PARASITISM AND STRESS LEVELS IN GREEN FROGS, *RANA CLAMITANS*, IN WESTERN MICHIGAN

FINAL REPORT - URGE 2008

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SEPTEMBER 15, 2008

Introduction

Emerging infectious diseases (EIDs) pose a major threat to amphibian populations worldwide (Daszak et al., 1999). Pathogens with the potential to directly influence amphibians populations through mortality or morbidity include viruses, fungi, and bacteria (reviewed by Pessier, 2002). Researchers believe that virulence of various pathogens is related to genetic diversity of hosts (host populations with greater genetic diversity suffer from lower infection rates; Pearman and Garner, 2005), and likely to background levels of stress on amphibians (amphibians under environmental stress are more susceptible to pathogens; Forson and Storfer, 2006). Unfortunately, little is known about the distribution of potential pathogens of amphibians and there are few studies which assess stress of amphibians in relation to parasitism or other environmental factors (Daszack et al. 2001; Rollins-Smith, 2001). For example, it is generally not known whether an infection by one pathogen increases susceptibility of amphibians to other pathogens.

In the Great Lakes region, amphibians sometimes suffer from red-leg disease, which can cause mortality and is associated with the presence of the bacterium *Aeromonas hydrophila* (Forbes et al., 2004). While prevalence of *Aeromonas* is variable, at the Pierce Cedar Creek Institute in Michigan it exceeds 80% on frogs – nearly five times the rate of colonization by this pathogen in Ontario and elsewhere (McCurdy and Krum, 2005; McCurdy and Lupek, 2006). The status of other pathogenic organisms which might threaten Michigan frog populations is unknown, although Greer et al. (2005) found that *Ranavirus* infection was present throughout central and northern Ontario and was likely responsible for mass-mortality in frog populations there (mortality rates from *Ranavirus* can exceed 90%; Chinchar, 2002; Harp and Petranka, 2006). In fact, a large-scale survey of 64 amphibian mortality and morbidity events across the

United States found that *Ranavirus* was directly responsible for 40% of these events, and in combination with other stress was associated with 9% of the other mortality events (Green et al., 2002). Still another pathogen, the chytrid fungus *Batrachochytrium dendrobatidis,* has been associated with severe declines of amphibians worldwide, including in North America (Blaustein et al., 1994; Kriger et al., 2006). Recent evidence suggests that *Ranavirus* and chytrid fungi are patchy in their distributions, but are moving rapidly across North America due to human activities (Morehouse et al.. 2003; Jancovich et al., 2005).

We investigated the distribution of two pathogenic organisms on/in Green Frogs, *Rana clamitans,* found at the Pierce Cedar Creek Institute (hereafter PCCI) in Barry County, Michigan. By using non-lethal sampling protocols developed within the past year (St-Amour and Lesbarrères, 2007), we tested Green Frogs (*Rana clamitans*) for infections by *Ranavirus* and colonization by *Aeromonas* bacteria. We also processed plasma samples taken from frogs to assess stress levels (as measured by plasma corticosterone level). We expected that frogs harboring pathogens would be under greater stress than uninfected frogs. We also expected that male frogs would exhibit higher rates of stress and parasitism than females due to immunosuppression associated with male-male competition and male sex hormones (Forbes et al., 2004). We predicted that frog in poorer condition would suffer from greater rates of parasitism and higher corticosterone levels as would frogs actively breeding.

Methods

Field Methods

We studied prevalence of two pathogens (*Aeromonas* bacteria, *Ranavirus*) in Green Frogs to determine occurrence of these pathogens and possible connections with stress-levels in frogs and

environmental conditions. Most samples were collected at four locations on the PCCI property: Hyla House pond, Wood Pond (also known as PVC pond), Back Pond, and Small Back pond (sites described in McCurdy and Krum, 2005). We also sampled from five other locations within 25-km of PCCI and two sites near Ann Arbor, Michigan. From prior studies, we know that the PCCI property is an excellent location for studies on Green Frogs as they are common throughout ponds on the property (McCurdy and Lupek, 2006). We also know from two prior studies that *Aeromonas* bacteria colonize frogs at unusually high levels (e.g., 90-95% for Green Frogs; McCurdy and Krum, 2005; McCurdy and Lupek, 2006).

