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# Prevalence, Clinical Signs, and Natural History Characteristics of Frog Virus 3-like Infections in Eastern Box Turtles (*Terrapene carolina carolina*)

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***Abstract.*—Ranaviruses, specifically Frog Virus 3-like virus (FV3), have been associated with chelonian mortality events and may threaten biodiversity. To help characterize the disease ecology of FV3 in chelonians, we sampled 606 Eastern Box Turtles (*Terrapene carolina carolina*) from Tennessee, Virginia, North Carolina, Alabama, and Georgia from 2007 through 2011. We collected whole blood and swabs of the oral mucosa from 458 adults (248 female, 199 male, 1 unknown sex) and 61 juveniles. Sex and age were unknown in 87 individuals. The prevalence of infection using quantitative PCR was 1.5% (n = 8; 95% CI = 0.8–2.9%), with half the animals diagnosed using whole blood samples and oral swabs, respectively. Three females and five individuals with unknown sex were FV3-positive, which corresponded to two adults, two juveniles, and four animals with unknown age. DNA concentration was significantly higher in extracts of blood samples than swab samples, except for FV3-positive animals. DNA purity (A260/A280 ratio) was non-significantly more variable in extracts of swab samples than blood samples. The only clinical signs significantly associated with infection were diarrhea and bone fractures. Results of this study indicate a low prevalence of disease in box turtles that is characteristic of an acute disease process. Based on this study and other published reports, Eastern Box Turtles are sensitive to FV3 infection, and this virus represents a potential threat to population sustainability.**

*Key Words*.—chelonian; infectious disease; qPCR; *Ranavirus*; *Terrapene carolina*

## Introduction

Ranaviral disease has been linked to several mortality events in amphibians and reptiles (Marschang et al 1999; Green et al. 2002; Johnson et al. 2008; Miller et al. 2011; Allender 2012a). These outbreaks in amphibians have been variable across the landscape, often leading to significant mortalities that threaten biodiversity (Miller et al. 2011). Reports in chelonians have been fewer than amphibians, but it is unclear whether this is a natural bias or underreporting. When reported, several species of chelonians, notably the Eastern Box Turtle (*Terrapene carolina carolina*), have had severe disease of short duration (Allender 2012a).

The status of Eastern Box Turtle populations was downgraded to Vulnerable status by the International Union on the Conservation of Nature and Natural Resources (IUCN. 2011. IUCN Red List of Threatened Species, Version 2011.2. Available from www.iucnredlist.org [Accessed 20 April 2012]) in 2011. Significant declines have been observed in numerous areas throughout its range, with an estimated 30% decline over the previous three generations (IUCN. 2011 *op. cit*.). Specific causes for the decline are not entirely known, but are attributed to human-induced factors including road and mowing mortality, collection, nest depredation, prescribed burning, disturbance of nest sites by off-road vehicles, and habitat loss (Nazdrowicz et al. 2008; Currylow et al. 2011; IUCN. 2011 *op. cit*.). While a combination of factors is likely playing a role in the population declines of the box turtle, disease outbreaks due to *Ranavirus* have been emerging across the eastern United States (US) in chelonians and are also listed as a

suspected cause of decline by the IUCN (Johnson et al. 2008; IUCN. 2011 *op. cit*.; Allender et al. 2012a).

*Ranavirus* is one of five genera from the family *Iridoviridae* and one of two genera from the family reported to infect reptiles. They are large, icosahedral, DNA viruses that have emerged as a significant cause of mortality events in free-ranging herpetofauna throughout the world (Mao et al. 1997; Green et al. 2002; Johnson et al. 2008). Ranaviral infection was placed on the World Organization for Animal Health list of reportable diseases for amphibians in 2010 (OIE. 2013. Listed Diseases 2013. Available from www.oie.int/animal-health-in-the-world/oie-listed-diseases-2013 [Accessed 14 June 2013]). Chelonians have been observed with sporadic ranaviral infections (Westhouse et al 1996; Mao et al. 1997; Chen et al 1999; Marschang et al 1999; Benetka et al. 2007), and disease events in box turtles are often associated with the type species of *Ranavirus*, Frog Virus 3 or frog-virus 3-like virus (FV3; Johnson et al. 2008; Allender et al. 2012a). Epizootics of FV3 have been scattered across several habitats and landscapes in the US; however, disease predictability has not been successful (Allender et al. 2012a). To date in the US, ranaviral disease has been diagnosed in seven species of chelonians; this includes ten outbreaks in Eastern Box Turtles across ten different states (Allender 2012a).

In the present study, we estimated the prevalence of FV3 in different populations of Eastern Box Turtles in the US. Our hypotheses were: (1) the prevalence of FV3 in ante-mortem samples collected from box turtles in the US will be low (< 5%); (2) FV3 is associated with an acute mortality in box turtles (3) the prevalence in wild animals presented to rehabilitation facilities would be higher than that found in wild caught animals; (4) the prevalence of ranaviral disease will not be associated with age, sex, or weight characteristics; and (5) clinical signs of conjunctivitis, ocular discharge, oral plaques, and respiratory distress will be associated with ranaviral infection in box turtles.

