A Natural History of *Ranavirus* in an Eastern Box Turtle Population

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Ranavirus is a genus in the family of Iridoviridae. Amphibians and fish had been the generally recognized victims of this highly virulent pathogen (Langdon and Humphrey 1987; Daszak et al. 1999; Green et al. 2002). More recently, however, *Ranavirus* was implicated in hitherto unexplained die-offs in chelonian populations (Johnson et al. 2008). In 2003, this virus struck one (but not the other) of two translocated (Belzer and Seibert 2009a) eastern box turtle (*Terrapene carolina carolina*) populations that we monitor in adjacent, northwestern Pennsylvania counties. The unaffected habitat, an 80-ha tract bisected by McCutcheon Run, is located in Mercer County, Pennsylvania. It is owned by the McKeever Environmental Learning Center. Its hiking trails are open to the public. The affected population's habitat (subject of this paper) is only 20 km away, to the northeast in Venango County, Pennsylvania. It is a privately owned 200-ha wildlife sanctuary with highly restricted access. A south-facing incline rises steeply from the sanctuary's southern boundary on the French Creek to a broad plateau toward the north. Extensive woodlands buffer the eastern and western boundaries (for more detailed site descriptions, see Belzer and Seibert 2009a). We have telemetrically monitored (Belzer and Seibert 2009b) box turtles in the Venango County sanctuary for a decade. It is the only chelonian population that has been intensively monitored before, during, and after a *Ranavirus* outbreak in natural habitat. Herein we share our observations, speculations, and insights that emerged as the disease spread through this population. We hope that details in this narrative will prove helpful to field biologists confronted with an outbreak of this unfamiliar disease.

Baseline Health Profile of the Affected Population

The Venango County population-median, from October 1999 to July 2003, was 35 box turtles (1999 census = 4 adults and 0 subadults; 2003 census = 32 adults and 34 head-started subadults). During those first 4 years at the sanctuary, this population suffered only 1 death. That was an adult who failed to survive its 2001/2002 winter brumation. During those same 4 years, 10 other members (all adults) of this mixed-age population developed garden-variety (e.g., non-*Mycoplasma agassizii*) respiratory, ocular, or aural infections that are common in wild box turtles (e.g., see de Vosjoli 1991; Dodd 2001). They were treated in our infirmary, recovered, and released back into the study habitat with unremarkable aftermaths. Even in cases where we discovered illness only after many weeks, with pathogenesis well established (Belzer 2008b), infirmary treatment (Belzer 2008a) enabled recovery of health.

A 2003–2006 Sudden-Death Event

By 2003, our observations of the Venango County study population during its first 4 years (and of our Mercer Coun-

ty study population during the concomitant 4, and previous 6, years) taught us that eastern box turtles are remarkably resilient and can recover from protracted periods of illness (Belzer 2008b) and from severe injury (Belzer 2008a). But on 24 August 2003, we found 1 of our Venango County turtles (who had been seen 3 days earlier to be alert and in apparent good health) near death. The turtle was now devoid of almost all muscle tone; its eye lids were closed, mouth gaping with a caseous tongue coating, and slobbering a clear exudate. We had never seen a turtle in such terrible condition. We immediately brought it to our infirmary and began the therapeutic regimen (warm water soaks, thermal gradient and subcutaneous Baytril injections; Belzer 2008a) that we had used successfully for respiratory infection in the past. The turtle died 30 hours later, on 25 August. The speed with which death befell this turtle was astonishing and puzzling to us. We wondered whether there could have been an unusually asymptomatic and prolonged infection that gradually brought the animal to the brink of death without signs of illness until near the end. We did not realize that we had just witnessed the first fatality from a rapid-death disease that would sweep through our population during the end of the 2003 and the 3 ensuing seasons.