To sample frogs, we made trips to field sites four or five days per week (rotating around sites to catch between 1 and 10 frogs at each site per visit and approximately 20 frogs in total). Frogs were caught by hand and sampling was typically done between 21:00 and 22:30. Each captured frog will be immediately transported to the aquatic lab at PCCI for processing. Frogs were returned to sites of capture after processing the laboratory (described below) and following a recovery period of at least 1-2 hours.

Laboratory Procedures for Handling Frogs

Within four hours of returning to the lab, each frog was anesthetized by immersing it in a 2% solution of MS-222 (a common drug used for short-term anesthesia of frogs; Beaupre et al., 2004), measured (snout-vent length), and weighed. To test for the presence of *Aeromonas* bacteria on frogs, we rubbed the abdomen and legs of each frog gently with a sterile swab and placed the swab in phosphate buffer (Taylor et al., 1999). Swabs were then plated within 12 hours onto Ryan's *Aeromonas* medium and incubated at 27ºC for 48h to detect *Aeromonas*

colonies (Forbes et al., 2004). Hand sanitizer and gloves were used between handling of different frogs to prevent the spread of pathogens and frogs were housed separately during processing.

Detection of *Ranavirus* infections cannot be done by simply swabbing the outside of frog, so we used a non-lethal method to test for the presence of the virus (St-Amour and Lesbarrères, 2007). To begin, we removed a single toe clip from each animal (unless they had already been marked by other researchers or was wounded, in which case no sample was collected). Toe clips were frozen immediately for storage. The toe clip from each frog will also served to mark each frog as sampled - preventing accidental re-sampling of the same individuals.

To assess corticosterone levels within frogs (corticosterone is a hormone associated directly with stress levels and indirectly with immunocompetence in frogs; Moore et al., 2005), we removed a small blood sample (~1 mL; <5% volume) from adult frogs under anesthesia. Blood samples were drawn directly from the heart, which is a standard, non-lethal technique that allowed for rapid recovery of frogs (Beaupre et al., 2004)). While corticosterone levels can be measured in a variety of ways, we opted to use an EIA Corticosterone Analysis kit from Cayman Biological Laboratories due to the lower cost of this procedure and inclusion of extensive controls within each kit.

To determine whether toe-clips collected from frogs were positive for *Ranavirus*, samples were transported to Albion College and screened using PCR protocols (St-Amour and Lesbarrères, 2007). PCR reactions included the use of universal primers to verify that extractions were successful. Primers for *Aeromonas* (Dorsch et al., 1994) are being used to amplify bacteria for sequencing and characterization.

Results

Parasitism

Overall, we captured 136 different frogs (58 females and 78 males) at PCCI between 13 May and 10 July, 2008 (in this report, we include data only for Green Frogs from sites on the PCCI property and exclude re-captures). Frogs began calling actively (indicating onset of mating) in late-May. Prevalence of *Aeromonas* on frogs captured was 96.9% (n = 128; swabs from all but four frogs sampled for *Aeromonas* harbored colonies). None of the 14 toe-clips from different frogs that have been processed to date (including at least three frogs from each pond sampled at PCCI) tested positive for the presence of *Ranavirus.*

Stress hormone levels

Of the 36 frogs currently assayed for corticosterone levels, we found a near-significant difference between males and females (with males having higher levels of plasma corticosterone; $t = 2.0$, df = 34, P = 0.058; Figure 1). There was no relationship between date of sampling and corticosterone levels in frogs (R = 0.10, $n = 36$, P=0.56). There was also no significant difference in mean corticosterone levels in frogs collected across four ponds at PCCI (F = 0.97, df = 3,32, P = 0.42; Figure 2). We found that frogs that were heavier for their given length (assessed as mass divided by snout-vent length) had lower corticosterone levels than frogs in relatively poorer body condition (R = -0.35, n = 35, P = 0.038; Figure 3). When combining various predictor variables (e.g., sex, length, mass, condition) into a Generalized Linear Model, none of the interaction terms were related significantly to corticosterone level.