## Materials and Methods

***Sample populations***.—We sampled turtles from two populations: wild caught, free-ranging turtles (hereafter free-ranging turtles) and wild-caught turtles presented to rehabilitation facilities (hereafter rehabilitation turtles). We actively searched for free-ranging turtles in their natural habitat, while we sampled rehabilitation turtles in an unnatural place, or when they were injured or diseased. We sampled free-ranging turtles from 2008 through 2011 from a population near Oak Ridge (OR), Tennessee, USA. We searched through visual encounters or canine search). We sampled rehabilitation turtles from 2007 through 2010 from five different wildlife rehabilitation centers (institutions): University of Tennessee (UT; Knoxville, Tennessee, USA), Wildlife Center of Virginia (WCV; Waynesboro, Virginia, USA), North Carolina State University (NCSU; Raleigh, North Carolina, USA), Alabama Wildlife Center (AWC; Pelham, Alabama, USA), and the University of Georgia (UGA; Athens, Georgia, USA).

***Sample collection and handling***.—We collected blood samples (less than 0.8% body weight) from the subcarapacial sinus using a 22-gauge needle and 3-mL syringe. We immediately placed the samples in a lithium heparin coated microtainer (Becton Dickinson, Franklin Lakes, New Jersey, USA) and mixed by gentle rocking. We sampled oral epithelium and mucus from within the oral cavity using cotton-tipped plastic handled applicators (Fisher Scientific). We then placed the swab tips in a 1.7-mL polypropylene microcentrifuge tube (Costar, Corning Inc., Corning, New York, USA) for storage. We labeled the blood and swab tubes with an identification number and remained on-site at -20º C, batch shipped on ice, and stored at -20º C until analysis. We did not evaluate samples of poor quality.

