# INVESTIGATING THE EPIDEMIOLOGY OF ADENOVIRUSES IN FREE-RANGING TURTLES IN ILLINOIS

BY

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THESIS

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## ABSTRACT

Adenoviruses (AdV) were first reported as a disease threat in chelonians during a mortality event in 2007 caused by a *Siadenovirus* later named Sulawesi tortoise adenovirus (STADV). Adenoviruses of another lineage, *Testadenovirus*, have been detected in multiple managed and free-ranging turtles in North America and Europe with variable or unknown relationships to clinical disease. This leaves a large gap in understanding the prevalence and impact of AdV in many turtle species. Therefore, a multi-species investigation to detect novel or existing adenoviruses in Blanding's turtles (*Emydoidea blandingii*), painted turtles (*Chrysemys picta*) and red-eared sliders (*Trachemys scripta elegans*) using conventional PCR across four counties in Illinois from 2016-2022 was performed. Ten AdV were identified across the three species, with STADV being the most common virus species detected. Subsequently, a highly sensitive and specific TaqMan quantitative PCR assay targeting the DNA polymerase gene of STADV was developed. The newly developed and validated qPCR assay was used to test oral-cloacal samples from Blanding's turtles, painted turtles and red-eared sliders sampled across three Illinois counties from 2017-2022. The prevalence of STADV was 2.4% for Blanding's turtle samples (n=14), 14.9% for painted turtle samples (n=24), and 45.1% for red-eared slider samples (n=37). Clinical signs associated with STADV detection included quiet, alert, responsive (QAR) mentation ($p=0.002$), pink mucous membranes ($p<0.001$), carapacial abnormalities ($p=0.036$), and plastron abnormalities ($p=0.003$). These results provide a baseline for the presence and diversity of AdV in free-ranging turtles in Illinois, including evidence that freshwater emydid turtles, such as red-eared sliders or painted turtles, may be the North American reservoir for STADV.

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# CHAPTER 1: INTRODUCTION

Global climate change has been associated with surges in disease outbreaks among wildlife (Cohen et al. 2020; Jones et al. 2008; Liang & Gong 2017). Reptiles and amphibians have been described as especially vulnerable, given that their poikilothermic physiology is intimately tied to ambient temperature and other environmental parameters (Gibbons et al. 2000; Hannah et al. 2002). The thermal mismatch hypothesis states that disease hosts adapted to cooler and warmer climates should be at greatest infection risk under abnormally warm and cool conditions (Cohen et al. 2020). Projections based on climate change models indicate ectothermic wildlife hosts from temperate zones may experience sharp increases in disease risk with climate change, based on the thermal mismatch hypothesis (Cohen et al. 2020).

Beyond climate, other anthropogenic impacts, such as habitat loss, degradation and fragmentation, introduction of invasive species, and the illegal pet trade have threatened the long-term persistence of reptile populations, including chelonians (Stanford et al. 2020). More than half of all turtle species are officially regarded as globally Threatened (Critically Endangered, Endangered, or Vulnerable) by the IUCN (Rhodin et al. 2018). Infectious diseases are a major cause of morbidity and mortality in chelonians, with key pathogens including *Mycoplasma* spp, ranaviruses, and herpesviruses (Allender et al. 2006; Brown et al. 1994; Brown et al. 1999; Brown et al. 2002; Jacobson et al. 2014; Johnson et al. 2007). Mass mortality events in chelonians have been associated with frog virus 3, and other iridoviruses in the *Ranavirus* genus have caused significant morbidity and mortality in fish, amphibians, and chelonians (Ariel et al. 2015; Cheng et al. 2014; Cullen & Owens 2002; Langdon et al. 1988). Herpesviruses are widespread across chelonian species and been associated with severe upper respiratory disease

resulting in significant morbidity (Gandar et al. 2015; Jacobson 2007; Jones et al. 2016; Sim et al. 2015; Teifke et al. 2000; Une et al. 2000). Infectious disease represents a conservation concern for chelonians and has contributed to the decline of populations in North America. In the 1980s, major declines in desert tortoises in the Mojave Desert of California, USA, were associated with mycoplasmosis (Jacobsen et al. 2014). Furthermore, aclinical to mildly clinical mycoplasmosis is widespread in desert tortoises and anthropogenic impacts may cause sufficient physiological stress to trigger outbreaks of clinical disease (Jacobson et al. 2014). Western pond turtles (*Actinemys marmorata*; WPT) have experienced population declines and the emergence of *Emydomyces testavorans* poses a previously unaccounted conservation concern for imperiled WPT alongside other threats from human activity (Lambert et al. 2021).

In the last two decades, adenoviruses of multiple lineages have been detected in chelonian hosts (Doszpoly et al. 2013; Farkas & Gal 2009; Franzen-Klein et al. 2020; Rivera et al. 2009; Salzmann et al. 2021; Schumacher et al. 2012). Increased attention to adenoviruses in chelonians was garnered in 2009, when a multispecies mortality event involving over 100 Sulawesi tortoises (*Indotestudo forstenii*), Impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*) was attributed to a novel adenovirus, Sulawesi tortoise adenovirus (STADV), in North America (Rivera et al. 2009; Schumacher et al. 2012). Concurrently, novel adenoviruses of other lineages were being detected in terrestrial and freshwater turtles in North America and Europe, with unknown relationships to clinical disease (Doszpoly et al. 2013; Farkas & Gal 2009; Franzen-Klein et al. 2020; Rivera et al. 2009; Salzmann et al. 2021; Schumacher et al. 2012). There remains a persistent knowledge gap about which infectious diseases circulate in populations of free-ranging chelonians and their clinical implications.

Blanding's turtles (*Emydoidea blandingii*) are state-endangered semiaquatic freshwater turtles that share wetland ecosystems with painted turtles (*Chrysemys picta*) and red-eared sliders (*Trachemys scripta elegans*), both of which are more abundant in the state of Illinois and across their range (Ernst 2009). There are few studies that have reported pathogen screening in Blanding's turtles, including one that identified a novel herpesvirus (*Emydoidea* herpesvirus 1 [EHBV1]) in northern Illinois (Allender et al. 2009; Lindemann et al. 2018; Lindemann et al. 2019; Winter et al. 2020). Disease surveillance of free-living painted turtles (*Chrysemys picta*) and red-eared sliders (*Trachemys scripta elegans*) in North America is even more sparse; however, Sulawesi tortoise adenovirus has been detected in apparently healthy free-living painted turtles in Indiana (Vincent et al. 2023). Detecting novel and historic infectious organisms in novel host species has important conservation implications, including potential risk for cross species transmission and resultant concerns for translocation and captive-rearing (head-starting) programs for endangered species, such as Blanding's turtles.

Blanding's turtle conservation work through habitat management, mesopredator control, nest protection and headstarting programs has curbed the likelihood of local extinction of the species in one county in Illinois (Glowacki 2018). Existing pathogen surveillance efforts in Blanding's turtles have also focused on populations in Illinois, however, adenovirus testing has not been reported in this species and the prevalence and epidemiology of adenoviral infections in North American turtles overall remains poorly understood (Doszpoly et al. 2013; Glowacki 2018; Farkas et al. 2009; Franzen-Klein et al. 2020; Salzmann et al. 2021). Elucidating the impact of AdV and other infectious agents on Blanding's turtles would enable biologists and conservation leaders to understand how to mitigate impacts of infectious disease, guide management decisions such as where to release headstart turtles to maximize survival, determine

whether some populations are naïve to disease and need to be protected, and resolve whether human impacts contribute to immunosuppression and resultant outbreaks of disease that impact survival. The three species examined in this thesis represent a broad spectrum of conservation status. Blanding's turtles are endangered and are a species of conservation concern, while painted turtles are abundant and may serve as a sentinel species for their less common counterparts. Finally, red-eared sliders are so abundant that they are considered invasive and nuisance animals in many parts of their current range.

This thesis aims to address key gaps in the taxonomy, diagnostic testing, and epidemiology of adenoviruses in Blanding's turtles, painted turtles, and red-eared sliders in Illinois, USA, with implications for chelonian species on a global scale. The following objectives will be presented:

1. Describe the detection of novel and existing adenoviruses using consensus PCR in Blanding's turtles, painted turtles and red-eared sliders in Illinois, USA.
2. Develop and validate a quantitative TaqMan PCR for detection of Sulawesi tortoise adenovirus.
3. Describe the prevalence and epidemiology of Sulawesi tortoise adenovirus in free-ranging Blanding's turtles, painted turtles and red-eared sliders in Illinois, USA.

# CHAPTER 2: LITERATURE REVIEW

## *Blanding's Turtle Natural History*

The Blanding's turtle (*Emydoidea blandingii*) is an endangered, semiaquatic chelonian in the northeastern United States and the Great Lakes region (Ernst 2009). Identifiable by the yellow mandible, throat, and dark, domed shell, Blanding's turtles are medium-sized chelonians with males slightly larger than females (Henning & Hinz 2016). Their geographic distribution extends to west-central Nebraska in the west, central Illinois in the south, and eastern Ontario in the northeast; a few sporadic populations can be found in New England and Nova Scotia (Henning & Hinz 2016). In Illinois, Blanding's turtles frequent the northern part of the state; however, there is evidence of sporadic populations along the Illinois River valley down to Cass County and along the eastern part of the state (Henning & Hinz 2016). The Blanding's turtle is an opportunistic omnivore and will consume crustaceans, insects, snails, leeches, earthworms, small fish and amphibians, and plant material (van Dijk & Rhodin 2011). Natural predators of Blanding's turtles include raccoons, skunks, opossums, foxes, mink and coyotes, with raccoons having a major impact on nesting success (Urbanek et al. 2016).

Blanding's turtles are declining range-wide secondary to habitat destruction and fragmentation, predation, and vehicular mortality. Therefore, they are classified as endangered by the IUCN Red List of Threatened Species (van Dijk 2011). They are estimated to inhabit just 22% of their historic range in Illinois (King 2013). Based on mark-recapture surveys at five separate sites in Illinois, historic estimates of population sizes range from 25 to 135 adult individuals (King 2013; Rubin 2000; Rubin et al. 2001; Dreslik et al. 2011; Kuhns et al. 2006; Glowacki 2015). Without intervention, even the largest populations are expected to become

extirpated in the next 50 years; however, interventions beginning in 2010 have resulted in minimal likelihood of extirpation at certain sites (Kuhns 2006; Henning et al. 2016; Glowacki et al. 2018; Thompson et al. 2020).

Blanding's turtles frequent wetland habitats; however, they will make use of upland habitats, especially for nesting (Henning et al. 2016). Despite their site fidelity and tendency to return to the same areas annually, they differ from other aquatic chelonians because they may make long distance movements, sometimes moving more than 0.6 miles daily (Henning & Hinz 2016). One radiotelemetry study found daily movements between activity centers ranged from 1-230 m per day (Rowe & Moll 1991). Home range sizes are highly variable from 54 acres to more than 200 acres. The home ranges of Blanding's turtles broadly overlap, with as many as 23 adults per acre; however, that is likely an anomaly (Henning & Hinz 2016). Three populations in northeast Illinois had 0.3, 0.1, and 0.4 turtles per acre (Rubin et al. 2004). While preferred wetland habitat types are shallow with soft organic substrates, such as marshes, ponds, and shrub swamps, there is regional variation observed and turtles have been found using all wetland types (including lakes and rivers) within their home ranges (Ernst 2009; King 2013). On average, individual turtles used 6.5 different wetland sites per year, and up to 20 different sites annually in Maine (Beaudry et al. 2008).

The active season for Blanding's turtles generally falls between March and October. They have a polygamous mating strategy with multiple paternities being common (11-56%) along with repeated paternities (70-83%) (Refsnider 2009). Not only do females mate with multiple males, but they will store sperm for several years (Gist 2002). Nesting in Illinois occurs between late May and early July; site selection becomes important when considering that eggs developing at temperatures below 25.6ºC become male, while those above 30ºC become female

(Ewert & Nelson 1991). Females produce a maximum of one clutch per year and typically lay 10-14 eggs per clutch; females reproduce in 33-80% of years (Congdon et al. 1993; Congdon et al. 2000). Nests within residential landscapes have higher mortality rates than those in nonresidential areas, perhaps because residential areas support greater densities of predators such as domestic dogs and raccoons (Henning & Hinz 2016). Hatchling mortalities in Massachusetts are most commonly from eastern chipmunks (*Tamias striatus*), followed by cars, birds and domestic horses (*Equus caballus*) (Jones and Sievert 2012). Nest predation can be high at 15-100%, so nests protected with wire mesh tend to have much higher hatching rates (78%; Congdon et al. 1993; Congdon et al. 2000). Upon emergence, hatchlings will visually gravitate towards dark horizons, such as wooded areas, and utilize temporary wetland spaces before selecting permanent wetlands (Henning & Hinz 2016). Estimated survival to one year of age is variable but low (7-26%; Congdon 1993; Glowacki 2015; Golba et al. 2022; Kuhns 2010). Blanding's turtles typically overwinter partially buried in wetlands. High fidelity to nesting and overwintering sites has been documented (Henning & Hinz 2016). Their life history strategy, which includes delayed time to sexual maturity (14-20 years), longevity (expected >70 years), and low reproductive output, makes them vulnerable to extinction (Henning & Hinz 2016). Unfortunately, the major characteristics of their natural history also make Blanding's turtles, like other chelonians, prone to significant population declines as anthropogenic activities abound. In 200 years, 85% of wetland spaces were lost in Illinois, and from 1900 to 2000 there was a 325% increase in human population (King 2013). While wetland habitats are protected, it is challenging to protect the full extent of nesting areas and travel corridors used by individual turtles, since they frequently cross roads and railways to access adjacent wetlands and nesting areas (Henning & Hinz 2016). Vehicular mortality remains the most significant cause of adult

mortality, responsible for the loss of 11% of one northeast Illinois population from 2002 to 2006 in one report (Banning 2006). Habitat fragmentation, habitat degradation, agricultural activities, storm-water management, urbanization, garbage dumping, and forest succession threaten this species' survival (Henning & Hinz 2016). The small population sizes on a local level could also result in inbreeding depression, manifesting as nonviable eggs (up to 48% in one Illinois population) to declining genetic diversity (Sethuraman 2014).

The threat that infectious diseases may pose for Blanding's turtle populations has yet to be fully determined and infectious disease surveillance is ongoing in this species. In multiple other turtle species, population recovery has been hindered by disease and predation, even when active headstarting programs were in place (Mullin et al. 2020). Novel pathogens have been detected in Blanding's turtles from disease investigations, including *Emydoidea* herpesvirus 1 (EBHV1) and *Emydoidea* herpesvirus 2 (EHBV2) (Andersson et al. 2021; Lindemann et al. 2019). From 2017-2018, oral-cloacal swabs assayed from Blanding's turtles in Lake County, Illinois did not reveal any ranaviruses or tortoise intranuclear coccidiosis (TINC). Turtles in this study had a novel herpesvirus (EBHV1) detected, with detections occurring most commonly in May and not associated with clinical signs (Winter et al. 2020). *Salmonella tymphimurium* was rarely detected, and one turtle with nasal discharge had a novel *Mycoplasma* spp. (Winter et al. 2020). Other work includes seroprevalence studies for *Leptospira* sp. and evaluation of shedding of *Leptospira* sp. in urine (Grimm et al. 2015; Rockwell et al. 2019), establishing normal hematologic and biochemical reference parameters in free-living Blanding's turtle populations (Mumm et al. 2019), plasma electrophoretic profiles and relationship with demographic variables (Andersson et al. 2021), and using computed tomography to measure fat content and establish a body condition index in free-ranging Blanding's turtles (Newman et al 2019).

Conservation efforts for the Blanding's turtle in Illinois include land protection, mesopredator control, on-site protection of nests, off-site hatching of eggs, and headstarting programs (Crawford et al. 2018; Henning & Hinz 2016; King et al. 2021; Thompson et al. 2020; Urbanek et al. 2016). Blanding's turtle habitat is encased within many of the nature preserves established by the Illinois Nature Preserve Commission (Illinois Department of Natural Resources 2015). On-site protection of nests from predation through wire mesh cages after egg deposition has improved hatching rates to 67%, compared to 23% from unprotected nests (Kuhns 2010; Glowacki 2015). Likewise, incubating eggs off-site has improved hatching rates to 78%. Several forest preserve or conservation districts in Illinois, including those in Lake, DuPage, Kane, and McHenry Counties have worked to increase juvenile survival through "head-starting" where eggs are collected, incubated, and hatchlings are released to improve juvenile survival (Kuhns 2010). Control of predators in Lake County through trapping has also aided hatching success (Glowacki 2015; Urbanek et al. 2016). The Lake County Forest Preserve District (LCFPD) has been monitoring Blanding's turtles in response to population declines since 2004 (Glowacki 2018). In 2010, the population was projected to have a 95% chance of going extinct in the next 50 years without intervention, so the LCFPD created the Blanding's Turtle Recovery Program (BTRP) to address threats to the population. A comprehensive population health assessment program was added to the BTRP in 2016 to characterize health of the population and identify disease threats. The LCFPD's BTRP has successfully increased juvenile survivorship, recruitment and the overall population size at SBCP (Spring Bluff Chiwaukee Prairie), making extirpation at this site unlikely (Glowacki 2018). Therefore, these long-lived turtles may be amenable to population support through conservation activities. However, their life history

strategy suggests that the benefits of recovery efforts may not be reflected in the population for several decades.

*Painted Turtle Natural History*

Painted turtles (*Chrysemys picta*) are medium-sized, abundant, aquatic turtles native to North America, ranging from southern Canada to northern Mexico (Canadian Forest Service 2005; Green et al. 1987). They are widespread in Illinois, favoring freshwater, shoreline habitats. They are brightly marked, with conspicuous red and yellow markings on a dark black to greenish background, and a relatively flat shell (Ernst 2009). Females are distinguishable from males by their larger adult size, shorter claws, and position of the cloaca, which does not extend beyond the carapace (Green et al. 1987). Painted turtles do not have a special conservation status with the IUCN and are not state or federally listed, however they do co-habitate with Blanding's turtles and serve as a resilient species for comparison of pathogen occurrence and impacts.

