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Understanding early reproductive failure in turtles and tortoises

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Abstract

Turtles and tortoises (Order Testudines) are facing an extinction crisis, and ecosystems are at risk of collapsing with the loss of key roles they play. Hatching failure is a crucial barrier to population growth and persistence, but its causes are poorly understood, and it is unknown whether fertilization rates are declining as many populations become smaller and more female-biased. Here, we show that very few studies of turtle and tortoise hatching success consider fertilization rates, and those that do use unreliable methods to determine egg fertility. We also show that studies of hatching success are biased towards marine turtles, as opposed to freshwater and terrestrial species, and wild rather than captive populations. To address the lack of reliable methods for assessing fertilization rates in turtles and tortoises, a microscopy-based method (originally designed for bird eggs) for detecting perivitelline membrane (PVM) bound sperm and embryonic nuclei in the germinal disc of unhatched eggs has been developed and tested (in turtle and tortoise eggs). We demonstrate that this method provides unequivocal evidence of egg fertilization in five different turtle and tortoise species from both captive and wild populations, even after eggs have been left in wild nests for the full incubation period. This methodological approach represents a valuable tool for monitoring egg fertility and embryo survival rates in turtles and tortoises, with the potential to provide important insights into the underlying drivers of reproductive failure in threatened captive and wild populations.

Introduction

Turtles and tortoises (Order Testudines) are facing severe population declines: over 50% of species are threatened (includes all critically endangered, endangered and vulnerable species), of which 20% are critically endangered (Lovich *et al*., 2018; Rhodin *et al*., 2018; Stanford *et al*., 2020). Since 1800, at least seven species have gone extinct, three of which have been lost in the past few decades (Stanford *et al*., 2020). Many threats faced by turtles and tortoises impact their reproductive success and lead to reduced hatching success (Lovich *et al*., 2018). Low hatching rates have been reported for several threatened species, including the Leatherback turtle (*Dermochelys coriacea*) (50.4%; Rafferty *et al*., 2011), Olive Ridley turtle (*Lepidochelys olivacea*) (8%; Honarvar, O'Connor, & Spotila, 2008) and green turtle (*Chelonia mydas*) (20–60%; Booth, Staines, & Reina, 2022), and are predicted to decline further under climate change in several species (Fuentes, Hamann, & Limpus, 2010; Pike, 2014).

Several factors may impact hatching rates in wild turtle and tortoise species. Biotic drivers include low egg fertilization rates, potentially linked to male/sperm availability (Miller, 1985); genetically determined developmental abnormalities in embryos (Ingle *et al*., 2021); microbial/fungal infection of eggs (Peters, Verhoeven, & Strijbosch, 1994; Gleason, Allerstorfer, & Lilje, 2020; Carranco *et al*., 2022; McMaken, 2022); maternal condition (Rafferty *et al*., 2011; Duchak & Burke, 2022); and the density of females at a nesting site (Booth, Staines, & Reina, 2022). Habitat fragmentation may also indirectly lead to reduced hatching success and reproductive issues, via inbreeding depression and low genetic diversity (Ennen, Kreiser, & Qualls, 2010). Hatching success is also influenced by abiotic factors including rainfall, flooding, substrate composition and water potential, temperature, salinity and pollution (Ragotzkie, 1959; Mortimer, 1990; Wood & Bjorndal, 2000; Bilinski *et al*., 2001; Stanford *et al*., 2020; Limpus, Miller, & Pfaller, 2021), as well as elevation, slope and erosion of nesting beaches (Kraemer & Bell, 1980; Maneja *et al*., 2021).

Hatching failure is a problem for captive as well as wild turtle and tortoise populations. Captive conditions can negatively affect reproductive condition, potentially leading to male infertility or behavioural/copulation issues, low fertilization rates and decreased hatching success (Currylow *et al.*, 2017), and low levels of reproductive hormones (He *et al.*, 2010). In addition, translocation may stifle breeding success for several years (Currylow *et al.*, 2017). Hatching rates in captive marine turtles have been shown to be consistently lower than in the wild (Owens & Blanvillain, 2013), perhaps due to fertility issues, or captivity-related impacts on embryo survival (e.g. lack of essential fatty acids in maternal diet; Craven *et al.*, 2008; Owens & Blanvillain, 2013). However, in Gopher tortoises (*Gopherus polyphemus*), hatching success was found to be higher in captivity (58.8%) than in natural nests (16.7%), suggesting that low hatching success in the wild is attributable to both intrinsic (egg quality) and extrinsic (nest environment) factors (Noel, Qualls, & Ennen, 2012). Nevertheless, many captive breeding programmes are plagued by elevated hatching failure. For instance, the sole remaining female Yangtze giant softshell turtle (*Rafetus swinhoei*) produced eggs that showed no sign of development for over 8 years before her death in 2019, despite international conservation efforts, including artificial insemination (Lovich *et al.*, 2018; Liu, Li, & Zhang, 2019).

Identifying the underlying reproductive barriers to hatching success is crucial for improving success rates of both wild and captive conservation efforts (Bell *et al.*, 2003; Phillott & Godfrey, 2020; Dovč *et al.*, 2021). Hatching failure can result from either fertilization failure or embryo death, and these two issues may have different causes (Hemmings, West, & Birkhead, 2012). Unfertilized eggs are indicative of parental fertility, copulation or behaviour problems, whereas embryo mortality is more likely due to genetic or developmental problems and/or environmental factors directly affecting the egg or embryo (Hemmings, West, & Birkhead, 2012).

