Pertanika J. Sci. & Technol. 31 (5): 2601 - 2619 (2023)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Fusarium solani Species Complex (FSSC) in Nests of Hawksbill Turtles (*Eretmochelys imbricata*) with High Hatching Success in Melaka, Malaysia

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ABSTRACT

Hatching failure is one of the threats to the declining sea turtle population. Sea turtle egg fusariosis, an emerging fungal disease, has been linked to lower hatching success in sea turtle nests. The disease is associated with the presence of members of the *Fusarium solani* species complex (FSSC). Samples of cloacal mucus, nest sand, eggshells, and eggs were collected from seven hawksbill turtles and their corresponding nests at Melaka's nesting beaches and hatchery site. FSSC was prevalent in the unhatched eggs (n = 32) from the seven study nests, colonising 96.9%. The remaining eggs from the study nests were found to have high hatching success, with a mean of 85.8 ± 10.5% (n = 7). It is unknown if the presence of FSSC contributed directly to embryonic mortality in this study. There are two possible roles of FSSC in sea turtle eggs: as a saprophyte or a primary pathogen. The presence of FSSC in the nest did not always compromise the hatching success of the entire egg clutch. FSSC was not detected in the sand samples of all nests, even though all nests contained *Fusarium*-colonised eggs. The concentration of FSSC in the sand might influence the infection rate of sea turtle eggs and their hatching success. Best practices for hatchery must be in place to achieve high hatching success for sea turtle conservation.

Keywords: Fungal infection, *Fusarium solani* species complex (FSSC), hatchery management, hatching success, hawksbill turtle

ARTICLE INFO

Article history:
Received: 10 September 2022
Accepted: 25 January 2023
Published: 27 July 2023

DOI: https://doi.org/10.47836/pjst.31.5.29

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INTRODUCTION

Of the seven extant sea turtle species worldwide, six are categorised as Vulnerable, Endangered, or Critically Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2021). Sea turtles face

ISSN: 0128-7680
e-ISSN: 2231-8526

various threats throughout their life cycle, and the failure of their eggs to hatch is one of the threats to the population decline of sea turtles. The reported sea turtle hatching success in different nesting sites in Malaysia has been ranging from around 60% to 80% (Chan, 2013; Mutalib & Fadzly, 2015; Salleh & Sah, 2014), with the study site, Melaka, having a hatching success of around 55% (DoF Melaka, unpublished data), which is at the lower end of the range. The variability in hatching success reflects various factors influencing hatchling production (Lindborg et al., 2016).

Sea turtle eggs are vulnerable to multiple human and natural threats, such as climate change (Howard et al., 2014); poaching activities (Chan, 2006); sea water inundation (Pike et al., 2015); nest destruction by beach erosion (García et al., 2003); predation (Heithaus, 2013); and microbial infection (Sarmiento-Ramírez et al., 2010). There are concerns that high fungal density in nests could cause infections of sea turtle eggs and deplete respiratory gases, resulting in reduced hatching success (Bézy et al., 2015; Sarmiento-Ramírez et al., 2010). Sea turtle egg fusariosis is an emerging fungal disease that causes egg failure and mortality, making it a high priority for a study. This disease has been associated with *Fusarium keratoplasticum* and *F. falciforme*, members of the *F. solani* species complex (FSSC) (Smyth et al., 2019). FSSC is a diverse complex comprising at least 60 phylogenetically distinct species with overlapping morphological and cultural characteristics. Infections caused by the members of the FSSC are generally referred to as *F. solani* infections (Short et al., 2013; Zhang et al., 2006).

The genus *Fusarium* has a widespread impact on causing diseases in humans and animals, and it is also known to contain many plant-pathogenic fungi (Leslie & Summerell, 2006). It causes many plant diseases, such as root rots, stalk rots, and vascular wilt diseases. *Fusarium* species have been recovered as the pathogens of several economically important host plants, including bananas, legumes, maise, peas, and sweet potatoes (Summerell et al., 2003; Zhang et al., 2006). FSSC has been implicated as the causative agent of human mycoses, causing fatal infections, particularly in immunocompromised patients (Zhang et al., 2006). *Fusarium* species has also been found to act as an opportunistic pathogen colonising the eggs of the Iberian rock lizard, which died during incubation and spread to infect adjacent live eggs (Moreira & Barata, 2005). In addition, FSSC was commonly found to occur in sea turtles' lesions. Many captive baby loggerhead turtles in the Bahamas were infected with FSSC on their shells and skins (Rebell, 1981). FSSC was also isolated from the lesions of loggerhead turtles in the rescue centres in Italy (Cafarchia et al., 2020). FSSC had also been reported as the agent of a cutaneous infection in an immunosuppressed loggerhead sea turtle, with the source of infection being the sand of the tank where the sea turtle was kept (Cabañes et al., 1997).

