HEALTH ASSESSMENT OF SPOTTED (CLEMMYS GUTTATA) AND PAINTED (CHRYSEMYS PICTA) TURTLES IN CAPE COD, MASSACHUSETTS, U.S.A, WITH DETECTION OF A NOVEL ADENOVIRUS

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HEALTH ASSESSMENT OF SPOTTED (*CLEMMYS GUTTATA*) AND PAINTED (*CHRYSEMYS PICTA*) TURTLES IN CAPE COD, MASSACHUSETTS, U.S.A, WITH DETECTION OF A NOVEL ADENOVIRUS

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Abstract: Freshwater turtles face numerous anthropogenic threats worldwide. Health assessments are a key component of chelonian population assessment and monitoring but are under reported in many species. The purpose of this study was to characterize the health of spotted turtles (*Clemmys guttata*; n = 30) and painted turtles (*Chrysemys picta*; n = 24) at Camp Edwards, a military base in Cape Cod, Massachusetts, using physical examinations, hematology, plasma heavy metal analyses, and pathogen surveillance via PCR. Spotted turtles had a high prevalence of carapace (n = 27, 90%) and plastron (n = 14, 46.7%) lesions, and a previously undescribed adenovirus was detected in three animals (proposed as *Clemmys* adenovirus-1). Female painted turtles had lower plasma copper ($p = 0.012$) and higher strontium ($p = 0.0003$) than males, and appeared to be in a similar plane of health to previous reports. This initial health assessment effort provides useful baseline data for future comparison in these species. Conservation efforts on Camp Edwards should incorporate continued health surveillance of these populations to identify intervention opportunities and determine the conservation threats, if any, of the novel adenovirus.

INTRODUCTION

Wildlife health assessments, including clinical pathology, pathogen detection, and contaminant exposure, enable detection of disease outbreaks and identify management strategies that support positive conservation outcomes.[22] Chelonians are one of the most imperiled vertebrate groups and are susceptible to numerous pathogens and toxicants.[1,12] Due to their longevity, habitat use, and trophic level, freshwater turtles are considered sentinel species that can be examined to characterize environmental contaminant burdens.[6,12,15]

Spotted turtles (*Clemmys guttata*), an IUCN-endangered species, inhabit shallow bodies of water, including vernal pools and marshes in the northeastern United States.[20] Isolated habitats and population declines put spotted turtles at risk for inbreeding and increased susceptibility to infectious disease outbreaks.[13] Painted turtles (*Chrysemys picta*) inhabit varied wetlands including rivers, ponds, and marshes in the northern United States.[21] These species have specific niche differentiation but may share certain habitats due to overlapping ranges.[2] This potentially allows the more abundant painted turtle to serve as a surrogate for understanding the health of the less abundant spotted turtle, assuming they share similar susceptibility to disease.

Joint Base Cape Cod (formerly the Massachusetts Military Reservation), a Superfund site in Cape Cod, Massachusetts, has been the focus of significant research into the endocrine disrupting effects of contaminated groundwater in painted turtles.[6,15] However, association of painted turtle health metrics to the presence of specific contaminants has not been performed and spotted turtle health has not been investigated at this site.

The objective of this study was to conduct health assessments, heavy metal testing, and pathogen surveillance in spotted turtles and painted turtles at Camp Edwards, part of the Joint Base in Cape Cod, Massachusetts USA. Our specific hypotheses were (1) turtles would be detected with pathogens using qPCR (2) painted turtles would have measurable concentrations of lead in their plasma due to the area's historic military use.

MATERIALS AND METHODS

Live animal use and biosecurity protocols were approved through the University of Illinois

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Institutional Animal Care and Use Committee (Protocol 20258). Turtles were sampled in Cape Cod, Massachusetts, on Camp Edwards, between May and June 2021. Both species were captured in identical manners, including incidentally via hand-catch and with baited, partially submerged minnow traps that were checked once daily. Biosecurity was provided with clean nitrile gloves changed between individuals, and all sampling equipment was sprayed with dilute chlorhexidine solution to prevent cross-contamination between turtles.