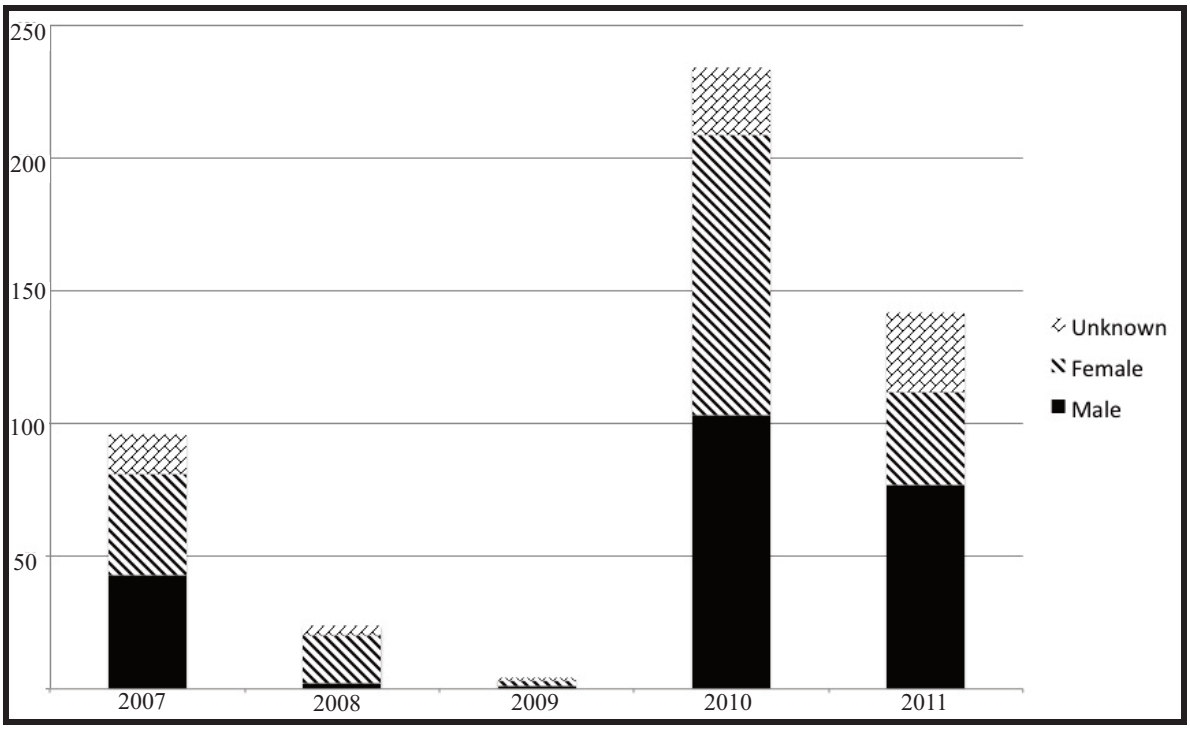


**Figure 1.** Number and the sex distribution of sampled Eastern Box Turtles by year.

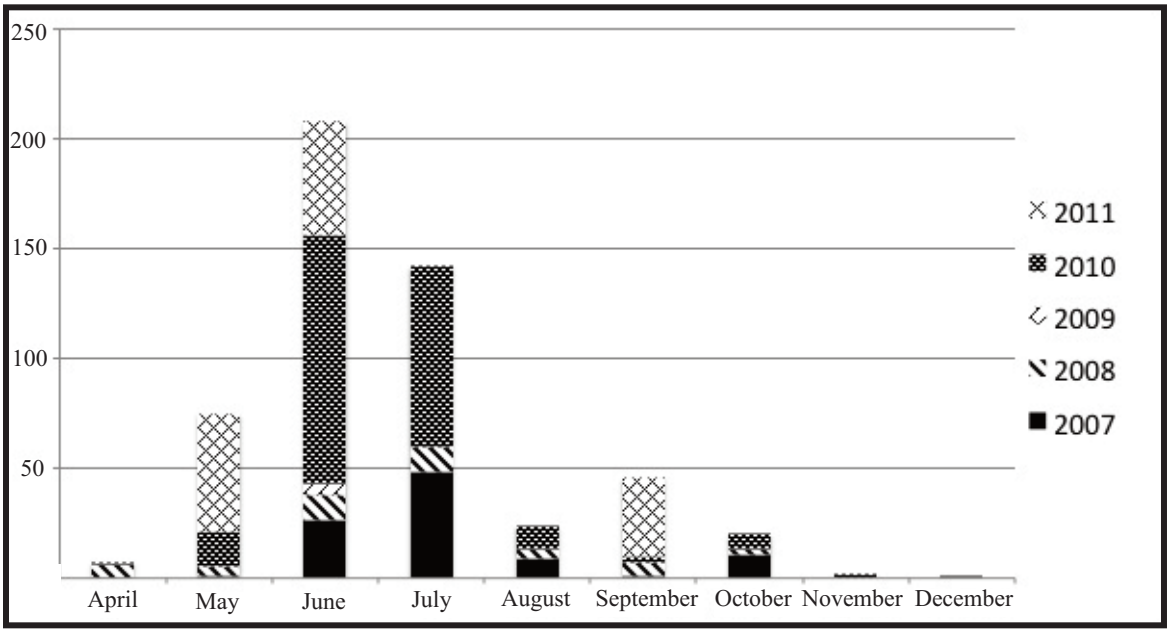


**Figure 2.** Number of sampled Eastern Box Turtles that were presented to rehabilitation facilities during each month each of the five by years (2007–2011) by the month sampled.

***DNA extraction and Polymerase Chain Reaction***.—We extracted DNA following manufacturer's instructions (QIAamp DNA blood mini kit, Qiagen, Valencia, California, USA). We determined the concentration and purity of DNA using a spectrophotometer

(Nanodrop spectrophotometer, Thermo Scientific, Wilmington, Delaware, USA). We performed quantitative PCR as previously described by Allender et al. (2012b). Briefly, we extracted DNA from whole blood and oral swabs using manufacturer's instructions (QIAmp Blood Mini Kit, Qiagen, Valencia, California, USA). We performed the TaqMan assay using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA) primer, and a probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein of FV3. We assayed all samples in three technical repeats using a real-time PCR thermocycler (7,500 ABI real-time PCR System, Applied Biosystems, Carlsbad, California, USA), analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, California, USA), and averaged the results.

***Clinical survey***.—We (admitting clinician or primary author) evaluated clinical signs and recorded our findings on a standard data sheet provided. We recorded the presence (1) or absence (0) of clinical signs compatible with those previously described for *Ranavirus* infection or other non-specific systemic disease. Specific clinical signs included aural abscess, cellulitis, conjunctivitis, cutaneous abscessation, nasal discharge/rhinitis, ocular discharge, oral abscessation, oral discharge/stomatitis, oral plaque, oral ulceration, and respiratory distress. Clinical signs for systemic disease not specifically reported in upper respiratory tract (URT) infections (at present) include fracture of appendage(s) or shell and diarrhea. Most turtles (n = 328) at OR had engaged their plastron hinge, making it impossible to perform a complete physical examination. Furthermore, 63 rehabilitation turtles had no clinical sign recorded.

***Statistical analyses***.—We tabulated descriptive statistics for all continuous variables. Normality was assessed using the Shapiro-Wilk test. We made comparisons using ANOVA/Kruskal-Wallis and t-test/Mann-Whitney U for within- and between-group differences for normally distributed and non-normally distributed data, respectively. We computed overall prevalence proportions (based on qPCR) and their 95% confidence intervals. We evaluated prevalence proportions for equal probabilities using a One-sample Chi-square test. We compared PCR results, natural history characteristics, and clinical signs using McNemar's Chi-square and Fisher's exact tests. We used Chi-square or Fisher's exact tests to evaluate the presence or absence of natural history characteristics or clinical signs by PCR test results. We calculated the Odds ratios for each clinical sign with FV3 status. Logistic regression models were created for any of the above variables that had a $P$ value $< 0.1$. We used the Hosmer-Lemeshow goodness-of-fit test to evaluate model fit. A stepwise approach was used to build the model. Statistical significance was considered when $P < 0.05$. We performed all analysis using statistical software (SPSS 20, IBM statistics, Chicago, Illinois, USA).

