AMPHIBIAN DECLINES IN THE GREATER YELLOWSTONE ECOSYSTEM: TOADS WITH PROTECTION FROM BACTERIAL DISEASE

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✦ INTRODUCTION

During the past forty years, biologists have become increasingly concerned about the decline and disappearance of various amphibian species throughout the world (Wyman 1990, Wake 1991). An example of an amphibian decline in western North America is that of the Boreal Toad (*Bufo boreas*). A previously widespread and abundant species, the Boreal Toad has undergone large population declines and is now a candidate for threatened and endangered status in the eastern half of its range (Stebbins and Cohen 1995, Federal Register 1995).

Decreases in Boreal Toad abundance and distribution have been observed in Colorado, eastern Wyoming, and eastern Utah (Corn et al. 1989). From 1971-82, eleven Boreal Toad populations disappeared from the West Elk Mountains of Colorado (Carey 1993). During this eleven-year period, numerous individuals were observed exhibiting symptoms of redleg, a well-documented disease in amphibians caused by the bacterium *Aeromonas hydrophila* (Russel 1898, Emerson and Norris 1905, Kulp and Borden 1942, Reed and Toner 1942, Dusi 1949, Hunsaker and Potter 1960, Hird et. al. 1981, Nyman 1986, Carey 1993). This disease was considered responsible for the decline and ultimate extinction of these eleven toad populations. These extinctions led Carey to propose a hypothesis regarding the disease process which is stated as follows: (1) Some environmental factor or synergistic effects of more than one factor changes sufficiently to cause sublethal "stress;" (2) This stress directly causes suppression of the immune system, or indirectly causes immunosuppression by effecting elevated secretion of adrenal cortical hormones; (3) Immunosuppression, coupled with the apparent effect of cold body temperatures on the ability of immune systems of ectothermic animals to fight disease, leads to infection by *Aeromonas* or other infectious agents, and to subsequent death of individuals and extinction of populations.

Another area where Boreal Toads have apparently declined is within the Greater Yellowstone Ecosystem (GYE). Field surveys during 1991 revealed Boreal Toads to be less widespread and less abundant than in the past, especially in the southern portion of the GYE (Peterson et al. 1992, Koch and Peterson 1995). This contradicts Carpenter's earlier description of Boreal Toads as being the most wide-spread amphibian in the Jackson Hole region (1953). During the 1991 field season, a interesting relationship was detected among toad breeding sites. At sites where toads were still breeding, there was an unusual water chemistry that included such

components as high conductivity and high acid neutralizing capacity (Peterson et. al. 1992); many of these breeding sites were also geothermally influenced.

Various factors have been implicated in amphibian declines worldwide including ultraviolet radiation, drought, pollution, introduced species, acid precipitation, and habitat modification (Blaustein & Wake 1990; Phillips 1990; Wyman 1990; Pechmann et. al. 1991). Drought in the late 1980's and early 1990's was responsible for reduced breeding success of Boreal Toads at some locations within the GYE (Bartelt, unpublished data). However, drought does not appear to explain why Boreal Toads have declined in other portions of the GYE. Other unlikely causes for Boreal Toad declines within the GYE are acid precipitation, due to intermediate pH and/or adequate buffering capacity of most amphibian sites in the Rocky Mountains (Corn 1992); introduced species like trout, because predatory fish have been shown to avoid consuming *Bufo* tadpoles under laboratory conditions, (Voris and Bacon, Brodie et.al. 1987); and habitat modification within the GYE does not appear extensive enough to explain the declines. Because redleg has been implicated in Boreal Toad declines elsewhere (Carey 1993), we suspect it may have contributed to declines within the GYE.

Interestingly, a negative correlation between densities of *A. hydrophila*, a causative agent of redleg, and conductivity in freshwater environments has been described (Hazen et al. 1978). If this relationship were to be demonstrated within the GYE, it could help explain why toad populations appear to be persisting within these high conductivity/geothermally influenced sites.

Based on the observations that Boreal Toads appear to be primarily breeding in areas of high conductivity and that densities of *A. hydrophila* may decrease with conductivity, we expected to observe low densities of *A. hydrophila* within these high conductivity/geothermally influenced sites. Our hypothesis was that the low bacterial densities may reduce the risk of infection and outbreak of the disease. Our overall objective is to determine if Boreal Toads are protected from infection(s) with *A. hydrophila* by breeding in areas of high conductivity/geothermal influence. However, before we can address our overall objective, we needed to determine the relationships among toad breeding, water chemistry, and densities of *A. hydrophila* in the field. We conducted field studies during the summer of 1997 to address the following questions. After addressing these questions, we will conduct laboratory studies in 1998 to determine if Boreal Toads are protected from infection(s) with *A. hydrophila* by breeding in high conductivity/geothermally influenced water.

Questions
(1997)

1. What is the relationship between toad breeding and water chemistry?
2. What is the relationship between bacterial density and water chemistry?
3. What is the relationship between bacterial density and water column depth?
4. Does water chemistry change with season?
5. Does bacterial density change with season?
6. What is the relationship between bacterial density and toad breeding?

(A summary of the results to these questions can be found in Table 1).

Finally, if we found low bacterial densities within our high conductivity study sites, we would conduct a series of laboratory investigations to determine if the components of the water are limiting bacterial growth.

✦ METHODS

STUDY SPECIES

The Boreal Toad (*Bufo boreas boreas*) is found throughout the western half of the North American continent, from southern Alaska south to northern New Mexico in the Rocky Mountains (Stebbins 1995). The boreal toad is widely distributed throughout both Yellowstone and Grand Teton National Parks, from the lowest elevations in both parks up to 2865m (9400 ft) near Togwotee Pass (historically) (Koch and Peterson 1995). A previously common and widespread species, the Boreal Toad appears to have declined in both distribution and abundance over much of its range in the Western United States, including the Greater

Yellowstone Ecosystem. In Colorado, eastern Wyoming, and eastern Utah, this toad species could no longer be found at 85% of the sites in which it historically occurred (Corn et al. 1989).