Monitoring fertilization rates also serves a broader goal of understanding the impacts of environmental change on turtles and tortoises (Order Testudines). Eggs are typically deposited by the mother in burrowed nests and left to incubate at ambient temperatures, and the effect of global warming on incubation temperature is therefore one of the most significant conservation concerns for many species (Stanford *et al.*, 2020). With the current rate of global warming, incubation temperatures are likely to exceed the range of 25–34°C required for viable embryo development in some populations close to the equator (Ackerman, 1977; Hawkes *et al.*, 2007; Hays *et al.*, 2017; Hays, Shimada, & Schofield, 2022). Even if embryos survive high incubation temperatures, many turtle and tortoise species have temperature-dependent sex determination, where embryos usually develop into males at cooler incubation temperatures and females at warmer incubation temperatures (Ewert, Etchberger, & Nelson, 2004; Valenzuela, 2004; Valverde *et al.*, 2010). A temperature-induced female bias at the population level could lead to a reduced egg fertilization rate, loss of genetic variation and decreased effective population size (Montero *et al.*, 2018; Hays, Shimada, & Schofield, 2022). These issues have already been identified in certain populations and require urgent monitoring and intervention if they are to be resolved (Hawkes *et al.*, 2007; Fuentes, Hamann, & Limpus, 2010; Booth *et al.*, 2020; Chatting *et al.*, 2021; Hays, Shimada, & Schofield, 2022). Monitoring rates of fertilization failure and embryo death may provide an early indicator of population sex bias (e.g. more unfertilized eggs due to insufficient males/sperm) or high levels of temperature-related embryo mortality, allowing more rapid conservation intervention.

Conservation interventions may themselves also influence hatching success (Marshall *et al.*, 2023). For example, nest relocations are commonly used to reduce threats such as tidal inundation (e.g. McElroy, Dodd, & Castleberry, 2015), but data suggest that manipulation of eggs may contribute to egg failure through increased embryonic death (Wyneken *et al.*, 1988). The impact of nest relocation on hatching success is unclear: some studies report lower hatching success compared to undisturbed nests (e.g. Garrett *et al.*, 2010; Candan, 2018), while others show improvements provided sites are carefully chosen (e.g. Wyneken *et al.*, 1988). Measuring the proportion of embryo death in relocated nests is therefore a useful tool for conservation managers, allowing them to assess how successful or disruptive relocation attempts are, and to measure the suitability of different nest relocation sites.

Despite the importance of distinguishing between fertilization failure and embryo survival as causes of hatching failure, few studies of turtle and tortoise eggs appear to do so. Undeveloped eggs with no visible embryo may be unfertilized, or they may contain an early stage embryo that died before it was visible to the naked eye. Embryonic development begins within the mother's oviduct prior to oviposition, so by the time a fertilized egg is laid, the developing embryo is already several days old. This means that embryo death can even occur prior to oviposition (Abella, García-Cerdá, & Marco, 2017). In published studies to date, undeveloped eggs are typically classified as unfertilized without further examination (e.g. Langer, Kapron, & Davy, 2020; Gane *et al.*, 2020a), and where attempts are made to determine fertilization success, potentially inaccurate macroscopic methods are typically used (Gárriz *et al.*, 2020; Phillott & Godfrey, 2020).

Recently, a small number of reptilian captive breeding programmes have trialled microscopic techniques, originally developed for birds (Birkhead *et al.*, 2008; Hemmings, West, & Birkhead, 2012), to help investigate infertility (Croyle, Durrant, & Jensen, 2015; Croyle *et al.*, 2016; Augustine, 2017), and the application of these methods has also been recommended for assessing fertility of wild sea turtle eggs (Phillott & Godfrey, 2020; Phillott, Godfrey, & Avens, 2021). The techniques allow detection of sperm on the perivitelline membrane (PVM) surrounding the yolk and embryonic nuclei in the germinal disc, thereby providing unequivocal evidence of fertilization. In birds, these techniques have revealed that approximately 52% of unhatched eggs are misclassified as 'unfertilized' using traditional macroscopic techniques (Hemmings & Evans, 2020). However, reptilian studies have so far only been successful in identifying PVM-bound sperm in captive populations of non-marine turtles and tortoises (Croyle *et al.*, 2016). The ability to identify embryonic nuclei in unhatched eggs has not

been demonstrated in any turtle or tortoise species, and the methods have not been tested on marine turtle eggs or on eggs that have been naturally incubated in the wild. To be useful for in situ conservation, these techniques must be applicable to unhatched eggs recovered from wild nests at the end of the incubation period (Phillott & Godfrey, 2020), which have often experienced decomposition, desiccation and insect infestations (Abella, García-Cerdá, & Marco, 2017).

