

## SHORT COMMUNICATION

# Transcending sea turtles: First report of hatching failure in eggs of an Amazonian freshwater turtle with symptoms of the fungal emerging disease fusariosis

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## Abstract

During the last few decades, fungal pathogens have caused devastating population declines across a broad range of taxa. A newly emerging fungal disease, sea turtle egg fusariosis, caused by members of the *Fusarium solani* species complex (FSSC), has been reported to be responsible for hatching failure in sea turtles worldwide. However, this has not been detected in fresh water turtle species. Here, using relocated clutches and artificial incubation, we report high hatching failure in eggs symptomatic of fusariosis in the yellow-spotted Amazon River turtle (*Podocnemis unifilis*) inhabiting a pristine environment in the Ecuadorian Amazon. In 2020, we screened 680 eggs of the yellow-spotted Amazon River turtle, relocated from wild nesting areas to artificial nests, for visual symptoms of fusariosis and to estimate hatchability despite infection. We selected 68 eggs sampled in 2019 to confirm *Fusarium* infection by PCR amplification of the TEF-1 $\alpha$  gene and sequenced seven of those amplicons on an Illumina Miseq to assess FSSC membership. We observed fusariosis symptoms in 42% of the 680 eggs. The proportion of symptomatic eggs within nests was negatively linked to the proportion of eggs that hatched. Hatchability was 8% for symptomatic eggs compared with 72% of asymptomatic eggs. Through PCR testing, 58% of symptomatic and 8% of asymptomatic eggs sampled in 2019 tested positive for *Fusarium* spp., and sequencing revealed that nine sequence variants from three asymptomatic and four symptomatic eggs corresponded to *F. keratoplasticum*, *F. solani* and *F. falciforme*, the three major FSSC pathogens reported in sea turtle egg fusariosis. Our study suggests that hatching failure in eggs linked to symptoms of fusariosis appears to be partially caused by *Fusarium* pathogens within FSSC in a freshwater turtle. Thus, fusariosis is more widespread among the Testudines than previously reported and is not limited to sea environments, findings of particular conservation concern.

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## KEYWORDS

Amazon, emerging fungal disease, freshwater turtle, fusariosis, *Fusarium*, hatching success, *Podocnemis unifilis*

## 1 | INTRODUCTION

During the last two decades, an unprecedented worldwide emergence of fungal pathogens has threatened animal and plant biodiversity (Fisher et al., 2012). Recently, concern has increased over the emerging fungal pathogens from the *Fusarium solani* species complex (hereafter, FSSC) in endangered sea turtles, as these pathogens cause hatching failure in the eggs of sea turtles worldwide (Smyth et al., 2019). FSSC members are ubiquitous soil fungi that are also known to be pathogenic to plants, invertebrates and vertebrates, including humans (O'Donnell et al., 2016; Zhang et al., 2006), and to be capable of infecting hosts in aquatic systems (O'Donnell et al., 2016; Salighehzadeh et al., 2019). The latter has been confirmed by a recent study reporting infection of *Fusarium keratoplasticum*, a member of FSSC, in wild eggs of a threatened Amazonian freshwater turtle (*Podocnemis unifilis*) incubated in artificial hatcheries (García-Martín et al., 2021). However, studies on FSSC pathogenesis in the order Testudines remains limited to sea turtles. Moreover, the threat posed by this pathogen to the 357 known species of freshwater turtles and tortoises (Rhodin et al., 2017) remains unknown.

Fungal colonization of sea turtle eggs was first reported by Wynecken et al. (1988), who described hatching failure of diseased eggs within natural and artificial nests. Since then, several studies have reported the presence of fungi (Phillott & Parmenter, 2001) and bacteria (Craven et al., 2007) in unhatched eggs and female cloaca, indicating that turtle eggs are not laid sterile and have a commensal surface microbiome. Fungi from the genus *Fusarium* were identified in 2010 as being pathogenic to sea turtle eggs (Sarmiento-Ramírez et al., 2010), and, in 2012, FSSC was recognized as a new fungal emerging infectious disease (EID) contributing to declines of sea turtle populations (Fisher et al., 2012). Infections caused by ~60 species belonging to the FSSC have been reported in sea turtles around the globe (Brofft Bailey et al., 2018; Candan, 2018; Sarmiento-Ramírez et al., 2014). FSSC has been isolated from both diseased and asymptomatic sea turtle eggs, and infection might be vertically transmitted from the cloaca of the mother or horizontally transmitted from the environment (Sarmiento-Ramírez et al., 2014). Given the large number of *Fusarium* species and their global range, this fungal disease is probably not limited to sea environments and might also be spreading to freshwater habitats (García-Martín et al., 2021). *Fusarium* is thought to thrive in water and damp environments (Smyth et al., 2019), and therefore, fresh-water turtle species might also be at risk. However, little surveillance of non-oceanic turtle species has been undertaken to date.