FSSC was found to be widely distributed, and many studies have isolated FSSC from sea turtle eggs in nesting sites around the world (e.g., Bailey et al., 2018; Neves et al., 2015; Phillott, 2004; Sarmiento-Ramírez et al., 2014). Sarmiento-Ramírez et al. (2010)

implied that FSSC poses an extremely high risk to the survival of sea turtles, following large numbers of failed loggerhead turtle eggs found having symptoms of fungal infection by FSSC. Despite Melaka being an important hawksbill turtle nesting rookery in Malaysia, in addition to the hawksbill turtle's status as a Critically Endangered species, there is a paucity of published research on hawksbill turtles in Malaysia. In Melaka, a previous study found FSSC on the surface of unhatched eggs and in the hatchery nest sand (Sidique et al., 2017). Nonetheless, many gaps remain because no study has taken samples from the nesting turtles nor the *in-situ* nest sand of beaches in Melaka, which could be the potential source of infection in sea turtle eggs (Keene et al., 2014). *Fusarium* species had been isolated from the cloaca of sea turtles in Pacific Costa Rica and Australia (Keene et al., 2014; Phillott et al., 2002).

Additionally, the fact that the same hatchery site and nest substrates are used across multiple nesting seasons in Melaka's hatchery could be a reason for concern, as pathogens might be able to accumulate in the sand (Patino-Martinez et al., 2012). Pathogens could be introduced by rangers and hatchery personnel when handling the eggs for relocation. Studies on FSSC and sea turtle eggs rarely mention the type of nests or the management strategies used (i.e., *in-situ* or *ex-situ* aspects of the nests). It is difficult to compare between studies because the solution to the problem will differ depending on the type of nest. The present study will address this gap, particularly in the contexts of hatchery management and relocation practices.

This study aims to determine the presence of FSSC in nesting sea turtles, nest sand, and eggs of hawksbill turtles in Melaka, as well as the hatching success of the study nests. The trend for the presence of FSSC across nesting site and hatchery site samples, along with egg mortality stages, can be used to assess the role of FSSC in unhatched eggs and whether it constitutes a threat to sea turtle eggs and therefore hatching success. The findings from this study will be used to propose several recommendations for the best practices for hatchery management.

MATERIALS AND METHODS

Study Site

The study was conducted at the hatchery and sea turtle nesting beaches in Melaka, which contain the highest number of hawksbill turtle nests in Peninsular Malaysia. Hawksbill turtles lay their eggs in various locations on the beaches, including the woody vegetation areas, open sand areas, grassy areas, and the beach's backshore. Among these locations, hawksbill turtles mostly prefer to nest in woody vegetation, especially within an area of sea lettuce (*Scaevola taccada*) (Salleh et al., 2018). Padang Kemunting hatchery, established in 1990, is an *ex-situ* site for incubating and hatching sea turtle eggs. Padang Kemunting is the prime nesting beach in Melaka. The hatchery is a permanent structure on the beach, and the substrate is replaced

every two years with sand from nearby beaches. The conservation of hawksbill turtles in Melaka is managed in collaboration with the Department of Fisheries Melaka (DoF Melaka) and the Worldwide Fund for Nature Malaysia (WWF-Malaysia). DoF rangers patrol sea turtle nesting beaches in Melaka every night during the nesting season, relocating any nests found to the Padang Kemunting hatchery for incubation. For this study, three beaches in Melaka, namely Padang Kemunting (2°18'28.9"N, 102°04'28.8"E), Kem Terendak (2°16'47.4"N, 102°05'49.9"E), and Tanjung Dahan (2°22'06.3"N, 101°59'24.3"E) (Figure 1), were patrolled with DoF rangers to conduct *in-situ* sample collection during August 2018. Study nests were relocated to Padang Kemunting hatchery for incubation, and *ex-situ* sample collection was carried out at the hatchery before and after the egg incubation.

