STRESS LEVELS IN GREEN FROGS IN RELATION TO COLONIZATION BY A WATERBORNE PATHOGEN

FINAL URGE REPORT TO THE PIERCE CEDAR CREEK INSTITUTE

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Introduction

Amphibians are in decline globally and the causes of the declines are poorly understood (reviewed by Green et al., 2002). In the Great Lakes region, the disease 'red-leg' has been implicated in mass-mortality of captive and natural populations of frogs (Forbes et al., 2004). This disease is typically associated with the presence of the bacterium *Aeromonas hydrophila*, which is ubiquitous in aquatic environments and frequently linked to red-leg disease (Bradford, 1991). At the Pierce Cedar Creek Institute, prevalence of *A. hydrophila* on frogs is several times higher than in other populations in the Great Lakes region (86.0%, n = 185; McCurdy and Krum, 2005), although the causes and consequences of high prevalence are unclear.

One hypothesis to explain the distribution of *Aeromonas* on frogs is that this parasite is opportunistic – proliferating and causing disease in hosts with weak immune systems. In fact, researchers have suggested that stresses associated with environmental change are a possible cause of red-leg disease and other emerging amphibian diseases (Rollins-Smith, 2001). Similar links between environmental change, stress, and parasitism have been suggested as explanations for sudden epizootics of malaria in birds and epizootics of trematodes in populations of invertebrates (e.g., McCurdy et al., 1998; McCurdy, 2001). Researchers have also suggested that opportunistic parasites can have sudden, drastic impacts to host populations and even alter the structure of ecological communities (e.g., McCurdy and Moran, 2004).

During the summer of 2006, we investigated links between stress, parasitism, and environmental conditions (breeding versus non-breeding) in green frogs, *Rana clamitans*. Using low-impact, non-destructive sampling techniques (catching and swabbing the skin of frogs and taking a small blood sample from each frog), we began a process to determine if levels of a stress hormone are related to either colonization by *Aeromonas* or reproductive activity. We expect that

frogs under greater stress (at any time of year) will be more likely to be colonized by *Aeromonas* bacteria and will harbor greater intensities of bacteria. We also expect that stress hormone levels and colonization by *Aeromonas* will be highest during the breeding season, when frogs are likely under the most stress (especially males, which suffer immunosuppression due to high levels of testosterone; McCurdy et al., 1998).

Methods

We studied prevalence and intensity of *Aeromonas hydrophila* on adult green frogs and collected blood samples from frogs to assess whether colonization by bacteria is correlated with levels of the stress hormone corticosterone (a sensitive hormone that is closely associated with stress and immune response in frogs; Moore et al., 2005). From our studies in 2005, we knew that green frogs are common throughout the property and that they harbor *Aeromonas* bacteria (McCurdy and Krum, 2005). We captured frogs between May 16 and July 12, 2006 by searching four known breeding ponds (locations described in McCurdy and Krum, 2005). Frogs began calling on May 24, 2006. Throughout this report, prevalence is defined as the percentage of animals harboring *Aeromonas* bacteria, whereas intensity refers to actual counts of bacterial colonies from animals colonized with *Aeromonas* bacteria.

When captured, each frog was immediately rubbed gently on the abdomen and legs with 3 sterile swabs (rubbed on frogs within separate 2.5 cm x 2.5 cm areas covered with disposable templates to record standardized intensity of bacteria). These standardized measurements were used to gauge intensities of bacteria (if present) on frogs. A fourth swab was then rubbed over the entire ventral side of the frog to maximize our ability to detect the presence of bacteria (assess prevalence) in cases where bacteria might not have been present within any of the smaller

templates. Each frog was then transported immediately to the nearby aquatic laboratory in a clean container with pond water. Swabs were stored individually in a phosphate buffer solution for transport to the lab (Taylor et al., 1999).

In the lab, each frog (typically 1-2 were captured per day) was anesthetized by immersing it in a 2% solution of MS-222. The frog was then weighed, measured (snout-vent length), and a small blood sample was taken (total volume = 0.25-0.5 mL; methods described in Beaupre et al., 2004). An externally-visible, fluorescent elastomer numbered tag was then injected into the frog to mark it and prevent accidental re-sampling of the same animals (Nauwelaerts et al., 2000). Blood samples were centrifuged to separate plasma, which was then stored at -20°C for further analysis. Swabs were inoculated onto plates containing Ryan's *Aeromonas* medium and cultured at 27°C for 48-hours (Ryan's medium includes an antibiotic which kills most bacteria other than *Aeromonas* strains; Forbes et al., 2004). Intensities of infection were be measured by counting colony-forming-units on plates using a colony counter. In cases, where a lawn of bacteria was present (>200 cfus per plate), the plate was recorded as TNTC (too numerous to count). These plates were assessed as having 'high' intensities of bacteria on them in our analysis.

