RH: Gray et al.—Cattle and FV3 Prevalence

Frog virus 3 prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, U.S.A.

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ABSTRACT: Ranaviruses have been associated with most of the reported larval anuran die-offs in the United States. It is hypothesized that anthropogenically induced stress may increase pathogen prevalence in amphibian populations by compromising immunity. Cattle use of wetlands may stress resident tadpole populations by reducing water quality. We isolated a *Ranavirus* from green frog (*Rana clamitans*, $n = 80$) and American bullfrog (*R. catesbeiana*, $n = 104$) tadpoles collected at 5 cattle-access and 3 non-access wetlands on the Cumberland Plateau, Tennessee, U.S.A. Sequencing confirmed *Frog virus* 3 (FV3); therefore, we compared its prevalence between tadpole populations inhabiting cattle-access and non-access wetlands, and among 3 seasons (winter, summer and autumn) in 2005. We found FV3 in both tadpole species and cattle land-use types; however, prevalence of FV3 was greater in green frog tadpoles residing in cattle-access wetlands compared to those in non-access wetlands. No difference in FV3 prevalence was detected between cattle land uses for American bullfrog tadpoles. A seasonal trend in FV3 prevalence also existed, with prevalence greater in autumn and winter than in summer for both species. In addition, we found that FV3 prevalence decreased significantly as Gosner stage increased in American bullfrog tadpoles. No trend was detected between FV3 prevalence and developmental stage for green frog tadpoles. Our results suggest that cattle use of wetlands may increase prevalence of FV3 in *Rana* tadpoles, although this effect may depend on species, season and tadpole developmental stage.

KEY WORDS: amphibian declines, anthropogenic stressor, emerging pathogen, *Frog virus* 3, cattle, water quality

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INTRODUCTION

Ranaviruses are responsible for some of the recent declines in amphibian populations (Carey et al. 2003, Greer et al. 2005), and are considered to be an emerging pathogen (Daszak et al. 1999). In the United States, ranaviruses have been implicated as the etiologic agent in the majority of reported amphibian die-offs (Green et al. 2002). Anuran die-offs known to be caused by *Ranavirus* disease have occurred in at least 15 U.S. states and 11 species (Converse and Green 2005). Ranaviruses exist worldwide and at all elevations (Daszak et al. 1999, Converse and Green 2005). Hence, this group of viruses is potentially a global threat to amphibian populations, especially if their prevalence is increased by anthropogenic stressors (Daszak et al. 2001, Carey et al. 2003).

Ranaviruses are most lethal to amphibian larvae (Gantress et al. 2003), with mortality rates often >90% (Converse and Green 2005). *Ranavirus* transmission has been documented via water bath (Brunner et al. 2004, Pearman et al. 2004, Harp and Petranka 2006), cannibalism (Pearman et al. 2004, Harp and Petranka 2006), proximity to infected individuals (Brunner et al. 2004), and exposure to virus-contaminated sediment (Harp and Petranka 2006). *Ranavirus* disease is characterized by systemic hemorrhage and tissue necrosis, ultimately resulting in organ failure within only a few days of exposure (Converse and Green 2005). The type species of the genus *Ranavirus* is *Frog virus* 3 (FV3), which was originally isolated from the northern leopard frog (*Rana pipiens*) in the mid-1960s (Granoff et al. 1965, Rafferty 1965). Since then, FV3 has been responsible for mass mortalities of wild and captive anuran populations in the United States, China and Thailand (Carey et al. 2003, Miller et al. 2007), and is known to be lethal in at least one European species (*R. latastei*, Pearman et al. 2005).

It is hypothesized that anthropogenic stressors will increase pathogen prevalence in amphibian populations by compromising their immunity (Carey et al. 1999, Daszak et al. 2003). For example, agricultural land use around wetlands may stress resident tadpoles by decreasing water quality. In particular, beef farming can decrease water quality in wetlands through deposition of nitrogenous waste by cattle (Hooda et al. 2000). Beef cattle often are given access to wetlands where amphibians breed to graze vegetation and drink water. Jancovich et al. (1997) reported emergence of the *Ranavirus*, *Ambystoma tigrinum* virus, in larval salamanders inhabiting a pond with cattle access. Therefore, for this study, we hypothesized that cattle access in wetlands would increase FV3 prevalence in resident larval anuran populations.