STUDY SITES

Over the past six years, about fifteen to twenty Boreal Toad breeding sites have been identified within the GYE. During the summer of 1997, we sampled 12 breeding and 15 non-breeding sites (Table 2, Figure 1). We originally planned to sample an equal number of breeding and non-breeding sites, but flooding during the spring of 1997, along with other factors, prevented this. The sampled toad breeding sites are located throughout the GYE. For each sampled breeding site, we selected a nearby amphibian site where toad breeding has not been detected. Some of the nearby amphibian sites were also chosen because toad breeding occurred there historically and is no longer occurring at that location. This sampling design allowed us to compare the water chemistry between known toad breeding sites to other amphibian sites where toad breeding does not occur.

SAMPLING SCHEDULE

We sampled the selected breeding and non-breeding sites at least once during the 1997 field season. We sampled a subset of eleven sites (five breeding and six non-breeding) additional times during the months of July and August. Eight of these sites were sampled two additional times and three sites were sampled one additional time. We collected these additional samples from the same location within each site that the initial samples were taken. This sampling scheme allowed us to observe differences in water chemistry and bacterial densities between breeding and non-breeding sites, and also allowed us to observe changes in bacterial density and water chemistry over the course of a breeding season.

WATER SAMPLES

Water samples for determining bacterial densities were collected using sterile, individually wrapped, 120 ml polypropylene bottles. We collected three water samples near observed amphibian egg masses (if present) or where breeding activity is known to occur based on past observations. Samples were collected just below the water surface, from the middle of the water column, and just above the bottom sediment. We used these samples to determine the mean bacterial density for each sampling site. Water samples were collected by first submerging the bottle and then removing the lid. Once the bottle was filled, the lid was replaced and the bottle was removed from the water column.

We collected two additional water samples to be used for chemical analysis from the same location within each study site that the samples were collected for determining bacterial densities. We stored these samples in 250ml and 500ml polypropylene bottles at 4°C until sending them off for chemical analysis. The water samples stored in the 250ml bottles were analyzed for alkalinity, ammonia, chloride, nitrate, sulfate, and total phosphorus. The water samples stored in the 500ml bottles were analyzed for calcium, potassium, lead, magnesium, silica, aluminum, and sodium.

WATER CHEMISTRY

Water chemistry analyses were performed for samples from each study site. In the field, we used a Solomat portable pH, temperature, and conductivity meter (MPM 2000, Solomat Corporation, Connecticut) to determine pH, temperature, and conductivity at each sampling site. Unfiltered water samples (250ml bottle) were collected and stored at 4°C until they were taken to the Enviro H_2O Lab, Pocatello Idaho. Unfiltered samples were analyzed for alkalinity, ammonia, chloride, nitrate, sulfate and total phosphorus. All samples were analyzed within two weeks of being collected following standard methods (APHA, 1995). Filtered water samples (500ml) were preserved with 1ml of nitric acid and stored at 4°C until taken to the Laboratory for Environmental Geochemistry, Idaho State University, Pocatello Idaho. Bottles used here were washed with nitric acid and rinsed with deionized water prior to sample collection. Filtered samples were analyzed for the following components: calcium, potassium, lead, magnesium, silica, aluminum, and sodium. All samples were analyzed within six months of being collected following standard methods (APHA, 1995).

ISOLATION AND QUANTIFICATION OF *Aeromonas hydrophila*

Approximately 200µl of each water sample was placed directly onto a plate of Rimler Shotts medium (specifically designed for isolation of *A.*

hydrophila (Shotts and Rimler 1973)) with a micropipetter. We spread this water evenly over the agar surface using an L-shaped rod and turntable. An additional 30ml from each sample was filtered using a millipore filtering apparatus. Bacteria were captured on a 0.45 μm filter with a grid printed on the surface. We placed these filtered disks on the surface of an additional Rimler Shotts agar plate. The two inoculated plates from each sample were placed in a 37°C incubator for 20-24 hours. After the incubation period, we counted bacterial colonies that appeared yellow (presumptive for *A. hydrophila*). From the number of bacterial colonies counted, we determined the colony forming units (CFU's) which is the number of bacteria/ml of water.

DATA ANALYSIS

We entered all our data into a spreadsheet program (Microsoft Excel) and then transferred it into SPSS for Windows 7.0 (SPSS Inc., Chicago IL.) or SYSTAT 6.0 student version (SPSS Inc., Chicago IL.).

To determine which "general" factor(s) are correlated with breeding we ran a backwards stepwise logistic regression with conductivity, alkalinity, pH, and temperature. Only those factors with significance levels of 0.05 or less were kept after each step. Prior to statistical analysis, conductivity and alkalinity were log transformed to avoid possible problems with curvelinearity. To test for curvelinearity we initially ran a backwards stepwise logistic regression without transforming any of the variables. The -2 log likelihood values obtained from the logistic regression can be used to assess how well the model fit the data and if the relationships between data and the binary response variable were possibly curvelinear (Ramsey and Schafer 1997). Smaller -2 log likelihood values indicate the logistic regression model fit the data better and that the binary response variables are more likely to be related to the explanatory variables by a direct linear relationship, which is an assumption of the logistic regression model (Ramsey and Schafer). The backwards stepwise logistic regression, without any of the variables transformed, revealed a -2 log likelihood value of 27.529. After log transforming both conductivity and alkalinity, we ran the logistic regression again. The -2 log likelihood value for this logistic regression was 25.334. This suggested that by log transforming both conductivity and alkalinity the model fit the data better. This also suggests that the relationship of breeding and non-breeding to conductivity and alkalinity was curvelinear. Therefore, we ran the backwards stepwise logistic regression with alkalinity and conductivity log transformed.

Once the "general" factor(s) correlated with toad breeding were determined, we ran a logistic regression on the water chemistry variables known to be associated with the "general" water chemistry measurement(s). Not all of the water chemistry measurements were included in this analysis due to the concentrations of some of the variables in solution being below the detection limit of the analytical procedures. If the water chemistry variable(s) could not be detected by the analytical procedure(s) at a minimum of fifty percent of the study sites, they were removed from the analysis. Nitrate, ammonia, chloride, total phosphorus, aluminum, and lead were excluded from this and subsequent analyses because the concentrations of these ions were below the detection limit of the analytical procedure at over half the study sites. This left sulfate, silica, magnesium, calcium, and sodium as the only water chemistry variables included in these analyses. Any values included in the analyses that were still less than the detection limit of the analytical procedure were given a conservative estimate (e.g, <10 mg/l was estimated to be 9.9mg/l) in order to perform the statistical procedures.

