	7.92	15066	547.85	1901.12	225.16	2.43	0.12	36.72
GL-5	15	5.49	7151	476.73	1302.87	400.00	1.19	0.31	1081.20
GL-6	7.8	3.69	6511	834.74	1763.13	800.00	1.04	0.45	913.24
GL-8	2.1	1.67	2601	1238.57	1559.99	409.96	3.02	0.26	5102.50

Results:

Minimal inhibitory concentrations for a total of 19 frogs were determined (Table 1). The average MIC of infected frogs was 421.57 µg/mL and was not significantly different than the average MIC of healthy frogs, 321.88 µg/mL (Fig 3A). Comparing the average MICs of frogs infected to those not infected yielded a p-value of 0.154, which is above the alpha value 0.05 (Fig 3A). Comparing the mean MICs normalized for body weight (p=0.487) and skin surface area (p=0.715) yielded no significant differences as well (Fig 3C, Fig 3D). Comparing average MICs that were normalized for protein concentration also yielded no significant differences

(p=0.292) (Fig 3B). When the infection load was plotted against the measured MICs, a linear regression analysis was performed, and the p-value calculated was 0.548 (Figure 4A). The MICs of frogs only infected with *Bd* were compared with infection load and a p-value of 0.855 was generated (Fig 4B). Therefore, the relationship between measured MIC and infection load was not statistically significant. Minimal inhibitory concentrations normalized for surface area were plotted against infection load and a linear regression analysis was performed, yielding a p-value of 0.458 (Fig 5A). This analysis was repeated with only frogs infected with *Bd* and yielded a p-value of 0.506 (Fig 5B). Since the result was above the accepted p-value of 0.05, it was determined to not be statistically significant. When MICs were normalized for body mass and plotted against infection load, the p-value calculated from linear regression was 0.914 (Fig 6A). This analysis was repeated with only frogs infected with Bd and yielded a p-value of 0.069 (Fig 6B). These values indicated no significant correlation.

A



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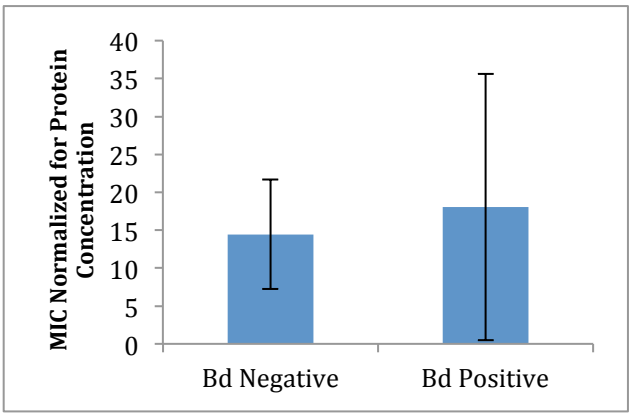

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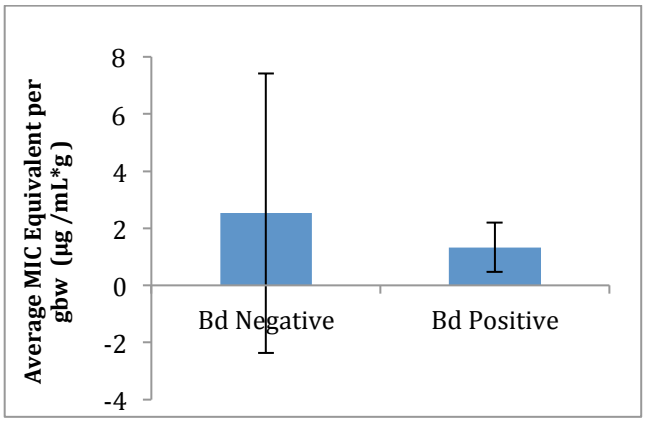

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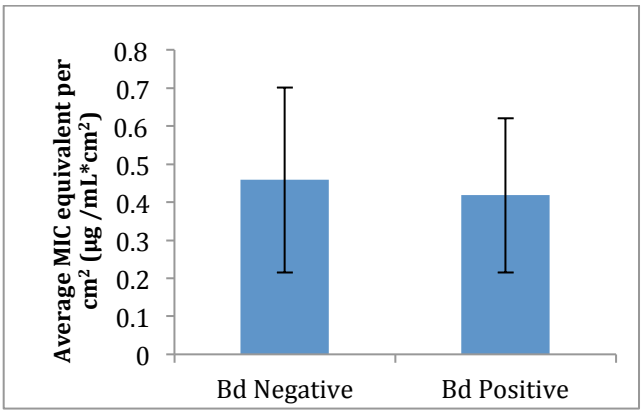
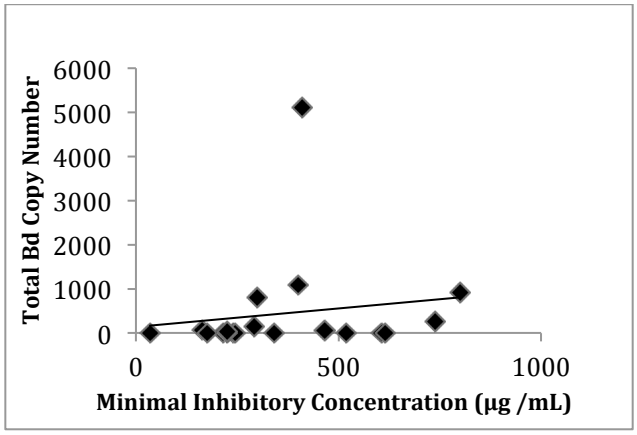


Figure 3. Comparison of mean MICs of frogs either infected or not infected with *Bd*. Error bars represent standard deviation. (A) Mean measured MICs (p=0.154). (B) Mean MICs normalized for protein concentration (p=0.292). (C) Mean MICs normalized for body weight (p=0.487). (D) Mean MICs normalized for skin surface area (p=0.715)