Each turtle was assigned an ID, and sex and age class were recorded based on previous studies.[4] Full physical examinations (PE) were performed and carapace photos were taken to prevent re-sampling as required by our permit. Whole blood was collected from the subcarapacial sinus (<0.8% body weight) and placed in lithium heparin microtainers. Samples with obvious lymph contamination were discarded. Cotton-tipped applicators were used to collect separate samples of shell and oral/cloacal mucosa, placed immediately in microcentrifuge tube, and then frozen at –20°C until analysis. Shell samples were collected by swabbing each animal in a similar manner starting with the entire carapace, then plastron, bridge, and any lesions. Following sampling, turtles were released at their coordinates of capture.

Hematologic analyses including packed cell volume, total solids, and total white blood cell (WBC) counts were performed for both species within six hours of sample collection. In painted turtles, WBC counts were determined using Avian Leukopets (Vet lab Supply, Palmetto Bay, FL 33157, USA). In spotted turtles, due to sample volume limitations of the collection permit, blood film WBC estimates were performed by averaging the number of leukocytes in 10 fields at 400x total magnification and multiplying this number by 2,000. Differential leukocyte counts were performed by a single observer (LA). Erythrocyte sedimentation rate (ESR) was performed in painted turtles and results were read at 60 minutes using digital calipers.

DNA was extracted from oral/cloacal swabs using Qiagen DNA Blood mini kits (Qiagen, Valencia, CA 91355, USA), according to the manufacturer's protocol. This protocol was also used for shell swab DNA extractions except for the addition of a lyticase digestion (60-minute incubation at 37°C with 12.5U of lyticase) immediately before the lysis step. DNA concentration and purity were assessed spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA 02451, USA) and DNA samples were stored at –20°C.

Table 1. Pathogens/co-pathogens tested in painted turtles (*Chrysemys picta*) from Camp Edwards in Cape Cod, Massachusetts and source for qPCR primers.

Pathogen	Source
Frog Virus 3 – ranavirus qPCR	Archer et al., 2017
Ambystoma tigrinum virus – ranavirus qPCR	Archer et al., 2017
Bohle iridovirus – ranavirus qPCR	Archer et al., 2017
Epizootic hematopoietic necrosis virus – ranavirus qPCR	Archer et al., 2017
Pan-ranavirus qPCR	Stilwell et al., 2007
Mycoplasmopsis agassizii qPCR	Archer et al., 2017
Mycoplasmopsis testudineum qPCR	Archer et al., 2017
Box Turtle *Mycoplasmopsis* sp. qPCR	Archer et al., 2017
Salmonella tymphimurium qPCR	Archer et al., 2017
Salmonella enteritidis qPCR	Archer et al., 2017
Intranuclear coccidia of Testudines qPCR	Archer et al., 2017
Human-pathogenic *Leptospira* spp. qPCR	Smythe et al., 2002
Coxiella burnetti IS1111 qPCR	Klee et al., 2006
Coxiella burnetti ICD qPCR	Archer et al., 2017
Emydomyces testavorans qPCR	Woodburn et al., 2019
Terrapene herpesvirus 1 qPCR	Archer et al., 2017
Terrapene herpesvirus 2 qPCR	Engel et al., 2019
Testudinid herpesvirus 2 qPCR	Archer et al., 2017
Emydid herpesvirus 1 qPCR	In house
Emydoidea blandingii herpesvirus 1 qPCR	Lindemann et al., 2018
Terrapene adenovirus 1 qPCR	Archer et al., 2017