To determine if the "general" factor(s) were highly correlated with mean CFU, we ran a backwards stepwise multiple regression on conductivity, alkalinity, pH, and temperature. Only those values with a significance level of 0.05 or less were kept after each step. Prior to statistical analysis, conductivity and mean CFU were log transformed to obtain normality. Temperature and alkalinity met the normality assumptions and were entered into the analysis without transformation. Values of pH were converted to hydrogen ion concentrations prior to analysis and each hydrogen ion concentration was multiplied by 10^7 to be recognized by SPSS. Once the "general" factor(s) highly correlated with mean CFU were determined, we ran a multiple regression analysis with the water chemistry variables known to be associated with these "general" water chemistry measurement(s). Prior to statistical analyses, chloride, silica, magnesium, sodium, potassium, and calcium were each $\log(x+1)$ transformed to obtain normality.

To determine the relationship between bacterial density and water column depth we used block design analysis of variance. Mean CFU values were log transformed to obtain normality prior to statistical analysis.

To determine whether bacterial density, temperature, pH, and conductivity vary significantly over the course of a breeding season, we used a repeated measures analysis of variance. Temperature met the assumptions of normality but, prior to statistical analysis, conductivity and mean CFU values were log transformed to obtain normality. Values of pH were converted to hydrogen ion concentrations prior to analysis and each hydrogen ion concentration was multiplied by 10^7 to be recognized by SPSS. The Geisser-Greenhouse Correction was used for the repeated measures analysis of variance with pH to correct for unequal variance. Mean pH values were calculated by taking the log of the mean hydrogen ion concentration.

Finally, to determine the relationship between toad breeding and bacterial density, we used a group comparison T test. Prior to statistical analysis, the mean CFU values were log transformed to obtain normality for the breeding and non-breeding sites.

✦ RESULTS

A summary of the results for each question can be found in Table 1.

Question 1- *What is the relationship between toad breeding and water chemistry?*

The majority of the Boreal Toad breeding sites, sampled within the Greater Yellowstone Ecosystem, had measurements of conductivity that were significantly higher than non-breeding sites. From the results of the backwards stepwise logistic regression we found that conductivity was the only variable that produced a significant model (Table 3). For each log unit increase in conductivity, the likelihood of a study site being a toad breeding site increased by a factor of 5.89. The approximate 95% confidence interval for this log likelihood ratio is 1.24 to 27.95. Toad breeding and non-breeding group membership as explained by conductivity is shown in Figure 2. This final model correctly classified 66.7% of the breeding sites and 86.7% of the non-breeding sites sampled during the 1997 field season.

Because conductivity is an indicator of the total number of ions in solution, we ran another logistic regression using only calcium, magnesium, potassium, sodium, silica, and sulfate as independent variables. We found that silica (Si) was the only variable remaining in the final model. For each unit increase in Si, the likelihood of a study site being a toad breeding site increased by a factor of 1.08. The 95% confidence interval for this log likelihood ratio is 0.96 to 1.23. This final model correctly classified only 33.3% of the breeding sites and 86.7% of the non-breeding sites sampled during the 1997 field season.

Question 2- *What is the relationship between bacterial density and water chemistry?*

We found that alkalinity, pH, and temperature were strongly and positively related to mean bacterial density. The results from the backwards stepwise multiple regression can be found in Table 4. Figure 3 shows the relationship between bacterial density and alkalinity and Figure 4 shows the relationship between bacterial density and conductivity.

Because alkalinity, pH and temperature were strongly related to mean CFU, we ran another multiple regression using pH, temperature and the two water chemistry variables most associated with alkalinity, calcium and magnesium. We found that all four contributed significantly and were each well below the cutoff to be removed in a stepwise fashion (Table 5). Therefore, this final model contains temperature, pH, calcium, and magnesium.

Question 3 – *What is the relationship between bacterial density and water column depth?*

Because developing toad tadpoles are commonly found at or near the bottom of freshwater habitats, we needed to determine if densities of *Aeromonas hydrophila* vary with water column depth. We found that the various levels of water column sampling had no effect on the density of bacteria measured. The water column samples collected from all the study sites revealed a median of 35 bacteria/ml with a range of 0 to 2630 just below the water surface, a median of 40 bacterial/ml with a range of 3 to 2760 from the middle of the water column, and a median of 25

bacteria/ml with a range of 0 to 2845 just above the bottom sediment. The lognormal bacterial densities were tested against each other and the calculated F-ratio was 0.321 with a probability of 0.727.

Question 4 – *Does water chemistry change with season?*

Because Boreal Toads are known to breed anytime from May to July (Koch and Peterson 1995) and even as late a August within the GYE (C.R. Peterson, unpublished data), we needed to determine if bacterial densities, water chemistry, and temperature varied significantly over the course of a single breeding season (May/June, July, August).

We found that the mean on-site temperature measurements for the four breeding and four non-breeding sites were not significantly different with respect to the time of year that the measurements were taken. The results for the repeated measures ANOVA can be found in Table 6. These results also indicate the four breeding and four non-breeding sites cannot be distinguished from each other by taking temperature measurements from the water. The mean on-site temperature measured for the four breeding sites sampled two additional times was 21.5 °C ± 7.32 SD in May/June, 20.5 ± 7.90 °C in July, and 24.05 ± 4.44 °C in August. For the four non-breeding sites the mean on-site temperature was 17.1 ± 7.49 °C in May/June, 20.3 ± 1.41 °C in July, and 17.9 ± 2.46 °C in August. The comparisons in temperature between the breeding and non-breeding sites during the May/June and August sampling period suggest a possible difference; however, the differences were not statistically significant.

Measurements of pH did not significantly vary over the breeding season within the four breeding and non-breeding sites. The results for the repeated measures ANOVA with pH can be found in Table 7. The mean pH value for the four breeding sites sampled two additional times was 8.60 in May/June, 7.80 in July, and 8.95 in August. For the four non-breeding sites the mean pH value was 6.83 in May/June, 7.62 in July, and 8.26 in August.