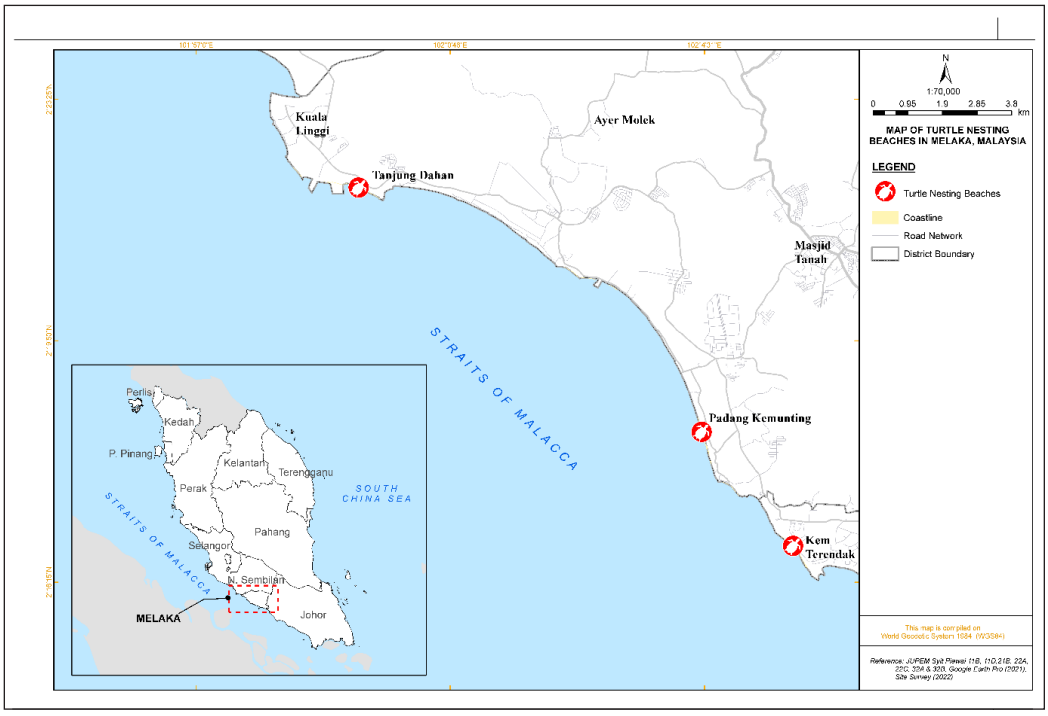


Figure 1. Locations for sample collection in Melaka, Malaysia

Sample Collection

Before Egg Incubation. At the nesting site, all eggs deposited by each nesting sea turtle were collected while wearing gloves and were arranged in a bucket along with the sand from the nest for relocation to the hatchery. All gloves and buckets were sterilised with 70% ethanol prior to usage. Three oviposited eggs were randomly collected straight from the turtle's cloaca and kept in separate sterile bags to prevent fungal contamination from contact with the sand (Sarmiento-Ramírez et al., 2010). Cloacal mucus secreted from the turtle during oviposition was aseptically collected on three replicates per turtle after around 20 eggs were deposited so

that potential contaminants introduced during the nesting process could be flushed off (Keene et al., 2014). An *in-situ* nest sand sample was aseptically taken after collecting all the eggs for relocation by scraping a sterile 50 ml centrifuge tube from the bottom to the top of the egg chamber (Keene et al., 2014). The eggs were then promptly transported to the hatchery site.

At the hatchery site, an egg chamber was dug to a depth of 45 to 50 cm while wearing sterile gloves. A hatchery nest sand sample was collected by a sterile 50 ml centrifuge tube from the bottom to the top of the egg chamber. After that, the eggs were reburied in the egg chamber for incubation.

After Egg Incubation. The nests were excavated after the hatchlings emerged from the nest (50 to 70 days). Sterile gloves were worn to excavate each nest (Bézy et al., 2015). Hatchery nest sand samples from the bottom to the top of the egg chamber were again collected. Five unhatched eggs were sampled from each clutch (except for one clutch in which there were only two unhatched eggs). Eggshell fragments from five hatched eggs were also sampled from each clutch.

Each sampling procedure before and after egg incubation was conducted aseptically to ensure that the samples collected were free from the human introduction of contaminants. The aseptic technique includes cleaning and disinfecting surfaces before use and sterilising equipment that comes into contact with samples (Bykowski & Stevenson, 2008). A total of seven clutches were sampled and labelled as nests A-G.

Hatching Success and Egg Mortality

During post-emergence nest excavation at the hatchery site, the remains of each nest were removed and placed next to the nest. The excavated materials were sorted into empty eggshells and unhatched eggs. The hatching success for each clutch was calculated as the percentage of successfully hatched eggs represented by the number of empty eggshells relative to the total number of eggs incubated in a clutch (Miller, 1999).

To avoid contamination, the unhatched eggs collected as samples at the hatchery site were opened in the laboratory under aseptic conditions during fungal isolation. Unhatched eggs were opened and examined for embryonic development signs and were categorised into the undeveloped stage, the early embryonic stage, the mid-embryonic stage, the late embryonic stage, and the advanced decomposition stage. Categories of early, mid, and late embryonic stages were identified based on Miller et al. (2017) and Rings et al. (2015).

Isolation and Identification of *Fusarium* Species

Each sand sample was diluted by serial dilution before plating onto Peptone Pentachloronitrobenzene Agar (PPA) plates to process the sand samples (including *in-situ* and hatchery nest sand). Cloacal mucus samples were also inoculated on PPA. Egg samples

(including eggs at oviposition and unhatched eggs) were surface-sterilised with sodium hypochlorite, 70% ethanol and sterile distilled water to remove the surface contaminants. After drying the egg with sterile filter paper, the egg's surface was swabbed with a sterile cotton swab to inoculate the PPA. The egg was then opened with a sterile scalpel blade (Wyneken et al., 1988). A sterile cotton swab was swiped through the egg's contents to inoculate PPA (Keene et al., 2014). Five eggshell fragments were randomly cut from each egg sample and cultured on a PPA plate. On the other hand, the eggshell samples of hatched eggs were also surface-sterilised before being cultured on PPA.

