Transcending marine turtles: first report of hatching failure in eggs of Amazonian freshwater turtles with symptoms of the fungal emerging disease fusariosis

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Abstract

In the last decades fungal pathogens are causing 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 around the world. However, this has not been reported in other non-marine turtle species. Herein we report high hatching failure from eggs symptomatic of fusariosis in the yellow-spotted Amazon river turtle (*Podocnemis unifilis*), inhabiting a pristine environment in the Ecuadorian Amazon. We assessed hatching success from eggs symptomatic and asymptomatic of fusariosis ($n = 680$ eggs), tested for *Fusarium* infection by PCR amplifying the TEF-1α gene (n= 68 turtle internal egg swab samples) and sequenced eight amplicons for screening of FSSC membership on an Illumina Miseq. Hatchability was 72% for asymptomatic eggs, whilst only 8% of symptomatic eggs hatched. Eight percent of asymptomatic and 58% of symptomatic eggs 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 already reported in sea turtle egg fusariosis. Our study therefore suggests that observed hatching failure of eggs showing symptoms of fusariosis is at least partially caused by *Fusarium* pathogens within FSSC in a freshwater turtle. This report highlights that fusariosis is more widespread among the Testudines order than previously reported and is not limited to sea environments, which is of particular conservation concern.

Introduction

In the last two decades there has been an unprecedented and worldwide emergence of fungal pathogens threatening animal and plant biodiversity (Fisher et al. 2012). Recently, there has been increasing concern over the emerging fungal pathogen *Fusarium* in endangered sea turtles, which causes hatching failure in eggs of sea turtles worldwide (Smyth et al., 2019). So far, reports of the pathogenesis and distribution of this pathogen has been limited to sea turtles, and it is unknown whether this pathogen also poses a threat to freshwater and terrestrial turtle species, of which there are 356 known species (Rhodin et al., 2017).

First reports of sea turtle eggs colonized by fungi were done 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 as pathogenic to sea turtle eggs (Sarmiento-Ramírez et al., 2010), and in 2012 *Fusarium solani* was recognized as a new fungal emerging infectious disease (EID) that may be contributing to population declines in sea turtles (Fisher et al., 2012). Infections caused by ˜60 species belonging to the *Fusarium solani* species complex (FSSC) have been reported in sea turtles around

the globe (Brofft Bailey et al., 2018; Candan, 2018; Sarmiento-Ramírez et al., 2014).*Fusarium* has been isolated from both diseased and asymptomatic sea turtle eggs, and infection may be vertically transmitted from the mother cloaca or horizontally transmitted from the environment (Sarmiento-Ramírez et al., 2014). Given the large number of *Fusarium* species and their global range, it is possible that this fungal disease is not limited to sea environments and may also be spreading to freshwater environments. Indeed, *Fusarium* is thought to thrive in water and damp environments (Smyth et al., 2019), and therefore fresh-water turtle species may also be at risk, yet there has been to date little surveillance of non-oceanic turtle species.

In this study, we screened 680 eggs from artificial nests of an endangered freshwater turtle species (*P. unifilis*) that inhabits the Amazon and Orinoco basin in South America for visual symptoms of fusariosis and assessed hatchability. We tested 68 eggs by PCR for*Fusarium* infection and sequenced eight of those amplicons on an Illumina platform to screen for members of FSSC.

Materials and Methods

Study site and sample collection

Sampling and data collection were performed from March to May 2019 and April to May 2020 in artificial nests relocated at the Tiputini Biodiversity Station (TBS) (037'05"S, 7610'19"W, 190-270 m a.s.l) situated in the Yasuni Biosphere Reserve in the Orellana province of Ecuador. After the incubation period (3 months) we determined symptoms of fungal infections by visual inspection. Eggs were cataloged as symptomatic eggs when covered with unusual colored spots (green, pink, greyish) and with a non-uniform shape (Figure 1a), and eggs without these traces were cataloged as asymptomatic. In 2019, we sampled inner eggshells of 68 eggs (29 asymptomatic, 39 symptomatic) using a sterile swab. In 2020, hatchability data of 680 eggs (394 asymptomatic eggs, 286 symptomatic) from 23 different nests was collected. See supplementary information for detailed information on sampling design and data collection.