Prevalence of ranaviruses in tadpole populations also may be influenced by natural environmental and developmental stressors. Maniero and Carey (1997) reported a decrease in T lymphocyte proliferation and serum complement activity at low temperatures in northern leopard frogs. Rollins-Smith (1998) also provided evidence that tadpole immunity increases with development. Therefore, we further hypothesized that FV3 prevalence would be greatest during months with colder temperatures and during earlier tadpole developmental stages.

The objectives of our study were two-fold. First, we wanted to compare FV3 prevalence between tadpoles collected at cattle-access and non-access wetlands and among 3 sample periods (15 February, 15 June, and 14 October 2005). Second, we wanted to relate FV3 prevalence with Gosner (1960) developmental stage. This information is fundamental to understanding if cattle access in wetlands is a possible stressor of amphibian immunity, and whether this anthropogenic land use interacts with environmental and developmental stressors.

METHODS

Our study was conducted at the Plateau Research and Education Center (PREC) on the Cumberland Plateau near Crossville, Tennessee, U.S.A. (36°00'59" N, 85°07'57" W). We chose green frog (*R. clamitans*) and American bullfrog (*R. catesbeiana*) tadpoles as our study species, because these species were relatively common at the PREC and have widespread distribution in the United States. In addition, these tadpole species are known to overwinter in Tennessee wetlands (Dodd 2004). We collected tadpoles opportunistically from 8 PREC wetlands using seine and dip nets during 3 sample periods (15 February, 15 June, and 14 October 2005) corresponding to 3 temperate seasons (winter, summer, and autumn, respectively). Four of our study wetlands had been exposed to cattle at a mean density of 19 head per 0.1 ha of water for >10 years. One additional wetland received cattle effluent via a natural drainage hence also was classified as a cattle-access wetland. Three other wetlands that we sampled never had direct cattle access nor were connected hydrologically to cattle effluent thus classified as non-access. All study wetlands were in close proximity to each other (< 0.4 km separation) and were similar in size (0.153–1.29 ha). In addition, they were permanently flooded in the center, <2 m deep, and had emergent shoreline vegetation composed of cattail (*Typha latifolia*), rushes (Juncaceae) and sedges (Cyperaceae).

We collected 80 green frog tadpoles from cattle-access and non-access wetlands (n = 47 and 33, respectively) in June and October 2005 (n = 40 each). No green frog tadpoles were captured in February 2005. We collected 104 American bullfrog tadpoles from cattle-access and non-access wetlands (n = 61 and 43) in February, June and October 2005 (n = 42, 41, and 21, respectively). Captured tadpoles were rinsed with sterile water and transported in separate containers to the University of Tennessee, where they were humanely euthanized within 24 hrs

using benzocaine hydrochloride. Gosner (1960) developmental stage was categorized following McDiarmid and Altig (1999). All collection and euthanasia procedures followed approved University of Tennessee Institutional Animal Care and Use Committee protocol #1421 and were authorized under Tennessee Wildlife Resources Agency Scientific Collection Permit #1990.

Complete necropsies were performed immediately following euthanasia. Fresh tissue specimens of all organs (liver, spleen, kidney, intestines, heart, gills, lungs, eye, reproductive tissue, brain, skin, muscle) were collected for viral culture and PCR. An identical suite of tissues also was collected and placed in 10% buffered formalin for paraffin-embedding and to archive specimens. All specimens were subsequently transported overnight to the University of Georgia Veterinary Diagnostic and Investigational Laboratory in Tifton, Georgia, U.S.A., for histological examination and viral testing.

To examine histopathological changes, formalin-fixed tissues were routinely processed and embedded in paraffin blocks. One or more 5 μm sections were cut from each block and placed on glass slides. Slides were stained with hematoxylin and eosin for light microscopic examination of viral inclusions and pathological changes.

For virus isolation, a single 10% tissue homogenate was made from all fresh tissue specimens for each tadpole, and filtered (0.45 μ) directly onto confluent monolayers of a variety of cell lines, including fathead minnow, epithelioma papilloma cyprini cells, white sturgeon skin and channel catfish ovary. Cultures demonstrating viral cytopathic effect were harvested and random isolates verified by electron microscopy (Figure 1).