Measurements of conductivity did vary significantly over the breeding season within the four breeding and four non-breeding sites. The results for the repeated measures ANOVA with conductivity can be found in Table 8. However, a Tukey test was unable to reveal the source of those differences (Table 9). The results also indicate that the four breeding and four non-breeding sites cannot be distinguished from each other by taking conductivity measurements of the water. A graphical representation of the results can be found in Figure 5. For the four breeding sites sampled two additional times, the median conductivity value was 339 µs/cm with a range of 28.7 to 1071 in May/June, 432 µs/cm with a range of 76.6 to 1445 in July, and 431 µs/cm with a range of 90.3 to 1264 in August. For the four non-breeding sites the median conductivity value was 42.6 µs/cm with a range of 15.9 to 138 in May/June, 58.2 µs/cm with a range of 20.8 to 206 in July, and 52.4 µs/cm with a range of 15.0 to 228 in August.

Question 5 – *Does bacterial density change with season?*

Densities of *Aeromonas hydrophila* did not significantly vary over the breeding season within the four breeding and four non-breeding sites. The results for the repeated measures ANOVA with bacterial density can be found in Table 10. However, it should be noted that bacterial densities at the South Entrance stream, below the horse corral, were measured at 273 bacteria/ml during June, increased to 2803 bacteria/ml during July, and decreased to 60 bacteria/ml during August. The results also indicate that the four breeding and four non-breeding sites cannot be distinguished from each other by taking measurements of bacterial density from the water. For the four breeding sites sampled two additional times, the median bacterial density was 83 bacteria/ml with a range of 15 to 2700 during May/June, 828 bacteria/ml with a range of 35 to 2803 during July, and 51 bacteria/ml with a range of 10 to 427 during August. For the four non-breeding sites the median bacterial density was 13 bacteria/ml with a range of 3 to 92 during May/June, 28 bacteria/ml with a range of 7 to 47 during July, and 18 bacteria/ml with a range of 8 to 105 during August.

Question 6 - *What is the relationship between bacterial density and toad breeding?*

Given that the occurrence of toad breeding is associated with high conductivity, and that densities of *Aeromonas hydrophila* increase with conductivity and alkalinity, we wanted to determine

if the occurrence of toad breeding is associated with bacterial density. The water samples collected for determining bacterial density at each study site revealed a median of about 83 bacteria/ml with a range of 20 to 2700 for all the breeding sites and a median of about 33 bacteria/ml with a range of 3 to 2723 for all the non-breeding sites. The lognormal bacterial densities were tested against each other and the resulting T value was 1.735 with a probability of 0.095. This indicates that the mean bacterial density measured at the toad breeding sites was not significantly different from the mean bacterial density measured at the non-breeding sites. However, it should be noted that Nez Perce Pond, a high conductivity (1185 µs/cm) non-breeding site, had a density of 2723 bacteria/ml. The next highest bacterial density measured at a non-breeding site was 128 bacterial/ml at pond 25. This is a difference of over 2500 bacteria/ml. If Nez Perce Pond is removed from the analysis, the median bacterial density is reduced from 33 bacteria/ml to 29 bacterial/ml with a range of 3 to 126 for the non-breeding sites. The resulting T is 1.586 with a probability of 0.024, indicating a significant difference.

✦ DISCUSSION

During the past forty years, Boreal Toads appear to have declined in abundance and distribution throughout much of the Greater Yellowstone Ecosystem (Peterson et al. 1992). The results of this study demonstrate that the remaining Boreal Toad breeding sites, located within the GYE, can be distinguished from non-breeding sites by examining the conductivity of the water. This observed difference in conductivity between toad breeding and non-breeding sites is similar to observations made during the 1991 field season (Peterson et. al. 1992). Conductivity correctly classified 67% of the breeding sites (eight of twelve) sampled during 1997 with an overall accuracy of 78%. Each log unit increase in conductivity increased the likelihood of a study site being a toad breeding site by a factor of 5.89. The only ion found to be significantly different between breeding and non-breeding sites in this study was silica. However, silica had a confidence interval around its odds ratio of 0.96 to 1.23 and could only correctly classify 33.3% of the breeding sites. Therefore, even though silica was the only variable remaining in the second logistic regression model, conductivity is a better predictor of toad breeding.

The logistic regression model with conductivity did not correctly classify four of the twelve toad breeding sites sampled during 1997. However, it should be noted that toad breeding could not be verified at McReynolds Reservoir (32.8 µs/cm) even though the breeding activities of Boreal Toads were detected at this study site in the early 1990's. The majority of the Boreal Toad tadpoles at the South Entrance stream below the horse corral (28.7 µs/cm) perished prior to metamorphosis due to infection and a substantial portion of the toad tadpoles at Pond 26 (152 µs/cm) perished shortly after hatching. This left Stamp Meadows, located on the Targhee National Forest, as the only low conductivity study site where Boreal Toads are known to breed successfully. Because three of the four toad breeding sites not correctly classified by the conductivity model had low tadpole survivorship, it may be more appropriate to label the eight toad breeding sites correctly classified by the conductivity model as successful breeding sites.

Unfortunately, measurements of water chemistry at toad breeding sites from over forty years ago were not taken. These data would have been useful in determining whether Boreal Toad breeding sites have always been associated with high conductivity within the GYE. However, we do know that Boreal Toads historically bred at Lower Moose Pond within Grand Teton National Park, and near Togwotee Pass within Bridger Teton National Forest (Carpenter 1953, and field notes). Boreal Toads were not detected at these two sites during the 1991 field season (Peterson et. al. 1992) or during the current study. During 1997, the level of conductivity measured at Lower Moose Pond was 15.9 µs/cm and the level of conductivity measured at Togwotee Pass Pond was 41.5 µs/cm. Both of these sites have levels of conductivity that are substantially lower than most of the Boreal Toad breeding sites sampled during 1997 (Figure 2). At present, we can only speculate as to whether Boreal Toads have only declined and disappeared from areas with low conductivity.