The habitats that painted turtles select include lakes, ponds, and slow-moving bodies of water with soft, muddy substrates where they can overwinter (Spalding 2020). However, they can also be found along rivers, marshes and seasonal wetlands. Home range sizes range from 10-29 acres (Spalding 2020). Juvenile dispersal appears to be low compared to adult movement between bodies of water. Terrestrial movement patterns are predominantly observed during the mating season, and most individuals traveling >400 m are mature females (Frazer et al. 1991). Overland travel appeared to be most common in the springtime for both sexes; however, males and juveniles are often found in the same location in successive years, compared to females (Frazer et al. 1991).

The active season occurs from March to November, with nesting season in May and June (Green & Pauley 1987). Sexual maturity is achieved at 3-5 years for males, and 6-10 years for females (Green & Pauley 1987). Painted turtles demonstrate a polygynous mating strategy, and nesting sites are often selected in sandy soil several hundred yards from the water's edge (Green & Pauley 1987). The mean clutch size is about 6.5 eggs and they may lay more than once in a single breeding season, with annual reproductive potential for an individual being around 13 eggs (Gibbons 1968). The growth rate is rapid for the first several years after hatching (Frazer et al. 1991). Adult painted turtles are omnivorous, while hatchlings and juveniles are more carnivorous. Predators of painted turtles include raccoons (*Procyon lotor*), otters (*Lontra canadensis*), mink (*Neovision vison*), and red foxes (*Vulpes vulpes*) (Canadian Forest Service, 2005).

Painted turtles' less specialized habitat selection, more flexible home range size, and greater fecundity compared to Blanding's turtles may make them more resilient to anthropogenic impacts. Despite this, habitat fragmentation and degradation and road mortality remain important threats to their survival. Baseline data for hematology and biochemistry profiles have been established for painted turtles (Carpenter 2018; Gibbons et al. 2019). A few studies have included painted turtles in pathogen screening; however, disease surveillance focusing on painted turtles has been limited. A health assessment of 40 free-ranging painted turtles at Kankakee Sands, Indiana identified Emydid herpesvirus 1 and Sulawesi tortoise adenovirus, but no *Mycoplasmosis* spp. or frog virus 3 were detected (Vincent et al. 2023). The identification of Sulawesi tortoise adenovirus 1 in these free-living turtles that share habitat with Blanding's turtles represents a significant disease concern, especially given that the impact of infection with STADV in these turtles is unknown. The implications of infectious disease in free-living painted

turtles remains largely unknown, representing a significant knowledge gap necessary to plan appropriate conservation strategies for this species.

*Red-eared Slider Natural History*

Red-eared sliders (*Trachemys scripta elegans*; RES) are a subspecies of pond slider native to the central United States, ranging from West Virginia through Texas to eastern New Mexico, and are considered the most ubiquitous and abundant turtle globally (Somma et al. 2019). They are medium-sized turtles distinguished by the bright red stripe behind the eye; however, older adults may lose this coloration and become melanistic. Beyond their endemic distribution, including Illinois, they are farmed commercially and maintained under human care as the most common pet turtle species (Global Invasive Species Database 2019; Somma et al. 2019). Through these activities and inadvertent escapes, they have colonized every continent except Antarctica, outcompeting other native freshwater turtles for food and basking sites (Cadi & Joly 2004; Polo-Cavia et al. 2010). In locations outside their original range, RES are considered a nuisance species because they act as competitors and vectors of disease for other native, threatened species (Somma et al. 2019). Furthermore, there are documented hybrids of introduced RES with other taxa of *Trachemys*, threatening the genetic integrity of more threatened species within this genus (Forstner et al. 2014; Parham et al. 2013; Seidel et al. 1999).

Red-eared sliders will inhabit most permanent bodies of water, especially those with soft, organic substrate bottoms, rich vegetation, and basking sites (Somma et al. 2019). In California, RES basking sites are related to sites with more human activity, steel mesh basking substrates, deeper water and shallower slopes compared to native western pond turtles (*Emys marmorata*; Lambert et al. 2019). While they prefer quiet waters, the extensive artificial canals and irrigation

ditches have allowed them to colonize habitats in California rapidly (Somma et al. 2019). In the Great Lakes, the RES is reproducing and overwintering in the basins of Lake Michigan, Lake Erie and Lake Ontario and overwintering success has been seen as far north as southern Ontario (Somma et al. 2019). The nesting season occurs from mid-May into early July in Illinois, and females may lay 5-18 eggs per clutch, with 2-3 clutches laid annually (Somma et al. 2019). Akin to most freshwater turtles, RES are dietary generalists as adults, progressing away from a primarily carnivorous diet as juveniles (Somma et al. 2019).

Given red-eared sliders are listed as one of the world's worst 100 invasive species (GISD 2019), their conservation is not a major concern. However, their shared use of wetland habitat, food items and other resources with Blanding's turtles in Illinois makes them a potential biosentinel species for infectious disease. Red-eared sliders are regarded as carrying a variety of pathogens; however, focused research on pathogens found in this species is sparse in the literature. Nesting areas of *Trachemys scripta* in the Mediterranean freshwater marshes can act as chronic reservoirs for fungal pathogens of sea turtles, namely *Fusarium* spp., and can act as potential vectors for the spread of *Fusarium* spp. among wild native species and even to humans (Rios et al. 2022). Red-eared sliders in the pet trade have been implicated in the spread of salmonellosis to humans (Lamm et al. 1972). Aplasca et al. (2019) included red-eared sliders in a health assessment evaluating turtles in a segment of the Bronx River from May-July 2012, and detected a *Mycoplasma* sp. and evidence for accumulation of various contaminants.

### *Adenovirus Taxonomy, Diversity and Molecular Characteristics*

Adenoviruses (AdV) occur in every major taxonomic class of vertebrates and are widespread (Benkő et al. 2003). The AdV are a diverse group, with over 50 serotypes classified

into seven species identified in humans (Benkő et al. 2003). Within the family Adenoviridae there are currently six accepted genera: *Mastadenovirus* members occur in mammals, *Aviadenovirus* species are found in birds, *Testadenovirus* species infect turtles, *Ichtadenovirus* includes a single virus recovered from a white sturgeon (*Acipenser transmontanus*), *Atadenoviruses* are believed to have cospeciated with squamate reptiles, but have also been detected in chelonians, birds, frogs, opossums, ruminant mammals, and tortoises, and *Siadenovirus* members occur in amphibians, chelonians, and birds (Benkő et al. 2022; Gibbons & Steffes 2013; Latney & Wellehan 2020; Marschang 2011). Phylogenetic analyses of AdV indicate that many elements in the branching patterns of these viruses are congruent with those of corresponding host species (Benko & Harrach 2003). Atadenoviruses demonstrated a large phylogenetic distance from avian and mammalian AdV, leading to the hypothesis that they had coevolved with reptilian hosts (Benkő et al. 2003). The subsequent discovery of many novel atadenoviruses in squamate hosts was enabled by developing a nested PCR with consensus primers, targeting the DNA polymerase gene (Wellehan et al., 2004). Siadenovirus originally contained only a frog virus (FrAdV-1) and turkey adenovirus 3, the latter of which is well recognized for its unusually broad host spectrum and pathogenicity (Beach et al. 2009; Davison et al. 2000). Siadenoviruses were later discovered in several bird species and Sulawesi tortoises, challenging the hypothesis that they had evolved with amphibian hosts. *Testadenovirus* is a relatively newly characterized genus, to be discussed further. Current taxonomy in the family *Adenoviridae* suggests a coevolutionary lineage of the virus with their hosts, with occasional host switches (Marschang 2011), serotypes of the Mastadenovirus C species, namely serotypes 2 and 5, are the best characterized regarding their molecular biology (Charman et al. 2019).

*Adenoviridae* are non-enveloped double-stranded DNA viruses with an icosahedral nucleocapside containing a medium-sized genome of 26-48 kilobase pair (kbp; Benko & Harrach 2003). Viral particles range in size from 80 to 110 nm. Within the genome, genes conserved across this family are often centrally located and encode proteins with functions involved in DNA replication. Genes are unique to specific genera of AdV, are typically located near the ends of the genome, and encode proteins such as minor coat polypeptide IX and core polypeptide V unique to mastadenoviruses, and LH3 and p32K proteins unique to aviadenoviruses (Gorman et al. 2005; Pantelic et al. 2008). The viral replication machinery comprises the preterminal protein (pTP), DNA polymerase, and the DNA-binding protein, encoded by early viral genes. Late genes including the major late transcriptional unit (MLTU), are expressed at the onset of viral replication and encode the capsid and packaging proteins needed for assembly of viral particles (Babich et al. 1980; Fraser et al. 1979; Zhao et al. 2014).

The AdV fiber protein is a main determinant of viral tropism (Penzes et al. 2014). In most AdV sequences thus far, 1-2 fiber genes and one-two fibers per penton are observed (Chiocca et al. 1996; Hess et al. 1995; Kajan et al. 2012; Marek et al. 2012). Structures formed by these fiber proteins form domains that attach to receptors at the host cell membrane, resulting in internalization via receptor-mediated endocytosis (Penzes et al. 2014). In the AdV currently characterized, internalization is mediated by an Arg-Gly-Asp (RGD) sequence in the penton base with integrins on the cell surface (Wickham et al. 1993). Following successful viral entry and import of the viral genome into the nucleus, the replication of the AdV genome occurs (Charman et al. 2019). Within the host cell nucleus, viral replication forms viral replication compartments (VRCs), concentrating factors beneficial to the virus and providing an environment conducive to viral processes (Hidalgo & Gonzalez 2019). These VRCs are believed to be the site of viral

genome transcription and RNA processing, and may act as sites of viral particle assembly and packaging (Hidalgo & Gonzalez 2019). Replication of AdV genomes is a prerequisite for the expression of viral late genes (Charman et al. 2019).

*Adenovirus Pathogenesis*

The pathogenesis of reptilian adenoviruses is not well characterized, in contrast to their mammalian and avian counterparts. Therefore, much of the following description is extrapolated from current knowledge in human and other mammalian adenovirus infections. Adenoviral infections are often opportunistic in many species, with environmental or nutritional stressors playing a role in developing clinical disease (Cook & Radke 2017). Reptilian adenoviruses vary regarding pathogenicity, and coinfections and immunosuppression likely precipitate severe systemic disease (Jacobson 2007; Rivera et al. 2009). Nonenveloped AdV virions shed by infected individuals are stable in the environment and can survive for long periods, not only tolerant of moderate temperature and pH changes; but also resistant to lipid solvents and simple household disinfectants (Cook & Radke 2017; Hernroth & Allard 2006). Due to their environmental stability, they have been known to accumulate in marine bivalves, and represent a major viral pollution source (Cook & Radke 2017). Primary virus shedding is primarily through the intestinal tract; however, aerosol transmission is possible and vertical transmission has been documented in host species including birds and cattle. In humans, adenoviruses generally cause gastrointestinal, ophthalmic, urinary and neurologic diseases (Cook & Radke 2017). Swimming pool-related outbreaks, including those causing keratoconjunctivitis and pharyngitis, are common.

Pathology associated with AdV infection results from viral replication in host nuclei and subsequent cell lysis (Cook & Radke 2017). However, contributors to pathology also include direct toxic effects caused by the host inflammatory response and high doses of structural proteins. Cellular entry of AdV triggers strong proinflammatory responses by activating Toll-like receptor signal transduction pathways (Gregory et al. 2011). Human adenoviruses have evolved mechanisms delaying the host cell's death and reducing proinflammatory responses to perpetuate their spread and immunopathogenesis (Cook & Radke 2017). One of these mechanisms is the E1A gene, which increases the efficiency of viral replication and represses transcription of certain proinflammatory and interferon-stimulating genes (Frisch & Mymryk 2002). AdV infection is multisystemic; tissues affected at necropsy include bronchial epithelium, liver, spleen and kidney. Inclusion bodies are formed with an accumulation of large amounts of capsid proteins that occur with viral replication inside the host cell nucleus, and may be observed histologically as basophilic structures in hematoxylin and eosin (HE) stained sections (Jacobson et al. 1996, Kim et al., 2002; Perkins et al. 2001). Virions form paracrystalline arrays that cause condensation and margination of nuclear chromatin. Virus particles are released when the host cell ruptures (Jacobson 2007).

Generally, AdV are host specific and are transmitted via the fecal-oral route or direct contact via oronasal secretions (Cheng et al. 2016). The incubation period from infection to clinical signs is reported to be 1-7 days in human AdV and may be dose dependent (Cheng et al. 2016). Most of these diseases are self-limiting; however, immunocompromised individuals are at greater risk for fatal consequences (Cook & Radke 2017). Recovery from infection is associated with developing serotype-specific neutralizing antibodies that protect against disease or reinfection; these antibodies do not cross react with other serotypes (Cook & Radke 2017).

Emergent strains where mutations have altered the hexon, fiber or penton (structural proteins) may be more likely to cause outbreaks, since those proteins are the targets for neutralizing antibodies (Houng et al. 2010). Outbreaks of AdV-associated respiratory illness in humans have occurred with emerging strains of AdV; while many patients were immunocompromised or with underlying conditions, outbreaks of infections have been reported in individuals with no pre-existing risk factors (Cook and Radke 2017).

*Overview of Adenoviruses in Reptiles*

Adenoviruses have emerged as potential threats to the health of reptiles. Before 2009, the only reported AdV in a chelonian host was an uncharacterized virus in a free-living leopard tortoise (*Stigmichelys pardalis*) (Gibbons & Steffes 2013). AdV-like particles had been previously identified in lizards and snakes and were characterized as belonging to the family *Atadenovirus* (Benko et al. 2002; Farkas et al. 2002; Wellehan et al. 2004). Atadenoviruses remain the most commonly reported genus of adenoviruses in squamate reptiles, and they have likely coevolved with this taxon (Farkas et al. 2002; Farkas et al. 2008; Wellehan et al. 2004).

While AdV-like particles have been recognized in crocodilians, they remain uncharacterized (Jacobson et al. 1984). In these taxa, AdV infection has historically been associated with hepatitis, enteritis, esophagitis, splenitis and encephalopathy (Jacobson et al. 1990; Jacobson 2007; Jacobson & Kollias 1986; Heldstab et al. 1984; Raymond et al. 2003). While reptiles do not always show evidence of disease, significant morbidity and mortality are associated with certain AdV infections. Clinical signs are primarily gastrointestinal and neurologic, and coinfections with other pathogens, including irido-, parvo-, and reoviruses, and coccidia, nematodes and microsporidia, have been reported (Doneley et al. 2014; Hyndman et al.

2011; Kim et al. 2002; Jacobson 2007; Moorman et al. 2009; Schilliger et al. 2016). Bearded dragons (*Pogona vitticeps*) are the most commonly reported reptile host of atadenoviruses, with many infections being attributed to agamid adenovirus-1 (Kubiak 2013; Doneley et al. 2014; Fredholm et al. 2015; Schilliger et al. 2016). Bearded dragons with adenoviral infection have been reported to present with poor growth, anorexia, and neurological signs such as head tilt and circling. Associated pathologic findings have included hepatitis, hepatic necrosis, intranuclear inclusion bodies in the liver and gastrointestinal tract, and interstitial nephritis (Jacobson et al. 2007; Julian & Durham, 1982; Kim et al. 2002). However, AdV have been detected in swabs from apparently healthy captive bearded dragons in the UK and free-ranging animals in Australia (Hyndman et al. 2019; Kubiak 2013). Coinfections with coccidia and dependoviruses have resulted in acute mortality of 30 hatchling bearded dragons following a short bout of weakness and lethargy (Kim et al. 2002).

While significant morbidity and mortality have been reported in snakes and lizards in association with AdV infection, both free-living and managed chelonians have more recently been reported as suffering from clinical disease (Farkas & Gal 2009; Garcia-Morante et al. 2016; Schumacher et al. 2012; Rivera et al. 2009). Despite the known presence of adenoviruses in chelonians, prevalence and clinical implications for infection have yet to be elucidated. The first report involved a single, moribund leopard tortoise (*Stigmochelys pardalis*) with biliverdinuria and episodes of hemorrhage, where coinfection with adenovirus and herpesvirus was identified; however, the adenovirus was not speciated (Gibbons & Steffes 2013).

Increased attention to adenoviruses in chelonians was garnered in 2007, after a mortality event involving a group of 105 Sulawesi tortoises (*Indotestudo forsteni*) was attributed to infection with a novel *Siadenovirus* (Rivera et al. 2009). Sulawesi tortoises are native to the

islands of Sulawesi and Halmahera in Indonesia, are classified as endangered by the IUCN Red List, and are listed under Appendix II of the Convention on International Trade of Endangered Species (CITES). They are threatened by habitat destruction and harvesting of wild individuals for consumption and the pet trade (Kuchling 1995).

One hundred and five illegally imported wild-caught Sulawesi tortoises (*Indotestudo forsteni*) were confiscated by the U. S. Fish and Wildlife Service upon entry into the United States in 2007 (Rivera et al. 2009). Thirty animals died before access to veterinary examination; the remainder of the animals were distributed to five institutions for aggressive medical care. The surviving 75 animals were in poor condition and clinical signs included anorexia, lethargy, mucosal and palatine erosions in the oral cavity, nasal and ocular discharge, and diarrhea, reflective of multisystemic disease (Rivera et al. 2009). Despite aggressive treatment with fluid therapy, nutritional support, antibiotics and antiparasitics, 62 of the 75 tortoises died, many of which succumbed within the first 3 weeks of treatment. The mortality rate was 82%, with 13 animals (17%) surviving to 20 months after presentation (Rivera et al. 2009). Clinicopathologic derangements on the complete blood count (CBC) before death from a subset of these animals showed anemia (33%), leukopenia (21%), and leukocytosis (21%), with the most common differential count abnormality being lymphopenia (37%). The most common biochemistry abnormality was elevated blood urea nitrogen (BUN) concentration (92%) and hyperglycemia (58%) (Rivera et al. 2009). Gross lesions on necropsy included oronasal fistulae, ulceration of the tongue and oral mucosa, hepatosplenomegaly, fibrinonecrotic membranes in the large intestinal lumens, and segmental discoloration of the small and large intestinal serosal surfaces (Rivera et al. 2009). Histology showed significant lesions in the gastrointestinal tract for 82% (41/50) of animals examined; necrotizing enterocolitis with pseudomembrane formation was a

consistent finding in animals that died early in the disease process (Rivera et al. 2009). Other lesions included hepatitis and/or hepatic necrosis, splenic necrosis, and myeloid necrosis in bone marrow. Viral inclusions were consistently identified in the intestinal epithelium, hepatocytes, and many other tissues for those individuals that lived longer with clinical support (Rivera et al. 2009).