## Results

***Sample populations***.—We sampled 606 turtles (free-ranging turtles: 367, 61.1%; rehabilitation turtles: 231, 38.9 %; institution unlisted: 8, 1.3%) from 2007 through 2011, with 109 (18.5%) turtles sampled in 2007, 87 (14.6%) in 2008, 7 (1.2%) in 2009, 261 (41.6%) in 2010, and 142 (24.1%) in 2011. The samples were from 458 (87.7%) adults, 61 (11.7%) juveniles, and 3 (0.6%) with unknown age recorded. Of the 522 samples from animals with age and sex recorded, 199 (38.1%) were male, 248 (47.5%) were female, and 75 (14.4%) were of unknown sex (Fig. 1). We collected samples throughout nine months of the year (month was not recorded for 51 individuals; Fig. 2). We collected samples from institutions that included 38 (6.3%) individuals from UT, 367 (61.1%) from OR, 125 (20.6%) from WCV, 47 (7.8%) from NCSU, 16

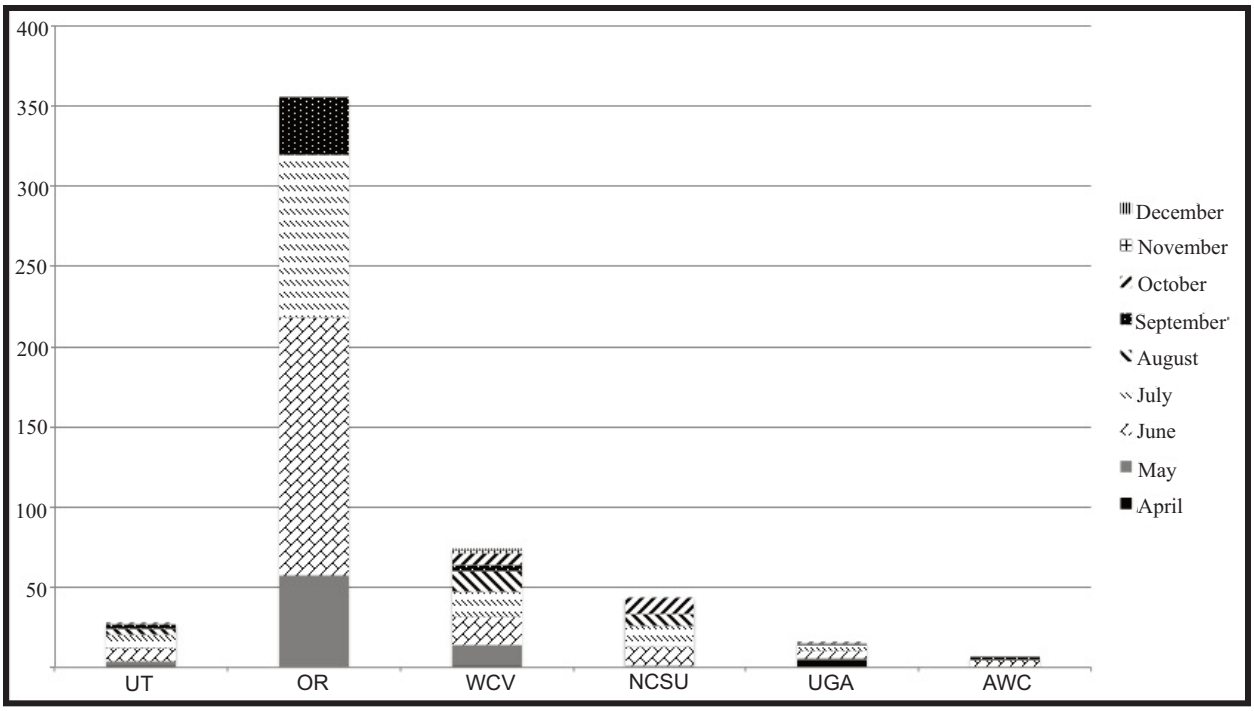


**Figure 3.** Proportion of sampled Eastern Box Turtles that were presented to rehabilitation facilities each month by the wildlife rehabilitation center (institution) location.

(2.8%) from AWC, and 9 (1.5%) from UGA (Fig. 3). We did not evaluate blood and swab samples for FV3 from 59 individuals from OR, 3 from WCV, and 1 from NCSU due to poor sample quality and thus we did not include them in the FV3 analysis.

We did not sample institutions, year, sex, and age classes with equal probabilities ($P < 0.001$). There was no difference in the age of turtles sampled by institution ($P = 0.698$), year ($P = 0.460$), or month ($P = 0.968$). Juvenile turtles were significantly associated with unknown sex ($P < 0.001$). There was no difference in the sex of turtles sampled by institution ($P = 0.093$) or by month ($P = 0.635$). There were significant differences in the sex of turtles when evaluating year (Fig. 1; $P = 0.007$). There was a significant difference in the month turtles were presented to rehabilitation facilities by year (Fig. 2; $P < 0.001$) and by institution (Fig. 3; $P < 0.001$).

