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Turtle Shell Disease Fungus (*Emydomyces testavorans*): First Documented Occurrence in California and Prevalence in Free-Living Turtles

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Pond Turtle Shell Disease is an emerging infectious disease associated with the fungus *Emydomyces testavorans* (*Emte*). Here, we report the first documented case of *Emte* in free-ranging Californian turtles. We assayed *Emte* in nine Western Pond Turtles (*Emys* = *Actinemys marmorata*; WPT) and three non-native Red-eared Slider turtles (*Trachemys scripta elegans*; RES) from the same pond in Santa Cruz County, California. Despite several WPT exhibiting significant shell lesions, scute defects, and pliable scutes, all tested negative for *Emte*. However, all three RES were *Emte*-positive, although two of these showed no shell defects. Given all tested RES were *Emte*-positive, it is possible that some WPT results are false negatives, either due to assay performance or insufficient shell tissue sampling. However, our *Emte* results in WPT are likely true negatives, and thus our observations contrast the high prevalence of *Emte* and shell disease in WPT from Washington. The emergence of *Emte* in California poses a previously unaccounted conservation concern for imperiled WPT. *Emte*'s presence in California is also an important opportunity to study the epidemiology and ecology of this pathogen in a contrasting environmental context to Washington state where shell disease has had pronounced conservation consequences. Given the recent discovery of Snake Fungal Disease in California and the substantial impact the amphibian chytrid fungus has had on California's amphibians, there is an urgent need to understand the spread, prevalence, and impacts of *Emte* and other emerging wildlife diseases in California and elsewhere.

EMERGING infectious diseases pose an increasing threat to aquatic vertebrates (Knapp et al., 2016; McKenzie et al., 2018). Pond Turtle Shell Disease is one such disease that was first discovered in wild turtles in 2009 (Haman et al., 2019). This infection presents similarly to Septicemic Cutaneous Ulcerative Disease (SCUD) commonly found in captive turtles (Haman et al., 2019). While various shell diseases in wild turtles are common (Lovich et al., 1996; Ernst et al., 1999; Hernandez-Divers et al., 2009; Christiansen et al., 2020), Pond Turtle Shell Disease is unusual in that other symptoms such as lethargy, emaciation, and skin lesions are typically absent despite severe and ongoing shell degradation (Haman et al., 2019; Adamovicz et al., 2020). Gross evidence of Pond Turtle Shell Disease manifests as shell pitting, lesions or hollow areas beneath keratin, marginal scute defects and pliability, and occasionally shell lesions penetrating into the coelomic cavity (Haman et al., 2019; Adamovicz et al., 2020). Unfortunately, Pond Turtle Shell Disease can be difficult to notice in cases without external lesions, and many severe cases are difficult to detect without logistically challenging and expensive micro-computed tomography (CT) scans (Haman et al., 2019).

Pond Turtle Shell Disease is so named because it was first observed and described from Western Pond Turtle (WPT; *Emys* = *Actinemys marmorata*) populations in Washington where it has impaired conservation actions for the species (Haman et al., 2019). WPT are state-listed throughout their U.S. range in Washington, Oregon, and California and are undergoing review for listing under the U.S. Endangered Species Act (USFWS, 2015). The hypothesized causative agent of Pond Turtle Shell Disease, the keratinophilic fungus *Emydomyces testavorans* (*Emte*), was recently described from shell lesions of several turtle species including WPT (Woodburn et al., 2019; Adamovicz et al., 2020). While the biology and epidemiology of *Emte* are primarily uncharacterized, it has been found in wild turtles in Washington state and Illinois as well as a diversity of captive turtle species (Woodburn et al., 2019; Adamovicz et al., 2020). Both Pond Turtle Shell Disease and *Emte* are believed to be widely underdetected, though PCR and qPCR molecular assays are facilitating more thorough screening of this pathogen and associated disease (Adamovicz et al., 2020).

