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Emydomyces testavorans Infection and Shell Disease Prevalence in Threatened Northwestern Pond Turtle Populations of the San Francisco Bay Area, USA

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***Abstract.*—In Northwestern Pond Turtles (*Actinemys marmorata*), the fungus *Emydomyces testavorans* (Emte) is of special interest due to its potential to have negative health impacts on both captive and wild turtle populations. The full effects of this fungus on Northwestern Pond Turtle fitness are not yet understood, and up to a few years ago, Emte was originally thought to be restricted to the northern range of the host in Washington State. In 2020, however, Red-eared Sliders (*Trachemys scripta*) living with Northwestern Pond Turtles in California were found infected with Emte, raising the need to expand pathogen monitoring in this region. We captured and tested Northwestern Pond Turtles for Emte across five populations in the San Francisco Bay Area of California, USA, between 2021 and 2023. We found a low prevalence of Emte positive qPCR samples across the Californian populations, and the pathogen loads of positive individuals were low compared to reference samples from Washington state. These observations suggest that *Emte* is broadly distributed in the San Francisco Bay Area, but concentrations of the fungus were low. Despite the low prevalence of Emte in California, we observed a high frequency of turtle shell disease, raising the need to further explore the etiology of shell disease in this area and a need to further nvestigate the genetic or ecological mechanisms behind these observations.**

Key Words.—shell disease; *Actinemys marmorata;* qPCR; disease ecology; California; CT scan

Introduction

Fungal diseases such as white nose syndrome in bats (*Pseudogymnoascus destructans;* Blehert et al. 2009), snake fungal disease in snakes (*Ophidiomyces ophiodiicola;* Lorch et al. 2016), and chytridiomycosis in amphibians (*Batrachochytrium* spp.; Rollins-Smith & Le Sage 2021) have negatively impacted the health and stability of vertebrate populations across the globe. A key tool in managing wildlife fungal diseases has been rigorous monitoring programs that can track the prevalence of pathogen infections across space and time (Morner et al. 2002; Guberti et al. 2014). In the event of outbreaks, information on the prevalence and severity of infections across the landscape can help managers design, model, and test the effects of management actions.

Turtles are among the most imperiled vertebrate groups on Earth (Rhodin et al. 2018). Fungal disease among freshwater turtles is an important issue affecting these declines. *Fusarium* spp. and *Emydomyces* spp. are recently discovered fungi that have been isolated from diseased eggs or shell tissue of freshwater turtles, respectively. Shell turtle egg fusariosis (STEF), caused by *Fusarium* spp., affects the eggs of both freshwater and sea turtles decreasing nest success (Smyth et al. 2019; Martinez-Rios et al. 2022). Early stages of *Emydomyces*-associated shell disease can manifest as bleaching of the epidermis, which can progress to pitting or ulceration of the shell to the loss of full thickness of bones with cyst formation (Haman et al. 2019). The disease can be insidious with normal-appearing shells on the surface, but with the presence of large cysts beneath

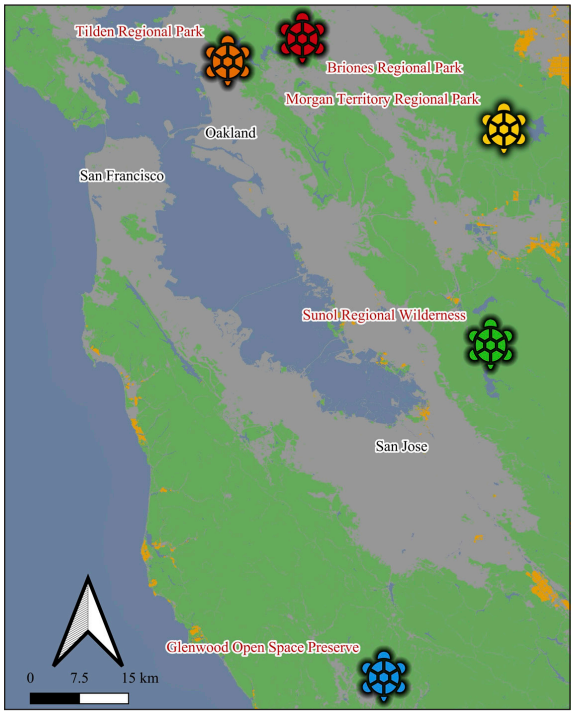

Figure 1. Map of sampling localities of Northwestern Pond Turtles (*Actinemys marmorata*) throughout the San Francisco Bay Area, California, USA.