We isolated and determined the concentration of *A. hydrophila* in the water for each of our study sites during 1997 because redleg has been implicated in Boreal Toad declines elsewhere (Carey 1993). Interestingly, a negative correlation between conductivity and densities of *A. hydrophila* in freshwater environments has been described (Hazen et al. 1978). From the results of our study,

we found *A. hydrophila* to be positively correlated with conductivity (Figure 4), and found that alkalinity, pH and temperature (°C) significantly predicted bacterial density just as well as temperature, pH, alkalinity, and conductivity combined. This observation is just the opposite of what we expected. Based on a previous study (1978), we predicted low bacterial densities within our high conductivity breeding sites. Therefore, we rejected our original hypothesis that Boreal Toads might be protected from redleg by breeding in water with very low densities of bacteria and that the low bacterial densities might reduce the risk of infection and outbreak of disease.

Because densities of *A. hydrophila* were positively correlated with conductivity, we needed to generate alternate hypotheses for how Boreal Toads could be protected from redleg within these high conductivity sites. One hypothesis is that the unusual water chemistry from the toad breeding sites may not allow *A. hydrophila* to "turn on" the virulence factors needed to successfully infect a host, even when an individual is immuno-compromised. Another hypothesis is that protection from redleg could be a product of the breeding sites being geothermally influenced. Sites which are geothermally influenced may maintain continuously higher temperatures than non-thermal sites and be less susceptible rapid changes in temperature. This could potentially reduce cold shock as an environmental stressor within these areas.

Because Boreal Toad tadpoles are commonly found at or near the bottom of freshwater habitats, we needed to determine if bacterial densities vary consistently with water column depth. We found that densities *A. hydrophila* did not consistently vary with depth among all the breeding and non-breeding sites. If we had found densities of *A. hydrophila* to be consistently higher at a given depth among all the study sites, we would have needed to consider this when deciding the level of bacteria to use for our infection studies in 1998.

During 1997 we found that densities of *Aeromonas hydrophila* did not significantly vary over the breeding season within the four breeding and non-breeding sites sampled two additional times. However, it is worth noting that the mean bacterial densities for the South Entrance stream below the horse corral were measured at 273 bacteria/ml in June, increased to 2803 bacteria/ml in July, and decreased to 60 bacteria/ml during August. The South Entrance stream below the horse corral is the only breeding site currently found in Yellowstone with conductivity measurements below 100µs/cm. The bacterial densities observed at this site in July are similar to the bacterial densities measured at study sites (breeding and non-breeding) with conductivity measurements greater than 400µs/cm. While sampling this study site in July, we observed approximately forty dead and decaying tadpoles. Some of these tadpoles could be identified as *Bufo boreas*, where others were identified as Columbia Spotted Frog (*Rana luteiventris*). The week prior to sampling this site in July, adult spotted frogs were apparently observed exhibiting symptoms similar to redleg (Debra Patla, personal communicaton). It is unknown whether *A. hydrophila* was responsible for this observed die-off in tadpoles and adult spotted frogs, because no swabs of the decaying tadpoles were taken due to the high probability of the causative agent being hidden by the growth of saprophytic fungi and bacteria. Also of interest, the temperature of the water at the time the dead tadpoles were found was 10.7 °C. Because the immune systems of ectothermic animals are apparently temperature dependent, (Kluger 1979), this "cooler" water temperature may have suppressed the ability of the tadpoles immune systems to function properly and led to some type of infection, and ultimately death.

Given that the majority the known Boreal Toad breeding sites in the GYE occur in areas with high conductivity and that densities of *A. hydrophila* were found to be positively correlated with conductivity, one might expect bacterial densities to be correlated with the occurrence of toad breeding. The results from this study found no significant difference in bacterial density between breeding and non-breeding sites. However, most of the observed variation in bacterial density for the non-breeding sites was driven by a high conductivity site known as Nez Perce Pond. This study site actually increased the mean bacterial density measured for the non-breeding sites by 178.6 bacteria/ml, and indicates that the levels of bacteria measured are more correlated with the chemical and physical (i.e., temperature) components of the water than with the presence of toad breeding.

In summary, the results of this study verified that the remaining Boreal Toad breeding sites found in the Greater Yellowstone Ecosystem are predominantly found in areas with high

conductivity in comparison to historical sites where toad breeding no longer occurs and other amphibian breeding sites where toad breeding has not been detected. Densities of *Aeromonas hydrophila*, a causative agent of redleg, were found to be positively correlated with conductivity, alkalinity, pH and temperature and are opposite than what we predicted. Because toad breeding has been shown to be associated with conductivity and densities of *Aeromonas hydrophila* increased with conductivity, we will test the effects of conductivity and bacterial density on the susceptibility of tadpoles to infection during 1998. We also plan to test the effect of temperature on the susceptibility of toad tadpoles to infection because many of the Boreal Toad breeding sites found in the GYE are also geothermally influenced.

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Table 1. Summary of the results.

Question	Dependent Variable	Independent Variables	Statistical Procedure	Results	Comments
1. What is the relationships between toad breeding and water chemistry?	breeding	most water chemistry variables	backwards stepwise logistic regression	1. Model with conductivity correctly classified 67% of breeding sites and 87% of nonbreeding sites. 2. Each log unit increase in conductivity increases the likelihood of a study site being a toad breeding site by a factor of 5.89	1. Some water chemistry variables were below detection limits. 2. Some variables were log transformed to correct for curvilinearity.
2. What is the relationship between bacterial density and water chemistry?	bacterial density	most water chemistry variables	backwards stepwise multiple regression	1. Mean bacterial density was strongly and positively correlated with alkalinity, pH and temperature. 2. Mean bacterial density was positively correlated with temperature, pH, calcium, and magnesium.	1. Some water chemistry variables were below detection limits. 2. Some water chemistry variables were excluded because they couldn't be normalized with transformations.
3. What is the relationship between bacterial density and water column depth?	Bacterial density	water column depth (top, middle, and bottom.	Block design ANOVA	No significant differences in bacterial densities at different depths.	Bacterial densities were log transformed to obtain normality.
4. Does water chemistry change with season?	PH, conductivity, and temperature	season (May/June, July, August)	Repeated measures ANOVA	Conductivity ($p=0.019$)	Only sampled at 4 breeding and 4 nonbreeding sites.
5. Does bacterial density change with season?	Bacterial density	season (May/June, July, August)	Repeated measures ANOVA	No significant changes.	Only sampled at 4 breeding and r nonbreeding sites.
6. What is the relationship between bacterial density and toad breeding?	Bacterial density	breeding/nonbreeding sites	T-test	1. No significant difference if all sites included ($p=0.095$). 2. Significant difference if Nez Perce Pond not included ($p=0.024$).	Nez Perce Pond is a very high conductivity nonbreeding site with very high bacterial density.