Mean mass for females (350 g; 95% CI = 338–362 g) and males (345 g; 95% CI = 335–354 g) was significantly higher than that of unknown sex turtles (202 g; 174–231 g; $P < 0.001$); there was no significant difference between females and males ($P = 0.770$). Adult turtles weighed 353 g (95% CI = 347–360 g), juveniles weighed 131 g (95% CI = 115–147 g), and unknown aged turtles weighed 203 g (range: 95–265). Adults were significantly heavier than juveniles ($P < 0.001$) and unknown age turtles ($P = 0.001$). Turtle mass was not significantly different by institution ($P = 0.717$).

***Molecular characterization***.—We collected 444 blood samples and 434 oral swab samples, for a total of 540 turtles with either a blood sample or oral swab or both (n = 400). Mean DNA concentration in blood samples was 161.9 µg/mL (95% CI = 142–180 µg/mL) and swab samples 20.7 µg/mL (95% CI = 19–22 µg/mL). DNA concentration of blood sample extracts was significantly higher than swab samples ($P < 0.001$), but there was no difference in purity between blood and swab samples ($P = 0.195$; Table 1). Purity of DNA (A260/A280) in blood samples was 1.81 (95% CI = 1.79–1.82) and swab samples was 1.91 (95% CI = 1.75–2.06).

**Table 1.** Descriptive statistics for DNA concentration and DNA purity (A260/A280 ratio) for extracts of blood and oral swab samples from Eastern Box Turtles from 2007 through 2011.

| | Blood | | | Swab | | |
|---|---|---|---|---|---|---|
| | Median | 10-90th %iles | Min/Max | Median | 10-90th %iles | Min/Max |
| DNA Concentration | 78.10* | 19.50–401.20 | 2.70–1,472.60 | 14.90* | 3.70–43.80 | 0.32–132.90 |
| DNA Purity | 1.84 | 1.65–1.89 | 0.78–2.77 | 1.83 | 1.40–2.15 | 0.00–25.70 |

*Statistically signficant higher in blood samples, $P < 0.001$.

There was a significant difference in DNA concentration of blood samples between NCSU (325 µg/mL) and UT (152 µg/mL; $P = 0.003$), and also among blood samples from OR (171 µg/mL; $P < 0.001$), WCV (101 µg/mL; $P < 0.001$), and AWC (25 µg/mL; $P < 0.001$). There were significant differences in the DNA concentration from swab samples between OR (26.9 µg/mL) and WCV (15 µg/mL; $P < 0.001$), OR and NCSU (7 µg/mL; $P < 0.001$), UT (21 µg/mL) and NCSU ($P = 0.003$).

***Frog Virus 3-like virus prevalence***.—We identified four positive swab samples and four positive blood samples, but no turtle was positive on both sample types. There was a significant difference in weight ($P = 0.021$) between FV3 positive (av. wt. = 325 g) and negative turtles (av. wt. = 198 g). When combining all cases that were FV3 positive in blood and swabs (n = 8), we found a significant association for institution ($P < 0.001$; Table 2) and month ($P = 0.045$) with infection. There were significant differences in FV3 status by institution ($P < 0.001$) and month ($P = 0.040$). There was no association of FV3 status with year ($P = 0.515$; Table 3), age ($P = 0.081$; Table 4), or sex ($P = 0.157$; Table 4). Median FV3 copy number (i.e., the number of amplicons produced during each polymerase chain reaction, which corresponds to one copy of virus) from blood samples in positive animals was 1,926 (range: 0–24,287) and median FV3 copy number from swab samples in positive animals was 2,505 (range: 0–29,893). There were no differences in DNA concentration in swabs ($P = 0.067$) or DNA purity ($P = 0.928$)

**Table 2.** Prevalence of Frog Virus 3-like virus determined by quantitative PCR in Eastern Box Turtles sampled from the southeastern US.

| Location | FV3-Like Positive | FV3-Like Negative | % Prevalence | 95% CI |
|---|---|---|---|---|
| Free-Living* | 1 | 308 | 0.30 | 0.0–1.8 |
| Rehabilitation* | 7 | 217 | 3.13 | 1.5–6.3 |
| UT | 3 | 35 | 7.90 | 2.7–20.1 |
| WCV | 2 | 120 | 1.60 | 0.4–5.9 |
| NCSU | 0 | 46 | 0.00 | 0.0–7.7 |
| AWC | 2 | 14 | 14.30 | 4.0–39.9 |
| UGA | 0 | 9 | 0.00 | 0.0–29.9 |
| Total | 8 | 532 | 1.5 | 0.8–2.9 |

*Significantly higher prevalence in rehabilitation turtles ($P = 0.01$) than free-living turtles.