Here we describe the first documented cases of *Emte* in free-ranging turtles in California from Santa Cruz County in a small population at a nature preserve. We document *Emte*'s prevalence on native WPT and introduced Red-eared Slider

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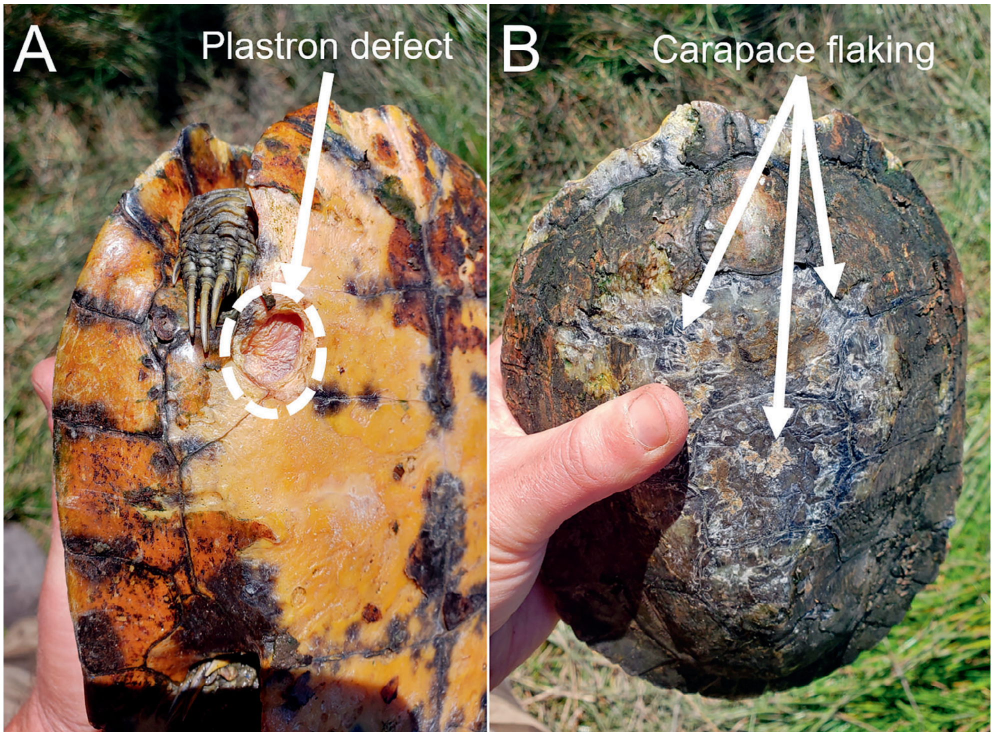

Fig. 1. The plastron (A) and carapace (B) of an adult female Red-eared Slider (*Trachemys scripta elegans*), the first free-ranging turtle to test positive for *Emydomyces testavorans* in California. The plastron defect is likely inconsistent with Shell Disease, whereas carapace flaking is possibly consistent with active infection. Photo credit M. Lambert.

turtles (RES; *Trachemys scripta elegans*), which threaten WPT and are globally invasive due to the pet trade (Lambert et al., 2019). The presence of *Emte* on non-native RES is particularly interesting because it may provide early evidence that RES could be a vector that introduces and maintains *Emte* into wild turtle populations and may also be a reservoir for this pathogen. We discuss the implications of this discovery and outline needs for future research.

MATERIALS AND METHODS

As part of ongoing efforts to study the impacts of introduced RES populations on native WPT, we are studying turtles in a pond at the Glenwood Open Space Preserve in Santa Cruz County, California that is managed by the Land Trust of Santa Cruz County. Our study site is a spring-fed pond that was expanded to serve as a stock pond. The ~80-hectare grassland and oak savannah preserve was previously a dairy farm, is presently grazed by cattle for endangered species conservation, and is embedded within a residential and urban landscape. The pond is isolated and has an area of 0.3 ha. The population size of either turtle species is unknown, although around 50 turtles are estimated to be in the pond. While the preserve was officially opened for public use in summer 2020, it has long experienced informal human activity. The Land Trust of Santa Cruz County is concerned about the WPT population's status and is actively managing the pond for WPT by maintaining suitable upland habitat for nesting and enhancing the available basking habitat by adding natural logs to the pond and deploying artificial basking platforms. The platforms were intentionally installed on the side of the pond furthest from the public trail to minimize disturbance from hikers.

We trapped turtles using ten wooden 0.5 m diameter 'mini-catfish' and four metal 1.0 m diameter hoop net traps secured along the pond's shoreline with wooden stakes and with the top 10% of the trap above water for turtles to breathe. Over two trap-nights from 29 June–1 July, 2020, we caught 14 WPT and 4 RES. This is the first time, to our knowledge, this population has been actively trapped for research. Multiple WPT showed signs of extensive shell damage including loss of superficial keratin and jagged defects along the free margins of the marginal scutes and divots in the plastron and carapace. This damage is possibly due to or exacerbated by mammalian predators like Coyotes (*Canis latrans*) and Domestic Dogs (*Canis lupus familiaris*), which are common in the area. However, we were concerned the extent of damage may also be consistent with Pond Turtle Shell Disease. RES shells generally appeared healthy except for one adult female that had a ~1 cm full-thickness plastron defect, as well as damage to the carapace and marginal scutes (Fig. 1). Given the shell damage in this turtle community, we brought a single WPT male with extensive shell damage and the female RES with the plastron defect to the San Francisco Zoo to be assessed for possible Pond Turtle Shell Disease and *Emte*.