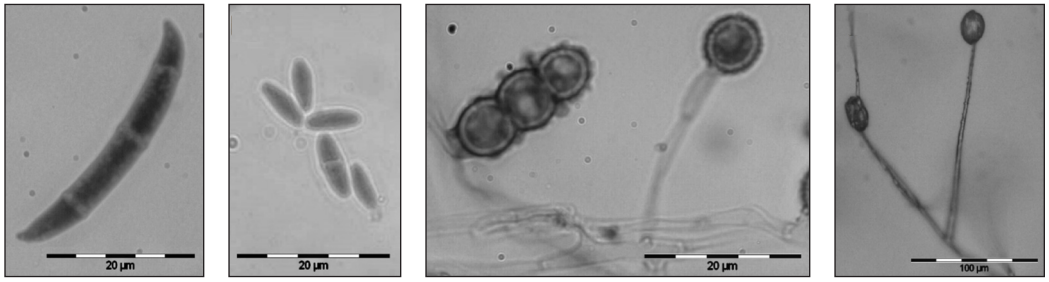
All PPA plates were incubated under standard conditions (alternating 12-hour periods of light and darkness) (Leslie & Summerell, 2006). Each fungus grown in PPA was subcultured onto Potato Dextrose Agar (PDA) to obtain axenic cultures and observe the fungi's macroscopic characteristics. Fungi were then subcultured onto Carnation Leaf-Piece Agar (CLA). For identification, the microscopic characteristics of the fungi growing on the leaf pieces in the CLA were observed and identified under a light microscope with reference to Leslie and Summerell (2006) and Nelson et al. (1983). The three types of culture media used in this study, PPA, PDA, and CLA, were prepared according to the standard recipes and procedures from Leslie and Summerell (2006).

RESULTS

Isolation and Identification of *Fusarium* Species

Fusarium was found in 64 of the 130 samples collected from seven nests. All cultures were identified as members of FSSC. Only FSSC was found in the samples in which it was identified based on its morphological characteristics.

By observing the microscopic characteristics of the CLA cultures, FSSC produced wide, straight and stout macroconidia. The macroconidial apical cells were rounded and blunt, while the basal cells had a notched end. The microconidia produced were oval, ellipsoid or fusiform. The monophialides were long and bearing microconidia. Chlamydospores were globose and formed singly or in short chains (Figure 2). Macroscopic characteristics were



(a) (b) (c) (d)

Figure 2. Microscopic characteristics of FSSC: (a) macroconidia, (b) microconidia, (c) chlamydospores in a short chain on the left and singly on the right, and (d) monophialides bearing microconidia

observed on PDA. Cultures of FSSC were white with sparse to abundant mycelium. Many cultures did not produce pigments, while some produced light brown pigments (Figure 3).

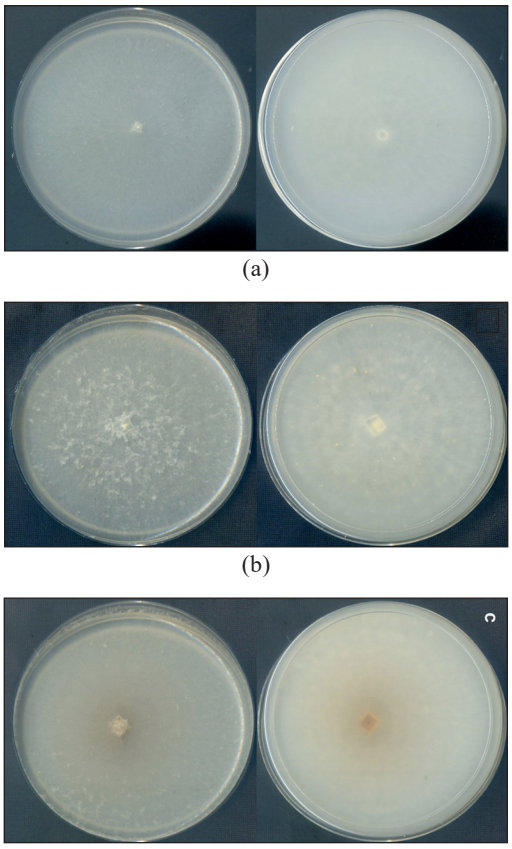


Figure 3. Macroscopic characteristics of FSSC on PDA. In each pair from (a) to (c), the left plate is the upper surface, and the right plate is the under the surface: (a) sparse mycelium; no pigmentation, (b) abundant mycelium; no pigmentation, and (c) sparse mycelium; light brown pigmentation

Determination of FSSC Presence in Samples

The sample types which contained FSSC included the eggshells of unhatched eggs, the contents of unhatched eggs, *in-situ* nest sand, the eggshells of hatched eggs, and hatchery nest sand after egg incubation. From all the sample types, the percentage of recovering FSSC was the highest in unhatched eggs, 96.9% of the eggshells (n = 32) and 84.4% of the content (n = 32). In contrast, FSSC only recovered from 5.7% of the eggshells of hatched eggs (n = 35). FSSC had a low occurrence in the sand samples as well, with only 28.6% of the *in-situ* nest sand (n = 7), 28.6% of the hatchery nest sand after egg incubation (n = 7), and none in the hatchery nest sand before egg incubation (n = 7) (Table 1).