Here, we first review the existing literature on turtle and tortoise hatching success to assess the extent to which studies to date have differentiated between fertilization failure and embryo mortality, and identify which methods have been used to do this. Then, we test the accurate method described above for determining fertilization success on unhatched eggs from three captive species, the Red-footed tortoise (*Chelonoidis carbonarius*), Galapagos giant tortoise (*Chelonoidis nigra*) and Spiny turtle (*Heosemys spinosa*), and three wild species, the Hawksbill turtle (*Eretmochelys imbricata*), the Green sea turtle (*Chelonia mydas*) and the Giant Aldabra Tortoise (*Aldabrachelys gigantea*) and present the first data on Testudines fertilization failure and early embryo mortality rates.

Materials and methods

Current understanding of turtle and tortoise egg fertility: Systematic review

We first conducted a systematic review of turtle and tortoise hatching success literature to establish the extent to which studies have attempted to discriminate between fertilization failure and embryo mortality as causes of hatching failure. We searched English abstracts from papers on Web of Science Core Collection and Scopus on 24 November 2021 using the following terms: ("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate") AND ("turtle" OR "tortoise" OR "testudines" OR "chelonia"). Our search may not have retrieved every paper published on this topic, but since the chosen databases are considered world-leading (Zhu & Liu, 2020), we assumed the retrieved papers represented most of the relevant published literature. Combined, the databases returned 374 records, but we were unable to include 44 papers (6.7%) due to lack of institutional access (23 papers) and/or because they were not published in English (21 papers; see Figure S1 for a depiction of the literature search and screening process following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA); Moher *et al.*, 2009; O'Dea *et al.*, 2021). We read the final set of papers in detail to determine (a) the methods used to determine egg fertility status, (b) the species studied and (c) whether the population was wild or captive.

Unhatched turtle and tortoise egg analysis

We obtained 45 eggs from captive populations of Red-footed tortoise *Chelonoidis carbonarius*, Galapagos giant tortoise *Chelonoidis nigra* and Spiny turtle, *Heosemys spinosa* via British and Irish Association of Zoos and Aquariums (BIAZA) UK Zoo members (Crocodiles of the World, Oxfordshire, and ZSL Whipsnade Zoo). Captive unhatched eggs were removed from incubators as part of standard zoo management procedures and transported to The University of Sheffield, UK, where they were refrigerated or frozen prior to dissection. All eggs were first examined for 'traditional' indicators of fertilization commonly used in the existing literature (see Material S3 and S4). Eggs were considered 'fertilized' according to traditional indicators if eggshell chalking/white spots were observed or visible embryo development was seen in the egg contents (Wyneken *et al.*, 1988; Dovč *et al.*, 2021). Eggs that displayed blood spots but no other signs of development were examined further to ascertain egg fertility (Table 1). Of the 45 captive eggs received, 27 showed no sign of development or had a blood spot only and were therefore examined microscopically (see below).

We also obtained 162 turtle and tortoise eggs from wild populations from the Republic of Seychelles (hereafter referred to as Seychelles) as they became available during the 2021/2022 and 2022/2023 nesting seasons (Table 1), supported by partnerships with conservation organizations on eight islands: Seychelles Island Foundation (Aldabra: 9.4237°S, 46.3433°E); Island Conservation Society (Alphonse Island: 7.0055°S, 52.7269°E); Nature Seychelles (Cousin Island: 4.3315°S, 55.6620°E); Save Our Seas Foundation: D'Arros Research Centre (D'Arros Island: 5.4180°S, 53.2962°E); Olive Ridley Project (Felicité: 4.3238°S, 55.8718°E); Frégate Island Foundation (Frégate Island: 4.5837°S, 55.9386°E); Marine Conservation Society Seychelles (Mahé: 4.6827°S, 55.4804°E); and North Island Company Limited (North Island: 4.3950°S, 55.2453°E). Seychelles has one of the five largest Hawksbill turtle populations (Hitchins, Bourquin, & Hitchins, 2004) and some of the world's longest sea turtle tagging and monitoring programmes (Allen *et al.*, 2010). For all three wild species, partners provided us with information collected during routine monitoring on parent/clutch identity, lay dates, unhatched egg collection dates and fates of other eggs in the clutch. At the end of incubation (~60 days after oviposition for the Hawksbill and Green sea turtles, and between ~6–8 months for the Giant Aldabra tortoise), we opened and visually assessed the contents of failed eggs during routine nest excavations, and randomly collected one or two eggs per clutch that showed no signs of development. Egg yolks were stored in either 5–10% formalin, depending on the availability of different formalin concentrations at different field sites, and transported to the University of Sheffield where they were examined up to 5 months after collection. Of the 162 eggs received, 131 were examined from 124 different clutches. The remaining 25 eggs were omitted due to having insufficient material for microscopic examination, or because they had visible development that was missed upon collection (Table 1).

Captive eggs were opened carefully by cutting around their shells with fine scissors. Frozen captive eggs were allowed to partially thaw before being opened, to ensure the perivitelline layer could be accessed through the albumin. Yolks from wild eggs were removed from formalin solution.