DNA extraction and Sequencing

DNA was extracted from 68 inner-eggshell swabs using the NucleoSpin® 96 Soil extraction kit (Macherey-Nagel, Germany) following kit instructions. The PCR amplification of the TEF-1α gene was performed using *Fusarium* -specific primers with the cycling conditions reported previously (Cobo-Díaz et al., 2019). Eight inner-eggshell swabs (four-symptomatic and four-asymptomatic eggs) were sequenced following the Illumina MiSeq sequencing methodology as described by Menke et al. (2017). More detailed information is provided in supplementary material.

Bioinformatics and phylogenetic analysis

Paired reads from Illumina MiSeq sequencing were processed with the 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 using the RESCRIPt (Robeson et al., 2020) and the NCBI database using BLASTnt (Table S1). For phylogenetic analyses we included *Fusarium* spp., isolated from sea turtles' eggs, crops, environment and humans, and in addition, we included*Fusarium* spp., from the Fusarium Tricinctum Species Complex (FTSC) and Fusarium Fujikuroi Species Complex (FFSC), to show the different complexes' clusters. We aligned and generated a Bayesian phylogenetic tree with 33 sequences and an outgroup sequence by using the MrBayes 3.2.6 (Ronquist et al., 2012) module within Geneious v.11.0.5 (Kearse et al., 2012). Detailed information on bioinformatics and the phylogenetic analysis is provided in the supplementary material.

Statistical analyses

To investigate whether infection status varied across nests we used a General Linear Model (GLM) with a binomial distribution within the R statistical software (RStudio Team; 2020). To investigate hatching success (hereafter hatchability) according to fusariosis symptom status, we performed a Generalized Linear Mixed Model (GLMM) with a binomial distribution using the R package "lme4" (Bates et al., 2015). To control for pseudo-replication, we entered nest ID as a random factor.

Results and Discussion

Several studies have reported and confirmed infections by *Fusarium* pathogens in sea turtles around the world and have investigated the relationship between FSSC infections and hatching success (e.g. Sarmiento-Ramirez et al., 2010; 2014). However, the negative effects of FSSC in freshwater turtles have not been reported until now. We found that all 23 nests of the yellow-spotted turtle monitored in this study had eggs showing symptoms of fusariosis, with an average of 42% of eggs symptomatic across nests (range 10-100%; Table S2). There was significant variation in the proportion of diseased eggs between nests ($X^2_{1,\ 22}$= 192.48; p-value < 0.001; adj-R^2 = 0.332) suggesting that some nests were more vulnerable to infection than others. Symptoms of FSSC infections in eggs of the yellow-spotted turtle were significantly associated with hatching success ($X^2_{1,\ 677}$= 149.86; p-value < 0.001; adj-R^2 = 0.525) with a 72% hatching success for asymptomatic eggs (n = 394), whilst only 8% of symptomatic eggs hatched (n = 286) (Figure 1b). In line with our results, experimental inoculation of *F. solani* in eggs of the sea turtle *Caretta caretta* resulted in a hatching success rate of 18% (n = 12) compared to 92% (n = 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).

Fusarium -specific PCR tests of 29 asymptomatic and 39 symptomatic eggs revealed that 59% of the symptomatic eggs tested positive for *Fusarium* (Table S3), whilst the remaining 41% tested negative (Table S3), suggesting that infected eggs may not only be afflicted by pathogenic *Fusarium* and that there are multiple causes of infection. Indeed, previous studies have reported infections of sea-turtle eggs caused by different fungi within *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 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, it is not well understood if colonization is systematically pathogenic and other species of *Fusarium* may 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 the pathogenic FSSC (PP= 1; Figure 2). 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. keratoplasticum* and *F.solani* isolates from human, crops and sea turtle egg infections. ASV_4 grouped with *F. solani* isolated from crops and *P. unifilis* eggs (PP= 0.98; Figure 2). 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. keratoplasticum* isolates from *P. unifilis* eggs (PP= 0.933; Figure 2). Our results add novel data to a recent study reporting the presence of *F. keratoplasticum* in *P. unilfilis* eggs in artificial nests of the same geographical region (García-Martín et al., 2021) by adding the incidence of the FSSC's *F. falciforme* and *F.solani* .