At the time this report was prepared, we are still working on a process to measure corticosterone levels from plasma samples using HPLC/UV detection methods (initial trials have been promising overall, but problems with the HPLC machine software have led to inconsistent results with control samples). Ultimately, data on prevalence and intensity of *Aeromonas* will be assessed in relation to species, sex, mass, body condition, date-of-capture, spatial location, and corticosterone level. A second set of samples is also planned for October, 2006 to assess condition, stress, and parasitism of frogs after the breeding season.

Data were analyzed using logistic regression analysis to assess importance of factors such as water temperature, capture date, frog mass, and frog length on levels of bacteria found on frogs ('low'=1-200 colonies/plate versus 'high'=TNTC/>200 colonies/plate). Multiple regression analysis was used to compare mean intensities of bacteria on frogs in relation to environmental factors. Chi-square tests were used to compare data on prevalence between our study and that of McCurdy and Krum (2005), and to compare levels of infection (low versus high) across sites and between males and females.

Results / Discussion

During the spring/summer of 2006, we captured 62 different green frogs within the PCCI property. *Aeromonas hydrophila* bacteria were common on the skin of green frogs captured over the spring/summer of 2006 in that 95.2% (n =62) of frogs harbored the bacteria (data from whole-body swabs). Although high, prevalence did not differ statistically from that found in a survey of green frogs made in 2005, when prevalence was 90.7% (n = 129) on frogs captured at the same ponds used in the current study ($X^2 = 1.2$, $df = 1$, $P = 0.28$). In another study of green frogs in eastern Ontario, Forbes et al. (2004) found that prevalence of *A. hydrophila* on epidermal samples of frogs was only 4%, which was more typical for prevalence values of *Aeromonas* on other species of frogs. Further monitoring of frog populations before and after the breeding season (discussed below) and from watersheds surrounding the PCCI would provide a better context in which to assess the causes and consequences of high prevalence of *Aeromonas* bacteria on frogs.

Although multiple samples were taken from small areas on each frog, colony-counts of bacteria frequently exceeded 200 cfu/s per plate (TNTC). We used a logistic regression model to

compare the frequency of these high-density plates to those with containing lower mean counts of bacteria (=low density). We found that the frequency of high-density plates pates increased in relation to date (i.e., plates assessed later in the season were more likely to harbor high densities of bacteria (model: $X^2 = 9.4$, df=4, $P = 0.05$; season: Wald $X^2 = 5.8$, df = 1, $P = 0.02$; Figure 1). Factors such as frog wet mass, frog length, and water temperatures were not related to the likelihood of plates being 'low' versus 'high' density (all $P > 0.05$). Sex was also not related to the frequency of high versus low plates ($X^2 = 0.6$, df =1, $P = 0.43$), and there was no significant difference in rates of 'low' versus 'high' colony counts across the four ponds samples ($X^2 = 3.0$, df = 3, $P = 0.39$).

Among plates where the number of colonies of bacteria could be assessed (mean density < 200 colonies per plate), we found no relationships between bacterial density and date, wet mass, length, or water temperature ($R^2 = 0.13$, $n = 27$, $P = 0.79$; P for all effect tests > 0.05). However, this sample is biased heavily toward the early breeding season because plates taken later (with very high densities of bacteria) could not be assessed (discussed above). Thus, densities did increase overall as the breeding season progressed, but the exact nature of the relationship between date and intensity of infection could not be established. We plan to sample frogs again during the fall of 2006 to compare counts of colonies, and ultimately stress levels, during the post-breeding period. Clearly, the presence of high, uncountable intensities of bacteria later in the season (above), suggests that densities of bacteria on frogs do increase seasonally.

Since the samples we collected so far were taken mainly after the breeding season began in 2006 (only 8 frogs were sampled before breeding calls were heard at ponds), it is difficult to make sound conclusions about the impact of breeding on counts of *Aeromonas* bacteria on frogs. Our results do suggest that there could be a link between breeding and densities of bacteria on

frogs in that the frequency of frogs harboring high densities of bacteria increased dramatically after breeding began (Figure 1). In another study, Forbes et al., (2004) found evidence that prevalence of *A. hydrophila* was highest in leopard frogs during the breeding season. Samples taken this fall (October 2006), well after the breeding season has finished, will help us examine a possible link between reproduction, stress, and parasitism. These data will also provide us with an opportunity to record stress-levels of frogs near hibernation, which can be associated with immunosuppression in frogs (Cooper et al., 1992).

