Evidence of Ranavirus Infections among Sympatric Larval Amphibians and Box Turtles

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ABSTRACT.—Ranaviruses are emerging as serious pathogens across ectothermic taxa, recently causing mass die-offs including entire chelonian populations. Amphibians may serve as reservoirs for chelonian infections. To assess this idea and determine whether chelonians in the Midwest are infected with (or at risk for) ranavirus infections, we tested for presence of ranavirus infections among sympatric larval amphibians ($N =$ 135) and Eastern Box Turtles (*Terrapene carolina carolina*, $N = 132$) earlier and later in the Box Turtle active season (between May and October 2009) in south-central Indiana. Three larval amphibians (2.2%; one *Ambystoma* sp. and two unidentifiable anuran larvae) and four Box Turtles (3.0%) tested positive for a ranavirus. Ranavirus infections were found in both early-season pond breeders (anurans) and then later-season pond breeders (salamanders), spanning the Box Turtle active period, but we did not detect mass die-offs. The majority (75%) of our ranavirus-positive Box Turtles were found later in the season. This represents the first documented case of ranavirus infection among a Box Turtle population in the Midwest and one of the few studies reporting ranaviruses across wild sympatric taxa. Our results suggest that ranaviruses persist sublethally in both larval amphibians and individual Box Turtles, allowing them to serve as reservoirs for this disease. Future studies should include sampling of all sympatric herpetofauna and investigate prevalence and persistence of the viruses to understand better the risks of cross-contamination.

Ranavirus is one of five genera of *Iridoviridae*, a family of large, double-stranded DNA viruses capable of infecting invertebrates and ectothermic vertebrates. Ranaviruses are well known to contribute to mass mortality events of fish and amphibians (Daszak et al., 1999; Hyatt and Whittington, 2002; Gray et al., 2009), and cases of the infection in reptiles are increasingly detected and reported (Marschang et al., 2005; Belzer and Seibert, 2011; Nazir et al., 2012; Farnsworth and Seigel, 2013). Jancovich et al. (2010) suggested that ranaviruses experienced a shift in host species from amphibians to reptiles, echoing the more historical shift from fish to amphibians and back (Picco et al., 2010). The ability of some ranaviruses to switch hosts, likely because of resemblances among strains, makes them a high conservation concern (Picco et al., 2010). There is a marked similarity between frog ranavirus (FV3) and ranaviruses isolated from turtles (Mao et al., 1997). Recently, the complete genomic sequence of a ranavirus isolated from soft-shelled turtles was reported as nearly identical to FV3 (Huang et al., 2009). Thus, it appears that the same (or at least very similar) viral species are capable of infecting both amphibians and turtles. Viruses displaying identical patterns on restriction enzyme analysis, and thus very closely related if not identical, have been isolated from sympatric species of fish and amphibians (Mao et al., 1999) and from sympatric species of amphibians and reptiles (Johnson et al., 2008), suggesting that one species may serve as a reservoir of infection for the other (Gray et al., 2009; Miller et al., 2011). Thus, it is reasonable to suspect that interclass transmission could occur anywhere the viruses exist and where amphibians and chelonians cohabitate.

Box Turtles are considered a terrestrial turtle; however, they are known to soak regularly in creeks, ponds, and wetlands during hot, dry weather (Stickel, 1950; Dodd, 2001). Where ephemeral ponds persist into the Box Turtle active season, sympatric larval amphibians can be found. Although Eastern Box Turtles (*Terrapene carolina carolina*; Crother, 2008) in the Midwest have high adult survival rates (Currylow et al., 2011), they are declining throughout their range as a result of road mortality, collection for the pet trade, habitat loss and

fragmentation, and most recently disease (Belzer and Seibert, 2011; Farnsworth and Seigel, 2013). Ranaviruses are implicated in mass mortality events of Box Turtles (De Voe et al., 2004; Johnson et al., 2010). In one outbreak, 23% of a repatriated Box Turtle population died within a 3-month period from ranavirus infections (Johnson et al., 2008) and another similar large die-off occurred in 2012 in Maryland (Farnsworth and Seigel, 2013). In experimental challenge studies, intramuscularly inoculated turtles showed high mortality rates following development of clinical signs of severe respiratory disease including nasal and ocular discharge and oral plaques (Johnson et al., 2008).