For PCR, a heminested procedure targeting the major capsid protein gene was performed on genomic DNA extracted from fresh and paraffin-embedded tissues following the protocol by Kattenbelt et al. (2000). In brief, the first round reaction mixture (25 μl, total volume) contained

50 – 100 pmol of primers FV3-991 (5'-CGCAGTCAAGGCCTTGATGT) and FV3-1571R (5'-AAAGACCCGTTTTGCAGCAAAC). For this first round, the thermal cycler program was 35 cycles, with an initial denaturization step of 5 minutes at 94 ºC, followed by 35 cycles of 1 minute at 94 ºC, 1 minute at 58 ºC, and 1 minute at 72 ºC. The second round reaction mixture (25 µl, total volume) contained primers P1050N (5'-TCAAGAGCGCCACGCTGGTGTA) and FV3-1571R. Only 0.5 µl of the first round product was carried over into the second round PCR. For the second round, the thermal cycler program was 25 cycles with an initial denaturization step of 10 minutes at 94 ºC, followed by 25 cycles of 1 minute at 94 ºC, 1 minute at 58 ºC, and 1 minute at 72 ºC. The PCR products were resolved by gel electrophoresis. The bands were isolated and submitted to SeqWright DNA Technology Services, Houston, Texas, U.S.A. for automated sequencing. The resulting forward and reverse sequences were assembled using LaserGene Sequence Analysis Package (DNASTAR, Inc). A GenBank Blast search was performed (http://www.ncbi.nlm.nih.gov/Genbank.html) on the consensus sequence (GenBank reference #DQ906048-49), and 100% identity with the FV3 capsid protein gene and FV3 complete genome was found.

Prevalence of FV3 was tested between cattle-access and non-access land uses and among sampling dates for each species using logistic regression (Stokes et al. 2000). We included an interaction term in the model to test for non-additivity of land-use types and sampling date main effects. We also used logistic regression to test for a trend in FV3 prevalence among tadpole developmental stages for each species. Odds-ratio statistics were estimated to quantify the likelihood of FV3 infection in each cattle land-use type, and among sample periods and developmental stages. All analyses were performed using the SAS® system at $\alpha = 0.05$ (Stokes et al. 2000).

RESULTS

A total of 80 green frog tadpoles and 104 American bullfrog tadpoles were captured and used in FV3 analyses. Other tadpole species that were captured but not collected included spring peeper (*Pseudacris crucifer*), northern cricket frog (*Acris crepitans*), pickerel frog (*R. palustris*), American toad (*Bufo americanus*) and Fowler's toad (*B. fowleri*). Prevalence of FV3 was greater in cattle-access (40%) than in non-access (15%) wetlands for green frog tadpoles ($\chi^2_{(1)} = 5.2$, $P = 0.02$, Figure 2). Odds-ratio estimates indicated that green frog tadpoles in cattle-access wetlands were 3.9X more likely to be infected with FV3 than those in non-access wetlands. No difference was detected in FV3 prevalence between cattle land-use types for American bullfrog tadpoles ($\chi^2_{(1)} = 0.08$, $P = 0.78$, Figure 2). Also, cattle land uses and sampling period did not interact for either species ($\chi^2_{(1)} < 0.98$, $P > 0.32$), indicating that land-use effects were consistent among sampling periods.

Frog virus 3 prevalence was greater in winter (57%) than in summer (15%) and autumn (24%) for American bullfrog tadpoles ($\chi^2_{(2)} = 15.9$, $P < 0.001$, Figure 3). Odds-ratio estimates indicated that American bullfrog tadpoles were 1.8X and 7.7X more likely to be infected with FV3 in autumn and winter than in summer, respectively. *Frog virus* 3 prevalence also was greater in autumn (45%) than in summer (15%) for green frog tadpoles ($\chi^2_{(1)} = 7.5$, $P = 0.006$, Figure 3). Odds-ratio estimates indicated that green frog tadpoles were 4.7X more likely to be infected with FV3 in autumn than in summer. No green frog tadpoles were captured during winter for comparison.

Frog virus 3 prevalence was different among developmental stages for American bullfrog tadpoles ($\chi^2_{(1)} = 12.8$, $P < 0.001$, Figure 4a). The predicted odds of FV3 infection decreased 28%

with each unit increase in Gosner stage. No difference in FV3 prevalence was detected among developmental stages for green frogs ($\chi^2_{(1)} = 0.71$, $P = 0.87$, Figure 4b).

DISCUSSION

Prevalence of FV3 was greater at cattle-access wetlands than at non-access wetlands for green frog tadpoles. To our knowledge, this is the first evidence of a relationship between an anthropogenic land use and an emerging wildlife pathogen. The potential stressors of cattle land use are unknown but may be related to water quality. In a concurrent study, Schmutzer (2007) documented that un-ionized ammonia concentration and turbidity were 3.2 and 4.6X greater, respectively, in cattle-access wetlands from March – August 2005. Dissolved oxygen also was 28% greater in non-access wetlands. We did not quantify physiological indicators of stress (e.g., blood cortisol) and immunocompetence (e.g., lymphocyte counts, serum complement), but decreased water quality in cattle-access wetlands may have stressed green frog tadpoles and facilitated FV3 infection. If this is true, we hypothesize that American bullfrog tadpoles are more tolerant of low water quality than green frog tadpoles. More research is needed to understand the relationships of water quality, immune function and FV3 infection among tadpole species.