In this study, we screened 680 eggs sampled in 2020 of an endangered freshwater turtle species (*P. unifilis*), relocated from wild nesting areas to artificial nests, for visual symptoms of fusariosis and assessed

hatchability. We subsequently tested 68 eggs sampled in 2019 for *Fusarium* infection by PCR and sequenced seven amplicons on an Illumina platform to screen for members of FSSC.

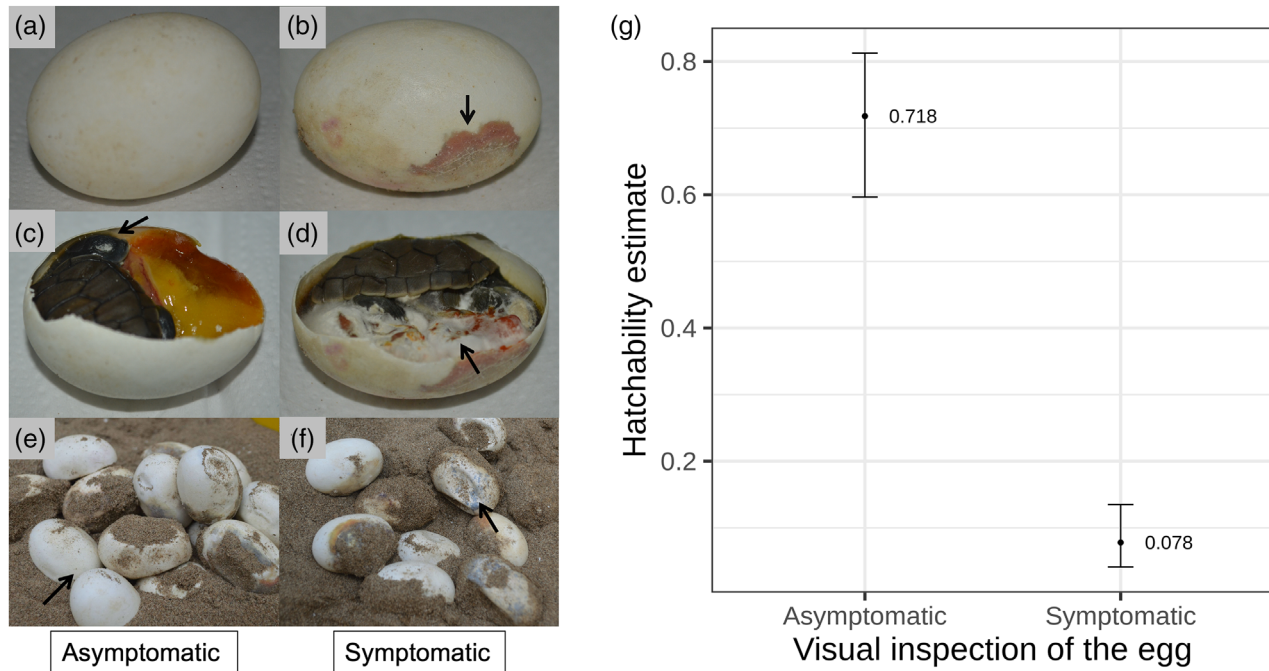
## 2 | MATERIALS AND METHODS

### 2.1 | Study site and sample collection

Sampling and data collection were performed in 2019 and 2020 at the Tiputini Biodiversity Station (TBS) situated in the Yasuni Biosphere Reserve in the Orellana province of Ecuador. We surveyed nests of the yellow-spotted river turtle relocated from wild nesting areas to artificial nests at the TBS. The artificial nests resembled natural conditions since they were filled with the same sand from the river, and eggs were located at the same depth as in wild nests and naturally exposed to sun and rain. After the incubation period, eggs were determined as fusariosis symptomatic when covered with unusual coloured spots (green, pink, greyish) and having a non-uniform shape, and eggs without these traces were catalogued as fusariosis asymptomatic (Figure 1). In 2019, we sampled inner eggshells of 68 eggs (29 asymptomatic, 39 symptomatic) by using sterile swabs. In 2020, we determined the hatching success of 23 nests (680 eggs = 394 asymptomatic, 286 symptomatic). Because of COVID19 restrictions, the sampling of internal eggshells was not possible during 2020. Details about the sampling design and data collection are given in the [Supplementary Information](#).