Table 1 Hundred and eighty eight turtle and tortoise eggs were collected from six species; 175 eggs showed no signs of chalking or embryo development and were further processed to determine their fertility status

Species	Common name	Conservation status	Population context	Storage prior to dissection			No of undeveloped eggs	No of eggs with blood spot	No of eggs examined
				Refrigerated	Frozen	Formalin-fixed			
Chelonoidis carbonarius	Red-footed tortoise	NE	Captive	1	2	0	2	1	3
Chelonoidis nigra	Galapagos giant tortoise	NE	Captive	2	21	0	20	3	21
Heosemys spinosa	Spiny turtle	EN	Captive	1	0	0	1	0	1
Eretmochelys imbricata	Hawksbill turtle	CR	Wild	0	0	119	110	0	94
Chelonia mydas	Green sea turtle	EN	Wild	0	0	22	21	0	19
Aldabrachelys gigantea	Aldabra giant tortoise	VU	Wild	0	0	21	21	0	18
	Total			4	23	162	175	4	156

Four had blood spots and these were also examined to determine whether they were fertilized as blood spots may be of ovarian origin (all four eggs were successfully examined). All captive eggs are from Red-footed tortoise, Galapagos giant tortoise and Spiny turtle populations in UK zoos; all other eggs are from wild populations from the Seychelles. All eggs were dissected for examination of the perivitelline membrane (PVM) and/or germinal disc (GD), but not all had retrievable or usable PVM/GD, resulting in a total of 156 eggs successfully examined.

Egg contents were checked for signs of embryonic development and, if possible, pieces of the PVM were removed from directly above the germinal disc using forceps and small dissecting scissors, following the methods of Birkhead *et al*. (2008) and Croyle *et al*. (2016). Germinal disc material, if visible, was siphoned from the yolk surface with a micropipette. If the germinal disc could not be seen or the egg was degraded/infected, as much PVM as possible was extracted from the egg contents to maximize the chance of detecting embryonic cells microscopically. The PVM was washed in phosphate buffered saline (PBS) to remove excess albumin and yolk and placed, along with any germinal disc material, on a microscope slide. A nucleic acid dye, Hoechst 33342 (0.05 mg/mL), was then applied, followed by a coverslip, and slides were left for at least 10 minutes in the dark before examination (Croyle *et al*., 2016).

PVM and germinal disc material were examined at 100–400× magnification using a fluorescence microscope with UV illumination, a BP 340–380 excitation filter and LP 425 suppression filter (Birkhead *et al*., 2008). Photographs were taken for documentation. Care was taken to distinguish between sperm heads and microbes in microbially infected eggs, as Hoechst 33342 stains fungal and bacterial DNA, as well as sperm (Croyle *et al*., 2016; Phillott & Godfrey, 2020).

Examined eggs were classified as fertilized only if embryonic nuclei were identified in the germinal disc or adhered to the PVM. Eggs were classified as unfertilized if the majority of PVM from around the yolk was retrieved and clearly observable under the microscope, and very few/no sperm and no embryonic nuclei were found consistently across all pieces of PVM. If there was insufficient PVM to be confident of fertility status (i.e. abundant embryonic nuclei could not be found), the egg was classified as inconclusive, even if PVM-bound sperm were detected, since the presence of PVM-bound sperm does not provide definitive evidence of fertilization (Croyle *et al*., 2016).

Animal ethics and permits

This research was reviewed and approved by BIAZA to be carried out with UK zoo members, and by the Seychelles Bureau of Standards (SBS) to be carried out with Seychelles conservation organizations (Ref: A0157). Non-viable eggs were received opportunistically from these UK and Seychelles-based collaborators. Hawksbill turtle (*Eretmochelys imbricata*) eggs from Seychelles were authorized for export as per the agreement made with the Ministry of Agriculture, Climate Change and Environment, in accordance with Article 15 of the Convention of Biological Diversity. Additionally, permits were acquired for all eggs collected from species listed under the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES; Seychelles export permit #A1517, #A1615, #A1622; UK import permit #615170/01, #631966/01, #631966/02, #631966/03).

Results

Current understanding of turtle and tortoise egg fertility

We identified 286 studies that investigated turtle and tortoise hatching success. Of these, 76.62% (n = 222) did not assess the fertility status of unhatched eggs, and 6.64% (n = 19) specified that they worked with eggs with visible signs of development only (Material S1–S3). Only 15.73% (n = 45) attempted to classify the fertility of unhatched eggs (see Material S2 for a full list of these studies), and these used a range of different methods, all of which are likely to

overlook early stage embryo death (see Material S4 for additional information on methods used and their limitations). Most studies (~84%) investigating hatching success have therefore overlooked the potential role that egg fertility may play in interpreting and understanding mortality in unhatched eggs.

Our final set of 45 studies focused on 23 species, representing just ~7% of all extant Testudines (Fig. 1). Marine turtles were the best studied, with 5 of 7 marine turtles represented and at a much higher frequency than any other group (30 investigations of marine turtles in total, exceeding the number of freshwater turtle and tortoise studies (n = 23) combined; Fig. 1). The Loggerhead Turtle (*Caretta caretta*) was the most investigated species (13/45 studies (29%)). Freshwater turtles have the greatest number of species studied (total of 17 species), but at a relatively low intensity (≤2 studies per species). Tortoises are under-researched – represented by a single study only, on the Ploughshare Tortoise (*Geochelone yniphora*; Material S2; Bourou *et al.*, 2001). In addition, we found a strong bias towards the study of eggs collected from wild clutches (84.44%), and few studies examining eggs from captive populations (13.3%), or both captive and wild populations (2.22%).