For painted turtles, qPCR was performed using a Fluidigm platform to evaluate 21 pathogens using published or in-house primer-probe assays (Table 1) as previously described.[1] Serial dilutions of positive controls for ranavirus (FV3), pan-ranavirus, box turtle *Mycoplasmopsis* sp., *Mycoplasmopsis agassizii*, *Mycoplasmopsis testudineum*, *Terrapene* herpesvirus 1, *Terrapene* herpesvirus 2, emydid herpesvirus 1, and *Terrapene* adenovirus 1 were prepared from 10^7 to 10^1 copies per reaction. A non-template control was included on each plate. All reactions were then analyzed using Fluidigm real time PCR analysis software (Fluidigm, South San Francisco, CA 94080, USA), and all positive samples were verified in a simplex reaction in triplicate on a QuantStudio3 real time thermal cycler (Thermo Fisher Scientific, Waltham, MA 02451, USA). Samples were considered positive if all three replicates had a lower cycle threshold (C_t) value than the lowest detected standard dilution.

For spotted turtles, due to few published pathogen assays, conventional consensus PCR assays were run according to published protocols for existing adenoviruses,[23] *Mycoplasmopsis* spp.,[14] and herpesviruses,[19] inclusive of positive and

negative controls. PCR products were resolved on 1% agarose gels. Amplicons of appropriate size were processed for sequencing routinely. FV3 qPCR was run in a simplex reaction as described above for spotted turtle oral/cloacal swab samples. For both species, *Emydomyces testavorans* qPCR was performed on shell swab DNA samples as previously described.[24]

Painted turtle plasma samples were analyzed using inductively coupled plasma mass spectrometry (Perkin Elmer NexION 300x, Waltham, MA, USA 02451) for cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), strontium (Sr), molybdenum (Mo), silver (Ag), cadmium (Cd), lead (Pb), arsenic (As), and selenium (Se).

Descriptive statistics were tabulated for all continuous variables. Normality was assessed using the Shapiro-Wilk test, Q-Q plots, skewness, and kurtosis. Normally distributed data were compared for sex (unknown excluded), site, and the presence of physical examination abnormalities using an independent samples t-test. When data were not normally distributed, a Mann-Whitney U test or a Kruskal-Wallis one-way analysis of variance test were used. Statistical analyses were performed using SPSS (Version 28, IBM Statistics, Chicago, IL 60606, USA) and alpha levels were set at $p = 0.05$.

RESULTS

Thirty spotted turtles (16 adult females, nine adult males, two juveniles of each sex, and one juvenile of unknown sex) and 24 painted turtles (11 adult males, six adult females, six juvenile females, and two juveniles of unknown sex) were sampled. Six turtles were hand-caught, 48 turtles were found in traps, and no traps contained both species together. Spotted turtle PE findings included carapace abnormalities (Figure 1) such as flaking, erosions, and apparent trauma (n = 27, 90%), similar plastron abnormalities (n = 14, 46.7%), missing digits (n = 1, 3.3%), and blepharedema (n = 1, 3.3%). Four adult female spotted turtles were gravid on coelomic palpation (25%). Painted turtle PE findings included plastron abnormalities (n = 18, 75%), carapace abnormalities (n = 16, 66.6%), ectoparasites (n = 6, 25%), missing limbs (n = 1, 4.2%), and integument lesions (n = 1, 4.2%). Most of the adult female painted turtles were gravid (n = 5, 83.3%).

Clinical pathology parameters were tabulated for both species (Table 2). There were no differences in any of these parameters based on sex, age class, month, or physical examination findings. Plasma heavy metal testing was performed for 23 painted turtles (Table 3). Females had lower copper (mean: 1.16 ppm; $p = 0.012$) and higher strontium (mean: 0.612 ppm; $p = 0.003$) than males (mean copper: 1.55 ppm, mean strontium: 0.244 ppm). Heavy metal concentrations were not associated with age class or the presence of any clinical sign.