the external epidermal layer (Haman et al. 2019; Woodburn et al. 2019; Wright et al. 2021). While Koch's Postulates (i.e., the generally accepted criteria for attributing an infectious disease to a potential pathogen; Koch 1890) have not been established to associate the fungus *Emydomyces testavorans* (Emte) with shell disease in turtles, the isolation of Emte from turtles with shell lesions suggests the pathogen may contribute to the pathophysiology of shell disease in some cases (i.e., Emte-associated shell disease; Woodburn et al. 2019). Emte-associated shell disease was recently described in Northwestern Pond Turtles (*Actinemys marmorata)* in Washington, USA (Woodburn et al. 2019), and has also been found on several other turtle taxa spanning 20 genera, including other free-living freshwater species such as Coastal Plain Cooters (*Pseudemys floridiana*) from Florida, USA (Woodburn et al. 2021), and Blanding's Turtles (*Emydoidea blandingii*) from Illinois, USA (Fredrickson et al. 2024).

Among Northwestern Pond Turtles in the state of Washington, Emte-associated shell disease ranges in severity, with some individuals exhibiting mild keratinopathy and others extensive shell disease through the presence of severe lesions penetrating through the shell into the coelomic cavity (Woodburn et al. 2019, 2021). Although this fungus was originally thought to be restricted to the northern part of the Northwestern Pond Turtle range in Washington, Emte was recently detected on invasive Red-eared Sliders (*Trachemys scripta elegans*) in California using qPCR technology (Lambert et al. 2021). At the time, no Northwestern Pond Turtles tested positive for Emte, but the potential presence of the fungus throughout its range in Washington merits a need to continue monitoring turtles for this fungus. Following the discovery of Emte in freshwater turtles of the San Francisco Bay Area in 2020 (Lambert et al. 2021), we established an annual shell disease survey program to evaluate the prevalence of the fungus throughout five free-ranging turtle populations in the Bay Area. We trapped, tagged, and collected samples of Northwestern Pond Turtles across multiple ponds that are approximately equidistant from each other. Emte-associated infections can be difficult to detect using either CT scanning or molecular assays alone due to the subcutaneous infectious process of Emte; thus, we applied CT scanning and a customized Emte qPCR assay to identify and quantify infections on the shell. Our sampling regime has been beneficial to clarify the distribution of Emte in the San Francisco Bay Area of California, USA.

Materials and Methods

Field surveys.—We executed annual turtle trapping efforts between 2021 and 2023 in the San Francisco Bay Area, California, USA, to assess Northwestern Pond Turtle health, shell disease, and Emte prevalence. The actual species identity of *Actinemys* turtles in the Bay Area is in question, as turtles in this part of the range and along the southern edge of the Transverse Range in Los Angeles County have both species and an admixture of both *A. pallida* and *A. marmorata* genes (Spinks et al. 2010; USFWS 2023). In this paper, though, we will refer to turtles we caught as Northwestern Pond Turtles. We collected turtle samples at five widely distributed freshwater ponds located around the San Francisco Bay (Fig. 1). The sampling locations range from natural, livestock-grazed, and urban environments, most of which came from the East Bay Regional Park District (EBRPD). Sampling ponds were located within EBRPD Morgan Territory Regional Preserve, EBRPD Sunol Regional Wilderness, EBRPD Tilden Regional Park, EBRPD Briones Regional Park, and Glenwood Open Space Preserve. Most of the locations are open for public use.