Adenovirus DNA was detected from nasal flush, choanal swabs, choanal-cloacal swabs, and plasma samples antemortem, and various tissue samples (mucosal tissue, colon, small intestine, liver, lung, kidney, spleen) postmortem (Rivera et al. 2009). Other pathogen testing performed included consensus PCR analysis for herpesviruses in 13 tortoises, for intranuclear coccidia in 16 tortoises, and for chlamydiales in 5 tortoises, bacterial culture and sensitivity from coelomic effusion in 2 tortoises, and plasma samples for mycoplasma serology. For the adenovirus consensus PCR, the sequence of all PCR products was identical and consistent with a novel *Siadenovirus*. Although other pathogens including amoeba, nematodes, *E. coli*, *Aeromonas hydrophila* and *Chlamydia* sp. were also identified in the affected tortoises, this adenovirus was determined to be the causative agent for this mortality event, as animals that died later in the course of treatment only showed evidence of adenoviral infection (Rivera et al. 2009). Immunosuppression secondary to viral infection or other factors may have facilitated colonization with these other pathogens; it is also possible that concurrent disease may have exacerbated clinical deterioration in animals that died earlier on in the course of the disease.

This outbreak was the first description of a *Siadenovirus* infection in reptiles and represents a clinically important disease threat to endangered chelonians. Interestingly, hematopoietic neoplasia was identified in 69% of surviving tortoises that were AdV PCR-positive several months after the initial diagnosis. This included five lymphoma cases, three

myelomonocytic leukemia cases, and one lymphoid leukemia case (Rivera et al. 2009). While adenoviruses in chelonians are not generally regarded as being oncogenic, this represents a correlation of adenoviral infection with the subsequent development of neoplasia in surviving animals.

Since then, this same *Siadenovirus*, named Sulawesi tortoise adenovirus 1 (STADV), was identified in a multispecies exhibit involving deaths of two Impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*) at one of the five original facilities where the confiscated Sulawesi tortoises were sent for intensive veterinary care (Schumacher et al. 2012). Burmese star tortoises are critically endangered and are only found in Myanmar, and Impressed tortoises are endangered in Thailand and listed as vulnerable in Vietnam and Laos People's Democratic Republic (Cota et al. 2021: Praschag et al. 2020). This cross-species transmission and mortality underscores the fact that pathogenesis of these viruses in chelonians may include crossing species and causing significant clinical disease and death, especially with artificially inflated densities or when animals are in close proximities to each other.

Both Impressed tortoises died without premonitory signs within the same week, 8 weeks after the arrival of the Sulawesi tortoises (Schumacher et al. 2012). One of these animals was a clinically healthy, wild-caught, adult male, and the other was an approximately 5-year-old captive-hatched juvenile. Postmortem examination revealed diphtheritic plaques on the oral mucosa and pseudomembrane formation with colonic wall thickening and edema on the colonic mucosa in one animal, the other animal did not have significant gross lesions on necropsy (Schumacher et al. 2012). Histologic lesions in the adult wild-caught male were reflective of systemic infection with STAdV-1 and included severe ulcerative and pseudomembranous duodenitis and colitis, cholangiohepatitis with granulomas, cholestasis, acute interstitial

pneumonia, myocarditis, fibrinous splenitis, renal tubular necrosis, stomatitis, facial dermatitis, and multifocal necrosis of the bone marrow (Schumacher et al. 2012). Intranuclear inclusion bodies consistent with STAdV-1 were observed in the spleen, biliary epithelium, hepatocytes and endothelial cells in the liver of this animal; this was confirmed via *in situ* hybridization (ISH) and ultrastructural examination of viral particles was achieved via transmission electron microscopy (TEM) in this animal (Schumacher et al. 2012). The captive-born, juvenile specimen that lacked gross lesions had enteritis, interstitial nephritis, portal hepatitis and mild nonsuppurative meningoencephalitis. This animal also had intranuclear coccidian organisms, representing a coinfection that may have contributed to or been the primary cause of death in this animal (Schumacher et al. 2012). The Burmese star tortoise was a 4-year old captive-bred specimen that developed lethargy and anorexia 19 months after the arrival of the Sulawesi tortoises and 13 months after the last Sulawesi tortoises were removed from the collection. This animal succumbed to disease despite medical intervention. Postmortem examination was precluded by autolysis. In all three tortoises, adenovirus was identified via consensus nested polymerase chain reaction (PCR) testing and sequencing, which indicated that the virus was STAdV-1 (Schumacher et al. 2012). The mortality observed with the initial Sulawesi tortoise outbreak and evidence for horizontal transmission to these cases suggest that this virus has the potential to impact tortoise populations, including endangered species, significantly. Since that original outbreak, a health assessment of painted turtles in Indiana identified STADV in four free-living individuals (Vincent et al. 2023); the implications of this finding remain unknown. This underscores the importance for increased disease surveillance in free-living turtles to understand disease ecology and potential impacts on native populations and where these pathogens originate.

Almost simultaneously, adenoviruses of a novel lineage were detected in multiple species of turtles in North America and Europe. In 2009, an ornate box turtle (*Terrapene ornata ornata*) carcass in Hungary was submitted for virologic examination via PCR after postmortem examination revealed hepatocellular degeneration and intranuclear inclusion bodies on light microscopy (Farkas & Gal 2009). Molecular testing by PCR from tissues in this animal identified an adenovirus and *Mycoplasma*. The sequence of the fragment of the partial DNA-polymerase gene indicated a novel AdV that did not cluster with any known adenovirus genera, termed box turtle adenovirus 1 (BTAdV1) (Farkas and Gal 2009; Gibbons and Steffes 2013). Following this discovery, adenoviruses that did not belong to any of the recognized genera of the family Adenoviridae were detected via PCR and sequencing in a captive pancake tortoise (*Malacochersus tornieri*), four eastern box turtles (*Terrapene carolina carolina*) and two red-eared sliders (*Trachemys scripta elegans*) in the USA, and red-eared sliders (*Trachemys scripta elegans*) and yellow-bellied sliders (*T. scripta scripta*) (YBS) in Hungary (Doszpoly et al. 2013). In apparently healthy red eared sliders and yellow-bellied sliders sampled from four Hungarian shelter colonies, 31.8% of RES and 22.7% of YBS were detected with an AdV with sequences that clustered with BTAdV-1 and other variants of this same, novel lineage that was later classified within the new *Testadenovirus* genus (Doszpoly et al. 2013). The prevalence and sequence diversity found in the *Trachemys* in Hungary suggests that AdV may be common in *Trachemys* populations. Phylogenetic analysis of the partial DNA polymerase sequences demonstrated that this lineage deserved genus-level separation, which was later granted as the *Testadenovirus* genus (Doszpoly et al. 2013). The lineage did not show a clear pathogenic role; thus, it most likely coevolved with these testudinoid turtles (Doszpoly et al. 2013). Subsequently, Salzmann et al. (2021) detected Testadenoviruses in Hermann's tortoises (*Testudo hermann*i),

spur-thighed tortoises (*T. graeca*), Horsfield's tortoises (*T. horsfieldii*), sliders (*Trachemys* spp.), box turtles (*Terrapene* spp.) and a black pond turtle (*Geochlemys hamiltonii*) in Europe as part of a mass PCR screening. The clinical significance of many of these viruses remains unknown, and surveillance efforts in free-living chelonians are scarce. For example, wild eastern box turtles presented to a wildlife rehabilitation center in Virginia had a 55.7% prevalence of a *Testadenovirus* termed box turtle adenovirus (BTAdV) (Franzen-Klein et al. 2020).

Most chelonian adenoviruses taxonomically are classified within the genera *Testadenovirus* or *Siadenovirus*; however, *Atadenoviruses* have also been found in chelonians. While Atadenoviruses are traditionally considered to be found in snakes and lizards, they have been detected in a spur-thighed tortoise (*Testudo graeca*), a Horsfield's tortoise, and three tortoises of unknown species (Garcia-Morante et al. 2016; Salzmann et al. 2021). A 2-year-old female, spur-thighed tortoise that presented with poor body condition and weakness before death had an AdV identified as belonging to the *Atadenovirus* genus, which represents the first of chelonian adenoviruses to cluster with this genus (Garcia-Morante et al. 2016). Lesions included hyperplastic esophagitis and stomatitis with marked epithelial cytomegaly and basophilic intranuclear inclusion bodies. Electron microscopy revealed large numbers of 60-80nm, nonenveloped, icosahedral virions arranged in crystalline arrays within nuclear inclusions, morphologically compatible with adenovirus-like particles (Garcia-Morante et al 2016). Interestingly, this AdV infection was associated with lesions only in the upper gastrointestinal tract (Garcia-Morante et al. 2016). The demise of this animal was associated with the death of two other spur-thighed tortoises without premonitory signs; all of these animals presented 2 months following the introduction of four new tortoises (2 Mediterranean tortoises (*Testudo hermanni*), 1 spur-thighed tortoise, and 1 Russian tortoise (syn. Horsfield's tortoise, *Testudo*

*horsfieldii*) to this private collection, suggesting a potential source of disease spread (Garcia-Morante et al. 2016). Unfortunately, necropsies were not performed on the other animals associated with this outbreak; therefore, the contribution of AdV to the death of these other individuals is unknown.

*Diagnostic Methods*

Diagnosis of adenoviruses in chelonians has been accomplished through a series of traditional approaches including molecular detection (consensus nested polymerase chain reaction (PCR) and sequencing), direct visualization (electron microscopy, *in situ* hybridization (ISH), immunohistochemistry), and host response (histopathology) (Farkas & Gal 2009; Garcia-Morante et al. 2016; Rivera et al. 2009; Schumacher et al. 2012). In human medicine, virus isolation in cell culture is the best for diagnosing AdV infection; however, it can take as long as 3 weeks to obtain results (Pehler-Harrington et al. 2004; Shetty et al. 2003). Among reptiles, methods used for AdV infection diagnosis include virus isolation, electron microscopy, *in situ* hybridization, and the serologic assay plaque reduction neutralization (Heldstab & Bestetti 1984; Marschang et al. 2003; Perkins et al. 2001). Virus isolation requires further diagnostics for speciation, such as detailed strain identification or sequencing (Burrell et al. 2017; Wellehan et al 2004). Electron microscopy and ISH do not specify reptile adenoviruses to species, and the cross-reactivity of neutralizing antibodies to reptile adenoviruses is not unknown (Wellehan et al. 2004). The most commonly reported method for diagnosing adenovirus in reptiles is using a consensus PCR that amplifies a partial sequence of the adenoviral DNA polymerase gene (Wellehan et al. 2004).

*Molecular Methods*

Polymerase chain reaction (PCR) amplifies a target nucleic acid sequence (Sigma-Aldrich 2008). The first reported consensus PCR protocol for adenoviruses in reptiles was described by Farkas et al. (2002) to identify an *Atadenovirus* in a corn snake, corn snake adenovirus (SnAdV-1). This protocol did not work with gecko samples evaluated by Wellehan et al. (2004), so these authors described a different protocol for novel diverse adenoviruses, which amplifies a partial sequence of the DNA-dependent DNA polymerase gene. This consensus nested protocol has since been successfully used to identify adenoviruses in chelonians. Amplification results in 318 to 324 bp products before primer sequences are edited out (Wellehan et al. 2004). The Wellehan protocol can be utilized on appropriate antemortem or postmortem samples, including nasal flushes, oral/nasal mucosal tissue, choanal/cloacal swabs, plasma, colon, small intestine, liver, lung, kidney and spleen (Rivera et al. 2009). To obtain additional adenoviral genome fragments (from the genes of Iva2, penton base, or pVII) from positive samples, family or genus-specific consensus PCR assays have been used (Doszpoly et al. 2013). These degenerate, consensus primers were designed based on highly conserved amino acid motifs selected by aligning the hexon sequence of four siadenoviruses, known at that time from turkey, frog, and avian species (Davison et al. 2000; Doszpoly et al. 2013; Kovacs & Benko 2011; Pitcovski et al. 1998).

Once PCR is complete, the products are identified via gel electrophoresis or digestion of restriction enzymes (Wellehan & Johnson 2005). Validating the identity of the PCR product can also be accomplished by hybridizing the product with a labeled nuclei acid probe from the expected product (Wellehan & Johnson 2005). The most specific method to validate the identity of the amplified product is sequencing (Wellehan & Johnson 2005). Sequences are then

compared to those in a database such as GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl), and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) databases using TBLASTX for protein coding sequence or BLASTN for nonprotein coding sequence (Altschul et al. 1997).

Real-time or quantitative PCR (qPCR) circumvents the need for identification using gel electrophoresis and is a more sensitive and specific method for virus detection (Sigma-Aldrich 2008). In qPCR, the products are detected as the reaction proceeds, allowing for the quantification of target copy numbers, and providing more rapid results than conventional PCR. Real-time visualization of amplified DNA fragments is enabled using specific fluorescent DNA dyes or fluorescently labeled oligonucleotide (hydrolysis) probes (Kralik & Ricchi 2017). SYBR green-based, and TaqMan-based detection are the two most common ways this visualization is accomplished (ThermoFisher). For the TaqMan hydrolysis probe, a fluorescent reporter dye and a quencher are attached to the 5' and 3' ends of a Taqman probe. During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe; it emits its characteristic fluorescence once separated from the quencher. The SYBR green dye fluoresces when bound to double-stranded DNA. The green dye is released when the DNA is denatured, drastically reducing fluorescence. During extension/polymerization, primers are annealed, and a PCR product is generated. When polymerization is complete, SYBR green dye binds to a double-stranded product, resulting in a net increase in fluorescence detected by the system (ThermoFisher). Fluorescence is measured after each PCR cycle and intensity of the fluorescent signal reflects the amount of DNA amplicons in the sample at that time. The quantification cycle (denoted $C_q$) is the cycle at which the fluorescent signal surpasses the threshold value. $C_q$ values

are compared to a calibration curve of serially diluted standard samples with known concentrations or copy numbers for determination of absolute quantity of target DNA in the sample (Kralik & Ricchi 2017). The amplicon detection is carried out in a closed-tube format, eliminating the need for gel electrophoresis and potential for cross contamination. The main advantages of qPCR include the ability to perform fast and high-throughput detection and quantification of target DNA sequences in different matrices (no further manipulation is needed after amplification), wide dynamic range for quantification, and capacity to multiplex amplification of several targets into a single reaction (Klein 2002; Kralik & Ricchi 2017). A quantitative PCR (qPCR) described for diagnosis of box turtle adenoviruses in eastern box turtles was developed using primers designed to amplify a short segment of the BTAdV DNA polymerase gene (Franzen-Klein et al. 2019). Of the samples used to determine the specificity of the assay, the known BTAdV-positive samples reacted and were confirmed positive by the qPCR assay.

*Electron Microscopy and Virus Isolation*

The diagnosis of adenovirus has also been made by the presence of intranuclear, amphophilic inclusion bodies by light microscopy followed by visualization of virus particles by electron microscopy (Schumacher et al. 2012; Rivera et al. 2009; Farkas & Gal 2009; Garcia-Morante et al. 2016). Viral particles identified are nonenveloped, approximately 60-90 nm in diameter, and hexagonal in the transverse section, indicative of the icosahedral capsid symmetry. Viral particles have been observed to form paracrystalline arrays in nuclei, and host cell nuclei will show chromatin margination, clearing of the nucleoplasm, and abnormal nuclear shapes and sizes (Rivera et al. 2009; Farkas & Gal 2009; Garcia-Morante et al. 2016; Schumacher et al. 2012). Adenovirus particles in chelonians have been identified most frequently in the

reticuloendothelial cells of the spleen, myeloid cells of the bone marrow, hepatocytes, and esophageal epithelial cells (Farkas & Gal 2009; Garcia-Morante et al. 2016; Rivera et al. 2009; Schumacher et al. 2012). Attempts to perform virus isolation have been performed but have been unsuccessful (Farkas & Gal 2009; Schumacher et al. 2012).

*In situ hybridization*

*In situ* hybridization uses radioactively or chemically labeled probes to identify target DNA or RNA in a tissue sample by forming complementary base pairs (Brown 1998). When mixed with a tissue sample, the single-stranded probe will bind in situ to the expressed mRNA or DNA of interest (Brown 1998). *In situ* hybridization has validated viral inclusions in tissues in several cases of AdV infection in chelonians. In the case of the initial Sulawesi tortoise adenovirus (STAdV-1) outbreak, a digoxigenin-labeled riboprobe transcribed from the cloned fragment of the AdV DNA-dependent DNA polymerase gene generated a hybridization signal on cells in the liver, kidney, or spleen to have viral inclusions (Rivera et al. 2009). A fluorescein-labeled riboprobe, transcribed from the cloned 450-bp consensus sequence of the STAdV-1 DNA-dependent DNA polymerase gene, was later used successfully for ISH in the identification of the same virus in an Impressed tortoise (Schumacher et al. 2012). Tissue sections that gave hybridization signal in this animal included endothelial cells, hepatocytes, biliary epithelial cells in the liver, reticuloendothelial cells in the spleen, renal tubular epithelial cells, and epithelial cells in the testis (Schumacher et al. 2012).