### 2.2 | Sequencing, bioinformatics and phylogenetic analysis

We extracted DNA from 68 inner-eggshell swabs and tested for *Fusarium* infections by using *Fusarium*-specific primers (Cobo-Díaz et al., 2019). Seven inner-eggshell swabs were sequenced following the Illumina MiSeq sequencing methodology as described by Menke et al. (2017). Paired reads were processed with open-source QIIME2 software (version 2019.1, Bolyen et al., 2018) and the DADA2 pipeline (Callahan et al., 2016) to denoise the dataset from artefacts and to generate amplicon sequence variants (ASVs). We assigned taxonomy within QIIME2 by building a classifier with RESCRIPt (Robeson et al., 2020) and the NCBI database by using BLASTnt (Table S1). For phylogenetic analyses, we included *Fusarium* spp. from various sources and species complexes, namely the *Fusarium solani* species complex (FSSC), *Fusarium tricinctum* species complex (FTSC), and *Fusarium fujikuroi* species complex (FFSC). A Bayesian phylogenetic tree was generated using the MrBayes 3.2.6 (Ronquist et al., 2012) module within Geneious v.11.0.5 (Kearse et al., 2012). Details about sequencing,



**FIGURE 1** Illustration and hatching success of turtle eggs, incubated in hatcheries, classified as asymptomatic and symptomatic by visual inspection. Images show eggs of the yellow-spotted freshwater turtle: (a) asymptomatic of fusariosis; (b) symptomatic of fusariosis; (c) opened visually asymptomatic egg that tested PCR-positive for *Fusarium* showing a dead embryo with hyphae around the eye; (d) opened symptomatic egg with a dead infected embryo; (e) asymptomatic eggs in artificial nests; (f) symptomatic eggs showing greyish dots; (g) hatchability estimates and associated 95% confidence intervals according to the visual inspection of eggs (GLMM fitted with binomial distribution with nest ID as random factor)

bioinformatics, and the phylogenetic analyses are provided in the [Supplementary Information](#).