Unhatched turtle and tortoise egg analysis

We identified PVM-bound sperm and embryonic nuclei in samples from all five species we were able to examine: the captive Red-footed tortoise and Galapagos tortoise, and the wild Hawksbill turtle, Green sea turtle and Aldabra giant tortoise (Fig. 2). A single Spiny turtle egg that we obtained had a significant microbial infection which precluded its examination. However, microbial infection did not always prevent examination. We confidently identified sperm and/or

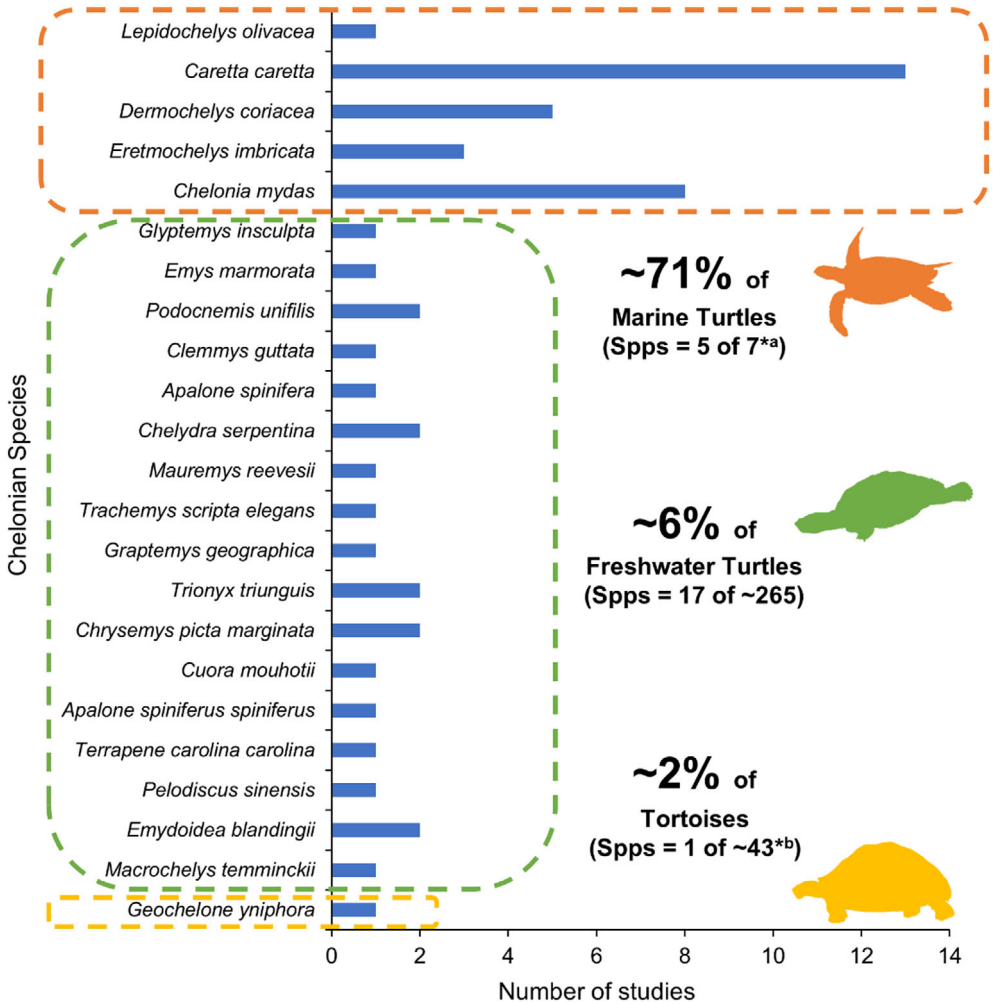

Figure 1 The taxonomic distribution of papers reporting on turtle and tortoise (Order Testudines) egg fertility. Data are from 45 papers in total, some of which report on more than one species. Twenty-three species have been studied in total; thus, approximately 292 (~93%) of turtle and tortoise species have yet to be investigated. Determining approximation of total number of extant turtle and tortoise species: *[a]Marine turtles: n = 7 (Rhodin *et al.*, 2018); *[b]Tortoises (i.e. terrestrial turtles): n = ~43 (Vlachos & Rabi, 2018); Freshwater turtles: Total number of extant species = ~315 (Pough, 2013) – (*a + *b) = ~265.