*Treatment*

Reports of treatment of AdV infection in chelonians are sparse, and published literature on successful treatment is even more elusive. Many chelonians identified as AdV-positive do not appear to have clinical disease and are apparently healthy (Doszpoly et al. 2013; Franzen-Klein

2020). In cases where AdV infection is determined to be pathogenic, these cases often die acutely (in some cases without known medical history, or known premonitory signs), and diagnosis is frequently achieved postmortem (Farkas & Gal 2009; Rivera et al. 2009; Schumacher et al. 2012). The outbreak of Sulawesi tortoise adenovirus (STADV) affecting 105 confiscated Sulawesi tortoises represents the only report where a pathogenic adenovirus was identified antemortem (Rivera et al. 2009). Even then, most animals succumbed to systemic disease despite aggressive therapy; only 13 out of 75 animals reportedly survived 20 months after initial presentation (Rivera et al. 2009). To the authors' knowledge, this is the only report of AdV-infected chelonians for which treatment was successful in a few select cases. Therapies were not described in detail; however, they were supportive and included antibiotics, antiparasitics, parenteral fluids, and nutritional support. Many tortoises died during the first 2-3 weeks after acquisition, with deaths continuing over 8 weeks after initiating treatment (River et al. 2009). In other cases where AdV-positive turtles have been identified, treatment may not be warranted. For example, a high prevalence of eastern box turtles had box turtle adenoviruses detected; however, clinical signs of the disease were absent (Franzen-Klein et al. 2020). Similarly, the pond sliders where multiple Testudinoid adenoviruses from a novel lineage were detected were healthy (Doszpoly et al. 2013). The finding of turtles testing positive for AdV without concurrent clinical disease signs supports the proposed coevolutionary lineage of adenoviruses with their hosts, where subsequent host switches may lead to significant morbidity and mortality.

# CHAPTER 3: DETECTION OF ADENOVIRUSES IN FREE-RANGING BLANDING'S TURTLES (*EMYDOIDEA BLANDINGII*), PAINTED TURTLES (*CHRYSEMYS PICTA*) AND RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) IN ILLINOIS, USA

**ABSTRACT:** Morbidity and mortality associated with adenoviruses (AdV) have been reported in snakes, lizards, and more recently, chelonians. AdV of a distinct testudinoid origin have been detected in managed and free-ranging turtles in North America and Europe. However, the prevalence and impact of AdV in free-ranging turtle populations have yet to be determined. A multi-species investigation to detect novel or existing adenoviruses in Blanding's turtles (*Emydoidea blandingii*; n=1359), painted turtles (*Chrysemys picta*; n=270) and red-eared sliders (*Trachemys scripta elegans*; n=205) was performed across four counties in Illinois from 2016-2022. Sequence-confirmed AdV were detected in painted turtles (13.0%; n=35), red-eared sliders (8.8%; n=18) and Blanding's turtles (1.8%; n=24). Ten AdV, including five novel AdV, were detected across the three species, with each species having 3-6 AdV detected. Three AdV were likely prey-related, while the remaining seven AdV were likely host-adapted AdV based on absence of clinical signs of disease. The majority of AdV detected were Testadenoviruses; however, the Siadenovirus Sulawesi tortoise adenovirus (STADV) was sequence-confirmed in 15 painted turtles, 10 red-eared sliders and one Blanding's turtle, all of which were free of clinical signs of disease. These results provide a baseline for the presence and diversity of AdV in free-ranging turtles in Illinois, including evidence for a North American reservoir for STADV.

## INTRODUCTION

Adenoviruses are nonenveloped double-stranded DNA viruses which replicate in host nuclei and are released when the host cell ruptures (Gibbons et al. 2013; Latney et al. 2013; Marschang et al. 2011). There are currently six accepted AdV genera: *Mastadenoviruses* found in mammals; *Aviadenoviruses* in birds; *Ichtadenovirus* from a white sturgeon (*Acipenser transmontanus*); *Atadenoviruses* in several taxa; *Testadenoviruses* unique to chelonians, and *Siadenovirus* in amphibians, chelonians, and birds (Doszpoly et al. 2013; Franzen-Klein et al. 2020; Gibbons et al. 2013; Latney et al. 2013; Marschang et al. 2011; Salzmann et al. 2021). Current taxonomy in the family Adenoviridae suggests these viruses coevolved with their hosts, with subsequent host switches (Marschang et al. 2011). Host-adapted viruses may not cause disease in their host species or only cause clinical disease in immunocompromised individuals (Benkö et al. 2003). However, understanding the behavior and pathogenicity of these viruses remains crucial, as subsequent host switches may lead to significant morbidity and mortality (Benkö et al. 2003; Marschang et al. 2011).

The first report of adenovirus infection in chelonians involved a single, moribund leopard tortoise (*Stigmochelys pardalis*) with biliverdinuria and episodes of hemorrhage (Gibbons et al. 2013). In 2007, a mortality event involving over 100 Sulawesi tortoises (*Indotestudo forsteni*), two Impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*) was caused by a novel *Siadenovirus*, named Sulawesi tortoise adenovirus (STADV; Rivera et al. 2009). Adenoviruses representing a novel lineage have also been molecularly detected in other chelonians, including captive pancake tortoise (*Malacochersus tornieri*;), eastern box turtles (*Terrapene carolina carolina*) and red-eared sliders (*Trachemys scripta elegans*) in the USA, and red-eared sliders (*Trachemys scripta elegans*), yellow-bellied sliders

(*T. scripta scripta*) and an ornate box turtle in Hungary (Doszpoly et al. 2013; Farkas et al. 2009). Eastern box turtles surveyed at a wildlife rehabilitation center in Virginia had a 55.7% qPCR prevalence of eastern box turtle adenovirus and/or ornate box turtle adenovirus (Franzen-Klein et al. 2020). These adenoviruses have since been classified into the new *Testadenovirus* genus, a group of testudinoid adenoviruses phylogenetically distinct from all other adenoviruses (Doszpoly et al. 2013; Farkas et al. 2009; Franzen-Klein et al. 2020; Salzmann et al. 2021). More recently, Salzmann et al. (2021) detected Testadenoviruses and Atadenoviruses in freshwater aquatic and terrestrial chelonians in Europe (Salzmann et al. 2021).

The Blanding's turtle (*Emydoidea blandingii*) is a semiaquatic chelonian native to the northeastern United States and Great Lakes region, and is considered endangered by the International Conservation of Nature (IUCN; Ernst CH et al. 2009; van Dijk et al. 2011). These turtles are declining throughout their range secondary to habitat destruction and fragmentation, predation, and vehicular mortality (van Dijk et al. 2011). While infectious diseases, namely ranaviruses, herpesviruses, and *Mycoplasma* spp. have contributed to significant morbidity and mortality in other free-ranging chelonians, the impact of pathogen prevalence on this species remains poorly understood (Berish et al. 2010; Brown et al. 1994; Butkus, et al. 2017; Dziadzio et al. 2018; McKenzie et al. 2019). Painted turtles (*Chrysemys picta*) are abundant and may serve as a sentinel species for their less common counterparts, and red-eared sliders (RES; *Trachemys scripta elegans*) are so abundant that they are considered invasive and nuisance animals in many parts of their current range. Despite their robust conservation status, infectious disease information in the literature for free-ranging painted turtles and red-eared sliders is limited. Red-eared sliders have been found to have a high prevalence and diversity of adenoviruses in Hungary (Doszpoly et al. 2013). STADV has been recently detected in free-ranging, apparently

healthy painted turtles in Indiana, USA (Vincent et al. 2023). The finding of this virus in a novel North American host species underscores the importance of additional work to address the knowledge gap regarding adenoviruses in free-ranging turtles in North America.

The prevalence and impact of AdV in free-ranging populations of turtles is not defined. Determining the prevalence and diversity of AdV in these three emydid turtles that occupy similar ecological niches but have significantly different abundances will build understanding of what viruses are present in the landscape. The study aimed to perform a broad scale, multi-species investigation to detect novel or existing adenoviruses using two-step conventional PCR in Blanding's turtles, painted turtles, and red eared sliders in Illinois.

## MATERIALS & METHODS

Oral-cloacal swabs were collected from free-ranging Blanding's turtles, painted turtles, and red eared sliders in three counties in Illinois, as part of an ongoing aquatic turtle disease surveillance program beginning in 2016.

### *Study Sites*

Blanding's and painted turtles were sampled at four sites in Kane County, IL, including Hannaford (HAN), Willoughby (WIL), Pingree (PIN), and Freeman Kame (FRE). Blanding's were also sampled at Spring Bluff (SB) and Illinois Beach State Park (IBSP) in Lake County and at Chiwaukee Prairie (C) in Kenosha County, WI. In Cook County, Blanding's turtles were sampled at Powderhorn Lake, Algonquin Woods, and Orland areas, and red-eared sliders were sampled in three areas: Powderhorn, Burnham, and Flatwoods. Samples were collected from mid-May to mid-October from 2016–2022. All animal sampling was permitted by the following orga- nizations: Illinois Department of Natural Resources (IDNR; Scientific Collectors Permits

[SCP] NH17.5065, NH18.5065; IDNR Endangered and Threatened permits SBT-16-062, 1199, 14-046, and 1042), the Wisconsin Department of Natural Resources (WIDNR; SCP: SCP-SOD-004-2013; WIDNR Scientific Research License SRLN-18-026), and the University of Illinois Institutional Animal Care and Use Committee (Protocols 15017, 15158, 18000, 18165, 20258).

*Capture Methods*

Turtles were captured with the aid of radiotelemetry (Blanding's only), in a hoop trap, or incidentally by hand during fieldwork. The turtles captured via telemetry were juveniles, subadults and adults being tracked as a part of a chelonian monitoring program. Telemetry, traps and incidental captures were used at all field sites.

*Physical Examination and Sample Collection*

Each turtle was assigned a permanent ID and mass, sex, and age class was recorded. Blanding's turtles were characterized as juvenile (<250 grams), sub-adult (250-750 grams), or adult (>750 grams). Sex was classified as male, female or unknown based on plastron concavity and position of the cloaca. Painted turtles were categorized as juveniles or adults based on total plastron length (TPL) using ranges established in painted turtle populations of Wisconsin (Ross 1989; St. Clair et al. 1994). Males with a plastron length greater than 85 mm or exhibiting mature secondary sex characteristics (e.g. elongated nails on the front feet) were classified as adults, while females with a plastron length greater than 130 mm were considered adults (Ross 1989; St. Clair et al. 1994). Red-eared sliders were classified as adults if the straight carapace length was > 100 mm. Sexing of red-eared sliders was based on secondary sex characteristics, with males identified based on elongated front claws and a cloacal opening past the caudal edge of the carapace. Physical examinations were performed, noting visual appearance of the eyes, nose, oral cavity, ears, legs, digits, shell, integument, and cloaca.

Combined oral-cloacal swabs were collected from each turtle using cotton-tipped plastic-handled applicators (Fisher Scientific, Pittsburgh, PA 15275, USA) by first swabbing all surfaces in the interior of the mouth, then taking the same swab and inserting into the cloaca and swabbing the interior surface. Swabs were labeled with a unique identification number and remained on ice packs until transport to the laboratory 2–6 hours later. Swabs were stored at -20 °C or -80°C until DNA extraction.

Following the manufacturer's protocol, DNA extractions from swabs were performed using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA 91355, USA). Quantity (ng/µl) and quality (A260:A280 ratio) of DNA were evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA 02451, USA). Two-step conventional adenovirus PCR was performed targeting the DNA-dependent DNA polymerase gene of AdVs as previously described (Wellehan et al. 2004). Reactions were performed in a 50 µl total volume using Taq Polymerase, 10× PCR buffer, MgCl2 (Taq DNA Polymerase Recombinant 500 U, Invitrogen, Carlsbad, CA), dNTP mix (10 mM dNTP mix, Invitrogen, Carlsbad, CA), and dH2O. Five microliters of template DNA from the primary PCR reaction were used in the secondary reaction. DNA extracts from an oral swab positive for Box Turtle Adenovirus 1 were used as a positive control, and DNase/RNase-free water was used as a negative control. The secondary PCR products were visualized by electrophoresis using a 1% agarose gel. A subset of the PCR products (33.3%) that produced a single, bright band of the appropriate size (approximately 320 bp) were Sanger sequenced in both directions (ACGT, Wheeling, IL 60090) and compared to known sequences in GenBank using BLASTN.

## RESULTS

Oral-cloacal swabs from 1,359 free-ranging Blanding's turtle encounters, 270 painted turtle encounters, and 205 red eared slider encounters were evaluated via consensus PCR. The number of sequence-confirmed adenoviruses detected in the sampled population was highest in painted turtles (35/270; 13.0%), followed by red-eared sliders (18/205; 8.8%) and Blanding's turtles (24/1,359; 1.8%).

Blanding's turtle samples included 780 females, 382 males, and 197 turtles of unknown sex. The age classes sampled included 595 adults, 203 sub-adults, and 561 juveniles. The number of sequence-positives for adenoviruses detected in Blanding's turtles across all counties and years are reported in Table 3.1. Almost all adenoviruses detected were from turtles in Lake County (91.7%), while the remaining positives were from Blanding's turtles sampled in 2016 in DuPage County (see Table 3.1). No adenoviruses were detected in Blanding's turtles in Kane County. The six distinct adenoviruses detected in Blanding's turtles in Lake County included *Emydoidea* adenovirus 1, Box turtle adenovirus, Sulawesi tortoise adenovirus, two distinct novel *Mastadenoviruses*, and one distinct novel *Atadenovirus* (Table 3.2). *Emydoidea* adenovirus 1 was also detected in two turtles sampled in DuPage County in 2016. Turtles with sequence-confirmed positives included 4 adults, 5 subadults, and 15 juveniles; 17 were males, and 7 were females. No clinical signs of disease were seen in adenovirus positive turtles.

Adenoviruses detected in painted turtles sampled in Kane County (n=35) are reported in Table 3.3. Painted turtle samples in Kane County (2019-2021) included 129 males, 125 females, and 16 turtles of unknown sex (181 adults, 89 juveniles). Twenty positive animals were female, and 15 were male. Positives were found in turtles in May-July of 2019 and 2021 and June-July of 2020. Of the positives, 3 were juveniles, and 32 were adults. The three adenoviruses detected in

painted turtles included: Sulawesi tortoise adenovirus, a novel painted turtle adenovirus (91% identical to yellow-bellied slider adenovirus), and a second novel painted turtle adenovirus (81-82% identical to red eared slider adenovirus; Table 3.3). Several additional positive samples had weak products that could not be successfully sequenced. No clinical signs of disease were seen in adenovirus positive turtles.

Adenoviruses were detected in 18 red-eared sliders sampled in Cook County from 2021-2022 (Table 3.4). Red-eared slider samples included 99 males, 91 females, and 15 turtles of unknown sex (188 adults, 17 juveniles) sampled from May-October in 2021 and 2022. Sequence-positive animals with adenoviruses included 16 adults and 2 juveniles; 12 were males, and six were females. Four adenoviruses were detected in red-eared sliders: Box turtle adenovirus, Sulawesi tortoise adenovirus, Red-eared slider adenovirus, and Yellow-bellied slider adenovirus (Table 3.4). No clinical signs of disease were seen in adenovirus positive turtles.

## DISCUSSION

This multi-species disease investigation identified 10 adenoviruses across the three species examined; five were novel, and each host species had 3-6 different adenoviruses identified. Although three of the Blanding's turtle adenoviruses were likely prey-related, seven adenoviruses were identified across these three species. This study represents the largest scale surveillance for adenoviruses in free-living freshwater turtles, and is the first survey to report detection of adenoviruses in Blanding's turtles. The results indicate multiple, diverse adenoviruses circulating in free-ranging freshwater turtles.

Sequence-confirmed adenovirus prevalence was highest in painted turtles (13.0%), followed by red-eared sliders (8.8%), and Blanding's turtles (1.8%). Each turtle species had three

or more adenoviruses identified, including three novel AdV in painted turtles. Overall prevalence for AdV was higher in this study than a broad-scale screening effort for European chelonians under managed care, where overall prevalence was 2.3% (Salzmann et al. 2021). AdV in that study were detected in Hermann's tortoises (*Testudo hermanni*), spur-thighed tortoises (*T. graeca*), Horsfield's tortoises (*T. horsfieldii*), sliders (*Trachemys* spp.), box turtles (*Terrapene* spp.) and a black pond turtle (*Geochlemys hamiltonii*) (Salzmann et al. 2021). The prevalence of AdV in the present study was lower for all three species compared to rehabilitating eastern box turtles surveyed in Virginia, which had a 55.7% prevalence and identified both box turtle adenoviruses and ornate box turtle adenovirus (Franzen-Klein et al. 2020). However, that survey was performed using qPCR, which is usually more sensitive than other techniques, such as consensus PCR (Vidal et al 2011). The same study was also performed on animals that were sick or injured, while the majority of our sampled animals were apparently healthy (Vidal et al. 2011).

One study identified AdV with high prevalence (27.3%) and sequence diversity in RES in Hungary, suggesting that *Trachemys* spp. may be common AdV hosts (Doszpoly et al. 2013). Yellow-bellied sliders (*Trachemys scripta scripta*) had an even higher prevalence (31.8%) but were not examined in our study (Doszpoly et al. 2013). Salzmann et al. (2021) identified AdV in 42.9% of pond sliders (*Trachemys* spp.) in Europe, further supporting this theory (Salzmann et al. 2021). Our study's AdV prevalence in RES was 8.8%, lower than that identified in both European studies. The RES in Hungary were sampled live or dead and housed in "turtle shelters" after being illegally released into the wild (Doszpoly et al. 2013). All AdV positive RES in the present study were alive and apparently healthy, but without known medical history. Unfortunately, no clinical information was available from *Trachemys* spp. sampled in Salzmann

et al. (2021). The RES were all presumably free-ranging turtles, however, it is possible that some of the Cook County RES were released pets, given their close proximity to the Chicago metropolitan center. Unfortunately, it is unclear at what point the Hungarian RES were infected with AdV, whether it was during their former existence as pets, or during the period of being housed in a "shelter" where nosocomial infections may have occurred with other housed turtles.