Discussion

As in prior years, we found that prevalence of *Aeromonas* was very high on Green Frogs. In fact, it was higher on Green Frogs at PCCI than in 2005 (91%; McCurdy and Krum, 2005) and 2006 (95%; McCurdy and Lupek, 2006). Reasons for such differences are unknown, but prevalence remains consistently higher than in Green Frogs studied in eastern Ontario, where prevalence was 4% (Forbes et al., 2004). Because prevalence of *Aeromonas* approached 100%, we did not have sufficient variation in our dataset to assess whether or not presence of *Aeromonas* was associated to any degree with stress levels of frogs, although the fact that stress levels varied widely among frogs that all tested positive for colonization by *Aeromonas* suggests that such a correlation, if present, does not explain much variation in nature. Follow-up studies to accurately assess *intensity* of colonization by *Aeromonas* (e.g. McCurdy and Lupek, 2006) would be useful. It is worth noting that numbers of bacterial colonies on plates appeared higher than in prior years and increased later in the summer, as they did in 2006 (McCurdy, personal observation; McCurdy and Lupek, 2006). Although others have argued that colonization of *Aeromonas* on frogs is likely related to likelihood of an internal infection (Forbes et al., 2004), it would be interesting to investigate the link between colonization and actual infections by this bacterium in frogs since it is possible that onset of Red-Leg disease in frogs is tied to stress levels, but that presence of this opportunistic bacterium on the skin of frogs is not so linked.

As expected, we found that male frogs exhibited higher stress levels than females. This, however did not result in higher rates of colonization by *Aeromonas* on males. Similar results were found by Forbes et al. (2004) and McCurdy and Lupek (2006) who observed similar colonization rates on Green Frogs throughout the breeding and non-breeding seasons. We also found no link between breeding status and stress levels, as found by Zerani et al. (1999) for

captive *Rana esculenta* frogs in that there was no relationship between plasma corticosterone levels and capture date. We did find that frogs under poorer condition (lower mass for their body size) do appear to be under greater stress, which might translate to lower reproductive output for these frogs. Stress levels did not differ among study locations, but this result should interpreted cautiously as sample sizes for some sites were low (processing of plasma samples will continue over the coming year to further address this question). Given that some ponds were visited more frequently than others (e.g., Hyla House pond and Wood pond were visited frequently by another research group during our year of study) we might expect that frogs at these sites would have higher stress levels (if capture is stressful as it is in the laboratory; Zerani et al., 1999). We hope to collaborate with other researchers to assess whether factors such as frequency and seasonal timing of handling of frogs influence stress levels of frogs in natural populations.

We did not detect *Ranavirus* in any of the samples of frogs collected at PCCI or elsewhere in Michigan. These results must be viewed as preliminary as many samples remain to be run (the procedure is expensive and very time-consuming), but this result is consistent with our observation that mass mortality events were not observed in populations of Green Frogs at PCCI during daily visits to ponds by our research group (when present, *Ranavirus* can cause high mortality rates in host populations; Chinchar, 2002; Green et al., 2002; Greer et al., 2005; Harp and Petranka, 2006). The absence of this pathogen also precludes direct statistical comparisons with stress hormone levels. Thus, it is still unknown whether or not infections by *Aeromonas* might influence susceptibility to *Ranavirus* and relationships between stress and risk of parasitism are unknown. An experimental approach might help resolve the issue of a lack of variation in nature in prevalence of *Aeromonas* and *Ranavirus* on/in frogs, although experiments with live *Ranavirus* would pose a serious risk to natural populations of frogs (especially if done

by researchers who visit the field regularly). A compromise approach might be to establish field plots and assess how experimentally-induced environmental stress (e.g., pH, high water temperature, drawdown of ponds) influence likelihood of contracting pathogens that might already present at field sites.