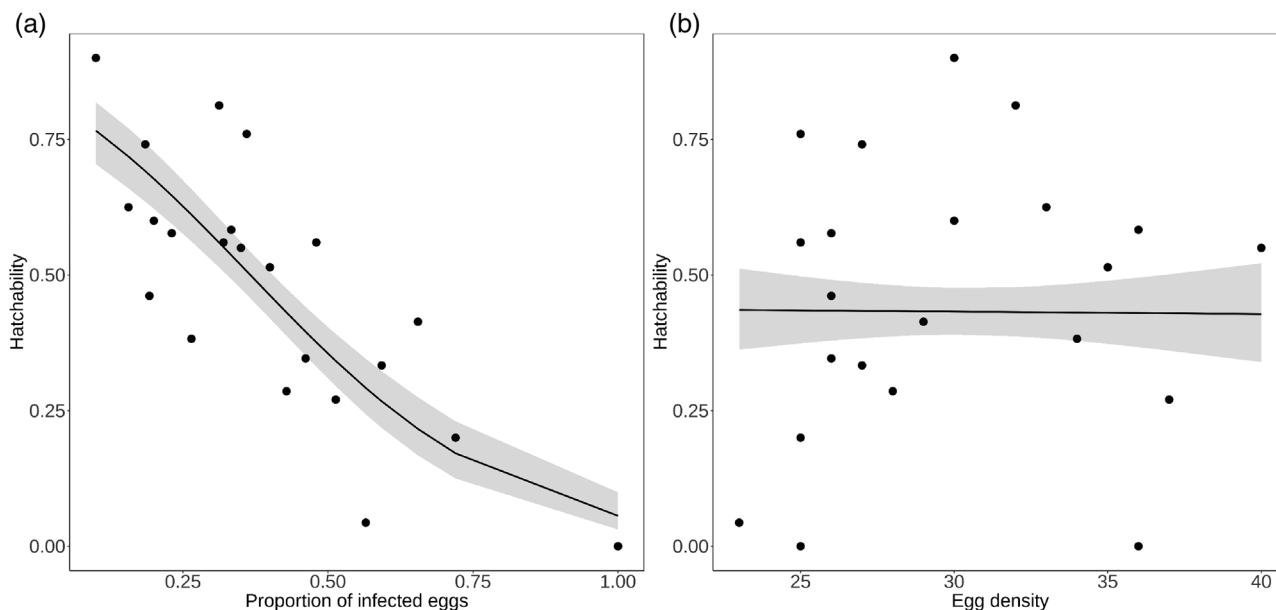
### 2.3 | Statistical analyses

We used a General Linear Model (GLM) with a binomial distribution with the R statistical software (RCore Team; 2020) to investigate variation in infection status across nests. We entered infection status as the response variable and nest ID as an explanatory variable. Subsequently, we investigated whether nest hatching success (hereafter hatchability) was linked to the proportion of eggs with symptoms of fusariosis. Moreover, we tested whether the latter was dependent on the number of eggs within a clutch (hereafter egg density). With these aims in mind, we built a GLM with a binomial distribution and the proportion of eggs hatched as the response variable. The explanatory variables were the proportion of symptomatic eggs, egg density and the interaction term. Next, we built a Generalized Linear Mixed Model (GLMM) with a binomial distribution, using the R package 'lme4' (Bates et al., 2015), to investigate hatchability according to fusariosis symptom status. To control for pseudo-replication, we entered nest ID as a random factor. We report  $p$  values,  $X^2$  of the model output and the adjusted- $R^2$  for each explanatory variable.

## 3 | RESULTS AND DISCUSSION

Several reports of hatching failure in nests of endangered sea turtles around the world have been linked to egg infections caused by *Fusarium* pathogens (e.g., Sarmiento-Ramirez et al., 2010, 2014). However, until now, symptoms of *Fusarium* infections have not been linked to hatching failure in other turtle species. In our study in the yellow-spotted Amazon turtle, we detected eggs showing symptoms of fusariosis infection in all 23 investigated artificial nests, with an average of 42% of eggs being symptomatic across nests (range 10–100%; Table S2). We found significant variation in the number of symptomatic eggs between nests ( $X^2_{1,22} = 192.48$ ;  $p < .001$ ;  $\text{adj-}R^2 = .332$ ) suggesting that some nests were more vulnerable to infection than others.

The proportion of symptomatic eggs among artificial nests was significantly linked to hatching success ( $X^2_{1,21} = 190.340$ ;  $p < .001$ ;  $\text{adj-}R^2 = .996$ , Figure 2a). Moreover, symptoms of FSSC infections in eggs of the yellow-spotted turtle were significantly associated with egg hatching success ( $X^2_{1,677} = 149.86$ ;  $p < .001$ ;  $\text{adj-}R^2 = .525$ ). After controlling for nest ID pseudo-replication using a GLMM, egg hatching success was 72% for asymptomatic eggs ( $n = 394$ ), whilst it was 8% for symptomatic eggs ( $n = 286$ , Figure 1b). In agreement with these results, experimental inoculations of *F. solani* in eggs of the sea turtle *Caretta caretta* resulted in a hatching success rate of 18% (2 out of



**FIGURE 2** Nest hatchability estimates and associated confidence intervals according to (a) the proportion of infected eggs and (b) the egg density within nests