Three apparently healthy (no clinical signs) spotted turtles (two males, one female) produced an identical 275bp adenovirus sequence that was 91.5–93% identical to sequences from box turtle adenoviruses (GenBank accession numbers MT900851.1 and EU828750.1) and 91.18% homologous to *Emydoidea* adenovirus 1 (GenBank accession number MW561636.1). We propose that this novel adenovirus be named *Clemmys* adenovirus 1 (GenBank accession number pending). All turtles tested negative for herpesviruses, frog virus 3, *Emydomyces testavorans*, and *Mycoplasmopsis* spp.

DISCUSSION

The goal of this research successfully generated data on health status, including identifying shell abnormalities in both species, quantifying plasma heavy metal burdens in painted turtles, generating baseline hematologic information, and detecting a novel adenovirus in spotted turtles. Though limited by small sample sizes and a narrow sampling window, these data will prove useful for future turtle health monitoring at sites with varying degrees of environmental contamination in Cape Cod and beyond. Baseline generation of hematology was provided, but due to sample size concerns, further analysis was not pursued and unfortunately comparisons between demographic factors cannot be performed.

Carapace and plastron abnormalities were the most common physical examination findings in spotted and painted turtles. Shell lesions can be attributed to a variety of different insults including primary or secondary bacterial and fungal infections, trauma, or metabolic imbalances.[5,24] Shell lesions of varying severity have been reported at a similar prevalence in other free-living freshwater turtles, and the importance of these lesions for health metrics appears to be situationally dependent on multiple environmental and physiologic variables.[9] In the present study, many shell lesions appeared relatively minor, however, superficial lesions can mask deeper damage to the shell and underlying tissues.[24] Aggressive swabbing was used to sample shells in this study, however with no positive *E. testavorans* results it is possible more aggressive sampling methods may be necessary to detect this pathogen in these species. Future health

Figure 1. Appearance of plastron abnormalities in spotted turtles (*Clemmys guttata*) at Camp Edwards, Massachusetts characterized as flaking (top left), erosions (top right, bottom left), and apparent trauma (bottom right). All animals were qPCR negative for *Emydomyces testavorans*.

investigations could incorporate computed tomography to better determine the extent and severity of these lesions.

Painted turtles had plasma heavy metal concentrations similar to whole blood values reported in other aquatic chelonians, however this may not reflect their true heavy metal burden since metals tend to accumulate in tissues.[11,22] Many metals bioaccumulate in the liver and kidney, whereas Sr and Pb accumulate in calcified structures, making noninvasive determination of toxic concentrations difficult.[12] Taking marginal scute samples as an alternative to sampling plasma concentrations has been proposed and may be accomplished, especially if scute notching is used for turtle identification in this population in the future.[12]

Three spotted turtles tested positive for a novel adenovirus. Adenoviruses are double-stranded DNA viruses that replicate inside the nucleus of host cells and are transmitted via the fecal-oral route.[10] Adenoviruses have been previously detected in other aquatic and terrestrial chelonians and their association with clinical disease is not always clear in this taxon.[16] Adenovirus-positive spotted turtles in the present study had no

Table 2. Descriptive statistics for spotted (*Clemmys guttata*) and painted turtle (*Chrysemys picta*) hematology from Camp Edwards in Cape Cod, Massachusetts. For data with a Gaussian (normal) distribution, mean and standard deviation are presented as measures of central tendency and dispersion. For data with a non-Gaussian distribution, medians and 25^{th}–75^{th} percentiles are presented.