While confirmed amphibian cases of ranavirus infections have been documented from many states across the United States, chelonian cases have been restricted to eastern and southern states including Tennessee, Texas, Florida, Georgia, Pennsylvania, New York, Maryland, and Kentucky (Allender et al., 2006; Johnson et al., 2008; Ruder et al., 2010; Farnsworth and Seigel, 2013). To date, only one study has screened for ranavirus infection among chelonians in the Midwestern region of the United States. Allender et al. (2010) used PCR to identify ranavirus in Blanding's (*Emydoidea blandingii*) and Painted Turtles (*Chrysemys picta*) in Illinois but found no turtles to be positive for the viruses. It remains unknown how geographically widespread the risk of ranavirus infection is for native chelonians, but the viruses are pervasive in amphibians and there is evidence that it can persist for long periods in ponds (Gray et al., 2009; Nazir et al., 2012). Infections of larval amphibians inhabiting ponds in the Midwest could put native chelonians at risk. We provide an initial assessment of the prevalence of ranavirus among Box Turtles and a number of potential reservoir species from central Indiana, United States.

MATERIALS AND METHODS

Study Area.—We sampled three semi-ephemeral ponds located across Morgan and Brown Counties, Indiana. The ponds each were separated by 10–16 linear km, 26 km total, north to south (midpoint: 39°20′27.14′′N, 86°18′55.79′′W). A concurrent radiotelemetry study on Eastern Box Turtles was being conducted throughout the study area (Currylow et al., 2012), and the three ponds were chosen due to their proximity

TABLE 1. Number of each species sampled for ranavirus in south-central Indiana. Samples are divided by species, sampling period (Earlier season: May/Jun; Later season: Jul/Aug–Oct), and associated pond number (for amphibian samples). Numbers in parentheses represent the number of samples in that category that tested positive for ranavirus infection.

Species	Earlier season sampling			Later season sampling		
Terrapene carolina carolina	66 (1)			66 (3)		
	Pond 1	Pond 2	Pond 3	Pond 1	Pond 2	Pond 3
Ambystoma spp. (A. maculatum, A. opacum, or A. jeffersonianum)	7	9	15	15	13	5 (1)
Notophthalmus viridescens (adult)	-	2	-	-	-	-
Lithobates catesbeianus/clamitans	12	-	8	1	1	1
Lithobates sylvaticus	-	21	1	-	-	-
Pseudacris crucifer	-	6	17	-	-	-
Unidentifiable anuran	2 (2)	1	-	-	-	-

and use by both transmittered and randomly encountered Box Turtles.

Sample Collection.—We collected whole larval amphibians using partially submerged minnow-traps during two sample periods between May and August 2009. We trapped each of the ponds on two separate occasions (each lasting 24 h) earlier in the season (late May/early June), and later in the season (late July/early August). In the field, we removed the captured larvae from the traps, separated larvae by class (salamander or tadpole), and immediately euthanized and preserved them by total emersion in fresh, site-specific tubes of 98% ethanol. We then transferred the larval amphibians to the lab where we identified them to genera (and species, where possible) via dissecting scope and mouthparts (tadpoles only). We decontaminated between sampling events and locations by saturating all equipment with bleach solution and then allowing it to air dry completely.

Using venipuncture of the subcarapacial sinus (Hernandez-Divers et al., 2002), we collected blood samples during the concurrent Box Turtle radiotelemetry study between May and October 2009. Upon sample collection, we recorded any signs of disease (e.g., oral, nasal, or ocular discharge, wheezing, basking behavior, etc.) in the turtles and used a marginal scute marking system (modified from Cagle, 1939) to assign a unique number to each individual turtle. We used standard aseptic field techniques (e.g., fresh gloves, needles) between animals and stored blood samples in individual vials of a lysis buffer solution until transferring them to the lab.

PCR and Sequencing.—We extracted DNA from small biopsies of amphibian liver tissue and the Box Turtle blood samples by digesting in 1% proteinase K (10 mg/ml) and 99% SNET buffer (20 mM Tris pH 8.0; 5 mM EDTA pH 8.0; 400 mM NACL; 1% SDS) solution overnight at room temperature. We used a standard proteinase K/phenol-chloroform procedure (Sambrook and Russell, 2001) and amplified viral DNA from an approximately 510-base pair (bp) portion of the *Ranavirus* major capsid protein (MCP) gene following the amplification procedures of Mao et al. (1996). The viral DNA used as a positive control was extracted from a ranavirus-infected *T. c. carolina* spleen (courtesy Zoo Medicine Diagnostic Laboratory, University of Florida, College of Veterinary Medicine). We used negative controls by substituting water for DNA in each PCR and subjected all samples to PCR analysis at least in duplicate. We ran and confirmed all putative positive samples at least three times, then excised and purified positive bands according to the manufacturer's protocol using a QIAquick Gel Extraction Kit (Qiagen).