No sequence-positive turtles in the present study had clinical signs of disease. Testadenoviruses have been associated with nonspecific clinical signs and death, but have also been detected in many apparently healthy turtles (Doszpoly et al. 2013; Farkas et al. 2009). Similar to our study, RES in Hungary infected with testudinoid AdV were all apparently heathy and free of clinical signs. Given that this lineage did not show a clear pathogenic role and was generally not associated with clinical disease in Doszpoly et al. (2013), it is most likely that it coevolved with these testudinoid turtles. Furthermore, eastern box turtles surveyed in Virginia had low viral quantities on qPCR, and infection was not associated with clinical disease, suggesting that the viruses identified are likely host-adapted (Franzen-Klein et al. 2020).

Novel adenoviruses arising from *Mastadenovirus* and *Atadenovirus* were also intermittently detected in the present study. It is possible that the *Mastadenovirus* and *Atadenovirus* representatives may have originated from rodent or passerine carcasses, respectively, that were then consumed by Blanding's turtles. Serial sampling would be required to determine if these were truly prey-associated or associated with the host.

Sulawesi tortoise adenovirus was well represented in the proportion of adenoviruses identified in painted turtles (42.9%; 15/35) and red-eared sliders (55.6%; 10/18). A single Blanding's turtle (4.1%; 1/24) was also detected with STADV DNA. Blanding's turtles and RES represent novel host species in which STADV has been detected. As part of a recent health

assessment of free-ranging painted turtles in Indiana, USA, four apparently healthy painted turtles had STADV detected (Vincent et al. 2023). That represents the first detection of STADV in the literature since the original outbreak, which involved three endangered tortoise species that experienced high mortality with infection (Rivera et al. 2009; Schumacher et al. 2012). None of the turtles in the present study that had STADV detected had any clinical signs of disease. Therefore, they may represent a host-adapted species for STADV and a potential North American reservoir or competent host for this virus, which poses an important conservation concern for chelonians given its potential to cause high mortality when jumping host species. The risk of STADV infection may be even higher with concurrent disease processes, immunosuppression, or poor husbandry practices.

Box turtle adenovirus has now been detected in Blanding's turtles, red-eared sliders and box turtles, representing a mixed species adenovirus. The ability to infect multiple hosts is not typical for adenoviruses, relieves selective pressure against virulence, and suggests that host jumping into additional naïve hosts may be plausible. Therefore, it may represent the most clinically concerning adenovirus detected. While this AdV is not known to cause large-scale mortality, coinfection with other common pathogens, such as *Mycoplasma* spp., may predispose to clinical disease or death (Farkas et al. 2009). Further investigation is needed using more sensitive molecular methods, such as qPCR, to characterize the epidemiology of AdV in free-living Blanding's turtles, painted turtles, and RES and determine if these pose a clinical disease threat.

Limitations of this study include using consensus PCR to survey populations of native turtles in Illinois for adenovirus. Consensus PCR is generally less sensitive and specific than qPCR. There is no evidence to support a latency period for adenovirus infected turtles and not all

turtles were sampled or followed up in multiple years; therefore, it is unknown if positive turtles in this study experienced fluctuating shedding of viral DNA or experienced any disease-related mortality. Only live turtles were sampled, and turtles experiencing clinical disease or death may be less likely to be detected. Lastly, not all PCR products were of appropriate quality or led to clean sequences. The investigators worked to ensure adequate representation across all species demographics; however, some of these numbers may be an underrepresentation of true virus prevalence in the population.

Both painted turtles and RES are abundant, cohabitating freshwater turtle species in Illinois and share wetland habitat with Blanding's turtles, which are less abundant across their range (Ernst et al. 2009). Furthermore, painted turtles and RES are commonly maintained in the pet trade and zoological institutions, and *Trachemys* spp. are farmed in high densities in the southeastern United States. This indicates high risk for mixing with non-sympatric species, potentially leading to virus transmission and host jumping (Doszpoly et al. 2013; Verneau, et al. 2011). The results of this study provide insight into the diversity and prevalence of AdV in free-ranging turtles in Illinois, including evidence for a North American reservoir for STADV. Further work to determine relationships between adenovirus detection, infection, and resulting clinical disease would be prudent to improve the safety of zoological collections that manage these species alongside others, and to develop conservation strategies that improve health in free-living turtles.

## TABLES

Table 3.1. Adenoviruses were detected in Blanding's turtles (*Emydoidea blandingii*) sampled in all Illinois counties from 2016-2022. Number of adenoviruses sequenced are reported as # positive/# sampled (% positive).

| County | 2016 | 2017 | 2019 | 2020 | 2021 | 2022 | Total |
|---|---|---|---|---|---|---|---|
| Lake | NA | 1/257 (0.4) | 1/148 (0.7) | 10/154 (6.4) | 2/150 (1.3) | 8/159 (5.0) | 22/868 (2.5%) |
| Kane | NA | NA | 0/55 (0) | 0/112 (0) | 0/79 (0) | 0/150 (0) | 0/396 (0) |
| DuPage | 2/95 (2.1) | NA | NA | NA | NA | NA | 2/95 (2.1) |
| Total | | | | | | | 24/1,359 (1.8) |

Table 3.2. Adenoviruses detected in Blanding's turtles (*Emydoidea blandingii*) sampled in Lake County from 2017-2022.

| Lake County | 2017 | 2019 | 2020 | 2021 | 2022 | Total |
|---|---|---|---|---|---|---|
| # Turtles sampled | 257 | 148 | 154 | 150 | 159 | 868 |
| # Adenoviruses sequenced (%) | 1 (0.4) | 1 (0.7) | 10 (6.4) | 2 (1.3) | 8 (5.0) | 22 (2.5%) |
| *Emydoidea* adenovirus 1 (MW561636) | | 1 | 7 | 2 | 7 | 17 |
| Box turtle adenovirus (EU828750.1) | | | | | 1 | 1 |
| Sulawesi tortoise adenovirus (EU056826.1) | 1 | | | | | 1 |
| Rodent *Mastadenovirus I* | | | 1 | | | 1 |
| Rodent *Mastadenovirus II* | | | 1 | | | 1 |
| Passerine *Atadenovirus* | | | 1 | | | 1 |

Table 3.3 Adenoviruses detected in painted turtles (*Chrysemys picta*) sampled in Kane County from 2019-2021. Painted Adeno 1 = 91% identical to yellow-bellied slider adenovirus. Painted Adeno 3/4 = 81% identical to RES adenovirus (JQ809465.1, JX307095.1). Painted Adeno 4 = 82% identical to RES adenovirus (JQ809465.1, JX307095.1)

| KANE COUNTY | 2019 | 2020 | 2021 | Totals |
|---|---|---|---|---|
| # Turtles sampled | 97 | 75 | 98 | 270 |
| # Adenoviruses Sequenced (%) | 3 (3.1) | 23 (30.6) | 9 (9.1) | 35 (13.0) |
| Painted Adeno 1 | 1 | 12 | 2 | 15 |
| Sulawesi tortoise adenovirus (EU056826.1) | 2 | 9 | 4 | 15 |
| Painted Adeno 3/4 | | 2 | 3 | 5 |

Table 3.4. Adenoviruses detected in red-eared sliders (*Trachemys scripta elegans*) sampled in Cook County from 2021-2022.

| Cook County | 2022 | 2021 | Totals |
|---|---|---|---|
| # Turtles sampled | 105 | 100 | 205 |
| # Adenoviruses sequenced (%) | 14 (13.3) | 4 (4.0) | 18 (8.8) |
| Sulawesi tortoise adenovirus (EU056826.1) | 6 | 4 | 10 |
| Box turtle adenovirus 1 (EU828750.1) | 3 | | 3 |
| Red-eared slider adenovirus (JX307097.1) | 3 | | 3 |
| Yellow-bellied slider adenovirus (JX307096.1) | 2 | | 2 |

# CHAPTER 4: DEVELOPMENT AND VALIDATION OF A QUANTITATIVE PCR ASSAY FOR DETECTION OF SULAWESI TORTOISE ADENOVIRUS

**ABSTRACT**: Pathogenesis of adenoviruses in chelonians may include crossing species and causing significant clinical disease and death. In 2007, a mortality event involving over 100 Sulawesi tortoises (*Indotestudo forsteni*) two Impressed tortoises (*Manouria impress*) and a critically endangered Burmese star tortoise (*Geochelone platynota*) was attributed to Sulawesi tortoise adenovirus (STADV; genus *Siadenovirus*). My study developed a TaqMan quantitative PCR assay targeting the DNA polymerase gene of STADV for use in clinical diagnosis and epidemiologic surveillance. This assay failed to amplify five closely-related chelonian adenoviruses, indicating high analytical specificity. The assay performed with high efficiency (slope = -3.337; $R^2 = 0.999$), low intra-assay variability, and low inter-assay variability (coefficient of variation <1.36% at all standard curve dilutions). Dynamic range included $1.00 \times 10^7$ to $1.00 \times 10^1$ target copies per reaction and limit of detection was $10^1$ target copies per reaction, though $10^0$ target copies per reaction were intermittently detected. This qPCR assay provides a valuable diagnostic tool for characterization of STADV epidemiology, including potential identification of the North American reservoir host.

## INTRODUCTION

Adenoviridae are non-enveloped double-stranded DNA viruses with an icosahedral nucleocapsid containing a medium-sized genome (Benkő & Harrach 2003). Within the family Adenoviridae there are currently six accepted genera: *Mastadenovirus* members occur in mammals, *Aviadenovirus* species are found in birds, *Testadenovirus* species infect turtles, *Ichtadenovirus* includes a single virus recovered from a white sturgeon (*Acipenser transmontanus*), *Atadenoviruses* are believed to have cospeciated with squamate reptiles, but have also been detected in chelonians, birds, frogs, opossums, ruminant mammals, and tortoises, and *Siadenovirus* members occur in amphibians, chelonians, and birds (Benkő et al. 2022; Gibbons & Steffes 2013; Latney & Wellehan 2020; Marschang 2011). Generally, adenoviruses are host specific and are transmitted via the fecal-oral route or direct contact with oronasal secretions (Cook & Radke 2017). Pathology results from viral replication in host nuclei and subsequent cell lysis (Cook & Radke 2017).

Adenoviruses have emerged as potential threats to the health of reptiles (Doneley et al. 2014; Farkas et al. 2002; Jacobson et al. 1984; Julian & Durham, 1982; Kim et al. 2002; Raymond et al. 2003; Wellehan et al. 2004). While significant morbidity and mortality have been reported in snakes and lizards in association with adenovirus infection, chelonians have more recently been reported as suffering from clinical disease (Farkas & Gal 2009; Garcia-Morante et al. 2016; Rivera et al. 2009; Schumacher et al. 2012). Increased attention to adenoviruses in chelonians was garnered in 2007 after a mortality event involving a group of 105 Sulawesi tortoises (*Indotestudo forsteni*) was attributed to infection with a novel *Siadenovirus* (Rivera et al. 2009). Among the survivors, 69% of adenovirus PCR-positive animals were diagnosed with neoplasia (5 cases of lymphoma, 1 case of lymphoid leukemia, and 3 cases of myelomonocytic

leukemia) several months later (Rivera et al. 2009). This same *Siadenovirus*, named Sulawesi tortoise adenovirus 1 (STADV), was identified in a multispecies exhibit involving deaths of Impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*) at the same facility (Schumacher et al. 2012). STADV has been subsequently detected in apparently healthy free-living painted turtles (*Chrysemys picta*) in Illinois (unpublished data) and Indiana (Vincent et al. 2023), underscoring the fact that pathogenicity of adenoviruses in chelonians may include crossing species and causing clinical disease and death.

Diagnostic methods used for adenovirus identification in chelonians have included molecular detection (consensus nested polymerase chain reaction (PCR) and sequencing), direct visualization (electron microscopy, in situ hybridization, immunohistochemistry), and host response (histopathology) (Farkas & Gal 2009; Garcia-Morante et al. 2016; Rivera et al. 2009; Schumacher et al. 2012). The most commonly-reported method for diagnosing reptile adenoviruses is a consensus PCR that amplifies a partial sequence of the adenoviral DNA polymerase gene (Wellehan et al. 2004). Quantitative PCR (qPCR) has not been previously developed to detect Sulawesi tortoise adenovirus (STADV) in chelonians and may offer increased sensitivity compared to conventional PCR. This method would also enable the quantification of target copy numbers, and provide more rapid results than conventional PCR and sequencing.

The purpose of this study was to develop and validate a TaqMan qPCR assay for the detection of Sulawesi tortoise adenovirus (Order *Rowavirales*, Family *Adenoviridae*, Genus *Siadenovirus*) in chelonians.

## MATERIALS AND METHODS

*Conventional PCR, sequencing, and cloning*

Oral-cloacal swabs were collected from free-ranging painted turtles in Cook, Kane, and Lake Counties, Illinois, as part of an ongoing aquatic turtle disease surveillance program beginning in 2016. DNA was extracted using a commercially available kit according to manufacturer's instructions (QIAamp DNA Mini Kit, Qiagen, Valencia, CA) and DNA quantity and purity was measured via a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE).

Two-step nested conventional adenovirus PCR was performed as previously described (Chapter 3) on the extracted DNA, targeting the DNA-dependent DNA polymerase gene of adenoviruses (Wellehan et al. 2004). Reactions were performed in a 50 µl total volume using Taq Polymerase (1 U), 10× PCR buffer (final concentration 1X), 50mM MgCl2 (final concentration 3mM), dNTP mix (final concentration 0.2mM for each nucleotide), forward primer (final concentration 0.2µM), reverse primer (final concentration 0.2µM), and dH2O (Taq DNA Polymerase Recombinant 500 U, Invitrogen, Carlsbad, CA; 10mM dNTP mix, Invitrogen, Carlsbad, CA). Five microliters of template DNA from the primary PCR reaction were used in the secondary reaction. Primers utilized in the primary reaction included outer forward (polFouter: 5'-TNMGNGGNGGNMGNTGYTAYCC-3') and outer reverse (polRouter: 5'-GTDGCRAANSHNCCRTABARNGMRTT-3',) primers. Primers used in the secondary reaction included inner forward (polFinner: 5'-GTNTWYGAYATHTGYGGHATGTAYGC-3') and outer reverse (polRouter) primers to produce an approximately 450 base pair product as previously described (Doszpoly et al. 2013). DNA from an oral swab positive for eastern box turtle adenovirus 1 (JN632569) was used as a positive control and DNase/RNase-free water was

used as a negative control. The secondary PCR products were visualized by electrophoresis using a 1% agarose gel. Products producing bands of the appropriate size were treated with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA), sequenced in both directions (ACGT Inc., Wheeling, IL 60090 USA), and compared to known sequences in GenBank using BLASTN.

A 445bp PCR product that was > 99% identical to the STADV sequence in GenBank (accession number EU056826.1) was cloned into a commercially-available plasmid containing an ampicillin resistance gene (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA), then transformed into competent *Escherichia coli* (One Shot Top 10 F Chemically Competent Cells, Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from bacterial cultures (QIAfilter plasmid Maxi kit, Qiagen, Valencia, CA) and the cloning products verified through sequencing in both directions (ACGT, Inc., Wheeling, IL). Plasmids were linearized using restriction enzyme digestion (EcoR1, Clontech Laboratories, Mountain View, CA) and visually confirmed on 1% agarose gel. Phenol-chloroform extraction and ethanol precipitation was then performed to remove restriction enzyme reaction components. Finally, DNA quantity and purity was measured via a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE). Target copy number was calculated using the following formula:

$$\#copies/\mu L = [(ng\ DNA/\mu L) \times (6.022 \times 10^{23}\ copies/mol)] / [(base\ pair\ length) \times (1 \times 10^{9}\ ng/g) \times (650\ g/mol\ of\ base\ pair)]$$

Ten-fold serial dilutions of the linearized plasmid stock solution were made from $1.00 \times 10^{7}$ to $1.00 \times 10^{0}$ target copies per reaction. This standard curve was used to evaluate primer-probe performance.

*TaqMan qPCR assay*

Four TaqMan-MGB (TaqMan primers, FAM dye labeled, Applied Biosystems, Carlsbad, CA) qPCR assays were designed based on the sequence of the STADV DNA polymerase gene segment in our plasmid using Primer3 (Table 4.1; Korresaar & Remm 2007; Koressaar et al. 2018; Untergrasser et al. 2012). Real-time qPCR assays were performed using a real-time PCR thermocycler (Quantstudio 3 real-time PCR System, Applied Biosystems, Carlsbad, CA). Each reaction contained 12.5 µl of 20x TaqMan Platinum PCR Supermix-UDG with ROX (TaqMan Platinum PCR Supermix-UDG with ROX, Invitrogen, Carlsbad, CA), 1.25 µl 20x TaqMan primer-probe, 2.5 µl plasmid dilution, DNA sample, or non-template control (NTC; sterile water), and dH2O for a final reaction volume of 25 µl. Cycling parameters were as follows: 1 cycle at 50 °C for 2 min followed by 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 60 s, and a final cycle of 72 °C for 10 min.

*Analytical specificity*

Analytical specificity was evaluated using chelonian oral/cloacal swab DNA samples that had previously tested sequence-positive for adenoviruses via conventional PCR, as described above (Wellehan 2004). Samples included in analytical specificity testing included Blanding's turtle adenovirus 1 (GenBank accession number MW561636.1) from a Blanding's turtle (*Emydoidea blandingii*), Eastern box turtle adenovirus (GenBank accession number JN632574.1) from an Eastern box turtle, a novel adenovirus (93% identical to *Terrapene* adenovirus 1 [EU828750.1], GenBank accession number pending) detected in spotted turtles (*Clemmys guttata*), and two novel adenoviruses detected in painted turtles (*Chrysemys picta*), one of which was 91% identical to yellow-bellied slider adenovirus (GenBank accession number JN632578.1),

while the other was 81 – 82% identical to red-eared slider adenovirus (GenBank accession numbers JQ809465.1 and JX307095.1).