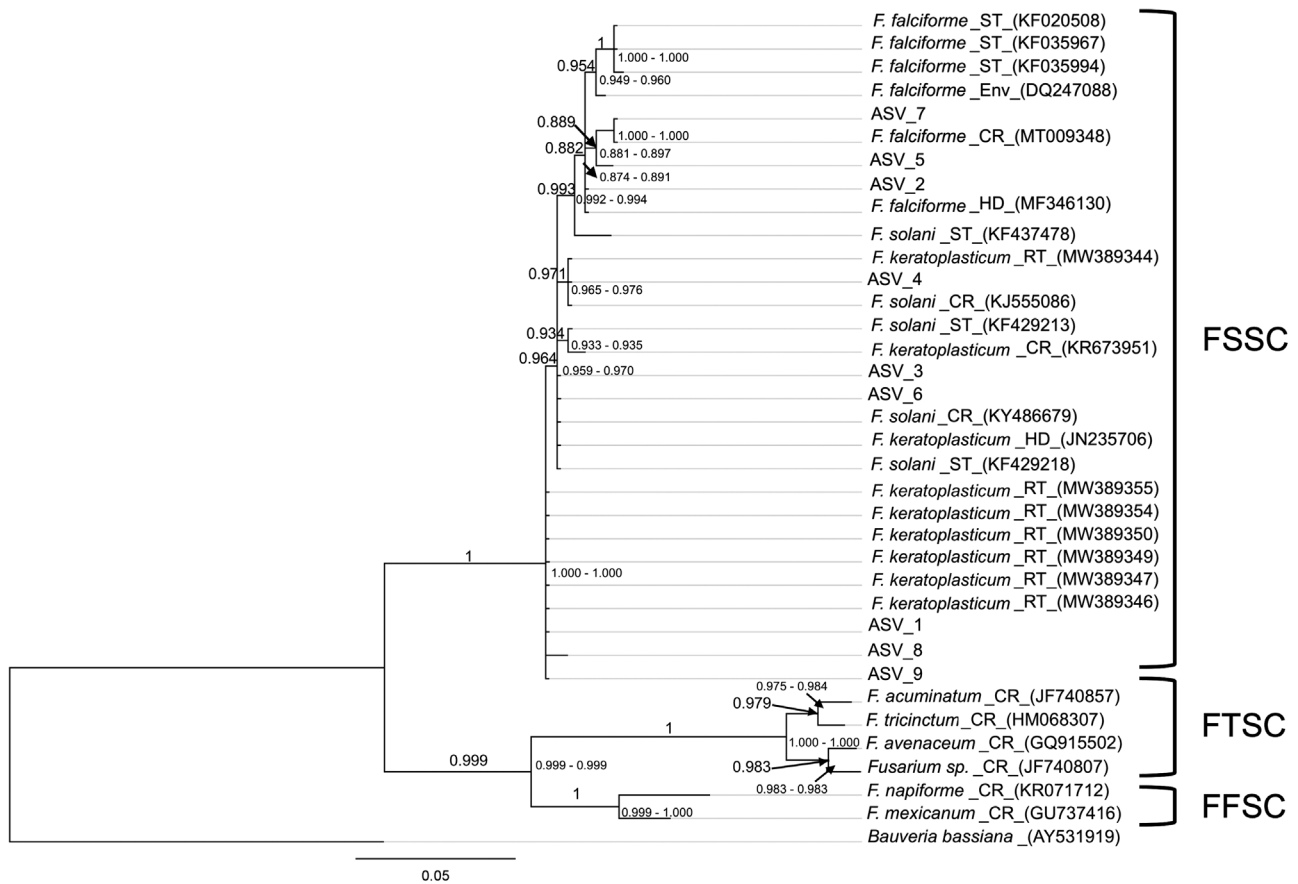
12) compared to 92% (11 of 12) for uninfected controls (Sarmiento-Ramirez et al., 2010). Similar disparity in hatching rates according to symptoms of fusariosis were also observed in the natural environment in endangered sea turtles, with up to 92% embryo mortality in diseased eggs (Sarmiento-Ramirez et al., 2014). Thus, we report, for the first-time hatching failure in artificial hatcheries, linked to eggs exhibiting symptoms that are typical of egg fusariosis but in a freshwater turtle species.

Within a clutch, fusariosis infections frequently started in damaged and non-viable eggs, which were the likely source of inoculum for neighbouring eggs (Phillot & Parmenter, 2001). Since higher egg density within a nest might increase the probability of a damaged/non-viable egg within the clutch, higher infection probability might be linked to egg density. However, we did not observe a significant effect of the interaction between egg density and the proportion of symptomatic eggs on hatching success within a nest ( $X^2_{1,19} = 0.169$ ;  $p = .681$ ;  $\text{adj-}R^2 < .01$ , Figure 2b), suggesting that the effect of fusariosis infection on hatchability was not directly related to egg density. *Fusarium*-specific PCR tests of 29 asymptomatic and 39 symptomatic eggs sampled in 2019 revealed that 59% of the symptomatic eggs tested positive for *Fusarium* (Table S3), whereas the remaining 41% tested negative (Table S3). These results indicate that infected eggs might not only be afflicted by pathogenic *Fusarium*, and that multiple causes of infection might be present. Indeed, previous studies have reported infections of sea-turtle eggs caused by various fungi, such as *Aspergillus*, *Rhizopus* and *Apophysomyces* (Candan, 2018; Santos Costa-Neves et al., 2015). Interestingly, we also found that 28% of asymptomatic eggs tested positive for *Fusarium* (Table S3) suggesting either that these eggs were at an initial stage of infection or that the colonization of *Fusarium* did not lead to the onset of pathogenic fusariosis. Indeed, although the pathogenicity of *F. solani* in sea turtles has been demonstrated, colonization with

this fungus might not be systematically pathogenic, and other species of *Fusarium* might not be pathogenic (Sarmiento-Ramirez et al., 2010).