A



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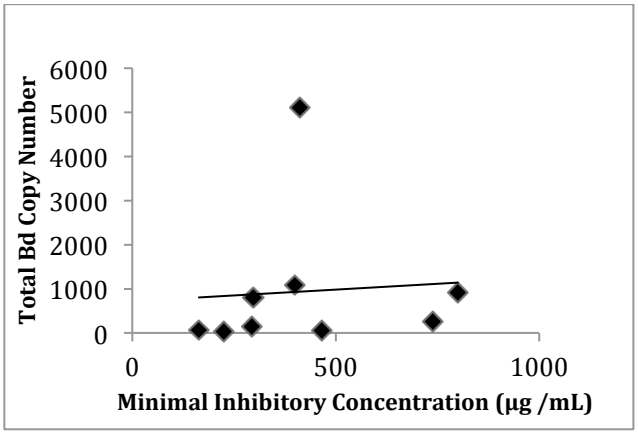
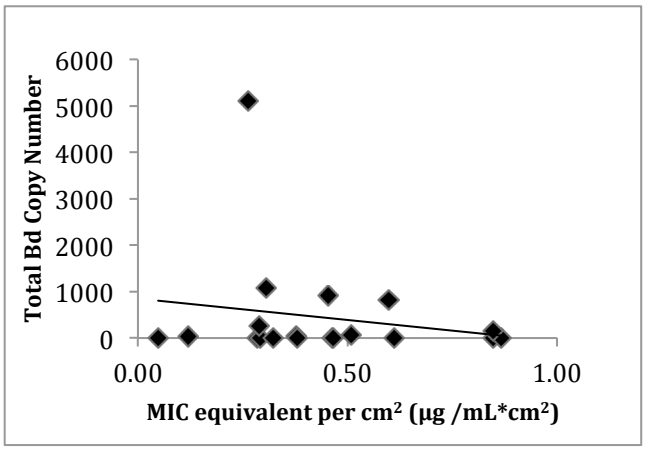


Figure 4. Comparison of measured MIC and *Bd* infection load for *L. luteiventris*.
(A) Plot of samples that were and were not infected with *Bd* (p-value=0.548).
(B) Plot of samples only infected with *Bd* (p-value=0.855).

A



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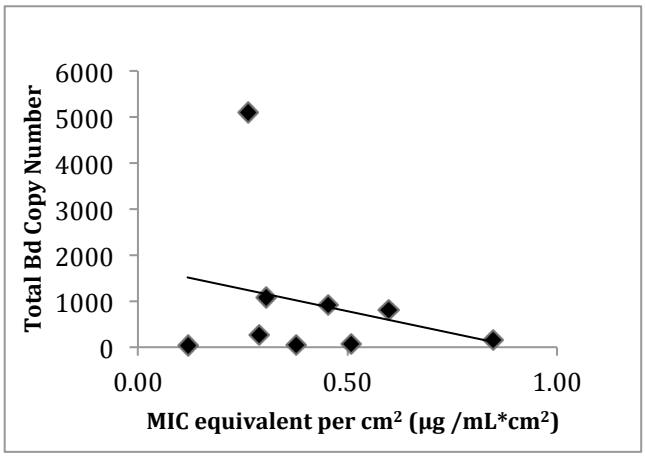
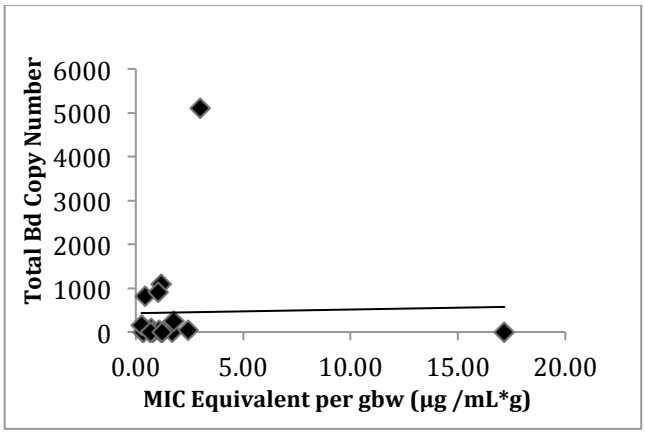


Figure 5. Comparison of MICs normalized for skin surface area (cm^2) and *Bd* infection load for *L. luteiventris*
(A) Plot of samples that were and were not infected with *Bd* (p-value=0.458).
(B) Plot of samples only infected with *Bd* (p-value=0.506).

A



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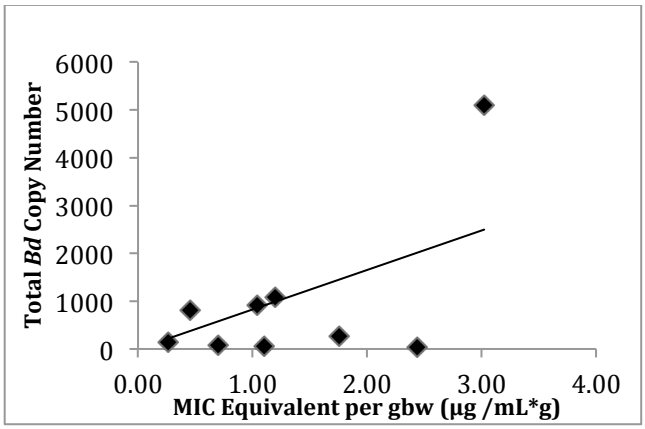

Figure 6. Comparison of MIC normalized for body mass (g) and *Bd* infection load for *L. luteiventris*.
(A) Plot of samples that were and were not infected with *Bd* (p-value=0.914).
(B) Plot of samples only infected with *Bd* (p-value=0.069).