We collected samples of shell material using sterile dental curettes from areas of damaged and abnormal keratin from both turtles (Haman et al., 2019) and submitted these to the Veterinary Diagnostic Laboratory at the University of Illinois for *Emydomyces* qPCR assay. A new curette was used for each animal, and, in animals with lesions, diseased keratin and debris were collected out of the shell cavities and other areas with abnormal loose or undermined keratin (Haman et al., 2019). In animals with no visible shell lesions, superficial keratin was curetted from the margins of the scutes at junction between the 1st and 2nd costal scutes of the carapace and between the abdominal and femoral scutes of the plastron to obtain sufficient sample material to cover at least 1 cm square. The sample materials were placed in sterile plastic containers without any additives and refrigerated until submission to the laboratory for PCR testing within 1–2 days. The amount of shell material collected varied among turtles depending on the extent of necrotic tissue and loose keratin available to scrape. The University of Illinois laboratory is currently the only institution with the capability of molecularly diagnosing *Emte*. Their method is described in Woodburn et al. (2021) and uses a highly specific qPCR assay with proprietary primer sequences that has an analytical sensitivity of 9.2 copies of target sequence per microliter.

Based on a positive *Emte* result from one of these two samples (see Results), we resampled the population from 17–18 August, 2020 to gain broader understanding of the prevalence of *Emte* in this turtle community. Due to wildfires in the area, we were only able to sample for a single trap-night. We captured eight WPT and two RES and collected shell scrape samples from all turtles using sterile dental curettes from any area with abnormal keratin or, in animals with no significant shell lesions, from the junction between the 1st and 2nd costal scutes of the carapace and between the abdominal and femoral scutes of the plastron (Haman et al., 2019).

We also collected additional shell tissue from the WPT that we brought to the San Francisco Zoo in July for treatment and which we were releasing back into the wild. Although this turtle tested negative for *Emte* originally (see Results) and appeared to have shell damage that was not clearly consistent with Pond Turtle Shell Disease, we collected this additional sample because we found a single small lesion in the carapace with necrotic material that had not previously been observed. We stored these samples as described above and shipped them to the University of Illinois for *Emte* assays.

RESULTS

Of the two July 2020 *Emte* assays, the RES (Fig. 1) but not the WPT tested positive for *Emte*. Veterinary examination (by AM) found the RES showed generalized superficial keratin flaking over the carapace with more extensive defects and irregular free margins to much of the cranial carapace and smaller areas of the caudo-lateral carapace (Fig. 1). The plastron showed numerous superficial pits and depressions, and parts of the free margins showed a jagged edge due to irregular defects along the margins. One 10x15 mm full thickness oval defect in the lateral margin of the left pectoral scute was healing with metaplastic calcification.

The WPT treated by the zoo showed extensive loss of superficial keratin in irregular patches over numerous marginal and some costal and vertebral scutes and multiple irregular defects along the free margins of the carapace and plastron. The left side of the carapace showed a depressed but stable fracture site across the second and third costal scutes and corresponding depression in the scutes over the left bridge. The plastron also showed an unstable but non-displaced transverse fracture beneath the pectoral scutes, which caused a partial separation of the right pectoral scute from the underlying bone. The fracture site was stabilized with bone cement patches. The areas of bone and keratin loss appeared to be generally healing normally with no progression of the shell lesions seen over the subsequent weeks while held at the zoo. The shell lesions were most consistent with damage inflicted by a canine predator.

Both RES samples from August 2020 tested positive for *Emte* despite neither showing any significant shell lesions. However, all nine WPT samples collected in August 2020 tested negative for *Emte*. No RES showed any notable shell lesions. In addition to multiple turtles with jagged marginal scute defects, the zoo-treated WPT had a single necrotic lesion and another WPT had shell bleaching consistent with Pond Turtle Shell Disease.