*Repeatability, dynamic range, limit of detection, and efficiency*

To evaluate repeatability, dynamic range, and limit of detection, assays were performed using five technical replicates for each standard curve dilution on two separate plates. Data assessed included cycle threshold (Cq) values of the plasmid dilutions and slope, y-intercept, and efficiency of the resulting standard curve which were calculated using commercial software (QuantStudio Design and Analysis Software version 1.5.2, Applied Biosystems, Carlsbad, CA). Intra-assay repeatability was determined by calculating the coefficient of variation (CV) for each STADV plasmid dilution ($10^7$–$10^0$ copies per reaction). Inter-assay variation was similarly calculated, using the values obtained from two duplicate plates for each assay. Dynamic range encompassed the range of standard dilutions over which the standard curve remained linear. Limit of detection (analytical sensitivity) was conservatively set as the lowest standard dilution where 95% of replicates successfully amplified. Efficiency curves were performed on adenovirus negative oral/cloacal swab DNA samples from wild painted turtles spiked with plasmid dilutions to ensure that this sample matrix would not interfere with assay efficiency.

*Statistical analysis*

In order to compare sensitivity of the qPCR assay for STADV to conventional PCR detection, data from red-eared sliders sampled in 2022 from Chapter 3 and Chapter 5 were evaluated via Pearson's chi square (significance set at $p<0.05$) and agreement was assessed with Cohen's kappa.

## RESULTS

*Analytical Specificity*

Assay STADV_107 amplified *Terrapene* adenovirus 1 and one of the painted turtle adenoviruses. Assay STADV_152 amplified one of the painted turtle adenoviruses and the spotted turtle adenovirus. Assay STADV_4 amplified Blanding's turtle adenovirus. These three assays did not undergo further validation testing. Only STADV_3 did not amplify any of the other adenovirus samples, so intra-assay variability, inter-assay variability, dynamic range, limit of detection, and efficiency in the presence of sample matrix (i.e., turtle oral/cloacal swab DNA) were evaluated for this assay alone.

*qPCR, standard curve, reproducibility*

Serial ten-fold dilutions of positive control plasmids were evaluated using TaqMan qPCR assay STADV_3. These generated amplification plots (Fig. 4.1A) and standard curves based on Cq values (Fig. 4.1B). The dynamic range for STADV_3 was $1.0 \times 10^7$ - $1.0 \times 10^1$ copies per reaction with an $R^2$ of 0.999 and slope of -3.337 (Fig. 4.1B). The limit of detection was $10^1$ target copies per reaction, though $10^0$ target copies per reaction were intermittently detected. Intra-assay CV for STADV_3 ranged from 0.22 % to 1.36 %, while inter-assay CV was between 0.2 % and 0.88 % (Table 4.2). The results indicate high reproducibility between assays at all dilutions.

When compared to the standard curves from plate 1 (slope -3.337, $R^2 = 0.999$, efficiency 99.362 %), and plate 2 (slope -3.361, $R^2 = 0.999$, efficiency 98.381 %), the standard curve spiked into adenovirus-negative turtle oral/cloacal swab DNA demonstrated equivalent performance (slope -3.403, $R^2 = 1$, efficiency 96.732 %). A standard curve slope between -3.6 and -3.1 (90–

110 % efficiency) with a coefficient of variance ($R^2$) above 0.98 is considered acceptable for most diagnostic qPCR assays (Broeders et al., 2014). The STADV_3 assay achieves levels of efficiency and variation within these acceptable ranges.

The qPCR assay detected significantly more STADV positives than conventional PCR, when using results from Chapters 3 and 5 ($X^2 = 71.689$; $p<0.00001$). Red-eared sliders in 2022 had 72.1% (31/43) detection rate for STADV with qPCR and 5.7% (6/105) with conventional PCR. There was no agreement between the two methods, (Kappa statistic =-0.453, CI -0.605- -0.300)

## DISCUSSION

Adenoviruses have been described in squamates, chelonians and crocodilians and have been associated with morbidity and mortality (Doneley et al. 2014; Farkas et al. 2002; Jacobson et al. 1984; Julian & Durham, 1982; Kim et al. 2002; Raymond et al. 2003; Wellehan et al. 2004). Chelonian adenoviruses have been more recently described, and associations with clinical disease have yet to be elucidated for many viruses (Farkas & Gal 2009; Garcia-Morante et al. 2016; Rivera et al. 2009; Schumacher et al. 2012). STADV infection was responsible for a large-scale mortality event involving over 100 endangered and critically endangered chelonians (Rivera et al. 2009; Schumacher et al. 2012). The high mortality despite treatment and subsequent development of neoplasia in survivors elevates this virus as an important disease concern for threatened or naïve chelonian populations (Rivera et al. 2009; Schumacher et al. 2012).

This study developed a quantitative PCR assay that allows for reliable and rapid (<24hr) detection of a 100 base pair segment of the STADV DNA-dependent DNA polymerase gene

with as few as 10 target copies per reaction. The assay is specific, highly efficient, and repeatable between runs. Improved identification of subclinical carriers may help zoological institutions that manage chelonians safeguard their collections, and may help biologists and veterinarians guide management decisions for conservation programs overseeing free-ranging chelonians.

More research is needed to examine the epidemiology and management implications of chelonian pathogens. In the case of STADV, to the authors' knowledge, no reported deaths have occurred since the original outbreak involving Sulawesi tortoises, Impressed tortoises and a Burmese star tortoise. The original cohort of affected Sulawesi tortoises originated from outside the United States, indicating that STADV infection may pose an international threat (Rivera et al. 2009). However, two-step nested conventional PCR and sequencing of oral-cloacal swabs have previously identified this virus in apparently-healthy painted turtles from the Midwestern United States (Vincent et al., 2023). This underscores the importance of testing subclinical animals to help elucidate the origin and epidemiology of this virus, including identifying potential reservoir hosts. The STADV TaqMan qPCR is reliable, sensitive and specific for the detection of the STADV DNA polymerase gene. The assay provides a valuable tool that should be used to understand further and characterize the epidemiology and disease ecology of Sulawesi tortoise adenovirus in North America and globally.

## TABLES & FIGURE

Table 4.1. Primer-probe sequences and product sizes for four TaqMan qPCR assays targeting the DNA-dependent DNA polymerase gene of Sulawesi tortoise adenovirus.

| Assay Name | Product Size | Primer-Probe | Sequence |
|---|---|---|---|
| STADV_107 | 98 bp | Forward Primer | 5'- AGC TAC TTC GAC CAC GAA ATT A -3' |
| | | Probe | 5'- TCT CCA TTT CAG CTT TAC CAC CAC CTG T -3' |
| | | Reverse Primer | 5'- CGA CTA CAA AGT GGA GGT ATG T -3' |
| STADV_152 | 103 bp | Forward Primer | 5'- GCT TTA CCA CCA CCT GTA GAA -3' |
| | | Probe | 5'- AAA GCG GAC GTC TTT GTT GGA CAA -3' |
| | | Reverse Primer | 5'- CAG TTT CGT CGT ATA GAG GTT CA -3' |
| STADV_3 | 100 bp | Forward Primer | 5'- ACA ACT GCG GAT GGA AAG T -3' |
| | | Probe | 5'- TTT CCA AGA TGG AAC ACG TGC TGT -3' |
| | | Reverse Primer | 5'- GCT ATG TTA ACC TCG ACG TAT TCT -3' |
| STADV_4 | 76 bp | Forward Primer | 5'- ACT AAC ATA CCT CCA CTT TGT AGT C -3' |
| | | Probe | 5'- AAA GCG GAC GTC TTT GTT GGA CAA -3' |
| | | Reverse Primer | 5'- CAG TTT CGT CGT ATA GAG GTT CAT -3' |

Table 4.2. Intra and inter-assay variability in cycle threshold values for the TaqMan qPCR SADV assay using a seven-point standard curve from 10-$10^7$ SADV target copies per reaction.

| | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| Target Copy Number | CT Mean | CT SD | CT CV (%) | CT Mean | CT SD | CT CV (%) |
| 10,000,000 | 15.318 | 0.034 | 0.22 | 15.37 | 0.0308 | 0.2 |
| 1,000,000 | 18.791 | 0.13 | 0.69 | 18.864 | 0.094 | 0.5 |
| 100,000 | 22.201 | 0.062 | 0.28 | 22.261 | 0.06 | 0.27 |
| 10,000 | 25.578 | 0.09 | 0.35 | 25.641 | 0.083 | 0.32 |
| 1,000 | 28.984 | 0.075 | 0.26 | 29.022 | 0.113 | 0.39 |
| 100 | 32.231 | 0.113 | 0.35 | 32.295 | 0.125 | 0.39 |
| 10 | 35.464 | 0.364 | 1.03 | 35.432 | 0.303 | 0.856 |
| 1 | 38.676 | 0.526 | 1.36 | 38.445 | 0.337 | 0.88 |

Figure 4.1. A. Amplification plot of the standard curve for TaqMan probe-based STADV_3 assay obtained with 10-fold serial dilutions from $1 \times 10^7$ to $1 \times 10^1$ target copies per reaction. B. Standard curve for a TaqMan probe-based STADV_3 assay with 10-fold serial dilutions from $1 \times 10^7$ to $1 \times 10^1$ target copies per reaction. This graph is plotted against logarithmic concentration of the serial dilution. Slope: -3.403, Y-Intercept: 39.026, $R^2$ : 1, Efficiency: 96.732 %.

A.

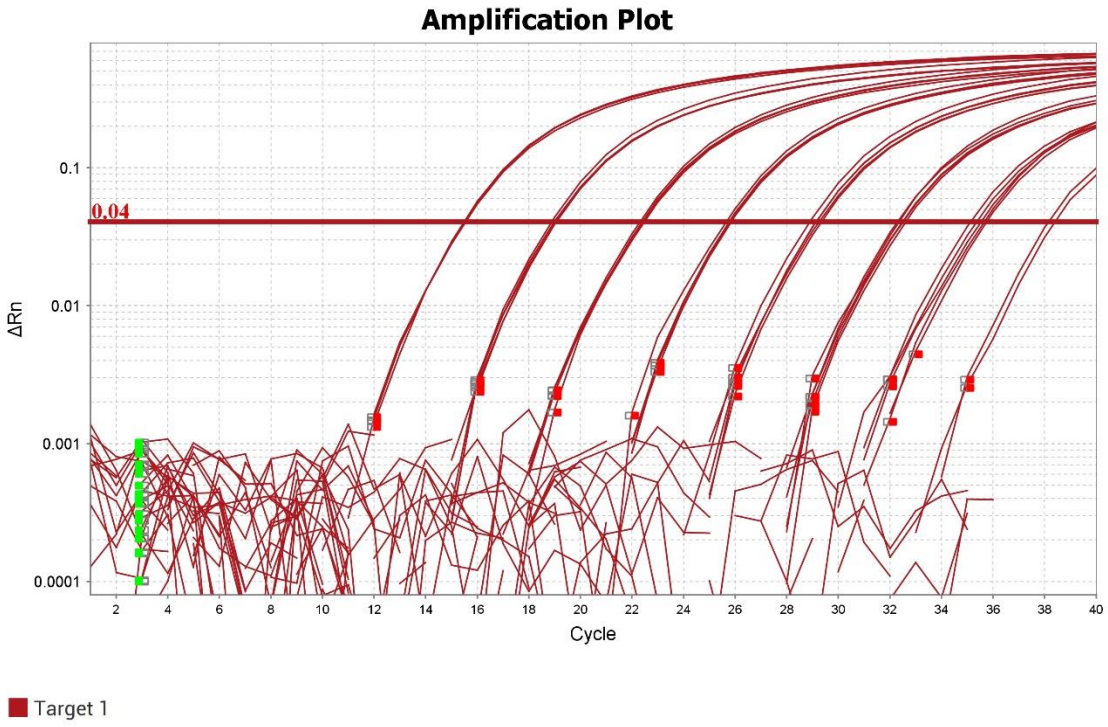
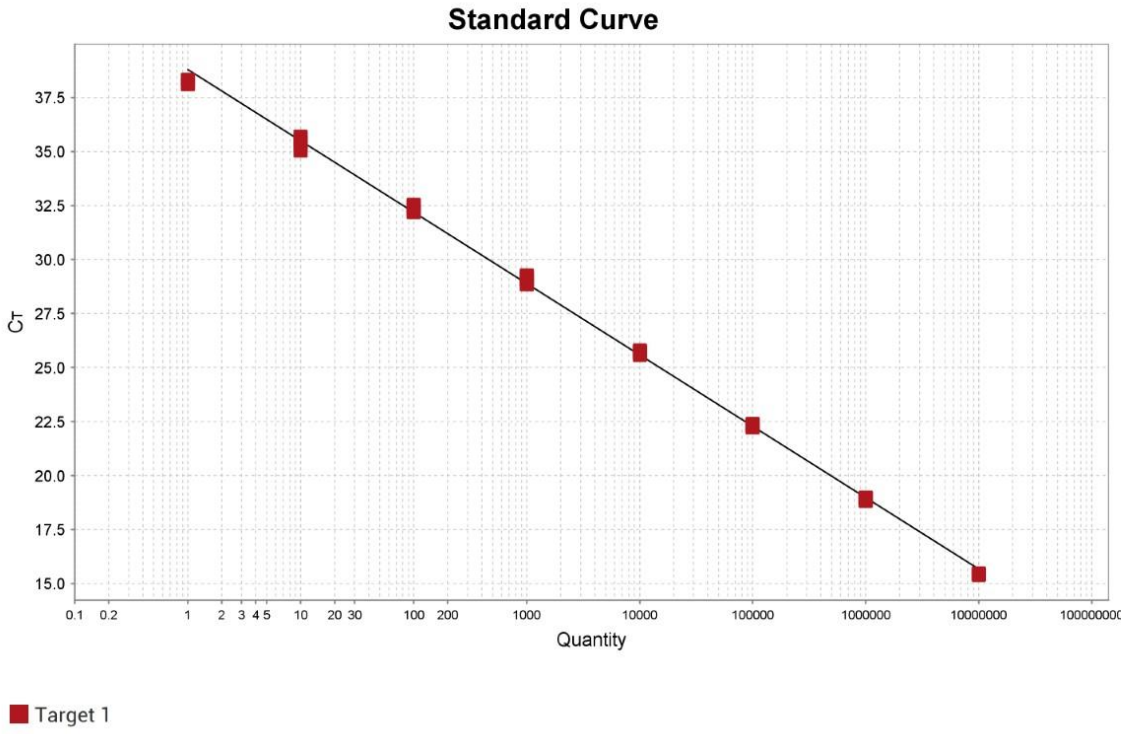


Figure 4.1. B. Standard curve for a TaqMan probe-based STADV_3 assay with 10-fold serial dilutions from $1 \times 10^7$ to $1 \times 10^1$ target copies per reaction. This graph is plotted against logarithmic concentration of the serial dilution. Slope: -3.403, Y-Intercept: 39.026, $R^2$ : 1, Efficiency: 96.732



# CHAPTER 5: PREVALENCE AND EPIDEMIOLOGY OF SULAWESI TORTOISE ADENOVIRUS IN FREE-RANGING BLANDING'S TURTLES (*EMYDOIDEA BLANDINGII*), PAINTED TURTLES (*CHRYSEMYS PICTA*) AND RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) IN ILLINOIS, USA

**ABSTRACT:** Sulawesi tortoise adenovirus (STADV) was associated with an outbreak of multisystemic disease with high mortality in three endangered specie of tortoises and has subsequently been detected in apparently healthy, free-living painted turtles. However, the impact of STADV on free-ranging populations remains unknown. The specific objectives of my study were to: 1) estimate the prevalence of STADV in free-ranging Blanding's turtles (*Emydoidea blandingii*), painted turtles (*Chrysemys picta*) and red-eared sliders (*Trachemys scripta elegans*) in Illinois using qPCR, and 2) determine if qPCR prevalence is associated with sex, age class, location, hematologic findings, or clinical signs across these three species. Oral-cloacal samples from 581 Blanding's turtles, 137 painted turtles, and 82 red-eared sliders sampled across three counties from 2017-2022 were evaluated via qPCR. The prevalence of STADV was 2.4% for Blanding's turtles, 14.9% for painted turtles, and 45.1% for red-eared sliders (n=37). Median viral copy numbers were 688.95 copies/ng DNA for Blanding's turtles, 17,131.15 copies/ng DNA for painted turtles, and 4,092.07 copies/ng DNA for red-eared sliders. Blanding's turtle subadults ($p=0.022$) and painted turtle adults ($p<0.0001$) were more likely to

test positive than their other age classes, while no age class association was found in red-eared sliders ($p=0.5$). In a multivariable model, significant predictors for STADV detection included species, with painted turtles ($p<0.0001$) and red-eared sliders ($p<0.0001$) more likely to test positive than Blanding's turtles, and year, with turtles sampled in 2021 less likely to test positive than those in other years ($p=0.005$). Clinical signs associated with STADV detection included quiet, alert, responsive (QAR) mentation ($p=0.002$), pink mucous membranes ($p<0.001$), carapacial abnormalities ($p=0.036$) and plastron abnormalities ($p=0.003$). STADV detection was not significantly associated with sex, location, month, hematologic findings, biochemical findings or protein electrophoretic profiles. However, subadult Blanding's turtles and adult painted turtles were more likely to have STADV detected, while age class was not associated with STADV detection in red-eared sliders. Widespread molecular detection of STADV in these three species suggests a possible origin for the virus in the original outbreak, underscoring the importance of epidemiology studies to aid in the management of free-ranging and managed chelonians.

## INTRODUCTION

Blanding's turtles (*Emydoidea blandingii*) are semiaquatic chelonians native to the northeastern United States and Great Lakes region, and are considered endangered by the International Conservation of Nature (IUCN; Ernst et al. 2009; van Dijk et. al 2011). The turtles are declining throughout their range secondary to habitat destruction and fragmentation, predation, and vehicular mortality (van Dijk et al. 2011). While infectious diseases, namely ranaviruses, herpesviruses, and *Mycoplasma* spp. have contributed to significant morbidity and mortality in other free-ranging chelonians, the impact of pathogens on this species remains poorly understood (Allender et al. 2009; Andersson et al. 2021; Berish et al. 2010; Brown et al. 1994; Butkus et al. 2017; Dziadzio et al. 2018; Lindemann et al. 2018; Lindemann et al. 2019; McKenzie et al. 2019; Winter et al. 2020). Painted turtles (*Chrysemys picta*) and red eared sliders (*Trachemys scripta elegans*) are commonly found in the same habitats as Blanding's turtles and are widespread in Illinois (Ernst et al. 2009). Despite their robust conservation status, pathogen prevalence is incompletely described for free-ranging painted turtles and red-eared sliders. Both species are frequently displayed in zoological institutions and kept as pets. Establishing baseline pathogen prevalence data and characterizing disease epidemiology will aid in the management of chelonians in managed-care, and in turn, support *in situ* and *ex situ* conservation efforts.