Analyte	Units	Species	N	Data Distribution	Central Tendency	Dispersion	Range
Packed Cell Volume	%	*C. picta*	22	Gaussian	18.9	4.4	10–28
Total Solids	g/dL	*C. picta*	22	Non-Gaussian	4.2	3.3–4.9	3.0–8.0
Total White Blood Cell Count	/mL	*C. picta*	22	Non-Gaussian	18,237	14,255–21,506	8,235–48,594
Heterophils	/mL	*C. picta*	22	Gaussian	1,720	893	209–4,272
Lymphocytes	/mL	*C. picta*	22	Non-Gaussian	6,625	4,235–8,420	1,751–15,064
Monocytes	/mL	*C. picta*	22	Non-Gaussian	535	384–849	0–2,864
Eosinophils	/mL	*C. picta*	22	Non-Gaussian	5,356	3,281–6,515	2,837–25,755
Basophils	/mL	*C. picta*	22	Non-Gaussian	3,106	2,363–4,348	824–9,634
Heterophil/Lymphocyte		*C. picta*	22	Gaussian	0.317	0.202	0.024–0.786
Erythrocyte Sedimentation Rate	mm	*C. picta*	18	Non-Gaussian	6.8	4.65–10.45	3.6–16.0
Packed Cell Volume	%	*C. guttata*	25	Gaussian	16.9	4.5	8–24
Total Solids	g/dL	*C. guttata*	23	Non-Gaussian	3.8	3.4–4.1	3.0–7.0
Total White Blood Cell Count	/mL	*C. guttata*	25	Non-Gaussian	13,000	8,000–17,000	4,400–32,400
Heterophils	/mL	*C. guttata*	25	Non-Gaussian	1,520	950–2,856	464–4,960
Lymphocytes	/mL	*C. guttata*	25	Non-Gaussian	8,640	5,490–13,724	3,256–25,920
Monocytes	/mL	*C. guttata*	25	Gaussian	651	436	0–1,620
Eosinophils	/mL	*C. guttata*	25	Non-Gaussian	752	324–976	0–2,268
Basophils	/mL	*C. guttata*	25	Non-Gaussian	464	244–960	0–1,860
Heterophil/Lymphocyte		*C. guttata*	25	Non-Gaussian	0.222	0.138–0.288	0.065–0.778

Table 3. Descriptive statistics for plasma heavy metal toxicant concentrations in 23 painted turtles (*Chrysemys picta*) from Camp Edwards in Cape Cod, Massachusetts, in June 2021.

Variable	Units	n	Mean	SD	Median	Minimum	Maximum
Cobalt	ppm	23	0	0	0	0	0
Nickel	ppm	23	0.253	1.134	0	0	5.446
Copper	ppm	23	1.347	0.392	1.375	0.733	2.1
Zinc	ppm	23	5.352	1.013	5.165	3.444	7.665
Strontium	ppm	23	0.436	0.307	0.289	0.192	1.3
Molybdenum	ppm	23	0	0	0	0	0
Silver	ppm	23	0	0	0	0	0
Cadmium	ppm	23	0.001	0.005	0	0	0.025
Lead	ppm	23	0.041	0.048	0.029	0	0.181
Arsenic	ppm	23	0	0	0	0	0
Selenium	ppm	23	0.079	0.165	0	0	0.587

apparent clinical signs of illness or clinical pathology changes to indicate a decreased plane of health, which may indicate that this is a host-adapted pathogen.[18] Biosecurity methods as described above were used to minimize cross-contamination of samples, and adenovirus-positive turtles were obtained on three different days at three different trap locations. Additional surveillance is recommended to determine whether this virus is associated with morbidity and/or mortality in spotted turtles.

Spotted and painted turtles had a low overall burden of tested pathogens in the present study, similar to previous reports.[22] This may be due to geographic isolation, low sample size, or our limited sampling window, as pathogen prevalence can shift due to season, year, and environment. Larger-scale multiyear surveillance efforts are recommended to better characterize pathogen occurrence and epidemiology in spotted and painted turtles from Cape Cod.

In conclusion, painted turtles from Camp Edwards appear to be in a similar plane of health to other reports in this species and spotted turtles were observed with a high prevalence of shell abnormalities and a novel adenovirus. Conservation efforts should be directed toward continued surveillance of these populations to identify trends that can determine the conservation threats, if any, of the novel adenovirus. Integrating health metrics into conservation efforts helps to define biological characteristics that are often missing from routine natural history studies. These can identify unseen threats such as physiologic disturbances, pathogens, and toxicant exposure, and ultimately inform more robust and successful conservation strategies.

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