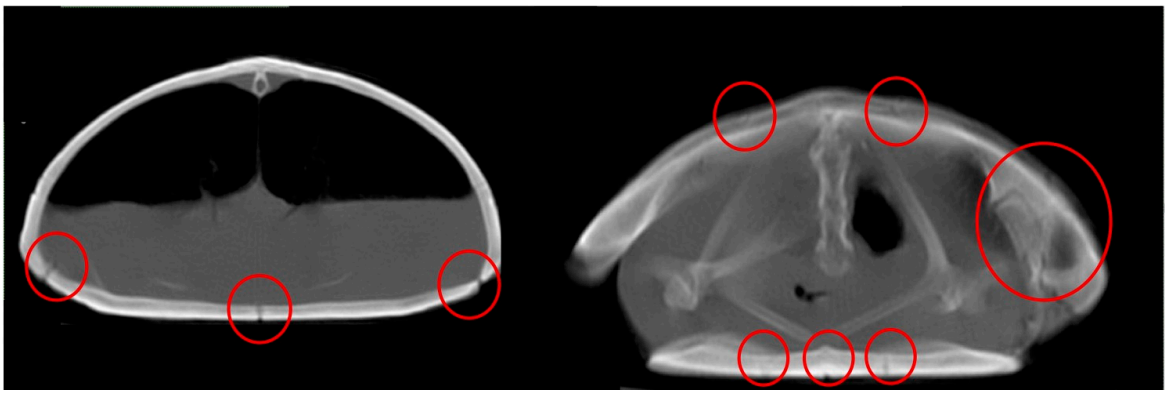

Figure 2. CT-scan image from a Northwestern Pond Turtle (*Actinemys marmorata*) taken in Washington state, USA. Axial slices showing anterior frame and mid-body frame showing naturally occurring bone fusions (red circles) that look like lesions. These fusions will show up in every turtle in the same area with two on the dorsal surface of the carapace close to the spinal column, one in the center of the plastron, and when the bridge is outlined.

We trapped Northwestern Pond Turtles by hand, dipnet, or hoop nets baited with canned sardines or cream corn. For hoop nets, we baited traps the night before sampling and checked the traps at least once the next day. As we collected turtles, we washed them using a clean toothbrush and 250 mL of sterile water to remove any mud and transient microbes (Culp et al. 2007). We then swabbed the entirety of the plastron and carapace of each turtle 30 times using the same swab (i.e., shell swabs; Guo et al. 2022). We used sterile dental curettes on abnormal areas and healthy scute joints to remove loose epidermal keratin from the shell (i.e., shell keratin scrapes). Of note, some turtle shells showed looser keratin resulting in more tissue collected. If the turtle was larger than 100 g, we proceeded to collect a cloacal sample by inserting a swab into the cloaca of the turtle and rotating it three times (i.e., cloacal swabs). We did not collect cloacal swabs in 2023. We placed the shell swabs, cloacal swabs, and shell keratin scrapes in sterile microcentrifuge tubes and stored them on dry ice until brought into the laboratory at Dominican University of California, USA, where the samples were stored in a -20° C freezer until DNA extraction was performed. Following Emte sampling, we measured both the weight (0.1 g) and plastron length (1 mm) of each turtle. We returned turtles to the pond immediately after we completed field processing. Reference samples from Washington State were collected in 2015 from turtles receiving treatment for Emte-associated shell lesions at the Oregon Zoo, Portland, Oregon, USA. Tissue from the infection site was collected using sterile cuvettes and the samples were stored in a 2-ml microcentrifuge tubes at -20° C until shipping to Dominican University of California in dry ice.