We also found that FV3 prevalence was greater during colder months in green frog and American bullfrog tadpoles. Although this appears to be the first documentation of a seasonal trend in FV3 prevalence, Rojas et al. (2005) reported an increase in *Ambystoma tigrinum* virus virulence at lower temperatures. Forbes et al. (2004) also reported an increase in *Aeromonas hydrophila* prevalence in 3 ranid species during colder months. These authors hypothesized that increased virulence and infectivity were related to a decrease in immune function at colder temperatures (Forbes et al. 2004, Rojas et al. 2005). Cold-induced immunosuppression in

amphibians has been suggested by others (e.g., Carey et al. 1999, Raffel et al. 2006). Mean water temperature in our study wetlands during June 2005 was 25.1 ºC (Schmutzer 2007). Although we did not measure water temperature during February and October 2005, it is reasonable to assume that water temperature decreased in the order of June, October and February, because Tennessee is located mid-latitude in the northern hemisphere. Mean ambient temperature at the PREC was 21.2, 14.2 and 5.1 ºC for these months, respectively (J. W. Hitch, PREC, unpublished data). Other mechanisms for higher FV3 prevalence in winter may be related to exposure duration or increased scavenging of infected tadpoles (Pearman et al. 2004, Harp and Petranka 2006).

Density of amphibian larvae has been suggested to be an important mechanism driving transmission of and infection by ranaviruses (Brunner et al. 2004). Relative abundance of green frog and American bullfrog tadpoles was 2.8X and 13.5X greater, respectively, in non-access wetlands from March – August 2005 and 2006 (Schmutzer 2007). We did not measure tadpole density in autumn or winter, but it is reasonable to assume that density during these seasons was lower than in summer because most tadpole species in Tennessee metamorphose before October (Dodd 2004). Thus, tadpole density likely was not a factor driving cattle land-use and seasonal FV3 trends in our wetlands. Tadpole density in our wetlands ranged from 0.01 – 0.5 tadpoles per m^2 (Schmutzer 2007). Similarly, Harp and Petranka (2006) reported that survival of *Ranavirus*-infected wood frog (*R. sylvatica*) tadpoles and time necessary to develop gross signs of *Ranavirus* disease were not related to tadpole density in a mesocosm experiment.

For American bullfrog tadpoles, FV3 prevalence decreased as Gosner stage increased. Several studies have suggested that amphibian larvae are more susceptible to *Ranavirus* infections than adults (Gantress et al. 2003, Brunner et al. 2005), perhaps due to the lack of MHC

class-I antigen expression in tadpoles, which is important in viral immune defense (Gantress et al. 2003). To our knowledge, however, this is the first field evidence of a trend in *Ranavirus* infection among Gosner developmental stages. This trend may be related to an increase in tadpole immune function with development (Rollins-Smith 1998). Several researchers have demonstrated an increase in lymphocyte numbers in *Xenopus laevis* during tadpole growth up to metamorphic climax (Rollins-Smith et al. 1984, Flajnik et al. 1987). Inasmuch as we observed this trend for American bullfrog tadpoles only, development of immune function during growth may be species-specific (Rollins-Smith 2001), and interact with cattle land use. Indeed, this hypothesis needs to be tested. An alternative hypothesis is that American bullfrog tadpoles may be fatally infected at earlier development stages, resulting in survival of mostly uninfected individuals at later developmental stages.

CONSERVATION IMPLICATIONS

An increase in FV3 prevalence in amphibian populations is a conservation concern if infected individuals experience mortality or decreased fitness (Carey et al. 1999). Most available research suggests that the mortality rate of FV3-infected tadpoles is >90% (Converse and Green 2005). We do not know if our infected tadpoles would have experienced mortality, because they were euthanized for collection. However, in a concurrent study, Burton (2007) observed that total capture of green frog metamorphs in pitfall traps at PREC cattle-access wetlands was 3.7X less than at non-access wetlands from March – August 2005. Histological findings in our infected tadpoles included mild to occasionally moderate degeneration of renal tubular epithelial cells, mild lymphoid depletion in the thymus and other lymphoid tissues, and occasional intracytoplasmic inclusion bodies in erythrocytes, but morbidity was not observed. We also did not observe tadpole mortality events at the PREC wetlands, although this could have been a