**Table 3.** Prevalence of Frog Virus 3 in blood and oral swab samples determined by quantitative PCR in Eastern Box Turtles from 2007−2011.

| Year | FV3 Positive | FV3 Negative | % Prevalence | 95% CI |
|---|---|---|---|---|
| 2007 | 1 | 107 | 0.9 | 0.2–5.1 |
| 2008 | 3 | 80 | 3.6 | 1.2–10.1 |
| 2009 | 0 | 7 | 0.0 | 0.0–35.4 |
| 2010 | 3 | 207 | 1.4 | 0.5–4.1 |
| 2011 | 1 | 131 | 0.8 | 0.1–4.2 |

based on FV3 status (Table 5). Logistic regression modeling for overall FV3 prevalence that included independent variables of institution, month, and age was not significant ($P$ = 0.260). Logistic regression modeling for FV3 prevalence in blood that included independent variables of year, month, and institution was not significant ($P$ = 0.338). Additionally, logistic regression modeling for FV3 prevalence in swab that included independent variables of age, institution, and sex was not significant ($P$ = 0.796). Mass was significantly lower in FV3-positive animals (198 g) than FV3-negative animals (325 g; $P$ = 0.012).

***Clinical examination***.—Two hundred and eight animals had full physical examinations performed. There were significant associations with FV3 prevalence and bone fracture (n = 3; $P$ = 0.045) and diarrhea (n = 1; $P$ = 0.017). The other clinical signs that were evaluated were not found to be significantly associated with FV3 prevalence: aural abscess ($P$ = 0.895), cellulitis ($P$ = 0.967), conjunctivitis ($P$ = 0.934), cutaneous abscessation ($P$ = 0.983), lethargy ($P$ = 0.902), nasal discharge ($P$ = 0.910), ocular discharge ($P$ = 0.910), oral discharge ($P$ = 0.934), oral plaque ($P$ = 0.967), or respiratory distress ($P$ = 0.910). FV3-positive turtles were 153 times (95% CI = 5–4,942) more likely to have diarrhea than in FV3-negative turtles.

There were 13 (6.3%; 95% CI = 3.7–10.4%) animals with an aural abscess, 4 (1.9%; 0.8–4.8%) with cellulitis, 8 (3.9%; 1.9–7.4%) with conjunctivitis, 115 (55.8%; 48.5–61.9%) with bone fracture, 11 (5.3%; 2.9–9.2%) with lethargy, 11 (5.3%; 2.9–9.2%) with nasal discharge, 11 (5.3%; 2.9–9.2%) with ocular discharge, 8 (3.9%; 1.9–7.4%) with oral discharge, 4 (1.9%; 1.9–7.4%) with oral plaques, and 11 (5.3%; 2.9–9.2%) with respiratory

**Table 4.** Prevalence of Frog Virus 3 in blood and oral swab samples determined by quantitative PCR in Eastern Box Turtles (*Terrapene carolina*) based on age class and sex from 2007–2011.

| Variable | FV3 Positive | FV3 Negative | % Prevalence | 95% CI |
|---|---|---|---|---|
| Female | 3 | 168 | 1.80 | 0.6–5.0 |
| Male | 0 | 218 | 0.00 | 0.0–1.7 |
| Unkown Sex | 1 | 69 | 1.45 | 0.2–7.7 |
| Adult | 2 | 398 | 0.50 | 0.1–1.8 |
| Juvenile | 2 | 55 | 3.50 | 0.9–11.9 |

**Table 5.** Descriptive statistics for DNA concentration and DNA purity (A260/A280 ratio) for extracts of blood and oral swab samples from Eastern Box Turtles from 2007 through 2011 with or without molecular evidence for Frog Virus 3.

| | FV3 Positive | | | FV3 Negative | | |
|---|---|---|---|---|---|---|
| | **Median** | **10–90th %ile** | **Min/Max** | **Median** | **10–90th %ile** | **Min/Max** |
| Blood DNA Concentration | 22.50 | 14.10–100.00 | 14.10–124.70 | 97.20 | 19.40–495.40 | 2.70–1,472.60 |
| Blood DNA Purity | 1.75 | 1.57–1.86 | 1.57–1.88 | 1.84 | 1.67–1.89 | 0.78–2.77 |
| Swab DNA Concentration | 41.30 | 11.00–56.80 | 1–57.20 | 14.50 | 3.50–43.85 | 0.00–132.60 |
| Swab DNA Purity | 1.94 | 1.85–2.14 | 1.85–2.20 | 1.83 | 1.39–2.14 | 0.00–25.70 |

distress. Other clinical signs seen in single animals included tongue discoloration, rear limb ataxia, edema of the neck and forelimbs, and skin lacerations. There were three animals that were positive for FV3 in blood that had clinical signs recorded. None of the FV3-positive turtles had aural abscess, cellulitis, conjunctivitis, lethargy, nasal discharge, ocular discharge, oral plaque, or respiratory distress. However, all three animals had a bone fracture and one of the animals had diarrhea. None of the turtles with FV3-positive swab samples had clinical signs recorded.