From our sequencing of amplicons (from three asymptomatic and four symptomatic eggs), we obtained nine *Fusarium* amplicon sequence variants (ASVs) belonging to pathogenic FSSC (posterior probability  $PP = 1$ ; Figure 3). From the obtained ASVs, ASV\_1 and ASV\_3 were present in both asymptomatic and symptomatic eggs, ASV\_4 and ASV\_5 were each present in two different symptomatic eggs, and the remaining five ASVs were only present in asymptomatic eggs (Table S4). ASV\_3 and ASV\_6 grouped with *F. keratoplaticum* and *F. solani* isolates from human, crops, and sea turtle egg infections. ASV\_4 grouped with *F. solani* isolated from crops and *P. unifiilis* eggs ( $PP = 0.98$ ; Figure 3). ASV\_2, \_5 and \_7 grouped with *F. falciforme* isolates from the environment, crops, human and sea turtle egg infections ( $PP = 0.915$ ; Figure 2). In contrast, ASV\_1, \_8 and \_9 grouped together with six *F. keratoplaticum* isolates from *P. unifiilis* eggs ( $PP = 0.933$ ; Figure 3). Our results concerning the incidence of the FSSCs *F. falciforme* and *F. solani* add novel data to a recent study reporting the presence of *F. keratoplaticum* in *P. unifiilis* eggs in artificial nests within the same geographical region (García-Martín et al., 2021).

Although both our study and that of García-Martín et al. (2021) were conducted on harvested eggs incubating in artificial nests, the source of pathogenic FSSC is likely to be vertical transmission from the mother or horizontal transmission from the natural environment. In a natural setting, various factors such as temperature, substrate type and soil moisture can influence the spread of fungi in damaged eggs (Norris et al., 2020). Whereas infection might spread more easily within artificial nests (since eggs are incubated in sand from the original nesting site) than in natural settings, our combined results suggest a possible *Fusarium* infection outbreak in a remote area of the Amazon. However, because of field work disruption during 2020 as a



**FIGURE 3** Bayesian phylogenetic tree of the *Fusarium* spp. based on sequence information of the TEF-1 $\alpha$  gene. ASV 1 to ASV 9 were isolated from the yellow-spotted Amazon River turtle (present study) and visualized together with *Fusarium* spp. isolated from various sources (CR = isolates from crops; HD = isolates from species causing diseases in humans; Env = isolates from the environment; RT = isolates from the yellow-spotted river turtle; ST = isolates from sea turtle eggs). Strains are grouped according to the corresponding *Fusarium* complexes: *Fusarium solani* species complex (FSSC), *Fusarium tricinctum* species complex (FTSC) and *Fusarium fujikuroi* species complex (FFSC). The tree was rooted with a TEF-1 $\alpha$  sequence from *Beuveria bassiana* (AY531919). The branch labels show the posterior probability values (PP), and node labels show the 95% confidence intervals based on the probability range

consequence of COVID19, no sampling or testing for FSSC membership of the mycobiome was possible in the clutches of 2020. Thus, we are aware that a direct causal relationship between FSSC and hatching failure cannot be demonstrated. Nonetheless, our data demonstrates that the symptoms typical of egg fusariosis are linked to hatching failure in this freshwater species developing in hatcheries and that eggs showing identical symptoms in 2019 were infected with FSSC strains. These findings have important implications for turtle conservation efforts worldwide, since egg harvesting and artificial incubation are major strategies applied by turtle conservationists. Further studies are necessary to understand the epidemiology and distribution of the *Fusarium* pathogenic fungi that are likely to be causing hatching failure of endangered non-marine turtle species worldwide.

#### AUTHOR CONTRIBUTIONS

S.S., M.G. and A.S.C. conceived the idea; all authors contributed to the study design; D.R., M.L.T. and A.S.C. led sampling and field work logistics. A.S.C. collected the data; A.S.C. and M.G. led the

analysis; A.S.C. and M.G. led the manuscript writing. All authors contributed critically to the drafts and gave final approval for publication.

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## CONFLICT OF INTEREST

The authors declare no conflict of interests regarding this study.

## ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this specific study.

## DATA AVAILABILITY STATEMENT

Individual *Fusarium* TEF 1-alpha gene raw-sequences are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA745100 with accession number SAMN20145776.

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#### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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