Adenoviruses are nonenveloped double-stranded DNA viruses which replicate in host nuclei and are released when the host cell ruptures (Gibbons et al. 2013; Latney et al. 2013; Marschang et al. 2011). There are currently six accepted AdV genera: *Mastadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Atadenoviruses*, *Testadenoviruses* and *Siadenovirus* (Benko et al. 2022; Doszpoly et al. 2013; Franzen-Klein et al. 2020; Gibbons et al. 2013; Latney et al. 2013; Marschang et al. 2011; Salzmann et al. 2021). Current taxonomy in the family Adenoviridae

suggests that these viruses coevolved with their hosts, with subsequent host switches (Marschang et al. 2011). Host-adapted viruses may not cause disease in their host species or may only cause clinical disease in immunocompromised individuals (Benkö et al. 2003). However, understanding the behavior and pathogenicity of these viruses remains crucial, as subsequent host switches may lead to significant morbidity and mortality (Benkö et al. 2003; Marschang et al. 2011;).

Adenoviruses present a potential disease concern for chelonians. The first report in chelonians involved a single, moribund leopard tortoise (*Stigmochelys pardalis*) with biliverdinuria and episodes of hemorrhage (Gibbons et al. 2013). In 2009, the U.S. Fish and Wildlife Service confiscated a group of 105 Sulawesi tortoises (*Indotestudo forsteni*) upon entry into the United States (Rivera et al. 2009). Upon entry, this group experienced multisystemic disease signs or sudden death, with significant mortality (82%) despite aggressive treatment (Rivera et al. 2009). The causative agent was later determined to be a novel *Siadenovirus*, named Sulawesi tortoise adenovirus (STADV; Rivera et al. 2009). Most surviving tortoises presented with hemolymphatic neoplasia after recovering from the event (Rivera et al. 2009). Since then, this same virus has been implicated in the deaths of Impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*) at the same facility (Schumacher et al. 2012). All three species are listed as endangered or critically endangered (Rhodin et al. 2018). STADV has been subsequently detected in apparently healthy free-living painted turtles (*Chrysemys picta*) in Illinois (unpublished data) and Indiana (Vincent et al. 2023). This underscores that the pathogenicity of adenoviruses in chelonians may include crossing species and causing significant clinical disease and death.

Despite the occurrence of STADV in endangered tortoises and recently in apparently healthy free-living turtles in the Midwestern United States, the impact of this virus on free-ranging populations is still unknown. Quantitative PCR (qPCR) has recently been developed for the detection of Sulawesi tortoise adenovirus (STADV) in chelonians (Chapter 4) and offers a more sensitive and rapid method for detection than conventional PCR. Therefore, the specific objectives of this study were to: 1) estimate the prevalence of STADV in free-ranging Blanding's turtles, painted turtles and red-eared sliders in Illinois using qPCR, and 2) determine if STADV detection is associated with sex, age class, location, hematologic findings, or clinical signs across three species. I hypothesize STADV positive turtles will not show significant clinical signs of disease, indicating that STADV is likely a host-adapted virus in these emydid turtles.

## MATERIALS & METHODS

*Sample populations*

Oral-cloacal swabs were collected from free-ranging Blanding's turtles, painted turtles, and red-eared sliders in three counties in Illinois as part of an ongoing aquatic turtle disease surveillance program beginning in 2016, other aspects of the health assessments from those samples are reported previously (Andersson et al. 2021; Mumm et al. 2019; Winter et al. 2020). Blanding's and painted turtles were sampled at four sites in Kane County, IL, including Hannaford (HAN), Willoughby (WIL), Pingree (PIN), and Freeman Kame (FRE). Blanding's were also sampled at three sites in Lake County, including Chiwaukee (C), Spring Bluff (SB), and Illinois Beach State Park (IBSP). In Cook County, Blanding's were sampled at three sites, including Powderhorn, Algonquin Woods, and Orland areas, and red-eared sliders were sampled in three areas, including Powderhorn, Burnham, and Flatwoods. These samplings were performed from mid-May to mid-October from 2016-2022. All animal sampling was permitted

by the following orga- nizations: Illinois Department of Natural Resources (IDNR; Scientific Collectors Permits [SCP] NH17.5065, NH18.5065; IDNR Endangered and Threatened permits SBT-16-062, 1199, 14-046, and 1042), the Wisconsin Department of Natural Resources (WIDNR; SCP: SCP-SOD-004-2013; WIDNR Scientific Research License SRLN-18-026), and the University of Illinois Institutional Animal Care and Use Committee (Protocols 15017, 15158, 18000, 18165, 20258).

*Capture Methods*

Turtles were captured using radiotelemetry (Blanding's only), in a hoop trap, or incidentally by hand during fieldwork. The turtles captured via telemetry were juveniles, subadults and adults being tracked as a part of a chelonian monitoring program. Telemetry, traps and incidental captures were used at all field sites.

*Physical Examination and Sample Collection*

Each turtle was assigned a permanent ID and mass, sex, and age class was recorded. Blanding's turtles were characterized as juvenile (<250 grams), sub-adult (250-750 grams), or adult (>750 grams). Sex was classified as male, female or unknown based on plastron concavity, position of the cloaca, and/or nest incubation temperature for head-started juveniles. Painted turtles were categorized as juveniles or adults based on total plastron length (TPL) using ranges established in painted turtle populations of Wisconsin. Males with a plastron length greater than 85 mm or exhibiting mature secondary sex characteristics (e.g. elongated nails on the front feet) were classified as adults, while females with a plastron length greater than 130 mm were considered adults (Ross 1989; St. Clair et al. 1994). Red-eared sliders were classified as adults if

the straight carapace length was > 100 mm. Sexing of red-eared sliders was based on secondary sex characteristics, with males identified based on elongated front claws and a cloacal opening past the caudal edge of the carapace. Physical examinations were performed, noting visual appearance of the eyes, nose, oral cavity, ears, legs, digits, shell, integument, and cloaca.

Physical examination (Newman et al. 2019, Winter et al 2020), clinical pathology (Mumm et al. 2019), and protein electrophoresis (Andersson et al. 2021) values for samples tested utilized data acquired in concurrent projects.

*Polymerase chain reaction*

Quantitative PCR was performed as previously described (Chapter 4). Briefly, DNA was extracted from oral swabs using a QIAmp Blood Mini Kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, California, USA). A TaqMan assay was performed as described in Chapter 4. All samples were assayed in three technical repeats using a real-time PCR thermocycler (7500 ABI Real-Time PCR System, Applied Biosystems, Carlsbad, California, USA) and analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems). Negative controls and plasmid-derived positive controls were included on each plate. Quantities were produced by comparing each sample's cycle threshold value to that of a seven-point standard curve. Resulting quantities were normalized based on the DNA concentration of each sample.

*Statistical analyses*

Descriptive statistics were tabulated for all continuous variables (mass, viral copy number, PCV, TS, white blood cell count, etc.). Normality was assessed using the Shapiro-Wilk

test, Q-Q plots, skewness, and kurtosis. Mean, SD, and 95% confidence interval (CI) are reported for normally distributed data. Median and 10–90 percentiles are reported for non-normally distributed data. An independent samples *t*-test and a one-way analysis of variance were used to determine between- and within-group differences, respectively, in normally distributed data. A Mann-Whitney *U*-test and a Kruskal-Wallis one-way analysis of variance were used when data were not normally distributed. Pearson's chi-square and Fisher's exact tests were used to test for associations between STADV detection and age class, sex, year, location (County), and clinical signs. Odds ratios were calculated based on STADV-positive status. Statistical significance was considered when $p<0.05$. All univariate analysis was performed using Prysm statistical software (GraphPad Software, San Diego, California, USA).

Prevalence of STADV was estimated by calculating the 95% binomial confidence interval (Wilson, 1927) in total and by species (Blandings, Painted, RES), year (2017, 2019, 2020, 2021, 2022), month (May, June, July, August, September, October) sex (female, male, unknown), age class (adult, subadult, juvenile), and county (Lake, Kane, Cook). Variables with a significance of <0.1 in univariate analysis were included in a series of logistic regression models to evaluate the effects of independent variables (species, year, month, sex, age class, county) on the output variable (STADV detection). Reference levels in the models were established for each categorical variable: species (Blanding's), year (2017), month (May), sex (Female), age class (Adult), and county (Lake). Next, an information theoretic approach was used to determine which model from the candidate set was most parsimonious using the aiccmodavg package (Mazerole, 2017). Odds ratios (OR) were then calculated for significant predictors using epidisplay package (Chongsuvivatwong 2018).

Longitudinal samples from 50 turtles that were sampled serially (at least two or more years) were evaluated for STADV presence. Descriptive statistics were tabulated for age class, sex, species, county of sampling, and viral copy number (when individuals were positive).

## RESULTS

Oral-cloacal samples from 581 Blanding's turtles, 137 painted turtles, and 82 red-eared sliders were evaluated via qPCR. The sex and age class breakdown of the sampled turtles is shown in Table 5.1. Turtles were sampled in the years 2017 and 2019-2022 (Table 5.1). Turtles were sampled from May-October. Blanding's turtles were sampled in Kane and Lake Counties in Illinois, while painted turtles were sampled in Kane County (Table 5.2) and red-eared sliders were sampled in Cook County, Illinois, USA (Table 5.2).

In total, 75 turtles tested positive by qPCR for STADV for an overall prevalence of 9.3% (95% CI: 7.5-11.6%). These included 14 Blanding's turtles (2.4%; 95% CI: 1.4-4%; see Table 5.3), 24 painted turtles (14.9%: 95% CI: 10.2-21.2%; see Table 5.4), and 37 red-eared sliders (45.1%; 95% CI: 34.8-55.9%; Table 5.5).

### *Univariate analysis*

When evaluating STADV prevalence by species, for Blanding's turtles, subadults were more likely to be positive (5/84; 6.0%; CI: 2.6-13%; $p=0.022$) compared to adults (7/242; 2.9%; CI: 1.4-5.8%) and juveniles (2/232; 0.9%; CI: 0.24-3.1%). There was no significant difference in prevalence based on sex ($p=0.78$), county ($p>0.99$), month ($p=0.79$), or year ($p=0.32$). For painted turtles, the majority of positive turtles were adults (23/98; 23.5%; CI: 16.2-32.8%; $p<0.00001$) compared to juveniles (1/137; 0.7%; CI: 0.13-4.0%). There was no significant

difference in prevalence based on sex (p=0.65), month (p=0.71), or year (p=0.62). For red-eared sliders, there was no significant difference in STADV prevalence based on age class (p= 0.5) or sex (p=0.48). However, turtles sampled in September were more likely to be positive (p=0.003; 90%; 95% CI: 59.6-99.5%) than turtles sampled in May (25%; CI: 12-44.9%), August (36.7%; CI: 21.8-54.5%) or October (58.8%; CI: 36.0-78.4%). Furthermore, all turtles sampled in 2022 were more likely to test positive (p<0.001; 72.1%; CI: 57.3-83.3%) than those sampled in 2021.

Clinical signs not associated with STADV detection included: oral discharge (p=0.728), oral plaques (p=0.901), ocular swelling (p=0.654), ocular discharge (p=0.588), nasal discharge (p=0.427), tympanum abnormalities (p=0.905), appendage abnormalities (p=0.431), cloacal abnormalities (p=0.382), presence of ectoparasites (p=0.478), or diarrhea (p=0.346). Mucous membrane color was significantly different between positive and negative individuals with negative individuals having higher association with light pink (n=244; 46.7%) compared to positive individuals associated with pink mucous membranes (n=30; 52.6%) (p<0.001). Quiet alert, responsive mentation was more common in STADV-positive turtles (n=16; 29.6%) than negative individuals (n=50; 12.9%) (p=0.002). Additionally, turtles testing positive for STADV had more carapacial (n=49; 80.3%; p=0.036) and plastron abnormalities (n=49; 80.3%; p=0.003) than negative individuals (n=370; 68.5% and n=335; 62.2%, respectively).

Viral copy numbers for Blanding's turtles, painted turtles and red-eared sliders were non-normally distributed. The median viral copy number for Blanding's turtles was 689.0 copies/ng DNA, range: 109-27,277; 10–90 percentiles: 126.7-12,113. The median viral copy number for painted turtles was 17,131 copies/ng DNA, range: 85.69-32,456,794; 10–90 percentiles: 113.5-23,273,171. The median viral copy number for red-eared sliders was 4,092 copies/ng DNA, range: 140.8-5,628,929; 10–90 percentiles: 234.8-161,779. Viral copy number for painted turtles

and RES was significantly higher than for Blanding's turtles (p=0.0062 and p=0.01, respectively).

A total of 464 Blanding's turtles, 35 painted turtles and 75 red-eared sliders had hematology with differential counts performed within this study. STADV-positive Blanding's turtles had a lower eosinophil concentration (p=0.03) than negative Blanding's turtles. Painted turtles did not have any significant differences in hematology based on STADV status. Red-eared sliders that tested positive for STADV had higher packed cell volume (p=0.04) and eosinophil concentrations (p=0.01) than negative red-eared sliders in the univariate analysis. Protein electrophoresis was performed for 312 Blanding's turtles and 133 painted turtles but not red-eared sliders. There were no significant differences in plasma protein electrophoretic profiles between STADV-positive and negative individuals.

*Multivariable logistic regression*

The final data set for the model included 634 individuals representing 299 adults, 121 subadults, and 213 juveniles. There were 566 negative and 67 STADV positive turtles. There were 435 Blanding's, 120 painted turtles, and 78 red-eared sliders. There were 306 females, 236 males, and 91 turtles of unknown sex. There were 94 turtles sampled in 2017, 117 in 2019, 122 in 2020, 130 in 2021, and 170 in 2022. June (n=281) was the most sampled month, followed by May (n=144), July (n=130), August (n=55), October (n=13), and September (n=10). The top model accounted for 32% probability of best approximating model (aiccwt), while all of the top 5 models all included species and year. The final model included the following variables: species, year, and age class (p<0.0001). Significant predictors for STADV detection included species, with painted turtles (p<0.0001) and red-eared sliders (p<0.0001) more likely to test

positive than Blanding's turtles, and year, with turtles sampled in 2021 less likely to test positive than those in other years (p=0.005). Red-eared sliders had a 42.72 (95% CI: 19.27-94.67, p=<0.001) and painted turtles had a 10.04 (95% CI: 4.08-45.77, p<0.0001) times higher odds of having STADV DNA detected, respectively, compared to Blanding's turtles. Turtles sampled in 2021 were 2.08 times less likely to be detected with Sulawesi adenovirus DNA than turtles in 2017 (p=0.005), no other significant differences between years were observed. Despite being in the top model, no age class pairwise comparisons were significant.

*Longitudinal Analysis*

Longitudinal samples (n=162) from 50 turtles were evaluated for STADV presence. This included 12 samples from six red-eared sliders (each collected once during 2021 and 2022 field seasons), 24 samples from 11 painted turtles (each collected 1-2 times from 2019-2021 field seasons), and 126 samples from 33 Blanding's turtles (collected 2-9 times from 2017-2022 field seasons; n=13 from Kane County, n=20 from Lake County). The majority of Blanding's turtles sampled were adult females (n=28), while the remainder were juvenile males (n=5). None of the Blanding's turtles included as part of the longitudinal study had STADV detected at any time point.

Painted turtles sampled over time included nine adult males, one adult female, and one juvenile female. STADV was detected in 6/24 samples (25%; CI: 12-44.9%) representing 4/11 individuals; this included three adult males and one adult female. Two of the four turtles had STADV detected twice (one sampling event each across two years), while the remaining two had STADV detected in one year and not detected in the following year of sampling. Viral copy number was 4,169,966 copies/ng DNA in June 2019 and 24,513 copies/ng DNA in July 2021 for

one of the turtles in which STADV was detected repeatedly, while the second animal had an increase in viral copy number from 119 copies/ng DNA in July 2020 to 1,226,400 copies/ng DNA in June 2021. The animals with only a single detection event had low viral copy numbers (206.36-1,106.63 copies/ng DNA).

Red-eared sliders sampled over time in Cook County included four adult females and two adult males; two adult females and one adult male had STADV detected once out of two sampling events (3/12; 25%; CI: 8.9-53.2%), all were in August, September, or October 2022. Viral copy number ranged from 492-13,537 copies/uL. STADV was only detected in 2022 and not in 2021.

## DISCUSSION

This is the first epidemiologic investigation of Sulawesi tortoise adenovirus in any species, and the first study to employ qPCR to detect STADV in turtle samples. Of the three species evaluated, red-eared sliders had the highest prevalence (45.1%) for STADV detection, followed by painted turtles (14.9%) and Blanding's turtles (2.4%). The detection of STADV in red-eared sliders and Blanding's turtles represents two novel species hosts for this adenovirus.

Red-eared sliders and painted turtles were significantly more likely to test positive for STADV than Blanding's turtles. Adenoviruses appear to be commonly detected in *Trachemys* turtles, including red-eared sliders; however, previous surveillance in Europe has occurred without detecting STADV (Doszpoly et al. 2013; Salzmann et al. 2021). This is the first study to document STADV in free-living North American red-eared sliders.

STADV has been detected previously via consensus PCR in oral-cloacal swabs from four painted turtles as part of a disease surveillance study in a restored wetland habitat in

northwestern Indiana, USA (Vincent et al. 2023). The prevalence in that study (10%) is similar to the prevalence determined in this study (14.9%). While these prevalences are similar, consensus PCR which was used in the Vincent et. al (2023) study is often less sensitive than qPCR; therefore, it is possible that STADV prevalence is underreported in that sample population.