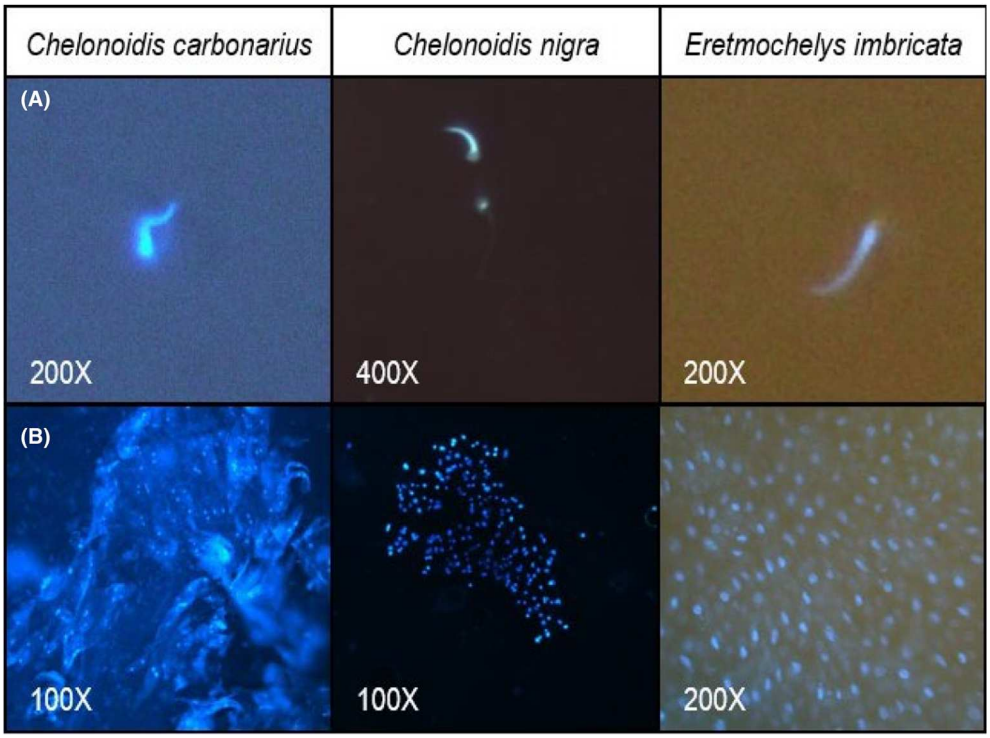

Figure 2 Stained nuclei of (Row A) PVM-bound sperm, and (Row B) embryonic cells and/or tissue and the respective microscopic magnification levels for the Red-footed tortoise (*C. carbonarius* – nuclei on perivitelline membrane), Galapagos tortoise (*C. nigra*) and Hawksbill turtle (*E. imbricata*).

embryonic nuclei in several infected eggs from the other species, aided by the fact that sperm heads and fungal/bacterial cells are morphologically distinct and easily discriminated (Material S5 to compare with an example of microbial nuclei). Microbial infections were more common in refrigerated eggs compared to eggs that were frozen or formalin-preserved, and overall, wild eggs fixed in 5% formalin were best preserved and proved easiest to examine. We were able to detect embryonic nuclei and PVM-bound sperm in undeveloped Hawksbill turtle eggs that had been left in nests for ~60 days after oviposition followed by another <5 months preserved in formalin.

Overall, egg fertility status was determined conclusively in 79% of all microscopic examinations (Fig. 3), and of these, only one sea turtle egg – collected from a wild Hawksbill turtle on Cousin Island – was found to be unfertilized (Fig. 3 and Table 2). The Aldabra giant tortoise had higher rates of infertility, with 28% of samples showing no evidence of fertilization (Fig. 3 and Table 2). All eggs with blood spots (Table 1) were identified as fertilized and, as expected, eggs with visible embryos and/or eggshell chalking (n = 23) consistently tested positive for the presence of embryonic nuclei.

Discussion

Here, we have shown that current understanding of the relative roles of fertilization failure and early embryo death in causing hatching failure in turtles and tortoises (Order Testudines) is limited, taxonomically biased, and suffers from methodological flaws. Most studies do not differentiate between fertilization failure and early embryo mortality, and when they do, the methods employed are inaccurate and/or unclear. Despite recommendations to use alternative methods (Phillott & Godfrey, 2020), studies have yet to adopt new techniques or test their efficacy across species/contexts.

Our literature review revealed biases in the study of hatching failure across turtles and tortoises (Testudines Order). Marine turtles are over-represented (especially *Caretta caretta*), tortoises are severely under-researched, and few studies consider captive populations or wild/captive comparisons. The bias towards wild populations is perhaps unsurprising; few captive breeding programmes exist for marine turtles due to the maintenance of healthy wild populations being prioritized, as well as species-specific challenges of maintaining sea turtles in captive conditions (Owens & Blanvillain, 2013). Wild marine turtle populations are also relatively easy to monitor, as their nesting seasons and locations are fairly predictable. In contrast, freshwater turtles and tortoises occur at lower densities, are more cryptic and have limited seasonal and daily activities (Zylstra, Steidl, & Swann, 2010), making them more challenging to study. The focus on wild marine turtles may also explain why visual assessment of egg contents has been most commonly used to assess egg fertility (see Material S3B) – this is the most practical method in the field. Indeed, several studies