During the 8 weeks spanning late-August to late-October of 2003, this rapid-death scenario repeated 12 times: an animal that we observed to be alert and eating and vigorously moving through habitat one day was moribund or dead a week or less later. A photo of one of our moribund victims is available online (retrieved 15 August 2009 from http://ebtct.org/node/142). A lone exception to the extreme rapidity of death, once disease was suspected, was a case that involved an adult male, who survived for 10 days in our infirmary before succumbing. His behavior, muscle tone, and other signs seemed quite normal in the field. However, one eyelid seemed to be paralyzed in a half-closed position. Although no other victim presented with this peculiar sign, closed eyelids were typical of the moribund state in previous cases; thus, we wondered whether the male's eyelid problem might be an early sign. Because previous deaths occurred so rapidly, with no evident prodrome, we took the precaution of immediately evacuating this turtle and beginning pre-emptory therapy. His eyelid never recovered function, remaining in its half-closed position until he neared death. Although his demise was more protracted than any other, he did finally progress into the state of general atony with closed lids that we saw shortly before death in other cases. A 13th death in 2003, occurring before the start of November, may have been a result of predation and, therefore, is omitted from our rapid-death count.

In early November 2003, the first of our turtles began digging under soil to begin winter brumation. Three of the survivors, who were still active on the surface after 1 November, died before the last population member got under soil's cover for the winter. In all, 15 turtles (23% of our population) were apparent victims of this rapid-death syndrome during the 2003 season. All of the turtles that were alive and under soil for winter brumation at the end of 2003 were alive and emerged from the soil at the start (April–May) of the 2004 season.

No deaths occurred during the first 3 months of the 2004 season, permitting us a false hope that this terribly lethal disease was gone. But on 28 July (4 weeks earlier than the onset of rapid-deaths in 2003), the first turtle of 2004 died of this syndrome. The disease killed 5 animals during the latter half of our 2004 season. Routine annual additions of head-started juveniles to the habitat, as part of our long-term repatriation studies (Belzer and Seibert 2007), had augmented the population, such that these 5 deaths represented 8% of the 2004 population.

One of the 2004 cases involved a juvenile who presented an oddity in the usual course of the disease. We saw the juvenile walking near a pond on 25 September. We offered him some chicken to assess his appetite (a practice we sometimes use if we wonder about an individual's vigor). He avidly ate the chicken and appeared to be in good health. In this same area, 3 days later, the sound of numerous flies buzzing around a mound of leaf litter attracted our attention. Approaching the buzzing sound, we noticed a small white mass amid the leaves. We were surprised to find that the white mass was the juvenile's head, thickly coated by fresh fly eggs. We had never seen such an occurrence before (or since). The numerous flies surrounding this animal prompted our expectation that it was dead. But after brushing away the eggs, we found that he was not only still alive, but appeared to be in good health with clear eyes and alert movement (28 September). On 2 October, however, we found his carcass at the pond's edge. In retrospect, we wonder whether undetected cytolysis, as an early aspect of the rapid-death disease, had begun in tissue somewhere in the juvenile's head on 28 September and whether proteinaceous byproducts from damaged cells had attracted the female flies. Dietary protein is required by females of many fly species to produce eggs, and they selectively seek out such nutrition (e.g., Belzer 1978a, 1978b). Moreover, decaying protein can stimulate gravid females to oviposit (W. Belzer, Princeton University, unpublished data, July 1968 [Box Turtle Conservation Trust]).

In 2005, the pattern of rapid-deaths repeated starting only after the season's first several disease-free months. The first 2005 case was found on 23 July, 5 days earlier than the first one in 2004. Three unambiguous cases of the rapid-death disease occurred during the latter half of the 2005 season. Including, in our 2005 census, the year's addition of displaced adults (Belzer 1996) and head-started juveniles (Belzer and Seibert 2007) to the habitat, these 3 deaths represented a 4% population loss for 2005 and a continuation of the declining trend in annual mortality. A fourth death in late-November, which might have been an end-of-season instance of this disease, could have been a case of early winter-kill and, thus, was omitted from our count.

In 2006, we found only 1 unambiguous rapid-death (10 July), a juvenile female on the edge of a swamp. This was the

earliest of all sudden-deaths during the four affected years (2003–2006). A second 2006 sudden-death was an adult male found (2 August) lying at the base of a 10-m cliff. Williams and Parker (1987) reported a box turtle death that may have resulted from falling off of a cliff, and because we could not rule out the possibility that our turtle fell from a considerable height, we excluded it from our count for this disease.