Discussion:

The comparison of the average MICs between frogs that were infected and not infected with *Bd* yielded no statistically significant results. There was also no significant correlation between MIC and infection load in this experiment. Since there is no significant difference in MICs, or a correlation between MIC and infection load, MICs may not be good indicators for measuring the susceptibility of frogs to chytridiomycosis. However, these results may have been affected by insufficient sample size, as not all 40 frogs were analyzed due to time constraints. Another contributing factor could have been the difference in the size of the frogs. Since glands in the skin secrete AMPs, the amount a frog produces could be influenced by body mass and relative skin surface area. To account for this the MICs were normalized for both of body weight and surface area. However, a significant correlation was still not observed between MIC per cm^2 and infection load or MIC per gbw and infection load. Another possibility for these results is that the correlations could have been affected by the presence of frogs that had not been infected with *Bd*. Frogs that produce AMPS that are ineffective in inhibiting fungal growth

might not have come into contact with the fungus yet. This would have resulted in a lower MIC being included in the group not infected with the fungus, and lowering the overall average. Because of this, analyses were performed where frogs that were not infected with *Bd* were left out of data set. However, even when these samples were excluded and MICs were normalized for body weight and surface area, there was no significant correlation with infection load. There was also no significant difference for averaged MICs normalized for body weight and skin surface area between frogs infected and not infected. If more data were to be collected and analyzed, a stronger trend between both MIC per gbw and MIC per SA could possibly be observed.

This study and others, such as Sheafor et al. (2008), have shown that AMPs from amphibians do have some influence on *Bd* growth. However, MICs in this study do not show any significant correlation with infection load, so the AMPs by themselves may not be very effective in preventing infection in *L. luteiventris*. The AMPs could be used in some alternative way to decrease susceptibility. There are still two other factors, water parameters and microbial community, that are included in the prediction model proposed for this project. The three factors could be influencing each other in order to increase an amphibian's resistance to chytridiomycosis. The type of peptides produced could be influenced by the water parameters, or the peptides could also be altering cutaneous microbial communities. In preliminary studies, Andrew Loudon analyzed the cutaneous bacterial communities from the frogs used in this research, and found that the cutaneous bacteria were different than those found in the collection site water. This could indicate that AMPs influence the type of bacteria growing on amphibian skin. Some of the bacterial colonies observed were also identified as having antifungal properties and, as

such, could have a major influence on the susceptibility of certain individuals to fungal infections. As these results are preliminary, more research is needed in this area. The more that is understood about the interactions of the multiple factors, the stronger the predictive value the model will have.

The ultimate goal of this research is to develop a prediction model and apply it to a variety of amphibian species in a variety of ecosystems. Once the model is created, it might be possible to take water, bacterial, and AMP samples, apply the values to the model, and predict the susceptibility of that community. Hopefully, this information could be used to aid in the preservation of dwindling amphibian populations, as well as prevent any further loss of biodiversity. An effort needs to be made to preserve amphibian populations and the ecosystems that they influence.

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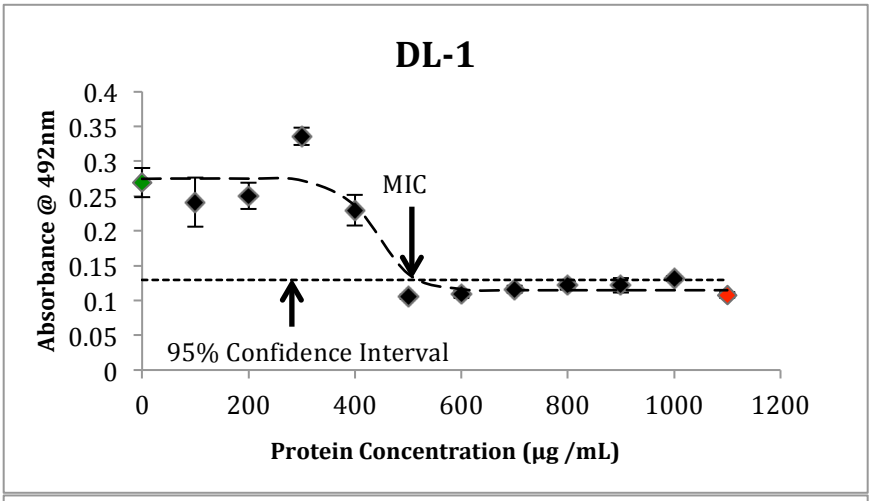
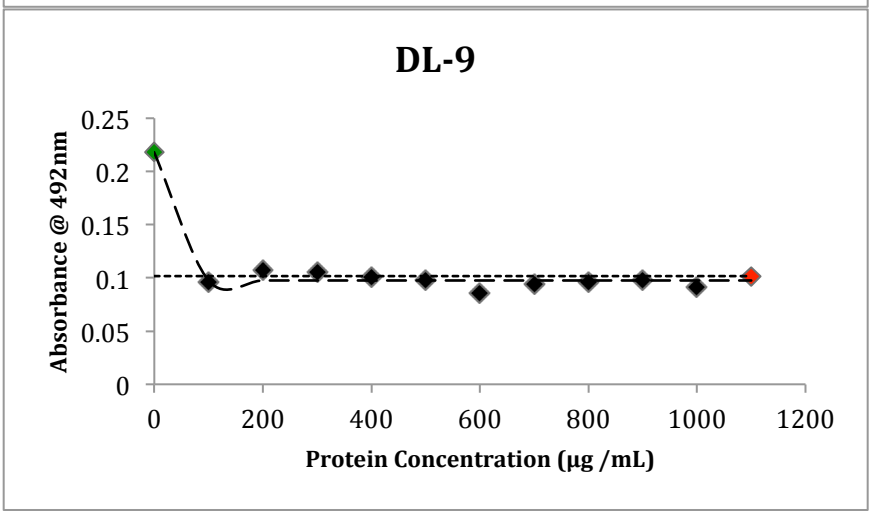
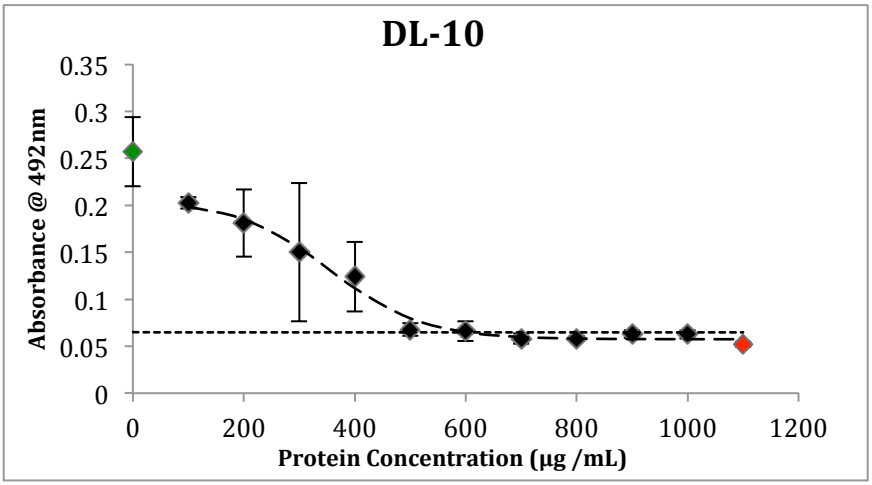
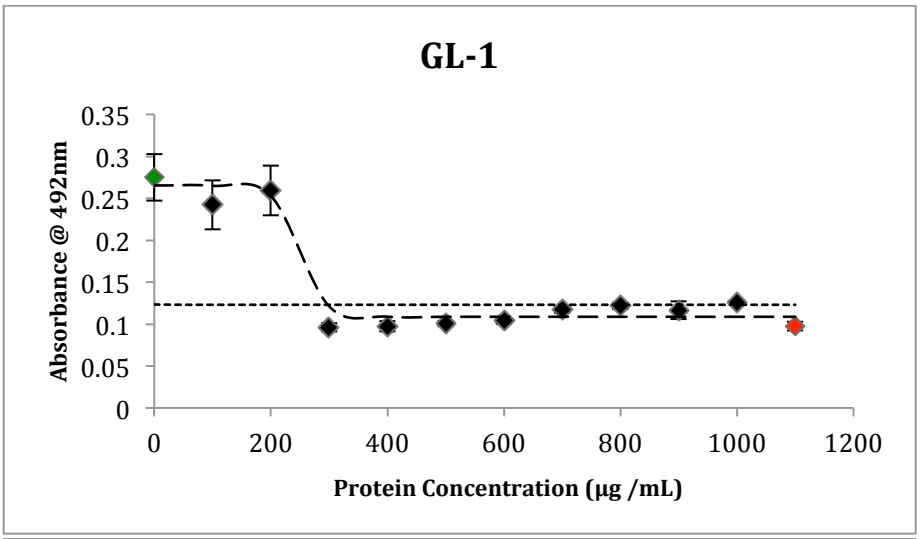
Appendix:

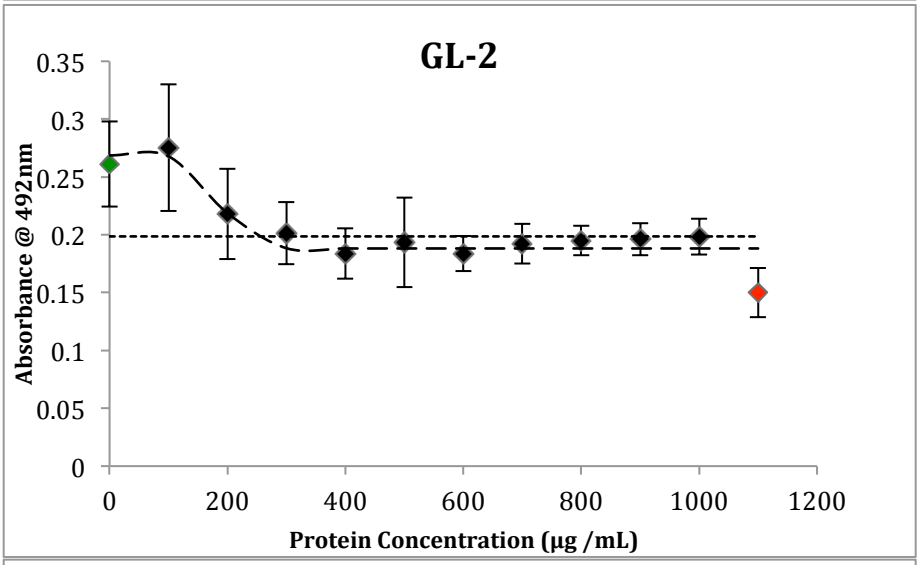
Graphs 1-19. Antimicrobial peptide assays for individual frogs. Green data points indicate positive control measurements and red data points indicate negative control measurements. Error bars represent standard error. The dotted straight line represents the 95% confidence interval used to calculate MIC.