## Discussion

As populations of box turtles decline, the opportunity for disease-related events to lead to catastrophic events increase. A disease that has a short duration of effect with high mortality may lead to such a catastrophic event. Thus far, over 70 reports of *Ranavirus* infections in free-ranging amphibians have indicated that mortality rates may be as high at 90% locally, with potential to cause local extirpations (Jancovich et al. 2003; Miller et al. 2011). Large events such as these have not been documented in chelonians, and it is unknown whether they are occurring or going unnoticed. However, there have been sixteen reported cases of ranaviral disease in chelonians, with 10 of them involving the Eastern Box Turtle (DeVoe et al. 2004; Johnson et al. 2008; Ruder et al. 2010 Allender et al. 2011; Allender 2012a). All of these reports represent single case reports or less than 10 individuals involved in an outbreak. This is in contrast to amphibians that breed in ephemeral ponds and mortality events are commonly seen in high densities. It would be of great concern if the same level of mortality was occurring in box turtles, but was just going unnoticed because of these natural history characteristics. Conversely, this behavior may prevent or slow the spread of disease if infrequent intra-species contact occurs. Our study characterized the prevalence of *Ranavirus* across several states in the southeastern US.

We confirmed a low prevalence of FV3 in free-ranging and rehabilitation Eastern Box Turtles. Not surprisingly, we found a higher prevalence in turtles presented to rehabilitation clinics. The rehabilitation population was a biased population, but this bias allows animals that are at highest risk to be identified with FV3 infection whereas these individuals would likely not be encountered if free-ranging animals. This bias allows the discovery of emerging threats to the population prior to catastrophic events in the wild. Regardless, prevalence in this study is similar to that reported for Gopher Tortoises (*Gopherus polyphemus*) and box turtles using an ELISA that detects antibodies to *Ranavirus* thereby reporting exposure to (and not necessarily infection of) *Ranavirus* (Johnson et al. 2010). It has been observed that the disease course is short (less than 30 days until mortality)

and mortality is high in experimental challenge studies in turtles with FV3 (Allender 2012a). Diseases with these characteristics tend to underestimate true prevalence because animals become infected, die, and are removed from the sample population prior to sampling. The prevalence in our study is low and may be due to these characteristics in that animals are infected and die prior to our discovery and the animals we sample were never exposed or infected. One limitation of our study is that free-ranging animals are sampled during discrete times that may cause us to miss outbreaks of less than 30 days duration and there is no field component to complement the rehabilitation turtle sampling. Continued monitoring is needed to document occurrence and course of disease in free-ranging populations that may aid in conservation and management efforts aimed at reducing the impact of this disease in susceptible populations.

We determined that the prevalence of FV3 across several states was variable, but was confirmed in Alabama, Tennessee, and Virginia. The prevalence was highest in Alabama, but is likely due to the small sample size tested from that state. Frog Virus 3-like viruses in Eastern Box Turtles have previously been seen in Tennessee and Virginia (Allender et al. 2011), but this is the first report from Alabama.

Larval and metamorphic amphibians of most species have higher documented mortality to FV3 than adult amphibians (Miller et al. 2011), but no such information is available for chelonians. In this study, we found that juvenile turtles were more likely to be infected with FV3 than adults. However, there were only 57 juveniles tested, while 400 adults were tested. Juvenile turtles are more difficult to find, and our results may represent a highly susceptible age class that should be monitored more closely. Juvenile box turtle survivorship is reported to be low (Dodd 2001), and threats that reduce it further may endanger long-term sustainability.

We found a statistically significant difference in prevalence of FV3 based on year, with 2008 higher than 2007, 2009, and 2011. However, looking at these numbers they are not expected to represent a biologically significant finding, as the prevalence remained low in all years. The year with the lowest prevalence also had the lowest number of animals tested. And even though there are several amphibian populations that have endured annual outbreaks of the disease (Fox et al. 2006; Carey et al. 1999), this pattern has not been seen in chelonians. The absence in certain years likely represents sampling bias rather than the virus being absent from the environment. This study found two cases in June, and one each in April and July, and four cases with no month recorded. Differences in infectivity of ranaviruses due to temperature have been shown for amphibians (Rojas et al. 2005) and turtles (Allender et al. 2012a), which likely will lead to differences in detection based on season and environmental temperature. Future studies need to identify the time of year and locations of known cases and begin ongoing surveillance of those locations to further characterize the epidemiology.

There were three females and no known males diagnosed with FV3 in the current study. Four positive animals had no sex recorded and were not included in the analysis. There has been no sex predilection described in other reports on amphibians or reptiles and the results of our study are likely only a statistical anomaly.