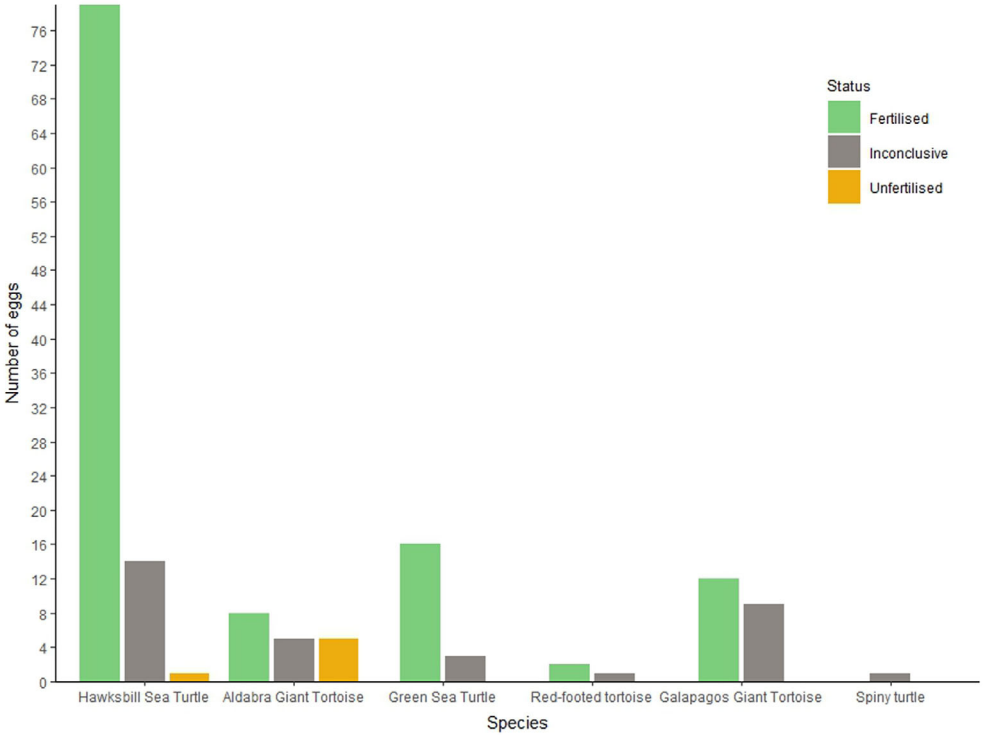

Figure 3 The fertility status of 156 undeveloped eggs across six different turtle and tortoise species. Eggs included those from captive populations from UK zoos (n = 25) and wild populations from eight different Seychelles islands (n = 131): Aldabra (n = 11), Alphonse (n = 3), Cousin (n = 26), D'Arros (n = 18), Felicité (n = 12), Frégate (n = 22), Mahé (n = 9) and North Island (n = 30). A total of 123 conclusive statuses were assigned (i.e. 79% of examined eggs), and 33 eggs remained inconclusive (21% of examined eggs). All eggs with blood spots (n = 4, all from UK zoos) were fertilized.

Table 2 The fertility status of 131 undeveloped eggs across three turtle and tortoise species from eight different Seychelles islands: Aldabra (n = 11), Alphonse (n = 3), Cousin (n = 26), D'Arros (n = 18), Felicité (n = 12), Frégate (n = 22), Mahé (n = 9) and North Island (n = 30)

	Hawksbill Sea Turtle			Green Sea Turtle			Aldabra Giant Tortoise		
	Fertilized	Inconclusive	Unfertilized	Fertilized	Inconclusive	Unfertilized	Fertilized	Inconclusive	Unfertilized
Aldabra	–	–	–	1	1	–	7	2	–
Alphonse	1	1	–	1	–	–	–	–	–
Cousin	21	4	1	–	–	–	–	–	–
D'Arros	10	4	–	–	–	–	1	1	2
Felicité	12	–	–	–	–	–	–	–	–
Frégate	18	3	–	1	–	–	–	–	–
Mahé	8	1	–	–	–	–	–	–	–
North Island	9	1	–	13	2	–	–	2	3
Total	79	14	1	16	3	0	8	5	5

explicitly state that they did not investigate egg fertility due to difficulties with accurately classifying undeveloped eggs (e.g. Pintus *et al*., 2009; Van Lohuizen *et al*., 2016), while others included a disclaimer about the accuracy of their methods (e.g. Gane *et al*., 2020a, 2020b). This highlights the need for a practical solution for both field and captive application.

Most studies investigating turtle and tortoise hatching failure have not discriminated between fertilization failure and early embryonic death as separate contributors to overall rates of egg failure. Consequently, our ability to fully understand the mechanisms underpinning early reproductive failure is limited. For example, Sinaei & Bolouki (2017) suggest that hatching failure in green sea turtles (*Chelonia mydas*) in Oman is associated with maternally transferred heavy metals. However, since the researchers did not investigate the fertility status of unhatched eggs, it remains unknown whether heavy metals primarily impact adult fertility (i.e. gamete production/quality), resulting in unfertilized eggs, or embryo development/survival via contaminated egg contents. We

attempted to move the field beyond this barrier by demonstrating the successful application of a new method across five species, including both captive and wild populations, and showing that they can be used to identify fertilization success and sperm availability. We have demonstrated that microscopic detection of PVM-bound sperm and embryonic nuclei in the germinal disc is usually (79%) successful, even in eggs that have passed their incubation time and undergone a degree of degradation. We have extended the work of others (including Croyle *et al*., 2016), to demonstrate unequivocal evidence of fertilization success (presence of embryonic nuclei from the germinal disc) in undeveloped eggs from several different species of conservation concern, including those retrieved from wild-incubated nests. Consistent with Croyle *et al*. (2016), we found identifying the germinal disc to be difficult (more so than in birds; NH pers. obs.), yet we were still able to detect embryonic cells in 79% of examined eggs, whereas Croyle *et al*. (2016) were not successful in detecting them in any of the eggs they examined. Moreover, while finding only PVM-bound sperm is not conclusive evidence of fertilization, it is nonetheless indicative of successful copulation and sperm availability – if few/no sperm are found, this may reflect issues with sperm production, transfer or survival within the oviduct (Croyle *et al*., 2016). The vast majority of successfully examined undeveloped eggs in our study were found to be fertilized (117/156 or 75%), indicating that traditional approaches significantly overestimate rates of fertilization failure. All eggs with blood spots (Table 1) were identified as fertilized and, as expected, eggs with visible embryos and/or eggshell chalking (n = 23) consistently tested positive for the presence of embryonic nuclei, suggesting that while using these methods alone may generate false negatives (i.e. overestimate fertilization failure), they are unlikely to generate false positives (i.e. lead to the misclassification of a fertilized egg as unfertilized). Although all examined eggs with blood spots were fertilized, our sample size was small (n = 4; Table 1) and further investigation is required to determine whether blood spots are a reliable indication of fertilization, considering that ovarian blood spots may be confused with embryonic blood islands (Dovč *et al*., 2021).