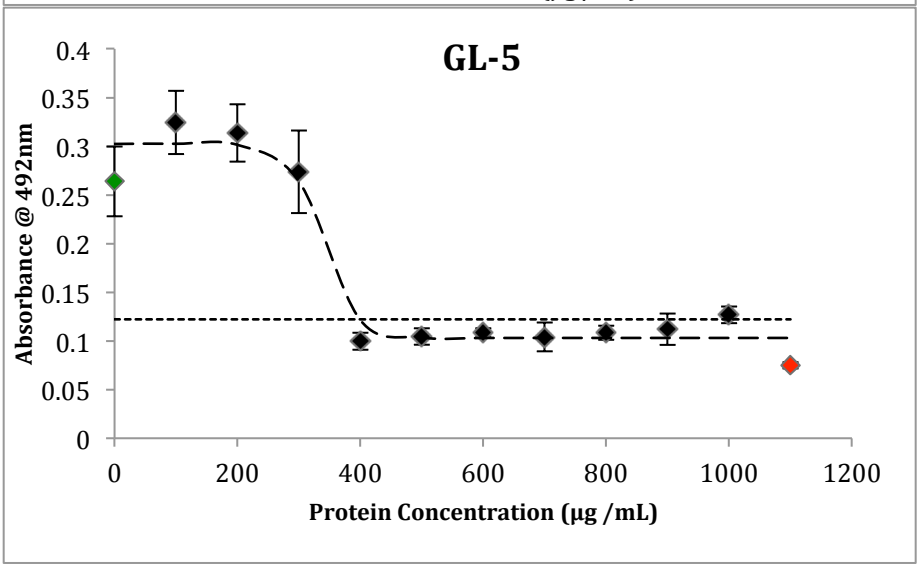


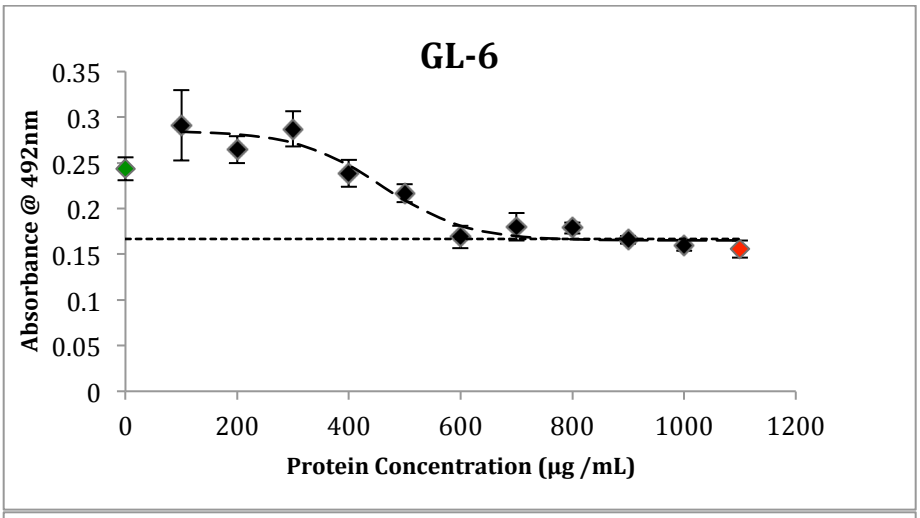


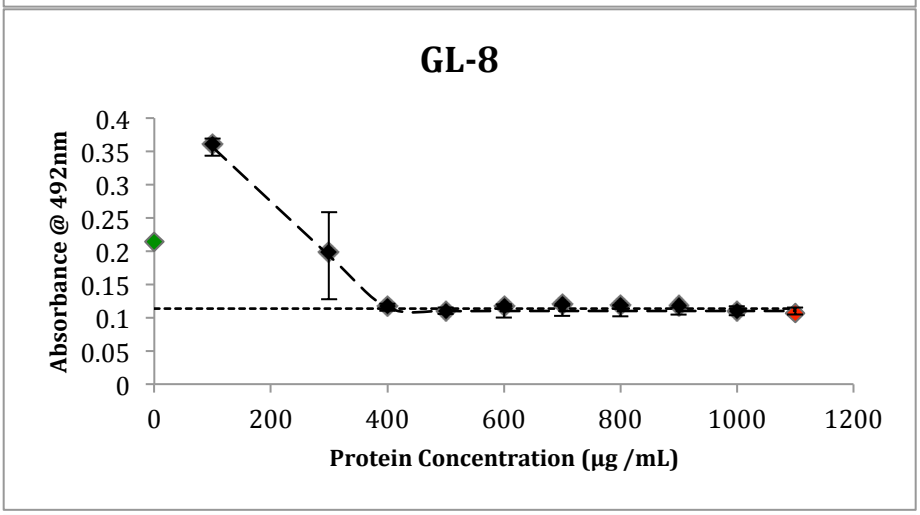



GL-1
Absorbance @ 492nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GL-2
Absorbance @ 492nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GL-5
Absorbance @ 492nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GL-6
Absorbance @ 492nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)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Absorbance @ 492nm
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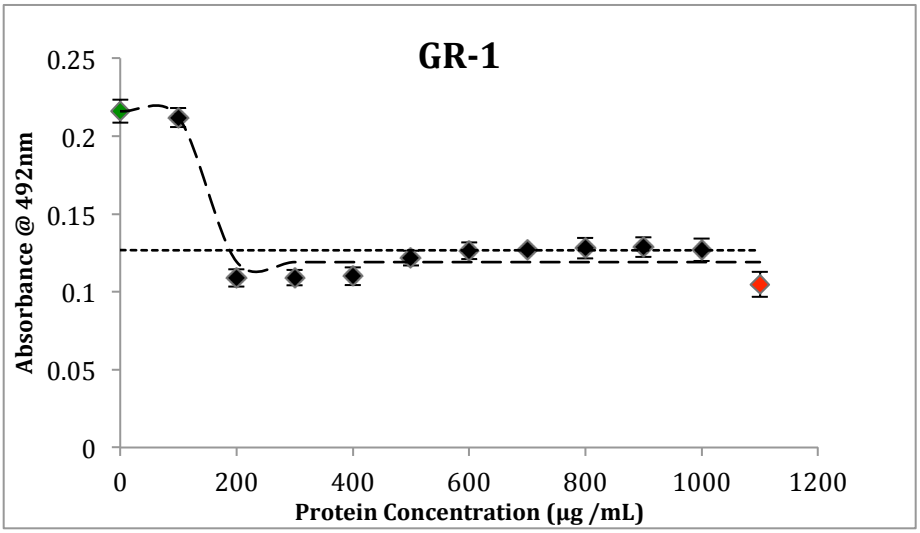