To date (1 November 2009), no sudden-death has occurred since the 10 July 2006 case at our study site. With the annual additions of adults and head-started juveniles since 2006, as part of our long-term repatriation study, the site's population for 2007 was 42 adults and 46 head-started subadults, for 2008, 44 adults and 51 head-started subadults, for 2009, 51 adults and 55 head-started subadults.

Of the 24 rapid-death victims, while the disease ran its course from August 2003 to July 2006, 18 (75%) were on the edge of water (pond, swamp, or seep) when found dead or moribund. Sudden-deaths occurred equally (12 each) between the sexes; 16 victims were juveniles, and 8 were adults. When we count all of the different adults and juveniles that had lived in this habitat for at least 6 months during the 4-year span of 2003–2006, the proportioned mortality rates were 28% of all juveniles and 20% of all adults exposed to the habitat during this epidemic. Because of our ignorance of the pathogen's means of transmission, contact that each turtle may have had with an infected vector, and other factors that might affect exposure, our simple analysis, suggesting that age-class is not statistically associated ($p = 0.62$) with dying from the pathogen (*Ranavirus*, see below), is not conclusive.

Finding the Cause

After the first two rapid-death cases in 2003, we realized that we were facing a disease with which we were completely unfamiliar; thus, using expedited delivery, we sent subsequent carcasses in a fresh, chilled state to various government and university veterinary diagnostic laboratories in Pennsylvania. The necropsy reports all came back with descriptions of extensive pathology: "severe fibrinonecrotic esophagitis, pneumonitis, acute hepatocellular necrosis, enteritis ... severe acute multifocal ulcerative enteritis and necrotizing splenitis ... congested lungs with acute hemorrhagic foci ... severe acute necrotizing splenitis, severe suppurative oropharyngitis... acute pulmonary hemorrhagia and hepatic inflammation".

No parasites or *Salmonella* were recovered from the moribund or dead turtles. The bacteria that were grown (e.g., *Morganella morganii*; *Streptococcus* non-entero group D; *Streptococcus* alpha hemolytic-L, *Providencia rettgeri*; *Chryseobacterium meningosepticum*-L, *Citrobacter* sp., *Clostridium perfringens*, *Fusobacterium russi*; *Aeromonas hydrophila, Stentrophomonas maltophilia*-L, *Vertivillium* sp-S) varied from turtle to turtle and were evidently opportunists that were secondary to the unknown infection that caused the massive tissue destruction. Toxicology screens of the turtles' organs were all negative.

In frustrating ignorance of the etiology and biology of the pathogen, we intensified our late-2003 field precautions in hopes of minimizing risk of spreading the disease. For example, 2 volunteers (who had previously helped with tracking) were excluded from the sanctuary such that only two workers (WRB and SS) would enter the habitat and approach turtles. We disinfected clothing and equipment with bleach after a session in the habitat, before we returned for the next round of fieldwork. We tried various hand cleaners (chlorhexidine acetate, didecyl dimethyl ammonium chloride, dimethyl benzyl ammonium chloride, isopropyl alcohol) on inner and outer surfaces of examination gloves and carried biohazard bags to receive used examination gloves for later sterilization and destruction. But we wondered how effective such measures could be if clean clothing/equipment might pick up infective forms of the pathogen from dew or surfaces of vegetation as we moved through the habitat.

In October 2003, Bob Wagner (University of Pittsburgh's Division of Laboratory Animal Resources, School of Medicine) suggested that the laboratory of Elliot Jacobson in Gainesville, at the University of Florida's College of Veterinary Medicine, a leader in uncovering emerging and cryptic reptile pathogens, might be able to shed light on what was decimating our population. After listening to our description of the disease's signs and rapid lethalness, and of local climatic conditions, Jacobson speculated that *Ranavirus* might well be the etiologic pathogen. He requested dead specimens for necropsy and PCR-tests. April Johnson, working in his laboratory, had recently identified *Ranavirus*-like particles using transmission electron microscopy in archived tissue samples from unexplained box turtle mass mortality events in Georgia during 1991 and in Texas in 1998 (Johnson et al. 2008). Her evidence suggested that this virus was the causative pathogen for those puzzling die-offs. Testing our specimens by PCR and virus isolation, she confirmed their suspicion that *Ranavirus* was killing our box turtles (Johnson and Jacobson 2004).