DISCUSSION

Our results represent the first reported cases of *Emte* in free-ranging California turtles. We detected *Emte* in all three non-native RES but none of nine WPT sampled from the Glenwood Preserve. The *Emte*-negative results from the sampled WPT are both interesting and surprising because we found gross epidemiological evidence consistent with Pond Turtle Shell Disease, including necrotic shell tissue and carapace bleaching (Fig. 2). Veterinary examinations did not identify lesions typical of Pond Turtle Shell Disease in the *Emte*-positive RES except perhaps irregular shedding of keratin flakes (Fig. 1). Yet, all *Emte*-positive RES were inhabiting the same habitat as the *Emte*-negative WPT. In WPT from Washington, *Emte* and Pond Turtle Shell Disease are hypothesized to be associated with time spent in captivity (Haman et al., 2019). The presence of *Emte* on non-native RES may offer early evidence that captive turtles, like many formerly pet RES (presumably at least some of the RES in this population were captive previously), may be a vector and reservoir of this pathogen into wild populations and among captive turtle facilities. At least three, non-mutually exclusive explanations could be behind our observed patterns: 1) limited detection methodology, 2) environmental variation, and 3) additional pathogens or cofactors.

The apparent conflicting evidence in our study could perhaps be because some or all *Emte*-negative results are false negatives. This this seems unlikely when an RES without lesions tested positive at the same time as an WPT tested negative using the same method. One explanation may be that species differences in behavior or physiology result in different fungal loads in the superficial keratin of the shells. *Emte* infections may be missed due to limitations of the qPCR assay or to inadequate sample collection for shell tissue. Given its reported sensitivity, the qPCR assay is unlikely the primary cause of possible false negatives (Woodburn et al., 2021). Because Pond Turtle Shell Disease can be challenging to observe externally and *Emte* infections can be highly localized rather than pervasive throughout the shell, it is possible to collect uninfected shell tissue when an infection is actually present. This may be particularly problematic if an infection has moved deeper into the shell towards the turtle's soft tissue (Haman et al., 2019; Adamovicz et al., 2020) and the shell has closed off behind the active infection; in this case, the infection would not be readily diagnosable externally through morphological or molecular means and would require a CT scan (K. Haman, K. Terio, pers. comm.). In Washington, Pond Turtle Shell Disease was observed in 25–50% of field-caught animals based on gross external morphological diagnosis and shell debridement but observed in >80% of WPT based on CT scans, demonstrating the value of CT scans for accurately diagnosing this disease (Haman et al., 2019). It is also possible that the WPT we sampled have cleared their *Emte* infections and are recovering from shell disease. An open-source *Emte* qPCR protocol (including the use of controls and pure culture dilutions to understand detection limits) and closer collaborations between veteri-

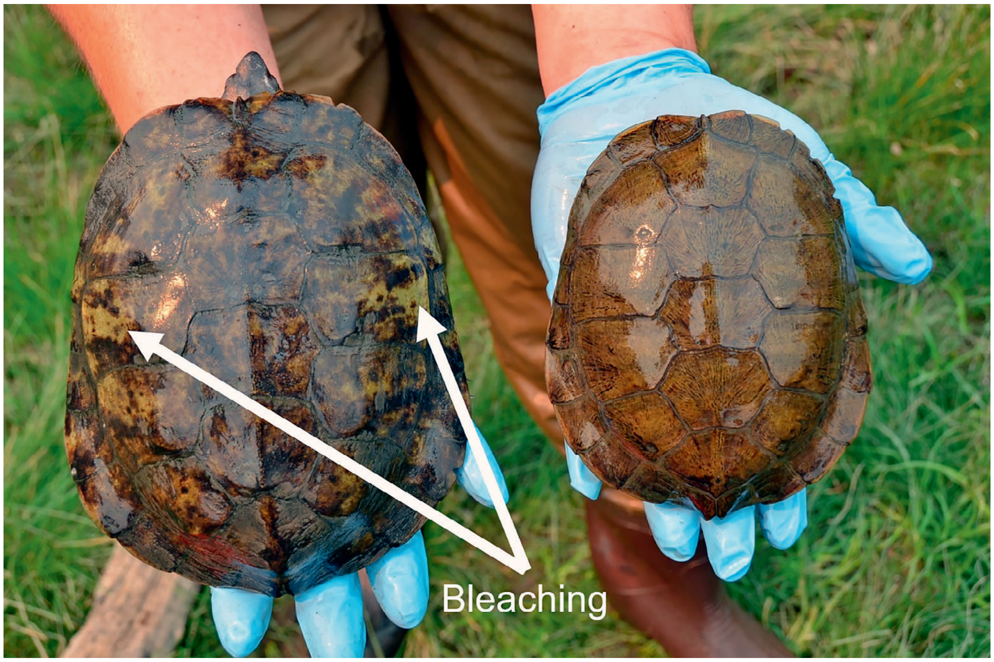

Fig. 2. Two Western Pond Turtles from the same pond. The larger turtle on the left has "bleached" markings that can be associated with an infection of *Emydomyces testavorans* and Pond Turtle Shell Disease. The smaller turtle on the right has a typical healthy carapace. Photo credit J. Bushell.

narians and ecologists, including for diagnostic imaging, will further address these challenges.