consequence of emergent vegetation along the shoreline limiting visibility or mortality events occurring when we were not present. Thus, we cannot make conclusions on whether an increase in FV3 prevalence increased the likelihood of population declines in our wetlands. Nonetheless, it is reasonable to assume that greater pathogen prevalence in a population will result in more disease occurrences. Currently, green frogs are not a species of concern in the United States; however if less common species are influenced similar to green frogs, cattle grazing in wetlands could represent a conservation concern. More information is needed on the fate of FV3-infected tadpoles in cattle-access wetlands.

The United States leads the world in beef production (USDA 2006). Currently, there are approximately 98 million head of cattle on 1.1 million farms in the United States, with an annual production of 12 million metric tons of beef and veal products (USDA 2006). Many of these farms contain wetlands and ponds that amphibians and cattle use simultaneously. Knutson et al. (2004) reported the importance of farm wetlands for amphibians, because they often represent the only remnant habitat available. Some studies have reported a negative correlation between cattle access in wetlands and amphibian species richness and abundance (Healy et al. 1997, Jansen and Healy 2003, Burton 2007, Schmutzer 2007). Indeed, more studies are needed examining the influences of cattle access in wetlands on resident amphibians. Given our FV3 results and the extensive cattle industry in the United States and other countries, beef farmers and land-use managers should consider fencing cattle from wetlands. Cattle use of wetlands and farm ponds likely represents an anthropogenic stressor of tadpole immunity for some species, and may contribute to global amphibian declines.

Our results also support previous concerns raised about the international transport of American bullfrogs for pet trade and aquaculture (e.g., Daszak et al. 2004). American bullfrog

tadpoles can be sublethally infected with FV3, which could result in the introduction of this pathogen into naïve amphibian populations if infected individuals are released intentionally or unintentionally. Pearman et al. (2005) reported 50-100% mortality of naïve Italian agile frog (*R. latastei*) tadpoles, depending on levels of genetic diversity. Their study illustrates the potential for catastrophic mortality events associated with introduction of FV3-infected American bullfrogs into naïve populations. *A priori* FV3 testing of live American bullfrogs should be considered as an export requirement in the United States.

Additional studies are needed on the impacts of water quality, temperature and larval development on immune function and *Ranavirus* infection. The interaction of these factors and larval density also needs to be determined. In addition, large-scale surveillance is needed to improve our understanding of *Ranavirus* distribution and identify infection hotspots. Studies should include uncommon species, which have a greatest likelihood of extinction.