Allender et al. (2006) identified the virus in a diseased, free-ranging eastern box turtle evacuated in October 2003 from its Tennessee habitat. Its presenting signs were very similar to those for our afflicted box turtles. Also, mirroring the aggressiveness of the virus in our Pennsylvania population, it killed the Tennessee turtle within 6 days of admission, despite intensive antibiotic therapy provided at the Avian and Zoological Clinical Service of the Veterinary Medical Teaching Hospital at the University of Tennessee. Jude Holdsworth (New York Department of Environmental Resources), a member of our project's advisory committee, reports (*pers. comm.*, 14 June 2006) that a dead Blanding's turtle (*Emydoidea blandingii*) from one of her New York State field sites was a confirmed victim of *Ranavirus*. A considerable diversity of chelonian species is now known to be vulnerable to this virus (e.g., see Benetka et al. 2007; McLeod 2009).

One reason why the 2003 virology cultures in Pennsylvania failed to discover the viral agent was because *Ranavirus* can be grown in cell lines incubated at 28C° but not at the higher temperatures (e.g., 42C°) routinely used in most laboratories (A. Johnson, School of Veterinary Medicine, Purdue University, *pers. comm.*, 28 September 2008).

In May 2004, April Johnson and April Childress from Jacobson's lab traveled to northwestern Pennsylvania and collected blood samples from almost all of our turtles living at the affected site. They also collected dead frogs and tadpoles found in the habitat. PCR and virus isolation revealed that the dead anurans had also been infected with *Ranavirus*. Restriction enzyme analysis of whole genomes of the turtle and frog isolates showed identical restriction patterns, suggesting they were infected with the same virus; thus, frogs might represent the disease vector for our outbreak. However, the turtle serology showed that only one individual (an aged female) had antibodies against the virus (Johnson et al. 2010). Our interpretation of their turtle-serology findings is that this virus kills so quickly that there is too little time for most victims to launch an effective immune response, but the authors point out other possible explanations for the serology findings, such as short-lived antibody production by sensitized leukocytes or a slowly developing immune response that might take many months (Johnson et al. 2010).

Mode of Transmission

The events surrounding one case, in the first months of the disease in our habitat, suggested to us that the pathogen might be transmitted by turtle-to-turtle contact. Before 4 September 2003, all deaths from the virus had occurred on the steep southern slope above the French Creek (see Fig. 1); none had yet occurred on the highland plateau to the north. A large male, whose predominant habitat-use had been up on the plateau, departed his usual haunts at the end of August on a relatively long excursion (>400 m) that took him down onto the steep slope where turtles had already died. We observed the male, on one of the days of that excursion, walking near one of our juvenile females who lived on the slope (who, at the time, appeared healthy, but died of the virus not long after, on 4 September 2003). By 3 September, before the female was known to have been infected, the male was off of the slope and back up onto the plateau; by 4 September, he was back in his usual habitation still further to the north. A photo of him during his trek back home from the southern slope is available online (retrieved 15 August 2009 from http://ebtct.org/node/142). He appeared to be in good vigor for the next few weeks, but we found him in a moribund state, on the edge of a swamp, on 25 September 2003. He died in our infirmary the night of 27 September from what proved to be a *Ranavirus* infection. The coincidence of his unusual excursion to a region of the sanctuary where all previous virus infections had occurred, and his proximity during that excursion to a female who was to later die of the virus, caused us to suspect that the male had made contact with the infected female and thereby contracted the virus.

Another circumstance that contributed to our (initial) suspicion that the virus was being transmitted by turtle-to-turtle contact involved a group of our turtles (adults as well as head-started juveniles) who were confined by fencing (described in Belzer and Seibert 2009a) at the sanctuary during the entire four-year course of this viral event. They were part of our ongoing study of subsequent habitat use by hard- versus soft-release turtles. Although free-roaming adults and juveniles were dying during August and September 2003, none of our confined turtles living in a 3-acre pen on the upland plateau had (yet) contracted the disease. It seemed reasonable that, if turtle-to-turtle contact was the means of transmission, we had the explanation for why none of the turtles living inside that pen had died. Therefore, we extended their confinement indefinitely, in hopes that their isolation from the at-large population would protect them. But by 1 October 2003, turtles inside the pen began to die.