CT Scans.—On 20 April 2021, we transported 11 Northwestern Pond Turtles from Glenwood Open Space Preserve to the Pacific and Santa Cruz Veterinary Hospital in Santa Cruz, California, USA, for scanning on a Lightspeed 16 Slice CT scanner (General Electric HealthCare, Chicago, Illinois, USA) to confirm/exclude internal shell infections. We held turtles in 37.85 L (10-gallon) tubs with water, then restrained them by wrapping the limbs within the shell with Vetrap™ (3M Co, Maplewood, Minnesota, USA) prior to scanning at the veterinary clinic. We scanned turtles in two groups: seven turtles and four turtles. We examined jpeg images of each turtle frame by frame using a CT scan assessment protocol used by the Washington Department of Fish and Wildlife (WDFW). Each frame displayed an axial slice of the turtle going from the anterior to posterior of the individual. Within each frame, normal anatomical bone fusions were identified to orient the researcher. These fusions stay in the same location and were often symmetrical on the scan and were in the same place on all turtles (Fig. 2). We used the assessment protocol from WDFW to identify any abnormal radiopaques within the bone of the turtle that were not consistent with a bone fusion. We marked these radiopaque images as suspect lesions and then examined them to determine if they were full-thickness lesions. Full-thickness lesions refer to lesions that penetrate through the bone into the body cavity (Fig. 3). What we called pock-marking was also noted on turtles and is differentiated from suspect lesions based on the half-moon shape of the mark, whereas suspect lesions tended to be more linear in nature (Fig. 3). We still noted pock-marks in the likelihood that they could be beginning signs of potential infection. It should be understood that we listed abnormalities on the shell

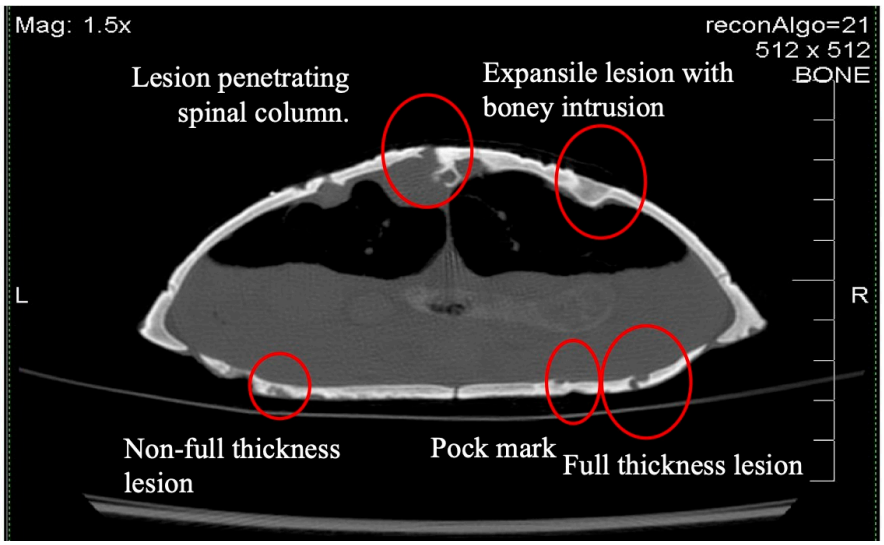

Figure 3. CT-scan image from a shell-rot afflicted Northwestern Pond Turtle (*Actinemys marmorata*) taken from Washington state. USA. This slice shows multiple suspect lesions as well as full thickness lesions (red ovals) that are penetrating through the shell. A full thickness lesion is seen penetrating through the spinal column, as well as multiple smaller lesions and two full thickness lesions penetrating through the plastron. There is also another lesion located on the right side of the carapace that would be considered an expansile lesion. The lesion would not be marked as full thickness because there is a bone barrier surrounding it, but the bone growth is penetrating the body cavity.

as suspect lesions within the assessment because of the difficulty of differentiating between lesions and an old injury. We also recorded abnormalities such as bone re-growth or healing, bone intrusions (i.e., expansile lesions), or soft tissue intrusions if present (Fig. 3). Any osteological abnormality was noted, as well as the location (i.e., plastron/carapace) and severity of the abnormality. We marked lesions that were on the spinal column, or the bridge of the shell as spinal or bridge lesions. We noted a count of the total number of lesions on both the carapace and the plastron throughout the scan, followed by a subsequent count of how many of those lesions we considered full thickness to try to create a severity rating system from no-infection to severe infection categories.