Table 2. The location of study sites within the Greater Yellowstone Ecosystem sampled during 1997. The number of each study site corresponds to the numbers displayed on the map (Figure 1) and the circles on the map represent the study site location.

Site	Toad Breeding Site	Yellowstone National Park	Grand Teton National Park	Targhee National Forest	Bridger Teton National Forest	Caribou National Forest	Remarks
1. Slide Lake	no	x					
2. Slough Creek pond	no	x					
3. Indian pond	yes	x					Historical?
4. Lodge Creek - Pool 3	no	x					
5. Lodge Creek Lagoon	no	x					
6. Harlequin Lake	no	x					
7. Paint Pot Toad Pool	yes	x					
8. Nex Perce Pond	no	x					
9. Tangled Creek	yes	x					
10. South Entrance thermal stream	yes	x					
11. South Entrance vernal pond	yes	x					
12. South Entrance permanent pond	no	x					
13. South Entrance stream by corral	yes	x					
14. Pond 25	no	x					
15. Pond 26	yes	x					
16. Taggart Lake Outflow	no		x				
17. Lower Moose Pond	no		x				Historical toad site
18. Leigh Lake	no		x				
19. Ditch Creek	yes				x		
20. Togwotee Pass Pond	no				x		Historical toad breeding site
21. Blackrock Oxbow	yes				x		
22. Stamp Meadow	yes			x			large (>50 adult) population
23. McReynolds Reservoir	yes			x			
24. Moose Creek Cattail Pond	no			x			
25. North Leigh Mill Pond	no			x			
26. Tin Cup Creek Oxbow	yes					x	
27. Tin Cup Creek Pond	no					x	

Table 3. Results for the backwards stepwise logistic regression of toad breeding verses temperature, conductivity, alkalinity, and pH. Each model represents a single step where one of the variables was removed. The final model (4) contains only conductivity.

Variable	Model	Coefficient	S.E.	Wald	df	Sig.	Odds Ratio	95% CI
pH	1	1.0910	0.8425	1.6670	1	0.1953	2.9774	0.57-15.52
Temperature		-0.1674	0.1332	1.5788	1	0.2089	0.8459	0.65-1.09
Conductivity †		4.2460	2.6692	2.5305	1	0.1117	69.8279	0.37-13064
Alkalinity†		-4.7912	3.0802	2.4196	1	0.1198	0.0083	0.00002-3.48
Constant		-5.4548	5.2275	1.0889	1	0.2967		
pH	2	0.7388	0.7634	0.9366	1	0.3332	2.0934	0.47-9.34
Conductivity†		2.8516	2.0976	1.8481	1	0.1740	17.3158	0.28-1056
Alkalinity†		-2.6219	2.1501	1.4870	1	0.2227	0.0727	0.001-4.92
Constant		-6.8043	4.9464	1.8922	1	0.1689		
Conductivity†	3	3.7829	1.8150	4.3439	1	0.0371	43.9418	4.30-448.3
Alkalinity†		-2.9692	2.0521	2.0935	1	0.1479	0.0513	0.0009-2.87
Constant		-2.4219	2.0098	1.4520	1	0.2282		
Conductivity†	4 (Final)	1.7727	0.7948	4.9740	1	0.0257	5.8867	1.24-27.95
Constant		-3.9623	1.7467	5.1458	1	0.233		

† log transformed variable

Table 4. Results for the backwards stepwise multiple regression of bacterial density verses temperature, alkalinity, pH and conductivity. Each model, beyond model 1, represents a step with one variable removed from the analysis.

Variables	Model	Sum of Squares	df	Mean Square	R-square	Adjusted R Square	F	Sig
Temperature,	1 Regression	11.070	4	2.762	0.589	0.514	7.885	0.00
Alkalinity, pH*,	Residual	7.722	22	0.351				
Conductivity†	Total	18.792	26					
	2 Regression	10.987	3	3.662	0.585	0.53	10.792	0.00
Temperature,	Residual	7.805	23	0.339				
Alkalinity, pH*	Total	18.792	26					

* converted to hydrogen ion concentrations
† log transformed variable

Table 5. Results for the backwards stepwise multiple regression of bacterial density verses temperature, magnesium, and calcium. Each variable was below the cutoff to be removed from the model. Therefore, the final model contains all three variables.

Variables	Model	Sum of Squares	df	Mean Square	R Square	Adjusted R Square	F	Sig.
Temperature	1 Regression	13.493	4	3.373	0.718	0.667	14.007	0.00
Magnesium†	Residual	5.298	22	0.241				
Calcium†	Total	18.792	26					
pH*								

† log transformed variable.
* converted to hydrogen ion concentration

Table 6. Repeated measures analysis of variance for temperature. Breed represents the between effects and season represents the within effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Breed	77.042	1	77.042	1.516	0.264
Error	304.958	6	50.826		
Season	11.523	2	5.762	0.235	0.794
Season*Breed	37.403	2	18.702	0.762	0.488
Error	294.367	12	24.531		

Table 7. Repeated measures analysis of variance for pH. Breed represents the between effects and season represent the within effects. The Giesser-Greenhouse Correction was used to adjust for unequal Variance.