Infectious diseases can occur as epidemics under certain circumstances and as sporadic outbreaks or endemic processes in others (Riley et al. 2019). *Mycoplasma agassizii* can occur at very high frequencies in some tortoise populations in the western USA; however, it only causes disease when it reaches high quantities within animals (Aiello et al. 2016; Sandmeier et al. 2018). Animals remain chronically infected, and the immune system appears to keep clinical infections infrequent; however, it is unclear whether episodes of clinical disease are precipitated by changes in immune function or by differences in strains of *M. agassizii* (Luzuriaga-Neira et al. 2021). Although American alligators will succumb to clinical disease from West Nile Virus, they also appear to be able to serve as amplifying hosts for WNV and therefore as possible reservoirs for the virus (Byas et al. 2022). This provides further evidence within reptile taxa that infectious agents can persist within hosts without causing clinical disease, and yet still have the potential to cause outbreaks when permissive host, pathogen, and/or environmental conditions occur. STADV caused significant mortality in the group of confiscated Sulawesi tortoises that were imported illegally into the United States. However, the detection of STADV in red-eared sliders and painted turtles with a high prevalence and absence of significant clinical signs of disease may represent an endemic presence of STADV in these populations of turtles, but ongoing repeated findings are needed. An additional consideration would be that the strain that caused the outbreak reported in Rivera et al. (2009) and Schumacher et al. (2012) could be

different from that found in the native emydid turtles described here; however, this relationship could only be elucidated with whole-genome sequencing or virus isolation, which was unavailable from the original studies and outside the scope of the present study.

The decreased prevalence of STADV in Blanding's turtles compared to RES and painted turtles despite sharing the same habitat is worth noting. The nature of the study design by which turtles are captured and sampled has limitations, including the likelihood that individuals experiencing more severe clinical disease and death are likely underrepresented, because they are not found. While much remains unknown, lower STADV prevalence in the Blanding's turtles may indicate that individuals experience more severe clinical disease or mortality when infected by STADV, resulting in much lower numbers of animals with the virus being detected. However, this currently appears unlikely at least in the Lake County Blanding's turtle population, given that surveys indicate the population is stable and recent measures have deemed it unlikely to be extirpated (Glowacki et al. 2018). Furthermore, the turtles are tracked via radiotelemetry and when they die, carcasses are frequently recovered and may be available for testing. Painted turtles and RES may serve as a host-adapted species for STADV, enabling viral persistence in the wetland habitats, while Blanding's turtles may become affected more sporadically or with more severe clinical disease. One similar disease model is that of *Cytauxzoon felis*, in which wild felids are the reservoir for this parasite and result in persistence of the infection in an area, while domestic cats that come into contact with them often succumb to infections within 9-15 days postinfection (Wang et al. 2017). However, there is mounting evidence that cats that survive nonlethal infection may also serve as an additional reservoir. Likewise, the low number of Blanding's turtles with STADV detected may represent a species that is more resistant to disease, or individuals that have survived a clinical course of disease and are now serving as an

additional reservoir. The lack of shedding observed in repeat sampling of telemetered turtles could be explained by several possibilities: (1) these animals are not persistently viremic but still harbor the virus as a host-adapted species and the viral copies present in oral-cloacal samples are below the qPCR assay's limit of detection of 10 copies/rxn, (2) these turtles undergo a period of clinical disease after which they become asymptomatic carriers where viremia and thus viral shedding rates may be variable, or (3) these turtles can clear the presence of the virus altogether and eliminate it from their system, resulting in lack of detection in sequential years. Future work could include development of serologic testing to determine past exposure if turtles are no longer actively shedding adenoviral DNA. However, there are challenges associated with validating and interpreting serologic assays in reptiles, including the need for specific anti-reptile immunoglobulins that recognize and bind the antibody being assayed (Jacobson et al. 2002).

In humans, adenoviruses are reported as being endemic throughout the year and can result in chronic and latent infections, which involves asymptomatic infection of lymphoid tissue (Usman et al. 2023). In chelonians, latency is less understood and turtles lack lymph nodes. Currently there is no evidence to support subclinical or latency of other DNA viruses such as herpesviruses in chelonians, as there is in mammals (Hardman et al. 2012). It is unknown if asymptomatic turtles experience disease-related mortality; the lack of follow up and associated challenges with monitoring wildlife disease make conclusions like this challenging. Likewise, it is unknown if chelonian adenoviruses can establish latency following infection and if so, where the virus localizes within the body during non-shedding periods. However, adenoviral latency could explain the persistence of STADV in the populations of RES and painted turtles.

No seasonal differences were detected for STADV-positive status in the logistic regression model. However, in red-eared sliders, turtles sampled in September were more likely

to be positive than turtles sampled in May, August or October, and was overall more commonly detected in the fall months. This is similar to other studies that have shown *Terrapene* herpesvirus 1 to have higher prevalence in fall than in summer or spring over the course of a 2 yr period (Kane et al. 2017). In contrast, *Emydoidea* herpesvirus 1 in Blanding's turtles was more common in the spring or summer and fall months (Lindemann et al. 2019). There were only 2-3 years evaluated in this study for red-eared sliders and painted turtles, and not all animals were sampled over the same years; therefore, a clearer pattern of seasonal emergence may have been observed if more years of detection were included in the study.

In our study, Blanding's turtle subadults were more likely to test positive than their adult or juvenile counterparts. Painted turtle adults were more likely to test positive for STADV than juveniles. There was no age class association with STADV detection in red-eared sliders. This finding in RES is similar to what was found in the epidemiologic study for *Terrapene* herpesvirus 1 (TerHV1) in eastern box turtles (*Terrapene carolina carolina*), in which age and sex were not significantly associated with positive status (Kane et al. 2017). Meanwhile, *Emydoidea* herpesvirus 1 in Blanding's turtles was only detected in adult females, despite the sampling population also including juveniles and male animals (Lindemann et al. 2019). Herpesviruses in desert tortoises and painted turtles also show a predilection for being found in adult animals (Johnson et al. 2005).

While the prevalence was low for Blanding's turtles, STADV has had high mortality in other endangered chelonians despite aggressive veterinary intervention, so the detection of STADV in this endangered species native to North America is concerning (Rivera et al. 2009; Schumacher et al. 2012). In contrast to the high mortality rate of the Sulawesi tortoises (82%) that died from multisystemic disease, no Blanding's turtles had any signs of clinical disease

(Rivera et al. 2009; Schumacher et al. 2012). Blanding's turtle populations are in decline, and infectious disease may be a contributing factor (Winter et al. 2020). The low prevalence documented in this study warrants further study, including additional longitudinal monitoring to determine if there is any disease-related mortality or subclinical effects on growth and reproduction associated with STADV and therefore any further impact on Blanding's turtle declines.

Clinical signs of STADV in affected Sulawesi tortoises included anorexia, lethargy, lesions in the oral cavity, nasal and ocular discharge, and diarrhea (Rivera et al. 2009; Schumacher et al. 2012). None of these clinical signs were observed in the STADV-positive turtles in our study. Unfortunately, clinical information from other studies surveying for adenoviruses have lacked clinical information, making it difficult to draw conclusions regarding the spectrum of clinical signs to be expected with adenoviral infections in chelonians (Salzmann et al. 2021; Doszpoly et al. 2013). It is possible that turtles infected with STADV may experience some degree of lethargy, making them more likely to present with quiet mentation. Furthermore, lethargy and immunosuppression may make them more susceptible to shell injures due to predation, or shell lesions from secondary opportunistic infections. An outbreak of shell lesions in river cooters (*Pseudemys concinna*) and yellow-bellied sliders (*Trachemys scripta*) was associated with bacterial infections, sepsis and trematode ova granulomatosis, indicating that shell lesions may present more commonly with secondary infections (Garner et al. 1997). However, the clinical findings of quiet mentation and shell lesions in positive individuals are vague compared to those reported for other adenovirus infections, and affected individuals may represent subclinical to aclinical animals. Likewise, clinical signs associated with herpesvirus infection in chelonians include ocular and nasal discharge, ocular swelling, oral plaques and

respiratory distress; however, multiple epidemiologic studies in emydid turtles have found no significant relationship between clinical signs and herpesviral detection (Kane et al. 2017; Lindemann et al. 2019). No clinical signs indicating overt active disease were found in the STADV-positive turtles in this study from any three species native to Illinois, suggesting that they may represent host-adapted species. Therefore, STADV testing of painted turtles, red-eared sliders or Blanding's turtles in zoological collections (especially if acquired from the wild or private collections) may be prudent. Identification of any positive subclinical carriers in the collection would allow for proper biosecurity measures to be implemented, safeguarding species that may be naïve to STADV or in which susceptibility to clinical disease has yet to be established.

In Vincent et al (2023), painted turtles with STADV had higher absolute beta globulins compared to negative turtles, with no other differences in clinical pathology seen. This was not the case for this study, in which no significant difference in beta globulins was seen for positive versus negative painted turtles. Furthermore, no significant hematologic or protein electrophoretic parameters were found to be significantly different between STADV-positive and negative turtles.

In conclusion, this was the first epidemiologic study for STADV and largest cross-sectional study to evaluate the prevalence of an adenovirus in free-ranging turtles. It showed that STADV prevalence in free-ranging freshwater turtles is widespread in northern Illinois and that the virus may be host-adapted in these turtles. When looking at age class, subadult Blanding's turtles and adult painted turtles were more likely to have STADV detected than in other age classes, while red-eared sliders showed no age class predilection. Sex and geographical influences were not found; however, further work to follow STADV detection over a longer time

period would be important to determine how the virus behaves in a natural system and if it putatively persists for extended periods of time. Red-eared sliders and painted turtles were significantly more likely to test positive for the virus compared to Blanding's turtles, and clinical signs associated with STADV-positive status were shell abnormalities and quiet mentation. The authors postulate that STADV may at least be endemic in these two species, with Blanding's turtles either more resistant to STADV, or more likely to succumb to severe disease if infected and therefore less represented in the surveys. Further investigations of STADV epidemiology in other North American chelonian species are needed to determine the threat that STADV poses in other ecosystems, if any. Based on this study, the authors recommend that zoo and wildlife veterinarians consider screening any native freshwater turtles entering collections in zoos and aquariums, or in other institutions where they will be mixing with other endangered, non-native chelonians.

## TABLES

Table 5.1. Number of turtles sampled by year in the sample population.

| Year | 2017 | 2019 | 2020 | 2021 | 2022 |
|---|---|---|---|---|---|
| # total turtles | 114 | 131 | 152 | 203 | 200 |
| Blanding's turtles | 114 | 81 | 117 | 112 | 157 |
| Painted turtles | 0 | 50 | 35 | 52 | |
| Red-eared sliders | 0 | 0 | 0 | 39 | 43 |

Table 5.2. Number of turtles sampled by County in Illinois, USA.

| Location | Total # turtles | Blanding's Turtles | Painted Turtles | Red-eared sliders |
|---|---|---|---|---|
| Lake | 379 | 379 | 0 | 0 |
| Kane | 339 | 202 | 137 | 0 |
| Cook | 82 | 0 | 0 | 82 |

Table 5.3. Proportions of Blanding's turtles (*Emydoidea blandingii*) testing qPCR positive for Sulawesi tortoise adenovirus by sex, age class and year.

| | N | All Years | 2017 | 2019 | 2020 | 2021 | 2022 |
|---|---|---|---|---|---|---|---|
| **County** | | | | | | | |
| Lake | 379 | 9/379 | 4/114 | 0/56 | 1/64 | 2/68 | 2/77 |
| Kane | 202 | 5/202 | | 2/25 | 0/53 | 3/44 | 0/80 |
| **Age** | | | | | | | |
| Adult | 242 | 7/242 | 3/56 | 1/48 | 0/44 | 3/46 | 0/48 |
| Subadult | 84 | 5/84 | 1/25 | 1/16 | 1/23 | 0/7 | 2/13 |
| Juvenile | 232 | 2/255 | 0/33 | 0/17 | 0/50 | 2/59 | 0/128 |
| **Sex** | | | | | | | |
| Female | 323 | 10/323 | 1/73 | 2/50 | 1/62 | 4/61 | 2/77 |
| Male | 163 | 4/163 | 3/35 | 0/21 | 0/34 | 1/51 | 0/22 |
| Unknown | 95 | 0/95 | 0/6 | 0/10 | 0/21 | | 0/58 |
| Total | 581 | 14/581 | 4/114 | 2/81 | 1/117 | 5/112 | 2/157 |

Table 5.4. Proportions of painted turtles (*Chrysemys picta*) from Kane County, IL, USA testing qPCR positive for Sulawesi tortoise adenovirus by sex, age class and year.

| | N | All Years | 2019 | 2020 | 2021 |
|---|---|---|---|---|---|
| **Age** | | | | | |
| Adult | 98 | 23/98 | 9/35 | 7/28 | 7/35 |
| Juvenile | 39 | 1/39 | 1/11 | 0/18 | 0/10 |
| **Sex** | | | | | |
| Female | 57 | 9/57 | 4/24 | 3/13 | 2/20 |
| Male | 77 | 15/77 | 6/23 | 4/22 | 5/32 |
| Unknown | 3 | 0/3 | 0/3 | | |
| Total | 137 | 24/137 | 10/50 | 7/35 | 7/52 |

Table 5.5. Proportions of red-eared sliders (*Trachemys scripta elegans*) from Cook County, IL, USA testing qPCR positive for Sulawesi tortoise adenovirus by sex, age class, location, month and year.

| | N | All Years | 2021 | 2022 |
|---|---|---|---|---|
| **Age** | | | | |
| Adult | 73 | 34/73 | 6/34 | 28/39 |
| Juvenile | 9 | 3/9 | 0/5 | 3/4 |
| **Sex** | | | | |
| Female | 39 | 19/39 | 2/19 | 17/20 |
| Male | 38 | 17/38 | 4/17 | 13/21 |
| Unknown | 5 | 1/5 | 0/3 | 1/2 |
| **Total** | 82 | 37/82 | 6/39 | 31/43 |

# CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The research within this thesis describes the steps taken to survey three free-ranging freshwater turtle species for adenoviruses, validate a sensitive and specific assay for the most clinically relevant adenovirus found, and perform an epidemiologic study using that assay in those same turtle species.

Ten adenoviruses representing four genera in Adenoviridae were detected via consensus PCR in Blanding's turtles, painted turtles and red-eared sliders in Illinois. The majority of these were Testadenoviruses and likely host-adapted, given the absence of clinical signs of disease in any of the turtles evaluated. Sulawesi tortoise adenovirus was the single most common virus detected across the three species, with painted turtles followed by red-eared sliders most commonly having STADV detected. Based on my study's findings, the authors propose that painted turtles or red-eared sliders represent the North American reservoir or aclinical host for STADV and may have been the source the original outbreak reported by Rivera et al. (2009) and Schumacher et al. (2012).

Following sequencing and confirmation of Sulawesi tortoise adenovirus in a painted turtle, a sensitive and specific quantitative PCR assay was developed. The results in this thesis demonstrate that the STADV TaqMan® qPCR assay is sensitive, specific, and reliable for detection of the DNA polymerase gene segment of STADV. Quantitative PCR may be used for detecting lower viral quantities in turtles during periods of latency or in subclinical carriers or reservoirs, which have yet to be identified and need to be investigated. While PCR has historically been utilized as the diagnostic modality for adenovirus infections, and still remains

useful for identifying novel adenoviruses, the evidence in this thesis provides a superior diagnostic technique for STADV, specifically.

The assay developed in this thesis was used to gain insight into the epidemiology of STADV in free-ranging Blanding's turtles, painted turtles and red-eared sliders. The prevalence of STADV via qPCR detection in these turtles was greater than what has been indicated by conventional PCR, suggesting that the assay is more sensitive and specific than conventional methods. Painted turtles and red-eared sliders were significantly more likely to have STADV detected, compared to Blanding's turtles. Clinical signs associated with STADV detection were not suggestive of active clinical disease. The authors postulate that STADV may at least be endemic in these two species, with Blanding's turtles either more resistant to STADV, or more likely to succumb to severe disease if infected and therefore less represented in the surveys.

Given what has been found regarding the prevalence of STADV in North American emydids, the authors recommend that zoo and wildlife veterinarians consider screening any native freshwater turtles entering collections in zoos and aquariums, or in other institutions where they will be mixing with other endangered, non-native chelonians. The detection of STADV in these turtles in the absence of major clinical signs of disease indicates they may serve as sources of transmission.

While this thesis adds significantly to the body of knowledge regarding adenoviruses in turtles, there is still much more that needs to be investigated. Additional future directions include the development of more sensitive and specific assays for other potentially clinically relevant turtle adenoviruses, such as the "mixed species adenovirus" found in box turtles, red-eared sliders and Blanding's turtles described in Chapter 3. The potential of this virus to be detected in multiple hosts is unlike that of typical adenoviruses and may represent another clinically

important disease threat. Using more refined testing methods, such as qPCR, in addition to other confirmatory diagnostics, such as in-situ hybridization, electron microscopy, or virus isolation, will continue to elucidate the prevalence and epidemiology of these viruses across the landscape.

Longitudinal sampling and sampling of individuals over longer periods of time would be important to help understand if adenoviruses, such as STADV, result in mortality in species like the Blanding's turtle. By nature of the study design, sampling methods bias towards animals that are more active; therefore, animals experiencing rapid mortality from disease may be underrepresented. A more direct method of understanding the pathogenesis of STADV infection, tissue tropism and testing would be an experimental study design to implement Koch's postulates, in which turtles are experimentally infected, live virus is recovered from these animals, and disease is experimentally demonstrated in additional subjects. Characterizing clinical signs, disease course, and histopathologic findings in experimentally infected turtles would be a valuable pursuit. This would aid in understanding of transmission and tissue tropism pattern along with pathology.

Information on prevalence of adenoviruses in free-ranging and managed populations of other North American turtles is needed to empower the conservation efforts of chelonians, including Blanding's turtle programs by helping biologists make critical translocation and headstarting decisions. Clarifying the epidemiology of AdV across the landscape and within aquatic chelonian communities is expected to not only help protect endangered chelonians in zoological collections, but also help guide conservation leaders in management decisions for maximizing the potential longevity of free-ranging populations.

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