Although frequent colonization by *Aeromonas* does not appear to cause harm to frogs at PCCI and *Ranavirus* does not appear to be present (yet) in Barry County, Michigan, researchers should be cautious when visiting ponds on the PCCI property and elsewhere. Given widespread evidence that *Ranavirus* and pathogenic fungi may be easily transported among ponds (Harp and Petranka, 2006; Jancovich et al.,2005), it would be prudent to continue quarantine practices on boats and waders already in place at the Institute to reduce the likelihood of introductions by such non-native species. High visitation rates and close proximity to roads, both conditions that are present at the Institute, are known to place amphibians at greater risk of parasitism and general population decline (Daszak et al., 1999, 2001).

Acknowledgements: We would like to thank Kelyn Carlson and Monica Yalamanchili for their help in the field and laboratory. Ryan Fouty, Rebecca Lodewyk, Denita Weeks, and Dr. Stephen Burton provided logistical support in the field and shared in several fruitful discussions about the biology of Green Frogs. We thank Rob Aicken, Matt Dykstra, and Michell Skedgell of the Pierce Cedar Creek Institute for their continual guidance and support throughout this project. Funding for this project was provided by an Undergraduate Research Grant for the Environment from the Pierce Cedar Creek Institute and the Foundation for Undergraduate Research, Scholarship, and Creative Activity at Albion College.

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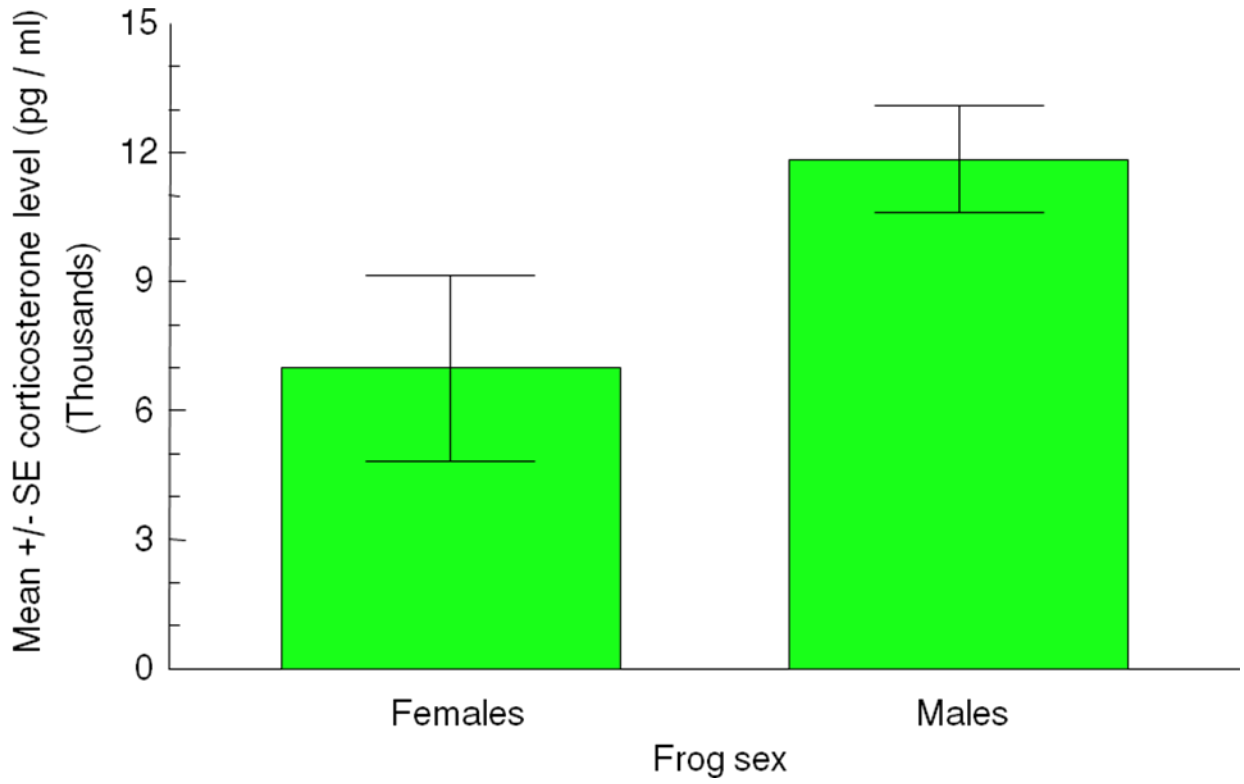