Our study utilized quantitative PCR to survey populations of chelonians for *Ranavirus*. This method is considered more sensitive than conventional PCR, allowing for the detection of potential subclinical disease states (Allender et al 2012b). However, there has been no evidence that box turtles exist in a subclinical state. We detected eight positive animals with four positives from blood and four positives from oral swabs. There are several factors that may have lead to one sample being positive and the other sample negative, such as inefficient DNA extraction, poor sample collection, inappropriate sample handling, or absence of viral shedding in the sample. In experimental transmission in

Red-eared Sliders (*Trachemys scripta elegans*), whole blood was found to be 100% sensitive and specific, while oral and cloacal swabs were 83% sensitive and 100% specific, which may explain a negative result from a swab and positive result from whole blood, but would not explain the reverse. Regardless, future investigations should continue to sample multiple tissue types to increase detection ability in a population.

Molecular factors may lead to false negatives including the DNA concentration and DNA purity. This study utilized a spectrophotometer that allowed for the quantification of DNA and assessment of its purity by using 1 µL of sample. This technology allows the user to assess whether the extraction was ideal for the developed PCR. If not ideal, then purification of the extract can be performed. However, it doesn't allow differentiation of viral DNA from host DNA. There was no statistically significant difference in DNA quantity or purity between blood and swab samples that were positive for FV3 in the current study, however DNA concentration was higher in positive swab samples rather than positive blood samples. Conversely, DNA concentration in positive blood was lower and DNA concentration of positive swabs was higher than the negative samples. Positive blood may have less DNA because of disease-related anemia or increased total solids in samples that dilute total DNA concentration. Positive swab samples may have had more DNA because there was increased cell sloughing that was disease-related or other pathogens such as bacteria, other viruses, and parasites.

Manufacturer's instructions indicate that the ideal range of A260/A280 is 1.7 to 1.9, and that this represents a pure sample. Swab extracts were much more variable than blood samples despite the fact that the median value was nearly the same as the median value of the blood extracts. This is likely due to less DNA in the sample or inhibitors with the cotton on the applicators. Conversely, blood samples were more frequently purified because of impure original extraction. This impurity is likely due to the overwhelming amount of host DNA and cell lysis that is required in samples with nucleated blood cells. All of these blood samples were isolated based on 200 µL of whole blood sample, however, recent manufacturer's recommendations indicate that 10 µL of whole blood is the current volume that should be used for samples with nucleated red blood cells. This will undoubtedly decrease not only the host DNA (as is anticipated by the manufacturer's changes), but it will consequently decrease the viral DNA. Alternatively, extractions can use higher volumes of blood, but require more reagents and time for extraction. Future studies should determine if smaller sample volumes can be optimized that maximize detection of FV3.

Several clinical signs have been associated with FV3 infection in turtles, including ocular discharge, oral discharge, respiratory distress, and oral plaques (DeVoe et al. 2004; Johnson et al. 2008). Unfortunately, none of those clinical signs were associated with positive status in our study. The absence of significant associations with clinical signs is likely due to the low prevalence of ranavirus and the relatively high number of clinical signs in non-infected animals. Despite this low prevalence, both bone fracture and diarrhea were significantly associated with FV3 infection in this study. It is valuable to clinicians at these or other rehabilitation clinics to not overlook infectious diseases in the face of a fracture. Diarrhea has high odds of occurring with FV3 infection. This likely represents a manifestation of systemic disease rather than a specific disease process. The failure of this study to identify other clinical signs indicates that other signs are non-specific and several other FV3-negative turtles were exhibiting oral plaques, ocular discharge, and nasal discharge. Additionally, this study utilized several collaborating institutions, and although given a specific checkbox examination sheet, may have introduced variability into assessment of each turtle and thus impacted the observed results. All future studies should continue to record clinical

signs on a standardized sheet as it may be possible to better determine associations with clinical signs in populations that have higher prevalence.

In summary, this was the largest cross-sectional study to estimate the prevalence FV3 in free-ranging and rehabilitating Eastern Box Turtles. It demonstrated that the prevalence of FV3 is low in both populations, and that temporal and geographical influences were minor. Findings that FV3 was in higher proportion in females and juvenile animals warrants further work, especially if there is a potential for vertical transmission. Clinical signs of bone fracture and diarrhea were significantly associated with infection. Since bone fracture is a common finding in turtles presented to rehabilitation facilities (Brown and Sleeman 2002; Schrader et al. 2010), this finding may have been biased by the types of presentation. Alternatively, it is possible that injured animals might change their behavior and/or become more susceptible to *Ranavirus* infection. Infection was detected both in blood and oral swabs, and future studies should continue this dual testing strategy for detection as a single sample was not consistent. These findings elucidate that FV3 infection has several signs of an acute systemic disease: low prevalence, few clinical signs, and no biologically significant sex-specific prevalence. The higher prevalence in juveniles is supported by other studies that show higher mortality in juvenile salamanders (Miller et al. 2011), but more work is needed to determine if this is true in experimental settings as well. Eastern Box Turtles are highly susceptible to infection based on published case reports, and based on results of this study, further work is needed to determine the threat to Eastern Box Turtle sustainability. However, due the number of published reports in box turtles and the high mortality observed in captive collections, future research is needed in this species.

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