We submitted 15 µl of the purified PCR product from putative positive samples to the Purdue Genomics Core Facility for analysis on an ABI 3730xl automated DNA sequencer (Applied Biosystems). Finally, we determined the likely origin of the sequences visually by assessing their alignment with expected *Ranavirus* sequences and by conducting BLAST searches for similar sequences in the NCBI nucleotide database.

RESULTS

We collected 135 larval amphibians (64 *Ambystoma* spp. [*Ambystoma maculatum*, Spotted Salamander; *Ambystoma opacum*, Marbled Salamander; or *Ambystoma jeffersonianum*, Jefferson Salamander]; 23 *Lithobates catesbeianus*, American Bullfrog [*Rana catesbeiana*] or *Lithobates clamitans*, Green Frog; 22 *Lithobates sylvaticus*, Wood Frog [*Rana sylvatica*]; 23 *Pseudacris crucifer*, Spring Peeper; and 3 unidentifiable anuran larvae) from the three ponds over the two time periods. We could not identify three anuran larvae because the condition of the animals at the time of collection was poor (deformed and torn), which caused the mouthparts to be unusable for identification. Moreover, the early collection dates prevented use of distinguishing morphological features present in later ontogenic larval stages. However, all three animals were distinguishable from the larger *Lithobates* species and therefore were likely to be *L. sylvaticus* or *P. crucifer*. Two adult amphibians (*Notophthalmus viridescens*, Eastern Newt) were found dead in the minnow traps and preserved for this study. We screened 132 *T. c. carolina* individuals (124 encountered randomly, 8 radiotelemetered) from which samples were collected during the same active season.

We detected three samples positive for ranavirus from amphibians (2.2%) and four positive samples from Box Turtles (3%; Table 1). We successfully sequenced a 510-bp portion of the MCP gene from each sample. The sequences in each of the seven individuals were identical to each other. BLAST analysis revealed that each sequence was identical to those from FV3 and other closely related or identical ranaviral strains.