Figure 1. Mean +/- SE corticosterone levels (pg / ml) for male (n = 9) and female (n = 27) Green Frogs captured at four ponds on the Pierce Cedar Creek Institute property in Summer 2008.

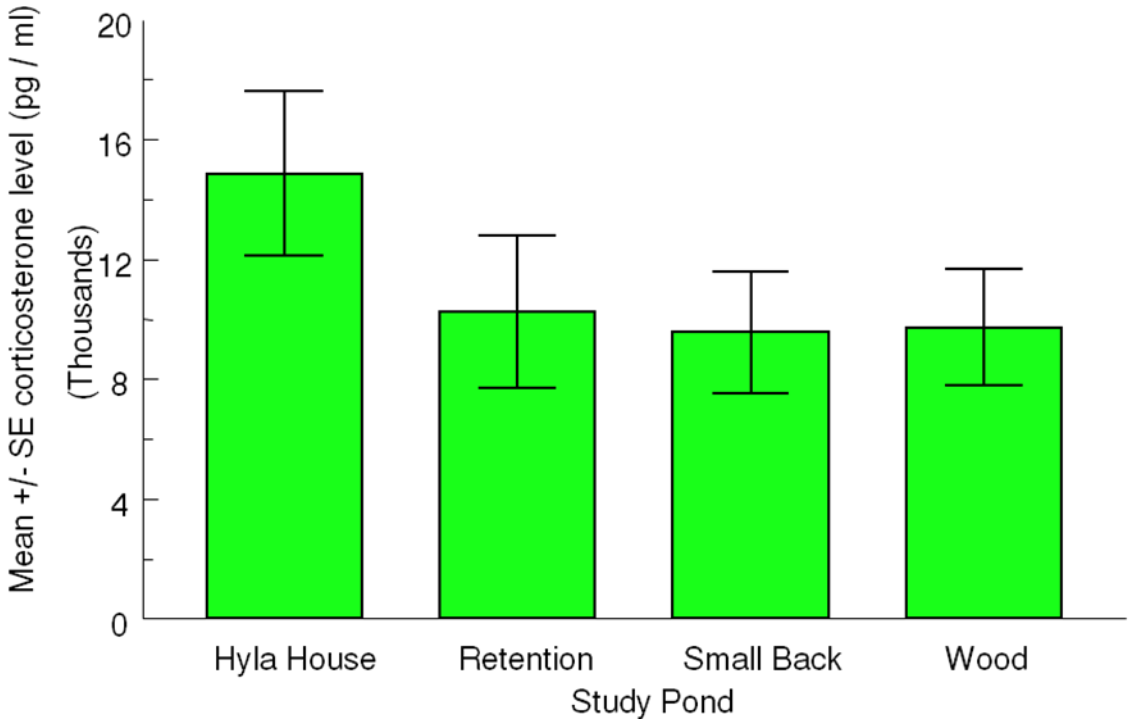

Figure 2. Mean +/- SE corticosterone levels (pg / ml) for Green Frogs collected at Hyla House Pond (n = 6), Retention Pond (n = 7), Small Back pond (n = 11) and Wood Pond n = 12) on the Pierce Cedar Creek Institute property in Summer 2008. Descriptions of study locations are from McCurdy and Krum (2005) and McCurdy and Lupek (2006).