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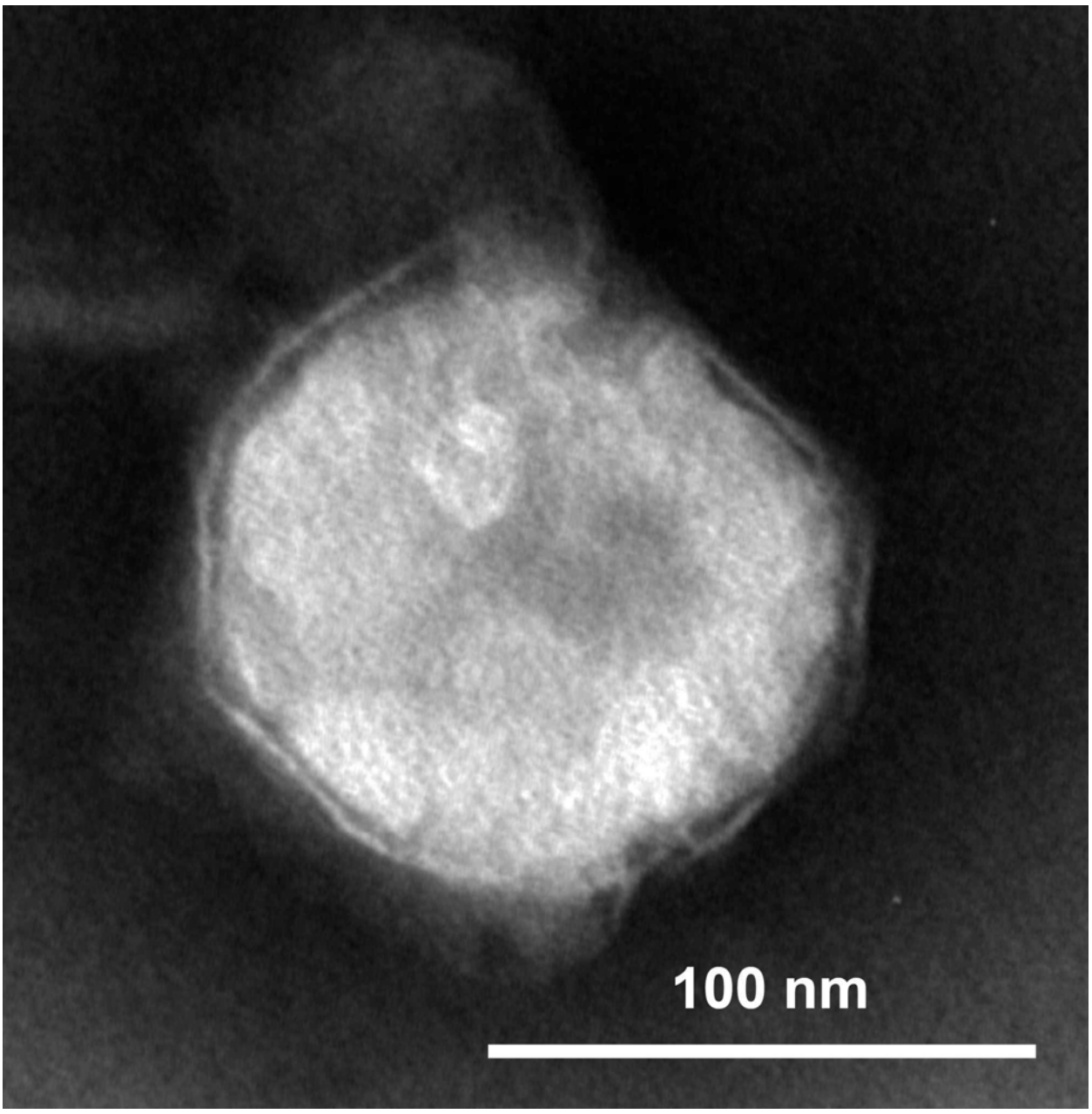
Figure 1. Electron microscopy image of a *Frog virus* 3 isolate from a green frog (*Rana clamitans*) tadpole inhabiting a cattle-access wetland, Plateau Research and Education Center, Tennessee, U.S.A., 2005.

Figure 2. *Frog virus* 3 prevalence in American bullfrog (*Rana catesbeiana*) and green frog (*R. clamitans*) tadpoles at cattle-access and non-access wetlands, Plateau Research and Education Center, Tennessee, U.S.A., 2005. Bars with unlike letters are significantly different ($P = 0.02$).

Figure 3. *Frog virus* 3 prevalence in American bullfrog (*Rana catesbeiana*) and green frog (*R. clamitans*) tadpoles collected on 15 February (winter), 15 June (summer), and 14 October (autumn) 2005, Plateau Research and Education Center, Tennessee, U.S.A. Bars with unlike letters are significantly different ($P < 0.02$).

Figure 4. *Frog virus* 3 prevalence in a) American bullfrog (*Rana catesbeiana*) and b) green frog (*R. clamitans*) tadpoles among Gosner developmental stages, Plateau Research and Education Center, Tennessee, U.S.A., 2005.