None of the samples from eggs at oviposition and cloacal mucus were found to have FSSC. Additionally, all samples of the surface of the eggs swabbed did not contain FSSC due to surface-sterilisation, which showed that the method of surface-sterilisation used was reliable and confirmed that the FSSC isolated from the unhatched eggs were indeed from the eggshells and the egg content instead of the surface (Table 1).

Hatching Success and Egg Mortality

In this study, the nests had a high mean hatching success, at 85.8 ± 10.5% (n = 7). A trend was observed between the hatching success and FSSC presence in sand samples. When FSSC was detected in *in-situ* nest sand or hatchery nest sand after egg incubation, nests C, D, and E had lower hatching success than the other nests (Table 2).

Table 1
The number and type of samples collected with FSSC presence in samples

Sample types	Number of samples collected	Percentage of recovering FSSC from each sample type (%)
Eggs at oviposition	21	0
Cloacal mucus	21	0
In-situ nest sand	7	28.6
Hatchery nest sand before egg	7	0
Hatchery nest sand after egg incubation	7	28.6
Eggshells of hatched eggs	35	5.7
Unhatched eggs:	32	
Surface		0
Eggshells		96.9
Content		84.4
Total	130	

Table 2
Hatching success of study nests and FSSC presence in different sample types for each nest

Nests	A	B	C	D	E	F	G
Nesting beaches	KT	PK	PK	PK	KT	KT	TD
Clutch size	117	101	103	157	112	177	150
Hatching success (%)	86.3	93.1	76.7	70.7	79.5	95.5	98.7
Eggs at oviposition	-	-	-	-	-	-	-
Cloacal mucus	-	-	-	-	-	-	-
In-situ nest sand	-	-	-	+	+	-	-
Hatchery nest sand before egg incubation	-	-	-	-	-	-	-
Hatchery nest sand after egg incubation	-	-	+	+	-	-	-
Eggshells of hatched eggs	+	-	-	-	+	-	-
Unhatched eggs (eggshells)	+	+	+	+	+	+	+
Unhatched eggs (content)	+	+	+	+	+	+	+

Abbreviations used: KT for Kem Terendak, PK for Padang Kemunting, TD for Tanjung Dahan
Symbols used: "+" for FSSC isolated from the samples, "-" for FSSC not isolated from the samples

For unhatched eggs containing FSSC, the highest percentage of egg mortality occurred during the undeveloped stage (51.6%), followed by the early embryonic stage (25.8%), which means that most eggs sampled died early in the incubation period. Less dead eggs were found at the late embryonic stage (16.1%) and advanced decomposition stage (6.5%). No mortality was observed in the mid-embryonic stage (Table 3).

Table 3
The proportion of unhatched eggs with FSSC in each egg mortality stage

Stages of unhatched eggs	Number of eggs	Percentage (%)
Undeveloped stage	16	51.6
Early embryonic stage	8	25.8
Mid embryonic stage	0	0
Late embryonic stage	5	16.1
Advanced decomposition stage	2	6.5
Total	31	100

DISCUSSION

The Source of *Fusarium* Species

From the analysis of FSSC presence in the samples collected, the results showed that nest sand samples in the hatchery did not contain FSSC initially, but the condition changed after the egg incubation to around one-third of the nests being infested with FSSC. When a nest is established for egg incubation, the nest environment will differ from that before incubation, becoming warmer and moister than the sand alone (Gammon et al., 2020; Wyneken et al., 1988). It is caused by sea turtles releasing a substantial amount of cloacal mucus and eggs to the nests during nesting (Wyneken et al., 1988). Furthermore, embryos generate heat during the metabolic process of development (Gammon et al., 2020). The changes in the nest environment during incubation might encourage the growth of fungi (Keene et al., 2014; Wyneken et al., 1988). However, nest conditions like temperature and humidity might not be the sole factor determining disease development. Other factors like embryo physiology and physical environments, such as natural immunosuppression and sand composition, might also play a role (Phillott & Parmenter, 2001; Sarmiento-Ramírez et al., 2010). For instance, sea turtle nests with clay/silt content were more susceptible to *Fusarium* invasion (Sarmiento-Ramírez et al., 2014).

In this study, *Fusarium* species were not detected in sand samples of all nests, even though they contained *Fusarium*-colonised eggs, which is also consistent with a previous study in Melaka by Sidique et al. (2017). Elshafie et al. (2007) and Neves et al. (2015) also isolated fungi from the sea turtle eggs but not from the corresponding sand samples. Some studies implicated that the source of *Fusarium* infection was the nesting sand because only eggs in contact with the sand were infected, while *Fusarium* species is also common as a soil saprophyte (Phillott et al., 2002; Sarmiento-Ramírez et al., 2014). FSSC was not isolated from some of the sand samples in this study, possibly because FSSC was not present at a detectable level in the sand. Wyneken et al. (1988) indicated that fungi might be present in the sand at very low levels and thus could be missed when the samples are collected. When FSSC was at detectable levels and isolated from the sand samples in this

study, the nests from which they were sampled were found to have lower hatching success (Table 2). It may indicate that the concentration of FSSC in the sand might influence the infection rate of sea turtle eggs and their hatching success. Thus, it is important to include the measurement of FSSC concentration in future studies.