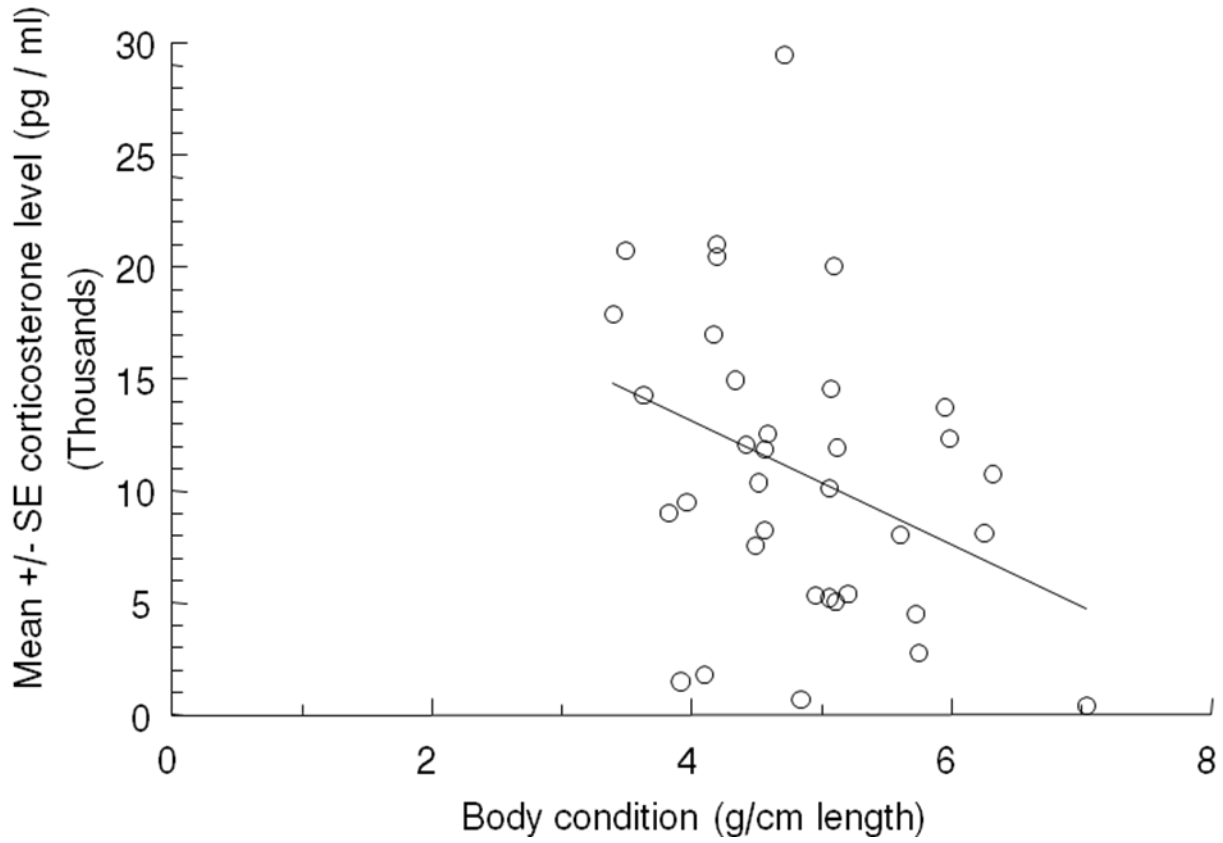

Figure 3. Relationship between body condition of frogs (determined as mass of each frog divided by its snout-vent length) and corticosterone level (pg / ml).