We observed no obvious differences in behavior between frogs that exhibited low versus high colony counts of bacteria (including no difference in the time it took them to recover from anaesthetia). We also cannot disregard the possibility is that heavily-colonized frogs were more likely to be captured due to a decreased ability to escape capture or increased visibility to researchers. We did attempt to minimize this confound by focusing a high catch-effort on a small number of randomly-selected frogs observed each day (as opposed to giving up on a particular frog in favor of another(s) that were more convenient to sample; McCurdy and Krum, 2005).

Given that *Aeromonas* bacteria have been associated with mortality in frog populations (e.g., Huys et al., 2003), and that links between *Aeromonas* and environmental disturbances have been established (e.g., Cunningham et al., 1996), further research on interactions between *Aeromonas,* frogs, and the connection of bacterial infections to red-leg disease are needed . The diversity of frog species, abundance of field sites, and high rate of prevalence of *A. hydrophila* within the PCCI property make it an ideal study site for experimental or observational studies of this amphibian pathogen. Experimental studies to assess the effect of bacteria on performance traits, survival, and reproductive output of frogs would be useful in determining the effect of bacteria on amphibian species.

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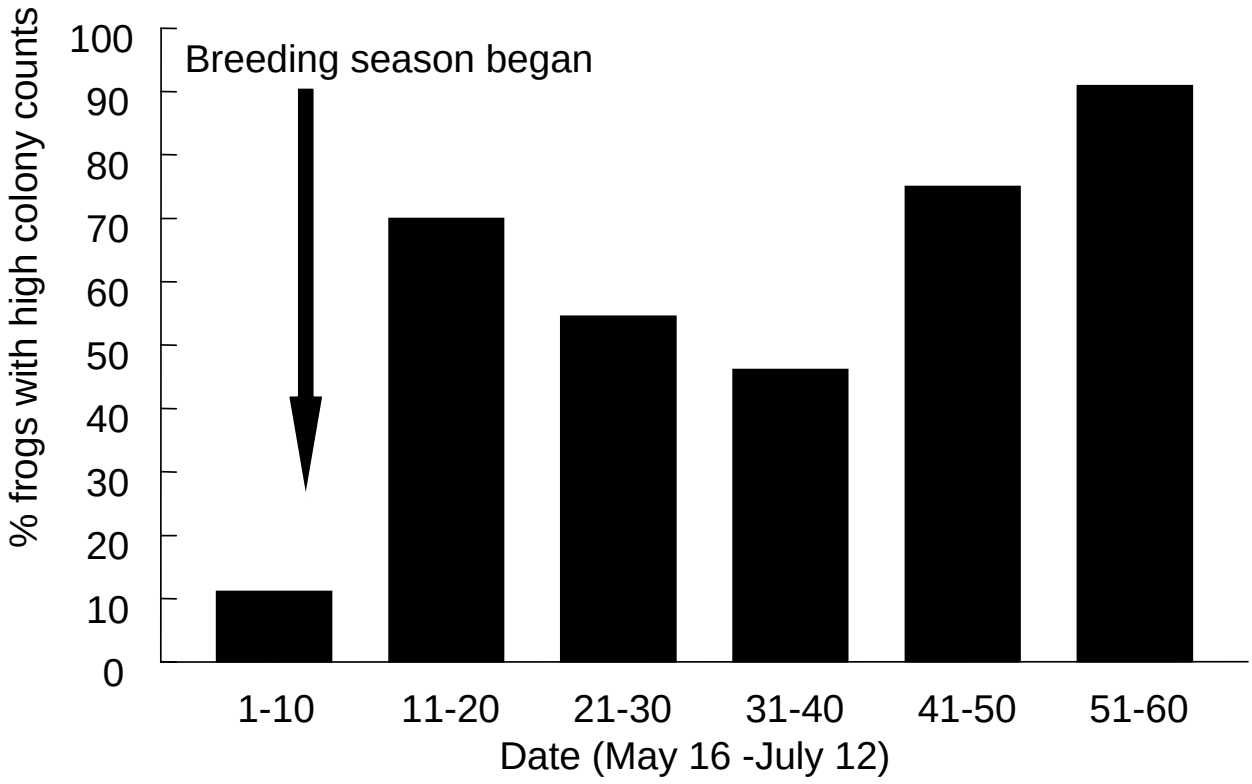

Figure 1. Histogram illustrating the percentage of frogs with low densities of bacteria (1-200 cfus / plate) versus high densities of bacteria (>200 cfus per plate) in relation to dates captured over the spring/summer of 2006. Frogs were caught at four breeding ponds on the property at the Pierce Cedar Creek Institute, Hastings, Michigan. Sample sizes for each set of dates ranged from 8-13 frogs.