400 Figure 1.



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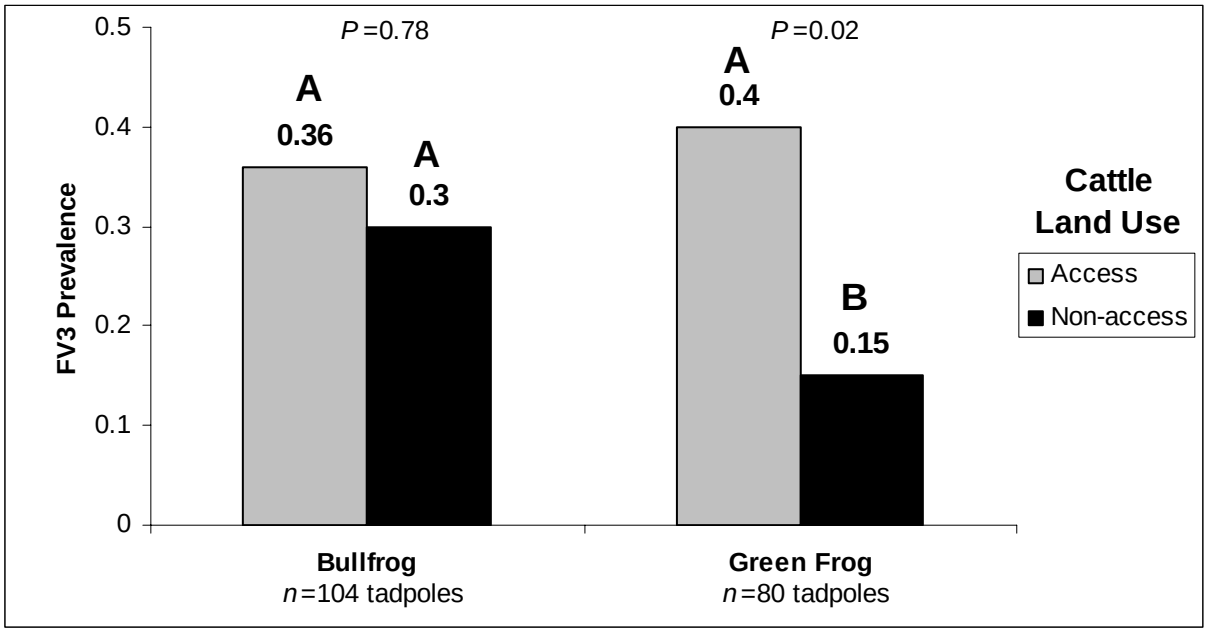
402 Figure 2.



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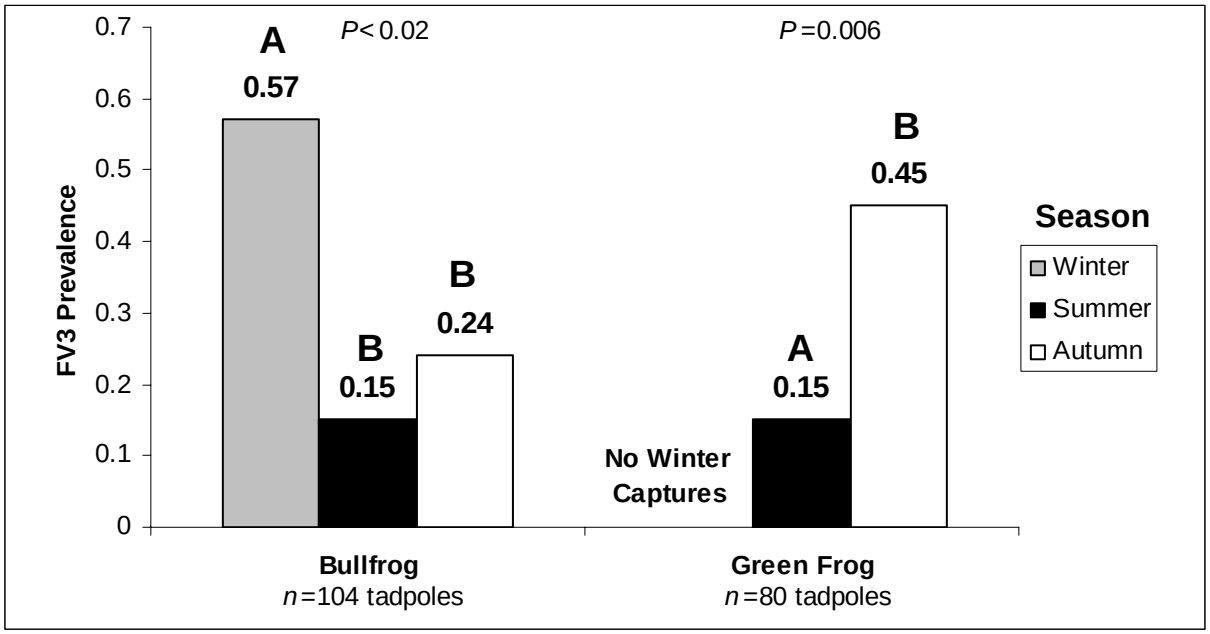
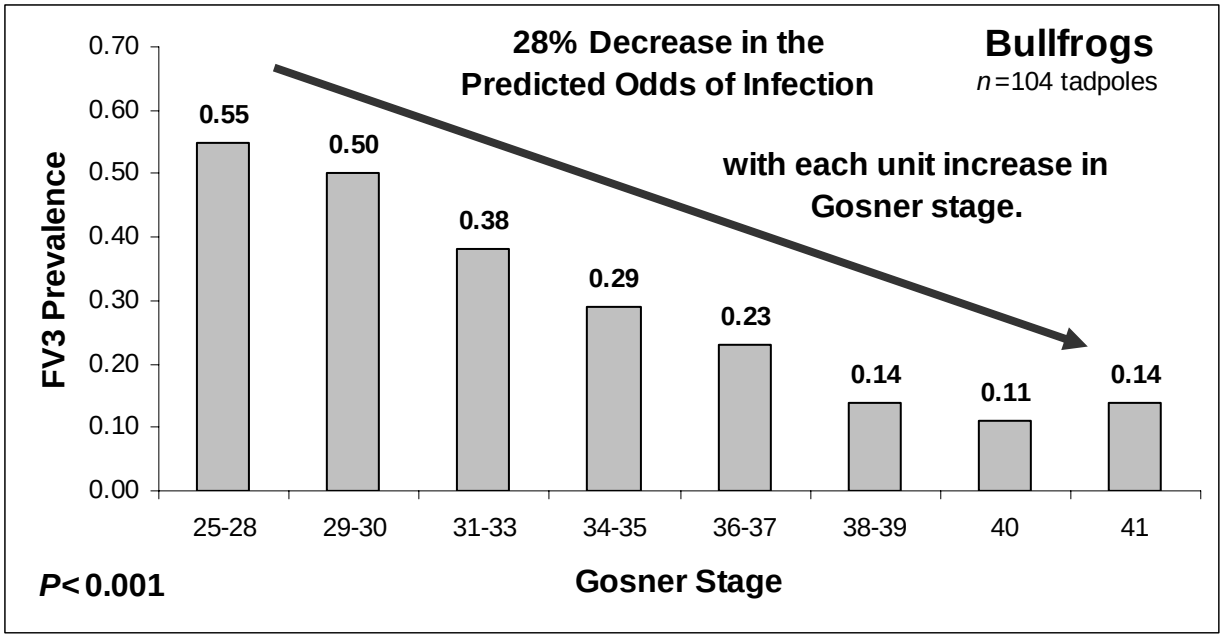
404 Figure 3.