Although some confined turtles contracted, and were killed by, the virus during the 2003 and subsequent seasons, others of the turtles confined with them survived in good health through all 4 years of the epidemic. This inconsistency of contracting the disease, among individuals living together inside a pen, seems to contradict our initial impression and, instead, suggests that *Ranavirus* might not easily be transmitted from turtle to turtle by proximity or contact. Further support for that notion may be that this study population is a low-density assemblage and turtle-to-turtle contact among the widely scattered, at-large segment of the population would be rather infrequent or, for some of those individuals, nonexistent; however, deaths from the virus were numerous, and a few of the afflicted turtles lived in areas where no other members of the population were known to have ventured. Survival of turtles living in an enclosure among ones that died might also suggest that the virus (at least in diluted doses) is not typically contracted from drinking water or dew on vegetation. The population's 2004 immunological profile provided to us (Johnson et al. 2010) suggests that survivors were not being protected by an active immunity against the pathogen that was killing conspecifics; rather, they evidently just had failed to contract the virus.

Amphibians are known carriers of *Ranavirus*, and the virus was identified in dead frogs from our habitat. This study site experienced record-setting rainfall during summer 2003. Elliot Jacobson (University of Florida College of Veterinary Medicine, *pers. comm.*, 29 October 2003) noted that exceptionally wet summers, particularly after several summers of drought (exactly the climatic circumstance in our habitat in 2003), might generate unusually robust amphibian populations. The increase in temporary pools in the habitat might have allowed them to spread more extensively than otherwise. Because necropsies routinely found extensive necrosis in the alimentary tract, ingesting living or dead amphibians harboring the virus seemed to us to be the probable means of transmission to our turtles. It seemed reasonable, given local climatic conditions, to suspect that

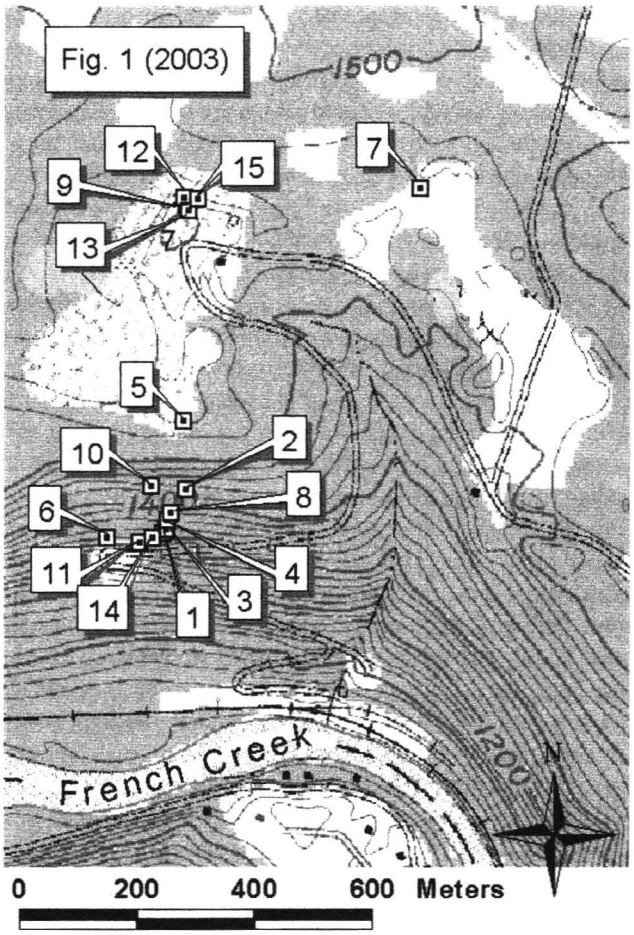

Figure 1. Chronologically numbered locations where *Ranavirus* victims were found in a moribund or recently deceased state during 2003: (1) 24 August; (2) 27 August; (3) 1 September; (4) 3 September; (5) 4 September; (6) 17 September; (7) 25 September; (8) 28 September; (9) 1 October; (10) 2 October; (11) 7 October; (12) 9 October; (13) 11 November; (14) 13 November; (15) 18 November.