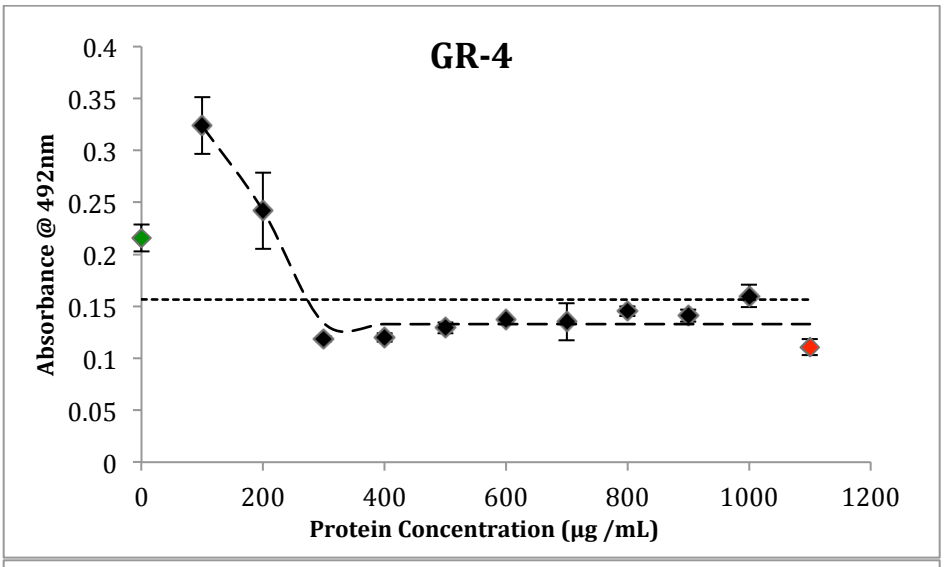
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GR-1
Absorbance @ 492nm
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)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Absorbance @ 492nm
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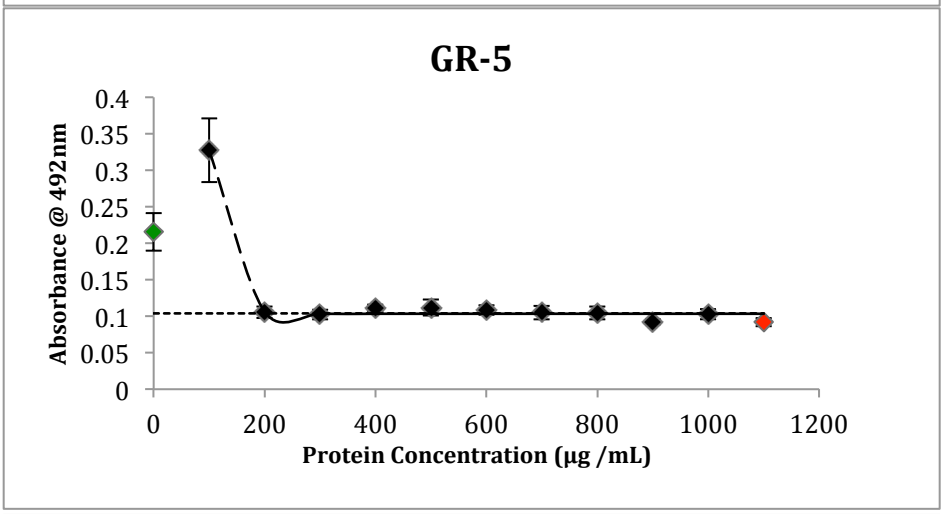
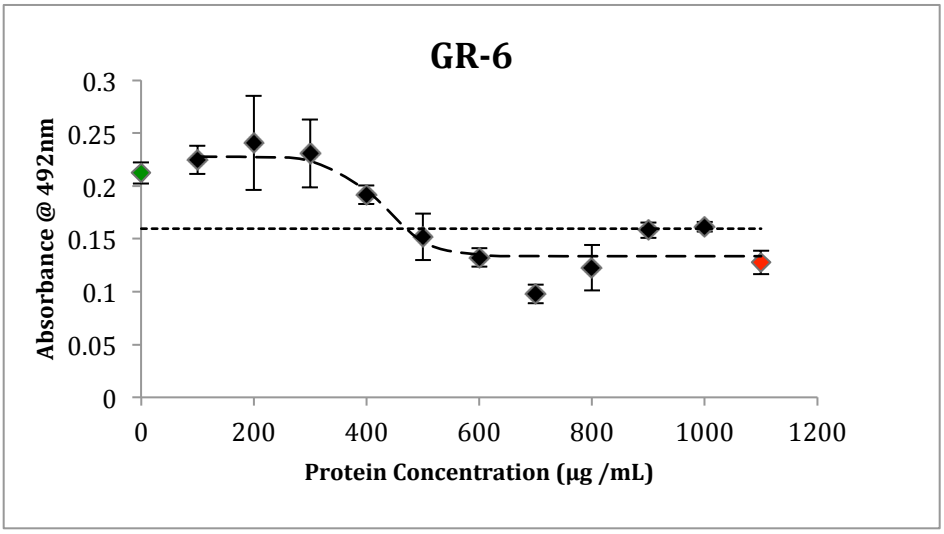
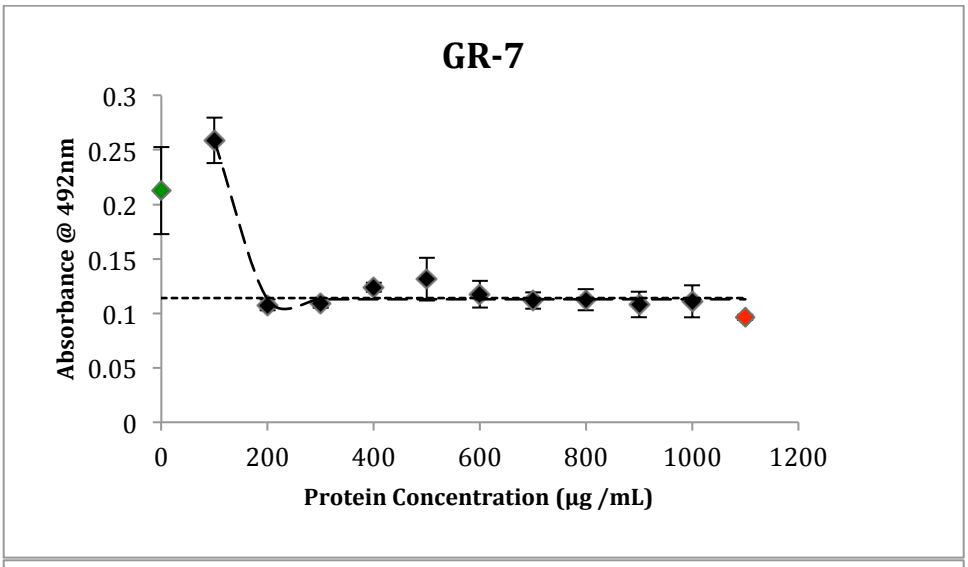
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GR-5
Absorbance @ 492nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GR-6
Absorbance @ 492nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