The study by Bézy et al. (2015) found that high fungal abundance in the nesting sand had negatively affected hatching success by which fungal decomposition of the organic matter diminished the oxygen supply in the nests. Nest sand in the study site by Bézy et al. (2015) was found to have high organic matter content. The organic matter content in nest sand could be an important factor in determining the level of microbial activity in the nests (Trullas & Paladino, 2007). Since FSSC was not detected in some of the nest sand samples in the present study, the organic matter content in the sand might be low in this case. Future studies should determine the organic matter content in the sand at this study site and the relationship between the concentration of FSSC and hatching success.

A study by Sarmiento-Ramírez et al. (2014) revealed the presence of the members of the FSSC, namely *F. falciforme* and *F. keratoplasticum*, in the eggs of six sea turtle species (i.e., green, loggerhead, hawksbill, olive ridley, leatherback, and flatback) in various nesting locations across the Atlantic Ocean, Indian Ocean, Pacific Ocean, and Caribbean Sea, implying that FSSC is globally distributed. In a different study by Sidique et al. (2017) in Melaka, *F. falciforme* and *F. keratoplasticum* had also been isolated from the sand of egg chambers in Padang Kemunting hatchery, confirming the presence of FSSC in Melaka, but like in the present study, they also did not manage to isolate FSSC from all corresponding sand samples. Thus, another potential circumstance for *Fusarium* species to come into contact with sea turtle eggs is via the oviduct of nesting turtles (Phillott et al., 2002). Although FSSC was not isolated from the cloacal mucus sampled from the nesting turtles in this study, microbial transfer from the nesting turtles to the eggs may still occur in the oviduct before oviposition. Microbes may not have been detected in the cloacal mucus sampled during oviposition because the phase of microbial shedding may have ended (Wyneken et al., 1988).

The ovipositor of sea turtles is often exposed to beach sand during the nesting process. Soil microbiota can accumulate on the ovipositor and enter the reproductive tract when the ovipositor retracts into the cloaca. When fungal spores are lodged in the sperm storage tubules, they could be transported along with spermatozoa and be enclosed in the egg (Phillott et al., 2002). In Australia, FSSC has been isolated from both the cloaca of nesting sea turtles and the exterior of unhatched eggs, suggesting that intra-oviductal contamination of eggs is possible (Phillott et al., 2002). *Fusarium* species have also been isolated from the cloacal mucus of olive ridley turtles in Pacific Costa Rica, though it was not isolated from the eggs (Keene et al., 2014).

FSSC was not isolated from the eggs at oviposition in this study. Although we did not find evidence of eggs being contaminated in the oviduct, FSSC could still be included in

eggs at a very low level or prevalence (A. D. Phillott, personal communication). Personnel handling nest relocation were also unlikely to introduce *Fusarium* species, as all contacts were properly sterilised. In short, FSSC could only be traced from the sand in this study but not all samples contained FSSC. As only seven clutches were sampled in this study, to increase the reliability, more samples and a longer study period are required to confirm the source of FSSC in Padang Kemunting hatchery, as well as the conditions that will lead to high FSSC concentrations in sand samples.

The presence of FSSC in sea turtle nests has been implicated in causing mass mortalities and low hatching success in sea turtle eggs (Sarmiento-Ramírez et al., 2010). However, even though FSSC was present and prevalent in the sampled unhatched eggs in this study, the nests from which they were sampled were found to have high hatching success. From this study, it was impossible to determine if FSSC contributed directly to egg mortality because *Fusarium* species recovered from diseased materials could be primary pathogens or saprophytes (Leslie & Summerell, 2006; Phillott, 2002). As a primary pathogen, *Fusarium* could cause diseases and mortalities in live sea turtle eggs (Sarmiento-Ramírez et al., 2010), whereas as a saprophyte, *Fusarium* may only colonise eggs after they have died (Phillott & Parmenter, 2001). Thus, the following sections will discuss the different mechanisms in which how these two roles of *Fusarium* might play out in this study.

Fusarium Species as a Primary Pathogen

This study discovered that FSSC was much more prevalent in the unhatched eggs than in the hatched eggs. FSSC strains might have penetrated the eggshells of unhatched eggs into the contents of the eggs. Mechanisms that FSSC might use as primary pathogens to cause the death of live eggs include producing enzymes to degrade the eggshells (Phillott, 2004). FSSC can also extract and reduce calcium content in the eggshell, impairing its functions as a barrier and thus allowing fungi to penetrate the egg. The loss of calcium in the eggshell could also lead to a calcium deficiency in the embryos, as embryos would be unable to obtain calcium from the eggshell for embryonic development, potentially resulting in embryonic mortality (Phillott et al., 2006). Once FSSC is inside a sea turtle egg, it might invade the embryonic tissues, such as the liver, heart, and gut (Phillott, 2004). In the study by Sarmiento-Ramírez et al. (2010), when sea turtle eggs were infected with FSSC, the size of the infected areas was observed to grow over time and developed into large necrotic lesions, causing embryonic mortality.