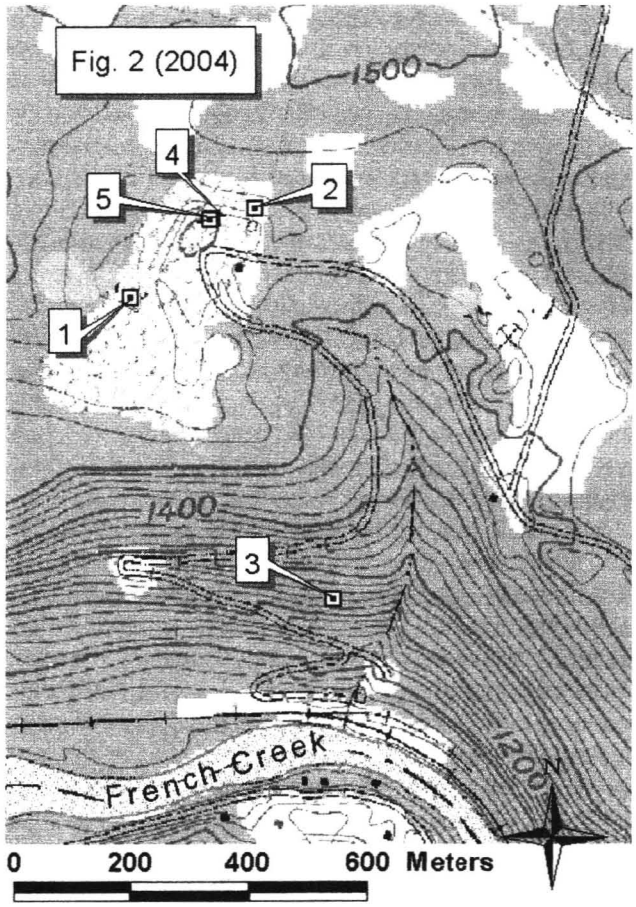

Figure 2. Chronologically numbered locations where *Ranavirus* victims were found in a moribund or recently deceased state during 2004: (1) 28 July; (2) 18 August; (3) 20 August; (4) 2 October; (5) 2 October.

an amphibian population increase in 2003 had augmented that food class for our turtles.

The finding of 75% of moribund or dead turtles at the edge of water would fit the amphibian-vector hypothesis. The slight predominance of juvenile over adult deaths might reflect the notion that juveniles are more carnivorous than adults and, therefore, would eat more of the living or dead amphibians available to them. It might also mean that juveniles are slightly more susceptible to the virus than are adults.

Examination of the pattern of death that spread through the habitat (Figs. 1–4) reveals that *Ranavirus* deaths began on the habitat's southern slope above the French Creek (Fig. 1) but reached the more northerly regions (further from the French Creek) only later in the 2003 season (Fig. 1). The southern limit of death in Fig. 1 also happens to be the closest that our box turtles got to the French Creek that year. The maps of ensuing yearly deaths (Figs. 2–4) show that in each successive year death events appeared in ever more northerly latitudes of the habitat, farther and farther from where the epidemic began. The very last death (2006) in this outbreak was the northernmost (farthest from the creek) case (Fig. 4). That pattern may suggest that the virus moved from the French Creek and spread northward up the steep slopes and then across the northern uplands of our study site. Perhaps infected amphibians living in the creek were able to more successfully infiltrate the upland habitat of the sanctuary because of the exceptional abundance in rain and temporary pools in 2003. The French Creek seemed to be an unlikely source of disease because it is known for its high water quality and species diversity (Pennsylvania Environmental Council 2001). However, the creek is a popular fishing stream, and infected fishing baits have been implicated in the spread of Iridovirus to new amphibian populations in the western United States (Hathaway 2004); therefore, species in the French Creek might have harbored the virus. The repeated annual pattern of no turtle deaths starting until the latter half of each season could fit a hypothesis in which juvenile amphibians, not metamorphosing and able to easily move overland until later in the season, might contract