GR-7
Absorbance @ 492nm
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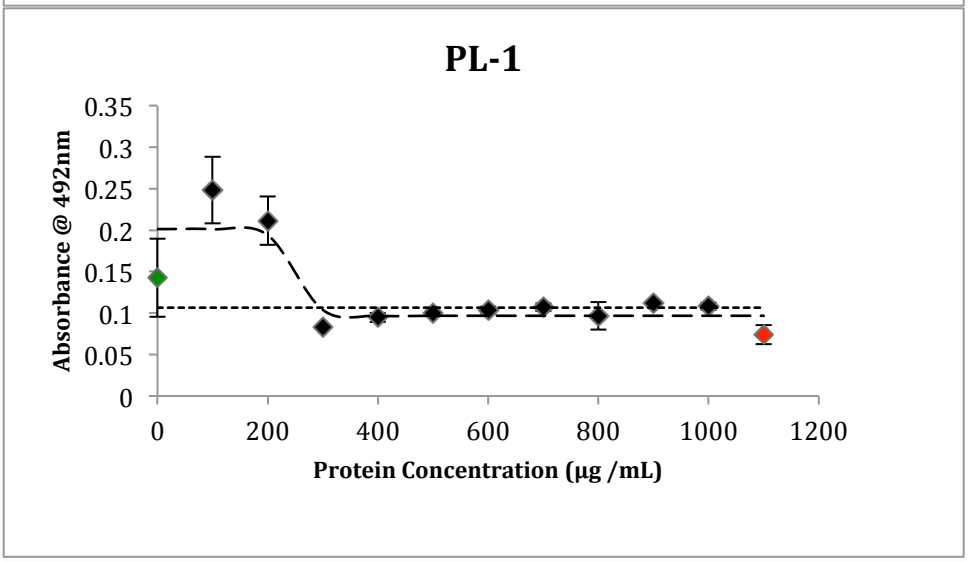
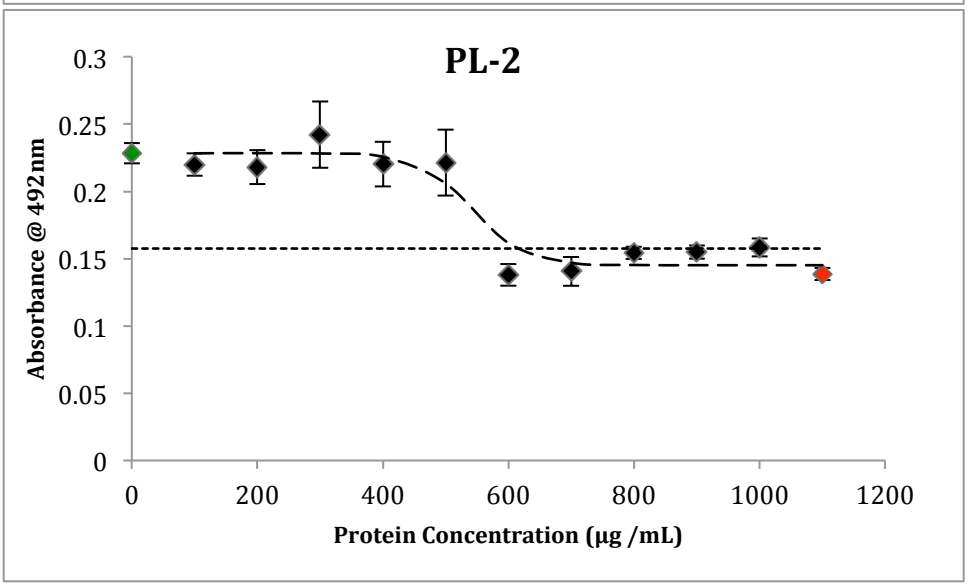
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


PL-1
Absorbance @ 492nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)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Absorbance @ 492nm
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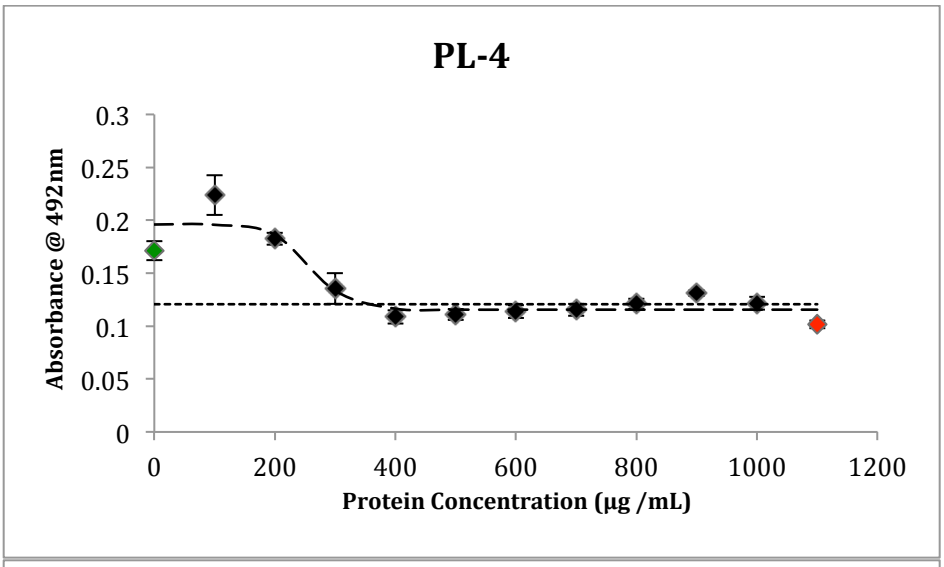
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