Another explanation may relate to geographic variation in ecology and epidemiology. Interestingly, the majority of wild Washington WPT spent substantial time in captivity (either because of headstarting or captive breeding) and many recaptured formerly captive individuals are *Emte*-positive and have Pond Turtle Shell Disease (Haman et al., 2019; Woodburn et al., 2019). It may be that some aspect of captivity—perhaps sanitary or microbiome differences—increases the likelihood of *Emte* infection or disease development (Haman et al., 2019). Even so, at least one wild WPT in Washington that never spent time in captivity was found to have Pond Turtle Shell Disease, indicating the disease can develop in the wild without a turtle having spent time in captivity (Haman et al., 2019).

Given Santa Cruz County is 1,100 km south of Washington state populations, the two areas experience dramatically different climates that likely affect fungal and turtle biology. While the presence of *Emte* in California is concerning, the environmental difference between regions may provide an opportunity to study the ecological conditions associated with *Emte* infections and Pond Turtle Shell Disease, including seasonal variation, which is an important factor in closely related *Ophidiomyces ophiodiicola*, the fungal pathogen that causes Snake Fungal Disease (McKenzie et al., 2018). We encourage collaborations between academics, veterinarians, and conservation practitioners to coordinate research on *Emte* and Pond Turtle Shell Disease among different environmental conditions.

While *Emte* is the hypothesized causative agent of Pond Turtle Shell Disease, this relationship is still correlational and has not fulfilled all of Koch's postulates, particularly an experimentally demonstrated link between *Emte* infection and disease (Haman et al., 2019; Woodburn et al., 2019; Adamovicz et al., 2020). It may be that other pathogens, or coinfections with multiple pathogens, cause Pond Turtle Shell Disease. DNA sequencing offers an important opportunity to further address this problem, and experimental evidence will be necessary to determine *Emte*'s contribution to Pond Turtle Shell Disease. In particular, metagenomics or amplicon sequencing could elucidate other microbes causing shell disease in imperiled turtles as these techniques do not rely on microbial culturing (McTaggart et al., 2019).

Introduced RES threaten WPT (Lambert et al., 2019) and the prevalence of *Emte*-positive RES observed here underscores the role RES may play in spreading pathogens (Silbernagel et al., 2013). To our knowledge, Pond Turtle Shell Disease and *Emte* have only been observed in a small number of RES, all of which were captive (Haman et al., 2019; Woodburn et al., 2019; Adamovicz et al., 2020). This may be due to RES being less susceptible to shell disease or researchers being less willing to spend resources in testing this invasive species for *Emte*. Regardless, our data highlight a critical need to further explore RES' role in spreading *Emte* and other pathogens both into wild populations and possibly among captive facilities like zoos or pet stores. Our data also highlight the necessity of surveying for *Emte* and Pond Turtle Shell Disease on non-native species like RES. Doing so may provide an early indicator of the spread of *Emte* into new locations and may highlight the role RES and other non-native species plays in the ecology of this disease. Just like globally invasive American Bullfrogs (*Rana catesbeiana*) may act as a non-susceptible reservoir and carrier of the amphibian chytrid fungus (Daszak et al., 2004), RES may vector *Emte* to imperiled populations of WPT and other turtle species.

WPT populations in northern California are considered relatively stable compared to the northern and southern regions of the WPT range (Thomson et al., 2016). However, turtles can suffer serious injury for years before showing evidence of decline, which means that proactive management before declines are observed are critical to turtle conservation (Van Dyke et al., 2019). We advocate for broad reconnaissance surveys of *Emte* and Pond Turtle Shell Disease, particularly in contrasting environmental conditions and in the presence and absence of RES. With the recent discovery of Snake Fungal Disease in California as well amphibian declines due to the chytrid fungus (*Batrachochytrium dendrobatidis*) in the state (Knapp et al., 2016), it is clear that California's herpetofauna are increasingly vulnerable to emerging infectious diseases. There is an urgent need to understand the prevalence, vectors, and ecology of *Emte* and its effects on WPT and other imperiled turtles.

DATA ACCESSIBILITY

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