qPCR Assays.—We performed DNA extractions on swab and shell scrape samples using the DNeasy Blood & Tissue kit (Qiagen N.V., Hilden, Germany) following the protocol of the manufacturer. We processed both types of samples similarly, except that we ran the proteinase K digestion step on shell and cloacal swabs for 1 h and on shell scrapes for 8 h. To detect Emte on the samples, we implemented quantitative PCR (qPCR) reactions in duplicate using a custom 28S rRNA assay as a qPCR assay is not currently freely available. We generated our custom assay following the protocol and using the DNA sequences described in Woodburn et al. (2019). We made each qPCR reaction using 5 µL of 1:10 diluted template DNA, 12.5 µL of 2X TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, Waltman, Maine, USA), 1.5 µL of 10 nM forward primers (5'-GAAGCGGCAGAAGCTCAAA-3') and reverse primers (5'-CCGAAGTGTCCTCTCCAAATTAC-3'), 1 µL of a custom PrimeTime® 3IABk FQ quencher qPCR probe (5'-6-FAM/TTGAATCT/ZEN/GGCTCTCATGCTGGG/3IABkFQ/-3'), and 3.5 µL of molecular-grade water for a total volume of 25 µL. We included two replicates of a 28S rRNA sequence gBlock (Appendix) in serial dilutions of 1,000,000 to 1 copies/µL or genome equivalents (GEs). We ran and analyzed these reactions on an Applied Biosystems StepOnePlus RealTime PCR (ThermoFisher Scientific) system using the following thermal profile program: 95° C for 20 s followed by 50 cycles of 95° C for 3 s and 60° C for 30 s. We classified as negative samples those whose average qPCR quantification fell below 1 GE after correcting for dilution effects. We included 12 known positive samples from known Emte infected Northwestern Pond Turtles from Washington in our qPCR pipeline to test the specificity of our assay.

Results

Between 2021 and 2023, we processed 345 Northwestern Pond Turtles from ponds across the San Francisco Bay Area, with varying levels of turtle

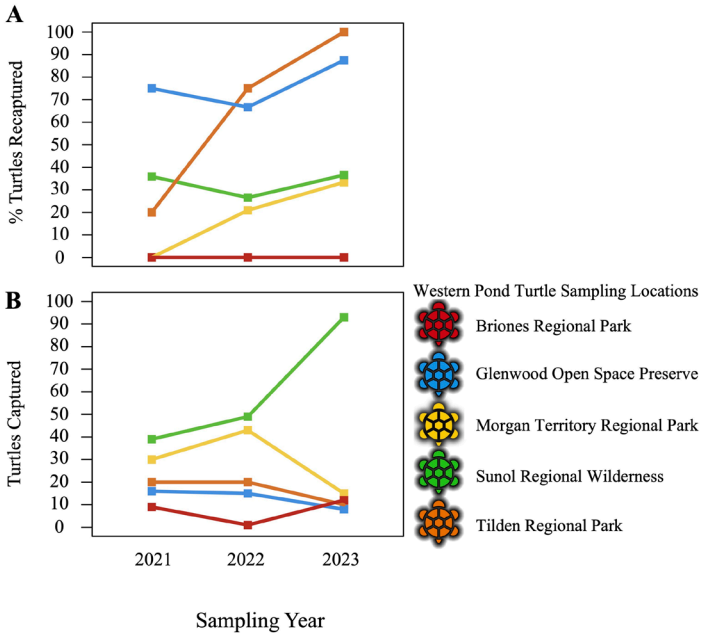

Figure 4. (A) Recapture frequency and (B) and capture rates of Northwestern Pond Turtles (*Actinemys marmorata*) over 3 y in the San Francisco Bay Area, California, USA.

capture success across our sampling sites (Fig. 4). Individuals (n = 260) were represented across all the sites. In 2021, we further evaluated 11 turtles collected from Glenwood Open Space Preserve for signs of Emte-associated shell disease via CT scans (Haman et al. 2019). Within the first scan containing seven individuals, there were only two turtles that appeared to have suspect shell lesions on the carapace and plastron. One turtle had several lesions or abnormalities on both the plastron and the carapace (Fig. 5). There was one full thickness lesion on the carapace of this individual. There also appeared to be bone healing from an injury the turtle had sustained on both the plastron and the carapace. Another turtle in that series had very few suspect radiolucent areas (i.e., areas with less dense bone tissue), but most notably one of the radiolucent areas appeared over the spinal column of the turtle. In the second scan with four turtles, three individuals in the series all seemed to have divots or pock marks on the plastron and carapace.