Thirty-four of the 132 sampled turtles presented one or more clinical signs of disease similar to ranavirus infection such as nasal and ocular exudates, conjunctivitis, oral discharge, wheezing, and characteristic basking behavior (De Voe et al., 2004; Allender et al., 2006; Johnson et al., 2008). However, one positive *T. c. carolina* sample was collected from an apparently healthy adult male turtle, demonstrating that asymptomatic carriers were present in our population. The other three ranavirus-positive samples obtained from *T. c. carolina* were from individuals exhibiting clinical signs (one female with nasal
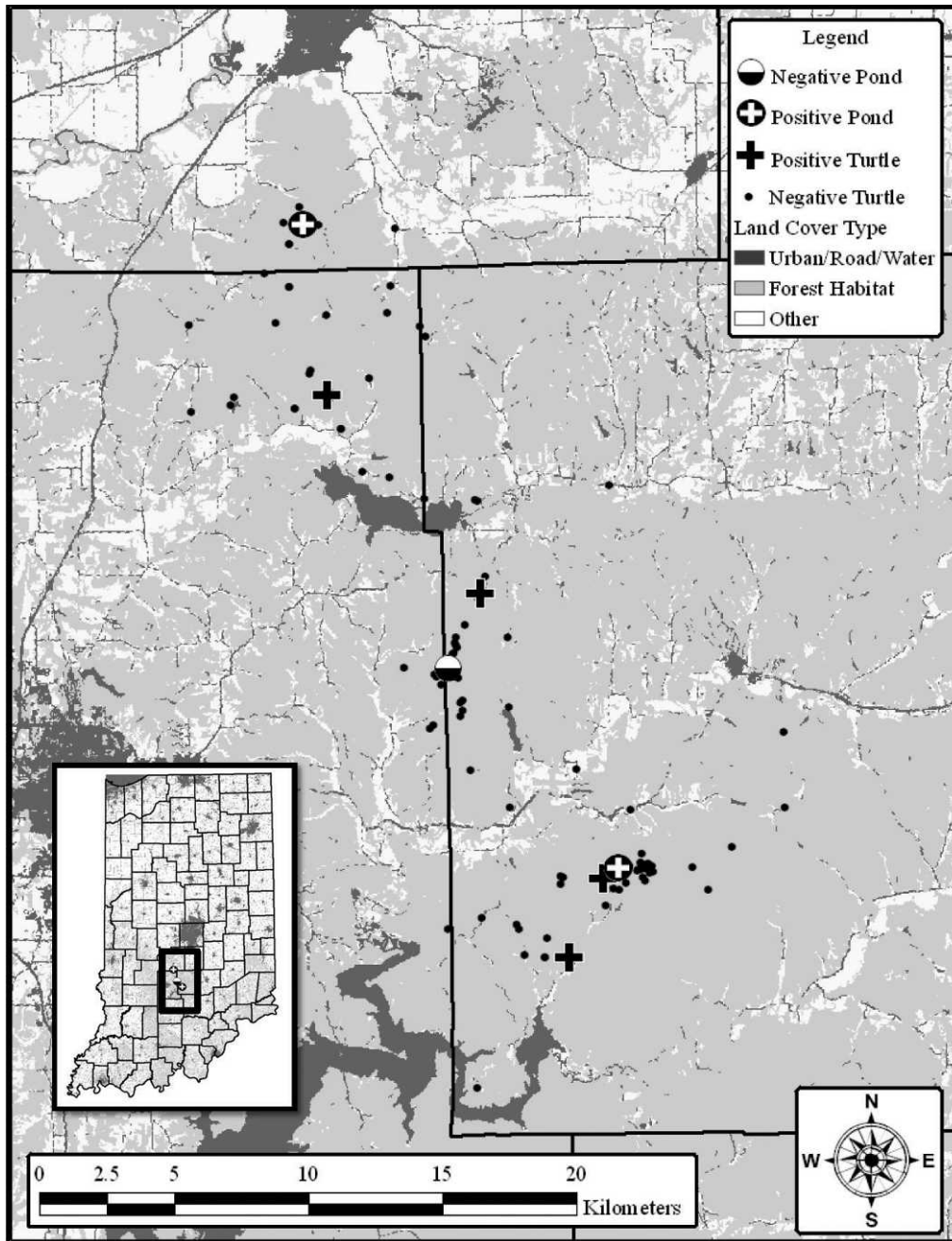
FIG. 1. Location and geographic distribution of *Terrapene carolina* samples testing positive/negative for ranavirus infection in relation to sample ponds in south-central Indiana, United States.

discharge and basking; one male with conjunctivitis and basking; one juvenile with conjunctivitis).

We collected three positive larval amphibian samples (one *Ambystoma* sp. and two unidentifiable anuran larvae) from two of the three sampled pond sites, the northernmost and southernmost (Table 1; Fig. 1). We collected all *T. c. carolina* samples within 10 km of a sample pond, but we found that not all positive *T. c. carolina* samples were near a pond that contained positive-tested larvae (Fig. 1).

DISCUSSION

Our study represents the largest reported cross-sectional study of ranaviruses in free-ranging Box Turtles and the first case documented from the midwestern United States. We screened amphibian larvae and Eastern Box Turtles genetically and found low, but detectable levels of ranavirus infections (i.e., 2–3%, respectively). These findings support previous reports of Eastern Box Turtle susceptibility to ranavirus infection, a known cause of mortality in a species undergoing widespread population declines (De Voe et al., 2004; Johnson et al., 2008; Belzer and Seibert, 2011; Currylow et al., 2011). However, cross-taxa transmission and sources of infection remain unclear, as well as strain virulence. Although we detected Ranaviruses in both amphibians and reptiles, our study populations did not experience mass die-offs. These findings suggest that disease prevalence in the United States might be higher than anticipated.

We provide an estimate of the percentage of turtles infected in a population using genetic assays. Other studies have used serology to estimate disease prevalence in chelonians (Johnson et al., 2010). Anti-ranavirus antibodies were detected in the Gopher Tortoise (*Gopherus polyphemus*) through the application of an indirect enzyme-linked immunosorbent assay. Johnson et al. (2010) tested 1,000 plasma samples of Gopher Tortoises from throughout the species range, finding that 1.5% tested positive. However, this was reported to be likely an underestimate of the true incidence of disease, as experimental studies found the virus to cause high mortality rates with a short disease course (Johnson et al., 2007). Similarly, our estimate of disease prevalence is likely conservative, as quickly fatal cases may not be detected in a cross-sectional study of this type.