Another mechanism that fungi might also use to affect embryonic development and egg hatching by secreting toxic compounds called mycotoxins. FSSC is known to secrete harmful mycotoxins, though it should be noted that not all FSSC strains produce mycotoxins (Azliza et al., 2014). No study has been done yet on whether FSSC produces mycotoxins on sea turtle eggs and its impact. Nevertheless, the mycotoxins of another fungal species

called *Aspergillus flavus* are extracted from the eggshells and eggs of dead sea turtles eggs. The level of mycotoxins was believed to be able to cause embryonic mortality (Elshafie et al., 2007).

Sarmiento-Ramírez et al. (2010) conducted pathogenicity tests on loggerhead turtle eggs with isolates from the member of the FSSC called *F. keratoplasticum* through inoculation challenge experiments. As a result of the experiments, *F. keratoplasticum* was reported as pathogenic to loggerhead turtle eggs because they fulfilled Koch's postulates. *F. keratoplasticum* and *F. falciforme* are the members of FSSC found to have high virulence in sea turtle eggs. They are also highly adapted to the host environment because their optimal growth temperature coincides with the optimal incubation temperature for sea turtle eggs (Sarmiento-Ramírez et al., 2014). An environment conducive to turtle egg incubation is also ideal for the growth and colonisation of these pathogens, thus presenting a significant threat to sea turtle eggs (Keene et al., 2014; Sarmiento-Ramírez et al., 2014; Wyneken et al., 1988). Since FSSC is pathogenic to sea turtle eggs, FSSC isolates recovered in this study may be likewise pathogenic.

Fusarium Species as a Saprophyte

Fusarium is soil-borne and can act as an opportunistic fungus that feeds on dead eggs (Moreira & Barata, 2005; Phillott & Parmenter, 2001). It is commonly recovered from diseased plant parts as a saprophyte (Leslie & Summerell, 2006). Unhatched eggs in this study were sampled after 50 to 70 days of incubation during post-emergence nest excavation. Most sampled unhatched eggs were in the undeveloped stage, and some were in the early embryonic stage (Table 3). It means that the eggs had ceased development since the beginning of incubation, which could range from day 1 to day 10 of the incubation period (Miller et al., 2017). Saprophytes and secondary pathogens are more likely to be recovered from samples that have been dead for a long time compared to newly colonised samples because the longer the period between mortality and analysis, the more likely it is for other fungal species that are not the primary coloniser to infiltrate the samples (Leslie & Summerell, 2006).

Opportunistic pathogens could use dead eggs to gain nutrient resources and then spread to kill other live eggs (Moreira & Barata, 2005). In the laboratory incubation of green and loggerhead turtle eggs in eastern Australia by Phillott and Parmenter (2001), the first appearance of FSSC was always on a dead egg, indicating a saprophytic role of the FSSC. The dead egg then acted as a nutrient source and a focus for spreading FSSC to other live eggs in the clutch (Phillott & Parmenter, 2001). In extreme cases, eggs were colonised, and none hatched (Phillott & Parmenter, 2001; Sarmiento-Ramírez et al., 2010). Similar observations were also seen in a laboratory experiment conducted by Moreira and Barata (2005) on the eggs of another reptile species, the Iberian rock lizard. *Fusarium*

was found colonising eggs that failed during incubation and spread to colonise adjacent eggs (Moreira & Barata, 2005). In these experiments, the eggs with healthy appearances did not show signs of fungal presence until they were colonised from the adjacent eggs. The spread of FSSC was suggested as an opportunistic invasion (Moreira & Barata, 2005; Phillott & Parmenter, 2001).

Since most of the unhatched eggs in this study had been dead from the early days of incubation, they could have also been the source of the colonisation of adjacent eggs. However, all nests containing FSSC in this study had a mean hatching success of around 86%, suggesting that FSSC in the unhatched eggs did not spread widely to kill other eggs. In the field experiment by Moreira and Barata (2005), fungi colonised dead eggs of the Iberian rock lizard but did not spread and affect other live eggs. As a result, it is feasible that *Fusarium* species present in the nest and acts as a saprophyte colonising dead eggs without compromising the hatching success of the entire egg clutch, which could be the case in the present study.

Nevertheless, both roles of *Fusarium* species as a primary pathogen or as a saprophyte have pointed to mass mortalities and low hatching success of sea turtle eggs in other studies, while the results of the present study indicated otherwise. A recent study by Bailey et al. (2018) discovered FSSC in sea turtle nests with high and low hatching success, but the reason for this discrepancy is unknown. Perhaps the nest's physical environment and conditions, as discussed above, such as organic matter content and composition, temperature, and humidity, could be important in determining whether FSSC would proliferate in sea turtle nests. Further studies are needed to determine the nest conditions in which FSSC would spread and infect sea turtle eggs.