Our qPCR assay sampling efforts resulted in 218 shell swabs, 177 shell scrapes, and 89 cloacal swabs. We also processed 11 shell scrapes from Washington State turtles as positive references. All the Washington samples processed returned positive

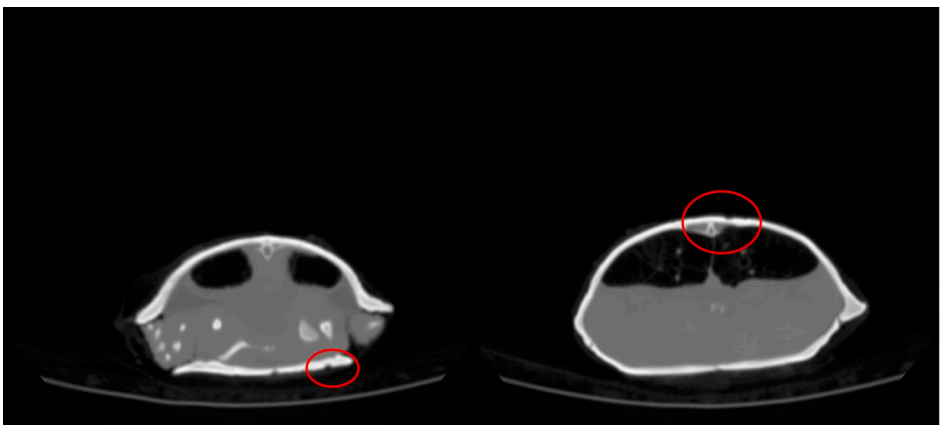

Figure 5. CT scans of the shell from a Northwestern Pond Turtle (Actinemys marmorata) collected from Glenwood Open Space Preserve, California, USA. This individual presents a pock mark radiolucency on the carapace near the spine and a small radiolucency (divot) on the plastron (red circles).

Table 1. Pathogen load in genome equivalents per µL (genome equivalents per µL), sample type, and pond location information of Northwestern Pond Turtles (Actinemys marmorata) that tested positive for Emydomyces testavorans (Emte) at sites in the Bay Area of San Francisco, California, USA. For reference, the results of known Emte positive samples from the state of Washington are also presented. The abbreviation WDFW = Washington Department of Fish and Wildlife.

State	Year	Sample ID	Emte Load (GEs/µL)	Sample Type	Pond Location
California	2021	DUC105	39.9	Shell Scrape	Glenwood
		DUC222	6.8	Shell Swab	Sunol
	2022	GWD13	1.2	Shell Scrape	Glenwood
Washington	2015	WA186	2,848.4	Shell Scrape	WDFW
		WA1053	10,208.4	Shell Scrape	WDFW
		WA216	1,825.7	Shell Scrape	WDFW
		WA805	30,751.4	Shell Scrape	WDFW
		WA218	35.5	Shell Scrape	WDFW
		WAB50084	439.5	Shell Scrape	WDFW
		WAA70215	165.6	Shell Scrape	WDFW
		WAB50082	1259.2	Shell Scrape	Oregon Zoo
		WAB50085	499.0	Shell Scrape	Oregon Zoo
		WA981	815.3	Shell Scrape	WDFW
		WAA50160	759.8	Shell Scrape	Oregon Zoo

qPCRs with Emte loads ranging from 35–30,751 GE/µL (Table 1). In contrast, we found low prevalence and loads of Emte throughout the San Francisco Bay Area. We observed one Emte positive qPCRs from Glenwood Open Space Preserve and one from Sunol Regional Wilderness in 2021, in 2022 we found only one positive, from the Glenwood Open Space Preserve, and we did not make any detections of Emte in 2023. Across all these sites, the loads of these samples remained close to the bottom of our standard curve (mean Emte load in California: 2.25 ± 1.35 GE/µL; Table 1).