Source	Sum of Squares	df	Mean Square	F	Sig.
Breed	163.408	0.5325	306.864	4.376	0.13
Error	224,075	3.195	70.133		
Season	222,625	1.065	209.022	3.971	0.089
Season*Breed	254.435	1.065	238.888	4.548	0.073
Error	336.403	6.39	52.641		

Table 8. Repeated measures analysis of variance for conductivity. Breed represents the between effects and season represents the within effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Breed	3.643	1	3.643	4.723	0.073
Error	4.628	6	0.771		
Season	0.152	2	7.59E-02	5.583	0.019
Season*Breed	4.01E-03	2	2.00E-03	0.147	0.864
Error	0.163	12	1.36E-02		

Table 9. Tukey test for repeated measures analysis of variance for conductivity.

source	Comparison	Difference	SE	q	$q_{0.05,12,3}$	Conclusion
Within effects (non-breeding)	May/June vs August	0.123	0.058	2.11	3.77	$\mu=\mu$
	May/June vs July	0.175	0.058	3.00	3.77	$\mu=\mu$
	July vs August	0.053	0.058	0.91	3.77	$\mu=\mu$
Within effects (breeding)	May/June vs August	0.183	0.058	3.14	3.77	$\mu=\mu$
	May/June vs July	0.189	0.058	3.24	3.77	$\mu=\mu$
	July vs August	0.005	0.058	0.086	3.77	$\mu=\mu$

Table 10. Repeated measures analysis of variance for bacterial density. Breed represents the between effects and season represents the within effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Breed	3.816	1	3.816	3.161	0.126
Error	7.242	6	1.207		
Season	0.58	2	0.29	1.241	0.324
Season*Breed	0.534	2	0.267	1.142	0.351
Error	2.803	12	0.234		

Figure 1. The location of 1997 sampling sites in the Greater Yellowstone Ecosystem.

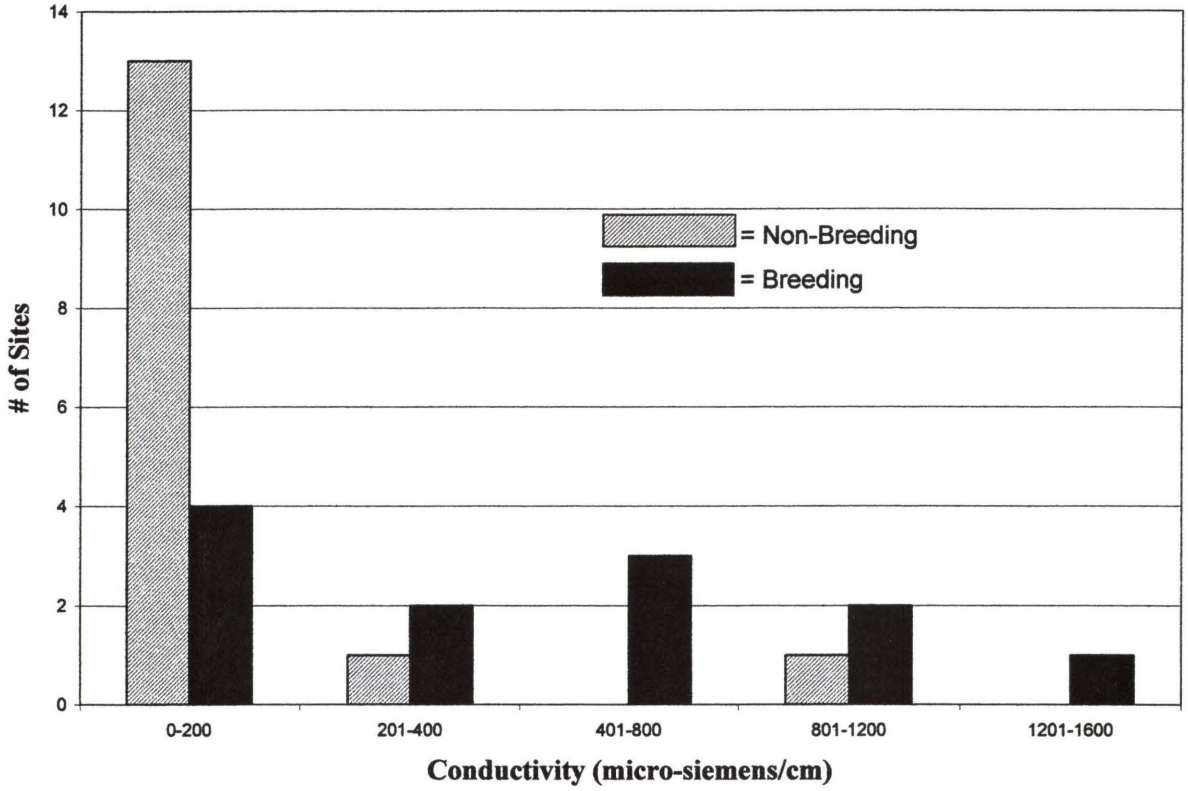

Figure 2. Breeding and non-breeding group membership as demonstrated by conductivity.

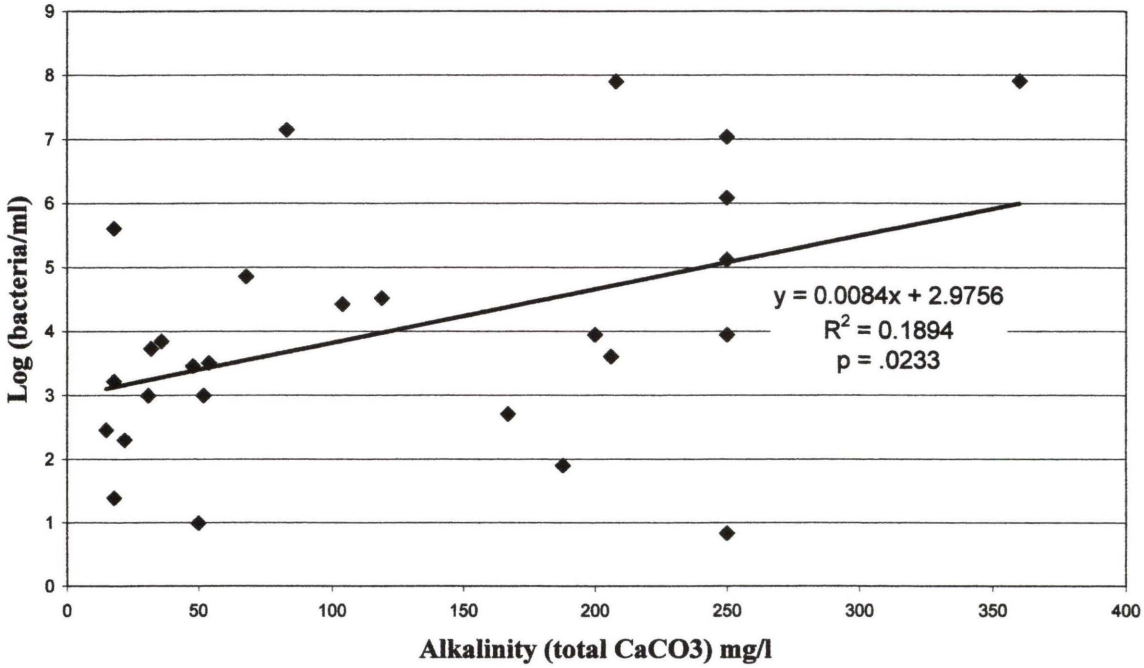

Figure 3. The regression of log(bacteria/ml) verses alkalinity.