Although both our study and that of García-Martín et al. (2021) were conducted on harvested eggs, the source of pathogenic FSSC is likely to be vertical transmission from the mother or horizontal transmission from the natural environment (since eggs are incubated in sand from the original nesting site). Whilst it is possible that infection may spread more easily within artificial nests than in natural settings, our combined results suggest a possible *Fusarium* infection outbreak in a remote area of the Amazon and that it is likely to be a major contributor of infectious hatching failure in this freshwater turtle species. This has important implications for turtle conservation efforts worldwide, since egg harvesting, and artificial incubation is a major strategy applied by turtle conservationists. Further studies are necessary to understand the epidemiology and distribution of *Fusarium* pathogenic fungi causing hatching failure of endangered non-marine turtle species worldwide.

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Conflict of Interest Statement

The authors declare no conflict of interests regarding this study.

Author contribution

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.

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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Figures

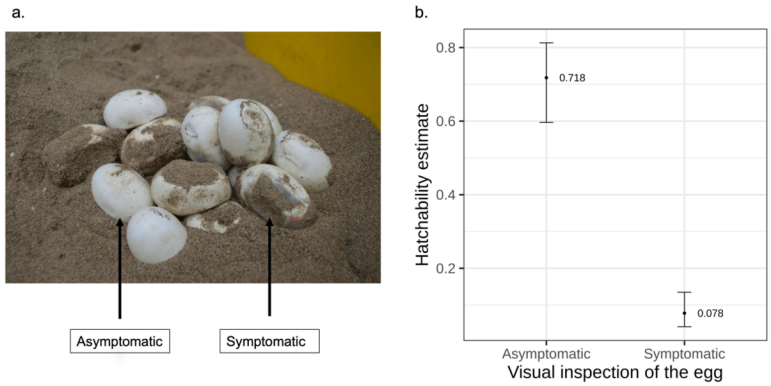

Figure 1. Illustration and hatching success of asymptomatic and symptomatic turtle eggs. a) Picture showing eggs of the yellow-spotted freshwater turtle which are asymptomatic and symptomatic of fusariosis; b) Hatchability estimates and associated 95% confidence intervals according to visual inspection of eggs (GLMM fitted with binomial distribution with nest ID as random factor).

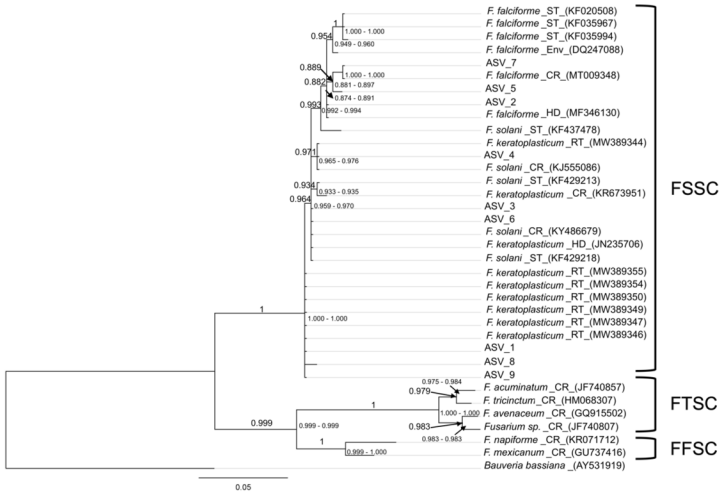

Figure 2. Bayesian phylogenetic tree of the *Fusarium*spp. based on sequence information of the TEF-1[?] gene. ASV 1 to ASV 9 were isolated from the yellow-spotted Amazon river turtle (present study) and visualized together with *Fusarium* spp. isolated from different sources (CR= isolates from crops; HD= isolates from species causing diseases in humans; Env= isolates from the environment RT= isolates from the yellow-spotted river turtle and 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[?] 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.