405

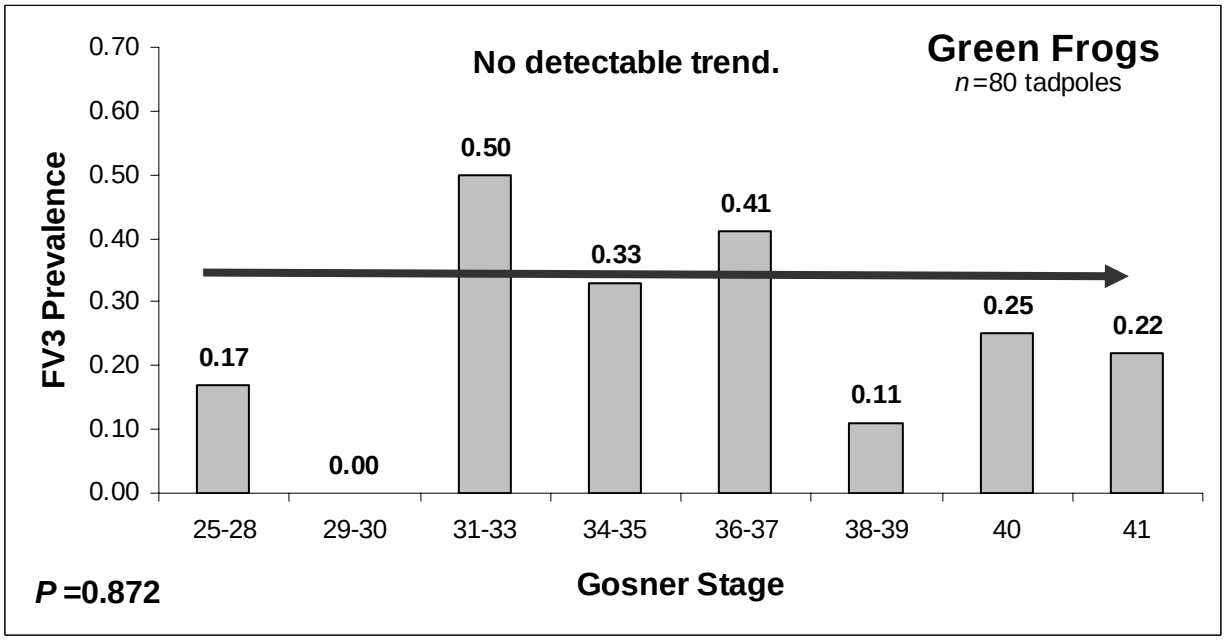
406 Figure 4.

407 a)



408

409 b)



410