Implications for Hatchery Management

Even though FSSC was present in the hatchery nests in the current study, the mean hatching success was around 86% (n = 7), as contrasted with the hatchery's consistently low hatching success, which ranged from 38.0% to 61.4% in the recent years from 2014 to 2018, with a mean of 55.5% (n = 2410) (DoF Melaka, unpublished data). Sea turtle egg relocation to hatcheries has been implied to cause low hatching success (Mortimer, 1999), but the results of this study show that relocation does not always have a negative impact on hatching success. Hatching success did not appear to be greatly affected by the reuse of the hatchery site and nest substrate across several nesting seasons at the current study site, which is also consistent with a study by Patino-Martinez et al. (2012). Thus, the high hatching success in this study might be attributed to the researcher's good handling practices for nest relocation as opposed to usual hatchery practices. High hatching success can be achieved in a hatchery when all precautions are taken and all protocols are followed closely while also being subjected to variations like environmental conditions that differ from site

to site. Eggs in this study were collected directly from the ovipositors during nesting by hands while wearing sterilised gloves. The eggs were gently placed in a sterile bucket and carefully transported to the hatchery without any shocking movement or delay. It would require frequent patrolling to relocate the eggs on time. Eggs were reburied gently in the hatchery in which the nest parameters approximate the natural nest of hawksbill turtles.

Rangers and hatchery personnel should avoid direct skin contact with sea turtle eggs by wearing gloves and cleaning the tools when handling eggs for a relocation because fungi or even other microbes may be transferred from nest to nest or from beach to beach through personnel handling different nests. Relocation practice is important and could contribute to the contamination of eggs if done incorrectly, yet it is rarely highlighted in studies. A comparison of hatchery manuals and guidelines from different locations shows inconsistencies in the relocation practices (Table 4). Using gloves and clean tools is often not documented or emphasised as precautionary measures during relocation. Although the use of gloves is documented in Malaysia's manual (Sukarno et al., 2007), it commonly does not happen in practice (personal observation and consultations). Hatchery manuals and guidelines should be revised and made compulsory for the use of gloves during the collection and reburial of eggs and change with new ones each time when handling different clutches.

Clean buckets or containers should also be used to transport the eggs to prevent cross-contamination. Retraining of personnel must be conducted regularly to ensure consistency and reliability. It is even more important at sites where personnel often change with each nesting season. As an additional mitigation, it is also important to ensure that hatchery sands are regularly refreshed by replacing them at every nesting season, if possible, to reduce fungal or other microbial contamination, especially at permanently placed hatcheries. While the present study only included seven clutches, good handling practices can result in high hatching success. Furthermore, maintaining hygiene practices could

Table 4
Summary of the relocation practices listed in hatchery manuals and guidelines

Practice	Reference
"Catch eggs by hand as they drop from the cloaca and place them gently in a bag or bucket."	Mortimer (1999)
"Hand should be clean and dry before handling the eggs."	Ahmad et al. (2004)
"Excavate the eggs by hand and place them in a rigid container."	North Carolina Wildlife Resources Commission (2006)
"Whilst wearing gloves the eggs are carefully placed in a plastic bag."	Couchman et al. (2009)
"Translocation of eggs from the original nest into a rigid container must be done carefully. Prepare equipment such as buckets or rigid containers and rubber gloves." [a]	Sukarno et al. (2007)
"Catch eggs as they are being laid by hand or into a clean plastic bag."	Phillott and Shanker (2018)

[a] Translated into English from original content in Bahasa Malaysia

protect staff from microbial infections and diseases such as *Fusarium* keratitis, which can affect immunocompetent individuals (Chang et al., 2006). It is best to leave sea turtle eggs undisturbed in their original location (*in-situ*), but since relocation (*ex-situ*) is highly necessary for sea turtle conservation in many of the nesting sites due to immense human and natural threats, strict practices must be in place.

CONCLUSION

In this study, FSSC is prevalent in the unhatched eggs but not in the hatched eggs. Sources of FSSC were traced to the sand samples, but not all samples contained FSSC, indicating that further studies are needed to confirm the source of FSSC and the conditions that will result in the spread of FSSC in sea turtle nests. This study also revealed that FSSC, recognised as the causal factor of an emerging fungal disease at other nesting sites, does not necessarily compromise the hatching success of sea turtle nests. Good hatchery management, stringent handling practices, and well-trained staff are the key to high hatching success. As this is one of the earliest studies in Malaysia to associate FSSC, hatching success, and egg mortality, more research is required in this regard in Malaysia.

ACKNOWLEDGEMENT

We thank the Department of Fisheries (DoF) Malaysia for permitting us to conduct this study in Melaka. We are thankful to Worldwide Fund for Nature Malaysia (WWF-Malaysia) for providing logistical and accommodation support and assistance in the field. Special thanks to the dedicated team of DoF rangers for guiding us in beach patrolling and locating the sea turtles. Thank you to the School of Biological Sciences, Universiti Sains Malaysia (USM), for providing the laboratory facilities and supplies needed for this study. We also thank Dr Rosnida Tajuddin and Dr Nik Izham for guiding techniques in isolating and identifying *Fusarium* species.

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