Discussion

We implemented field turtle surveys of turtles, CT scans, and molecular tools to evaluate the prevalence of Emte in the Northwestern Pond Turtle from the San Francisco Bay Area. Radiological analysis revealed mostly superficial lesions present in four of the individuals examined at one of our sites in 2021. Throughout two sites, and between 2021–2022, we detected a low prevalence of the fungus using qPCR assays among all individuals tested. We did not find any positive samples in 2023. Among all positive individuals, fungus loads were not as high in California as compared to Washington reference clinical samples. Although CT scans showed some evidence of suspect lesions on a few individuals, there was no indication of severe shell rot disease. Finding low prevalence of the Emte and no severe shell disease in Northwestern Pond Turtles from this one area of California provides insight into the status of Emte-associated shell disease in the southern portion of the range of the species. Our data highlights a potential need for enhanced biosecurity and further conservation efforts to help reduce the risk posed by Emte-associated shell disease.

The loss of populations of Northwestern Pond Turtles in Washington likely resulted in a genetic bottleneck (Gray 1995). This lack of genetic diversity could negatively influence the immunocompetence of wild Northwestern Pond Turtles in Washington, leading to increased disease prevalence. To truly understand the risk of Emte-associated shell disease across the range of Northwestern Pond Turtles, we recommend future research on immunogenetics and the ecological associations with Emte to measure the disease resistance potential throughout the range of the species.

Despite a lack of Emte detection in Northwestern Pond Turtles at our sites, we still observed numerous individuals with superficial shell lesions such as pitting, flaking, and bleaching in several sites over the duration of our surveys. We are concerned that other shell disease processes, either pathogenic (e.g., Turtle Frasevirus also can generate shell lesions; Waltzek et al., 2022) or environmental (e.g., eutrophication),

might be at play in Northwestern Pond Turtles from this region. Given that Northwestern Pond Turtles from the San Francisco Bay Area likely experience pressures from urbanization and invasive species (e.g., Red-eared Sliders; Lambert et al. 2019), it is relevant for future research to evaluate whether human activities are increasing the incidence of shell disease in freshwater chelonians. Comparing the genetic diversity of Emte DNA recovered from positive samples and other microorganisms isolated from free-living turtles might be an important next step in understanding the distribution and diversity of shell rot disease-causing pathogens.

Fungal associated shell disease, along with other prevalent wildlife diseases (e.g., chytridiomycosis), continue to concern conservation managers due to their potential to negatively impact threatened populations and derail ongoing conservation efforts. In this study, we generated protocols for the molecular detection of Emte in samples collected from turtles and CT scan interpretations to categorize radiolucencies. Using these methodologies, we were able to evaluate the plausible presence of Emte on one additional site in California and on the threatened Northwestern Pond Turtle, a species currently in consideration for listing under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2023). The unknown extent of the geographic range of Emte raises the need to continue to map its distribution along with shell rot disease frequencies across more regions in the range of Northwestern Pond Turtles.

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Appendix

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GATTGCCTCAGTAACGGCGAGTGAAGCGGCAGAAGCTCAAATTTGAAATCTGGCCTCCATGCTGGGGTCT-GAGTTGTAATTTGGAGAGGACACTTCGGGTGTGGCCACGGCAAAGTTCCTTGGAACAGGACGTCACAGAGGGT-GAGAATCCCGTTCAGAGCCGTTCGCGCCTTGCCCATGTGAAGTTCCTTCAACGAGTCGAGTTGTTTGGGAATG-CAGCTCTAAGTGGGTGGTAAATTTCATCTAAAGCTAAATATCGACCGGAGACCGATAGTGTACAAGTAGAGTGATC-GAAAGGTTAAAAGCACCTTGAAAAGGGAGTTAAATAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAACCA-GATTCGGTCTCGGGGGCTCAGCGGGCATGTGGTGCCCGTGTACTCCCCTGTGCTCCGGGTCAGCATCAGTTCTG-GCGGCTGGTCAAAGGCCTTTGGAATGTATCGTCCTTCCGGGGACGTCTTATAGCCAGAGGTGCAATGCAGC-CAGCTGGGACTGAGGAACGCGCTCCGGCACGGATGCTGGC

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