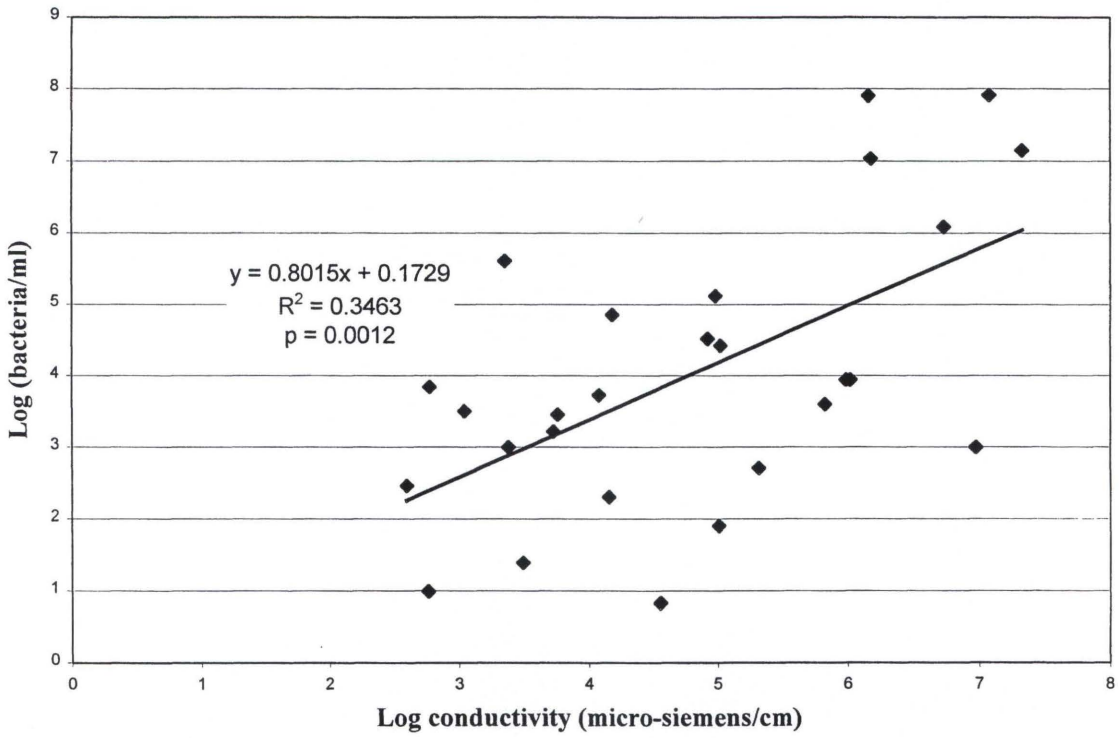

Figure 4. Regression plot for log (bacteria/ml) verses log (conductivity).

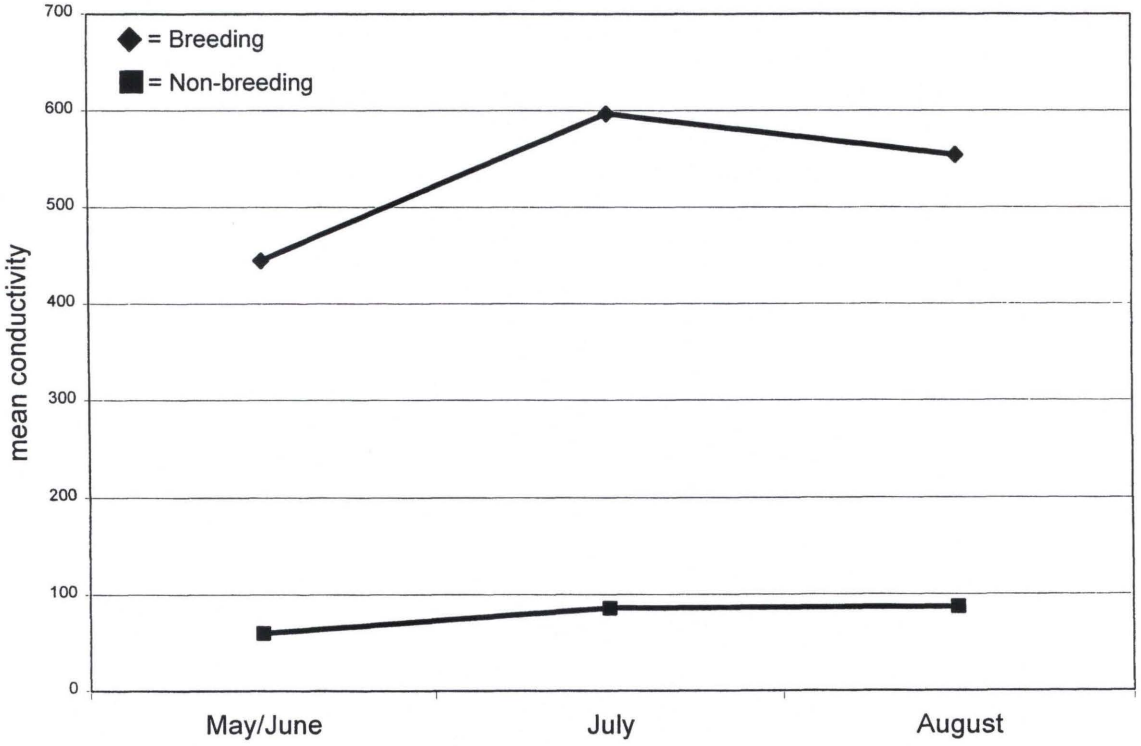

Figure 5. Changes in conductivity during 1997 within the four breeding and non-breeding sites sampled two additional times.