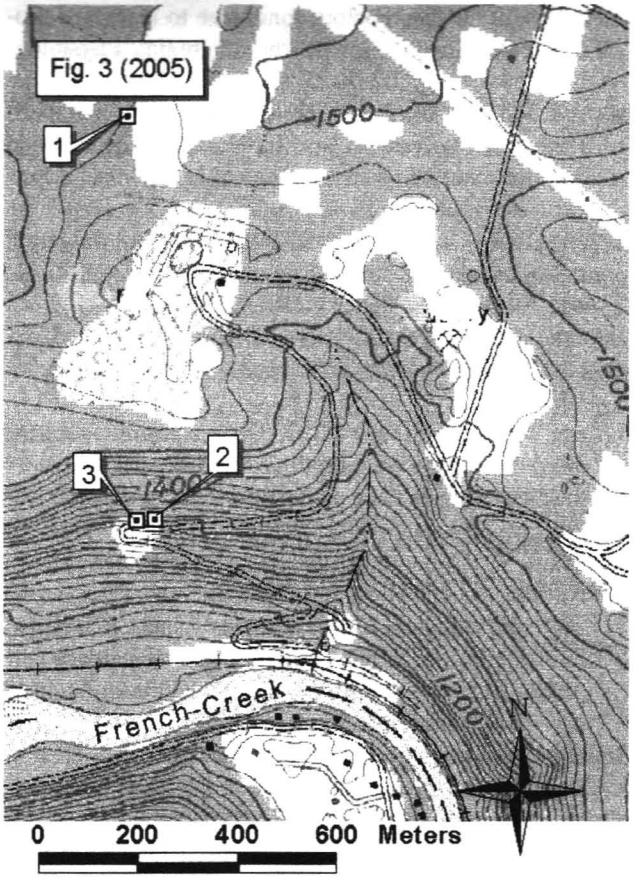

Figure 3. Chronologically numbered locations where *Ranavirus* victims were found in a moribund or recently deceased state during 2005: (1) 23 July; (2) 30 August; (3) 4 October.

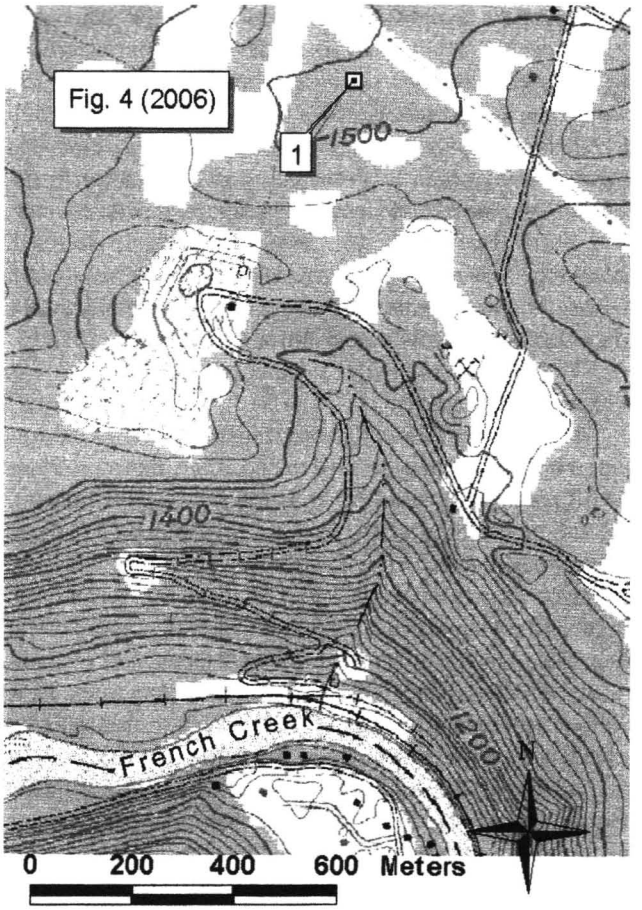

Figure 4. Location where we found the 2006 season's lone *Ranavirus* victim (recently deceased on 10 July).

the virus and disperse as an infected food source that became widely accessible to terrestrial turtles only in the later months of each season.