We sequenced a portion of the MCP gene to confirm infection with ranavirus. While all seven infected individuals, amphibians and turtles, had sequences that were identical, additional sequencing would be necessary to determine if they were indeed infected with identical viruses. The MCP is a fairly conserved gene among *Ranavirus* spp. and likely not sufficient for detecting differentiating viral strains (Schock et al., 2008). However, separate strains are not required to infect across taxa (Miller et al., 2011) and the jump can cause a change in virulence. The same ranavirus strain has been attributed to differing mortality rates in multiple species of amphibians and fish (Gobbo et al., 2010; Haislip et al., 2011). Further sequencing work is needed to better distinguish strains that infect sympatric species, and sublethal effects (asymptomatic carriers serving as reservoirs) should be investigated.

Clinical signs of disease in this study did not always correlate with ranavirus detection. Three of the four turtles that tested positive had clinical signs, and 31 of the turtles sampled displayed clinical signs of disease but did not test positive for a ranavirus. It is difficult to know whether the turtles with clinical signs, but PCR negative, were infected at levels below detection by the PCR, were able to mount an immune response sufficient to eliminate viremia, or were infected with other diseases. On the other hand, it was interesting that one apparently healthy turtle tested positive for a ranavirus. It has been speculated that an asymptomatic carrier state may be possible in turtles (Johnson et al., 2007); however, no studies have concluded whether this is the case. Johnson et al. (2007) found three out of four turtles died after intramuscular inoculation of ranavirus-inoculated cell culture. The surviving turtle continued to shed virus after clinical recovery, suggesting that it might be capable of carrying the virus asymptomatically. Unfortunately, the turtle was euthanized at the end of the study, and duration of shedding beyond that time point was not evaluated. An asymptomatic carrier state has been observed in amphibians (Brunner et al., 2004) and, therefore, it is likely also possible in turtles.

Asymptotic turtles could serve as a vehicle to transport the virus across populations and landscapes. Turtles within this population are known to maintain home ranges averaging 7.45 ha and sometimes up to 180 ha (Currylow et al., 2012). Male Eastern Box Turtles maintain larger home ranges than do females and often travel much farther within and across seasons. For example, one male was tracked traveling nearly 10 km in a single season (Currylow, 2011) and, in another population, a male was documented to travel farther over successive seasons (Kiester et al., 1982). It is plausible that this sort of transitory behavior by infected males (such as the one apparently healthy but positive male in this study) could explain the distribution we found across our 26-km sampling site (Fig. 1). This is particularly significant if the carrier becomes clinical and employs a common recovery behavior: soaking.

Turtles expressing signs of sickness are often found soaking in natural water bodies, and soaking turtles is a technique often used in the veterinary medicines to treat sick patients (e.g., Johnson et al., 2008). Twenty-six percent of the animals tracked over 4 yr were noted to be found in water at some point during tracking (Currylow et al., 2012). Many of these turtles were found repeatedly in creek beds and washes. For amphibians, we found only anurans to be positive earlier in the season and a single salamander later in the season (Table 1). This suggests that overlap in species larval development may extend the period of potential infection within a single pond as the larvae of the late-arriving species become infected before the early-arriving species metamorphoses and leave the pond. Therefore, the ponds may remain reservoirs for transmission of ranavirus throughout the turtle's active season despite the amphibian species turnover.

Ranaviruses continue to be a substantial threat for free-ranging chelonians. Half of the infected turtles in the current study were located 0.7–3.8 km from a positive sample pond at the time of sampling, a distance known to be within the typical home-range size for animals in this population (Currylow et al., 2012). Surveys for the virus in amphibian populations can help assess the risk to sympatric chelonian species. This study provides baseline information that contributes to a better understanding of the geographic distribution and prevalence of infection and disease. Additional studies are needed to further characterize the method of transmission between amphibians and chelonians, investigate the prevalence of asymptomatic carriers and their role in virus transmission, and evaluate the long-term impact of the sublethal viruses as well as the role of chelonians as asymptomatic carriers on populations and population declines.

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