PL-4
Absorbance @ 492nm
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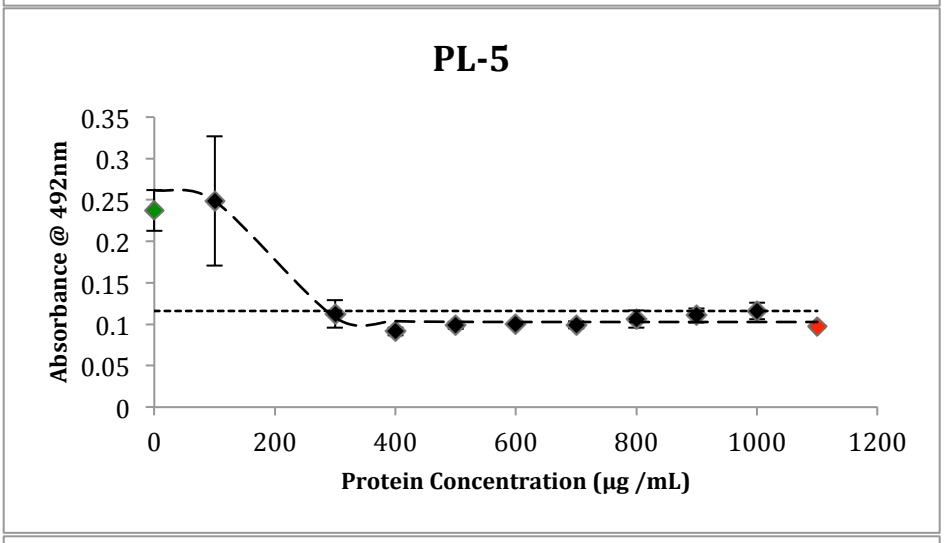
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)


PL-5
Absorbance @ 492nm
0.35
0.3
0.25
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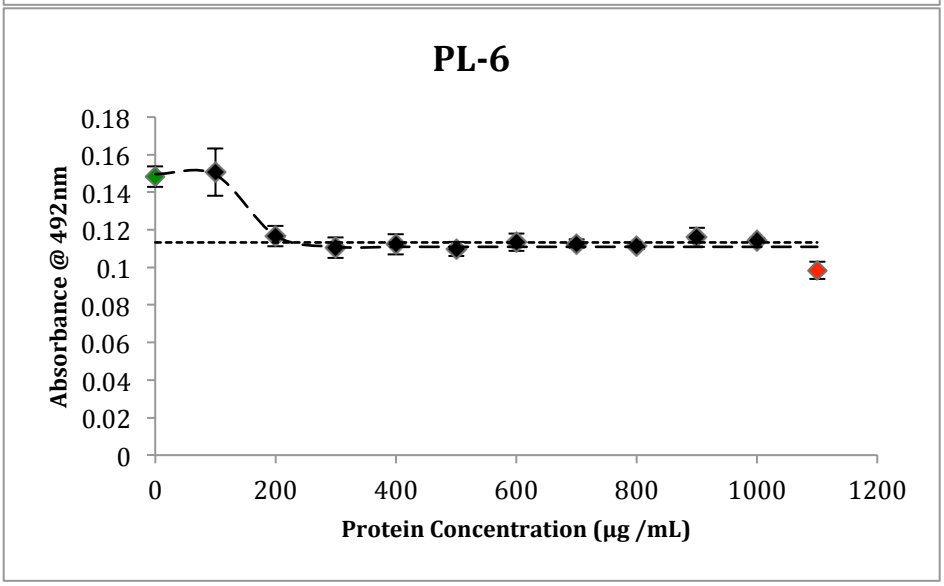
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)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Absorbance @ 492nm
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0.14
0.12
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0.06
0.04
0.02
0
0
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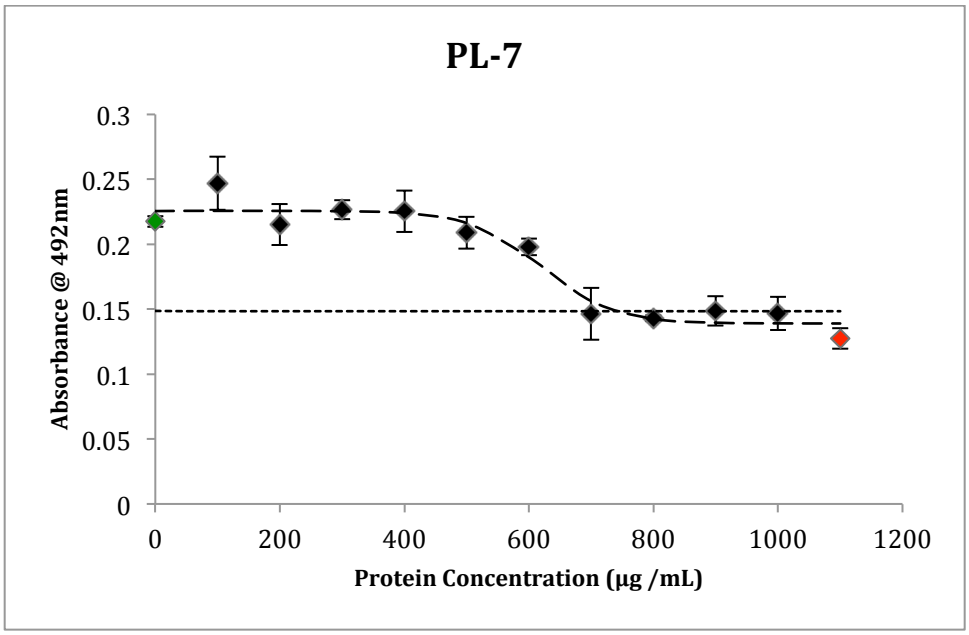
400
600
800
1000
1200
Protein Concentration (µg /mL)


PL-7
Absorbance @ 492nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
1200
Protein Concentration (µg /mL)