Although an array of our observations fit a hypothesis in which ingesting infected amphibians may have transmitted the virus to our turtles, laboratory support for the hypothesis is lacking (Johnson et al. 2007). Intramuscular injection of the virus produced the systemic disease seen in the wild, but virus introduced into the caudal esophagus (by a metal gavage feeding tube through an oral route) did not (Johnson et al. 2007). April Johnson (University of Florida College of Veterinary Medicine, *pers. comm.*, 6 September 2005 [School of Veterinary Medicine, Purdue University]) speculated that abrasion in the GI mucosa by bone fragments of an ingested amphibian might provide a route for viral entry not duplicated when the virus reaches the GI tract by oral gavage. If so, the hypothesis for turtles contracting the virus through an alimentary route might still stand.

Hypothesizing a mosquito intermediary (A. Johnson, University of Florida College of Veterinary Medicine, *pers. comm.*, 6 September 2005 [School of Veterinary Medicine, Purdue University]) could integrate an amphibian role in virus transmission to turtles with the laboratory finding that IM injection of the virus readily produces the disease. If infected amphibians increasingly spread through a habitat, and female mosquitoes obtain the virus from them, the mosquitoes might hypodermically introduce the virus into turtles during a subsequent blood meal. Extreme habitat wetness in 2003 and finding dead turtles predominantly near puddles, ponds, or small seeps would be in line with a hypothesized mosquito vector. However, the years following 2003 were relatively dry.

The owner of the sanctuary where this outbreak occurred reminded us that, in addition to the record breaking rainfall of summer 2003, a microburst on 21 July (a month before *Ranvirus* deaths first appeared) had uprooted numerous trees, dislodging much soil and producing heavy siltation on the southern slope above the French Creek. Perhaps unknown agents leaching out of the disturbed soil could have played some role in the emergence of the virus in 2003. Soil disruption might also change amphibian habitat use, which might contribute to the spread of the virus (A. Johnson, School of Veterinary Medicine, Purdue University, *pers. comm.*, 28 August 2008). We do not know whether similar soil disruption and heavy silt run-off accompanied the 1991 *Ranavirus*-associated mass mortality event in the Georgia box turtle population noted in Johnson et al. (2008).

Despite the insights provided to us by the laboratory studies of this newly recognized chelonian disease, we do not know yet how the virus entered and spread through our population nor do we know why the turtle death toll steadily declined and finally ended. Belzer (2008a) speculated that perhaps surviving turtles were genetically protected by virally incompatible cell receptors or, perhaps, that vector populations, or the viral abundance in them, steadily declined. Given the long-term monitoring planned for this population, we may be able to gain further insight into the natural history of the disease should it ever return to this habitat.

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We cannot overstate our gratitude for the generous and critical technical expertise devoted to unraveling this mysterious die-off among our turtles by April Johnson, April Childress, and Elliott Jacobson of the University of Florida's College of Veterinary Medicine; by Robert Wagner of the University of Pittsburgh School of Medicine; by Jason Brooks and Dan Shaw of the Pennsylvania Animal Diagnostic Laboratory at Penn State University; and by Robert Poppenga of the University of Pennsylvania's Toxicology Laboratory at the New Bolton Center. We are grateful for the special scientific permit issued in 1993 by the Pennsylvania Fish and Boat Commission, which makes our long-term fieldwork possible. Equally critical has been the kind permission granted by the owners of the private nature sanctuary in Venango County to carry on this and other important long-term studies in that habitat. Volunteer labor for tracking turtles and facilitating veterinary procedures during the serological sampling project in May 2003 was generously given by Ben Atkinson, Marci Ertola, Kathie Goodblood, Chris Hammond, Jude Holdsworth, and Elizabeth Wilson. April Johnson kindly drew our attention to errors in an early draft of our manuscript and suggested other improvements. Early stages of the epidemic's study were facilitated by a donation from the Audubon Society's Bartramian Chapter and by a Fall 2003 release-time stipend granted to WRB by Clarion University of Pennsylvania.

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