The apparent rarity of unfertilized Hawksbill eggs in our study suggests that reproductive behaviour and copulation problems, insufficient/defective sperm or oviductal-sperm incompatibility (Birkhead *et al*., 2008; Hemmings, West, & Birkhead, 2012) are unlikely to be a major problem for the wild Hawksbill turtle populations nesting on the eight Seychelles Islands in this study (Table 2). However, one Hawksbill turtle egg was found to be unfertilized (Table 2): a triple-yolked egg from an unusual nest on Cousin Island. The clutch presented several issues; 83% of eggs showed no signs of embryonic development based on macroscopic examination; eggs had irregular morphologies; and dwarfism was expressed in the few emerging hatchlings (Material S6). All three yolks in our triple-yolked egg sample remained intact for laboratory investigation and the PVM of each was thoroughly inspected, consistently revealing no embryonic nuclei or sperm. Considering that (1) the examined egg was unfertilized; (2) most other eggs in the clutch were undeveloped; and (3) there were many irregular sized eggs, it seems likely that the relatively high rate of hatching failure in this clutch was linked to issues with the parents' reproductive health.

While our sample sizes for Aldabra giant tortoises are relatively limited, our results are suggestive of higher variation in fertility status in this species compared to the sea turtle species we examined (Fig. 3 and Table 2). If this elevated variation is real, it may reflect differences in the reproductive dynamics of Aldabra Giant tortoises on different Seychelles islands. Unlike the relatively free-roaming Hawksbill and Green marine turtle populations, terrestrial Giant Aldabra tortoises are usually restricted to the sub-population of the island in which they inhabit, with little immigration or emigration. We examined Aldabra Giant tortoise eggs from Aldabra, North Island and D'Arros, (Table 2) where population numbers are estimated at +100 000, 150 and 36 respectively (Seychelles Island Foundation; North Island Limited Conservation Team and D'Arros Research Centre Team Pers. Comms.). These population numbers reflect rates of population growth, with Aldabra generally supporting good population growth, but North Island and D'Arros less so. Interestingly, data from Aldabra showed the greatest proportion of fertilized eggs (78% or 7/9 samples, with none conclusively determined as unfertilized). In contrast, D'Arros and North Island had significantly lower proportions of fertilized eggs (25% or 1/4; and 0%, respectively) and higher proportions of unfertilized eggs (D'Arros: 50% or 2/4 samples; North Island: 60% or 3/5 samples). This suggests that population growth on D'Arros and North Island may be stifled due to reproductive barriers such as suboptimal adult reproductive health, unsuccessful courtship behaviour and/or copulation failure. To understand the extent to which eggs are failing due to early embryo death or fertilization failure, along with the greater impacts it may have on population growth in Aldabra Giant tortoises, future research will benefit from collecting and analysing a larger sample size as well as behaviour and clutch data over several nesting seasons.

We used a range of different egg storage methods prior to examination, including refrigeration, freezing and formalin-fixation. This was largely due to logistical constraints, but nonetheless allowed us to make preliminary assessments of the suitability of different methods of storage. Refrigeration was the least effective: compared to frozen or fixed eggs, refrigerated eggs were more likely to develop or progress existing microbial infections. This may explain why captive eggs (typically refrigerated) were generally more difficult to analyse than wild eggs (all formalin-fixed) (Fig. 3). However, eggs from different species may react differently to different forms of preservation. For instance, in some Galapagos tortoise eggs, the yolk surface turned grey in colour after freezing, which made identifying the germinal disc impossible but did not affect microscopic examination of the PVM. This did not happen in frozen Red-footed tortoise eggs. Eggs appeared to be best preserved in 5% formalin; 10% formalin was also effective, but egg contents became somewhat more brittle and more challenging to dissect.

Importantly, embryonic nuclei and PVM-bound sperm detection was possible in undeveloped eggs that remained in the nest for ~60 days after oviposition, followed by <5 months stored in 5 or 10% formalin, addressing the concern raised by Phillott & Godfrey (2020) that the long incubation time (approximately 50 days) and nest cavity conditions of many turtle species may degrade failed eggs to the point that they cannot be examined.

In summary, we have identified important gaps in our understanding of turtle and tortoise (Order Testudines) hatching failure and demonstrated the applicability of tools for monitoring egg fertility and embryo survival to both wild and captive populations of threatened Testudines species. In terms of practicalities, we have shown that these tools can be used on wild-incubated eggs, with little need to disturb nests before hatching, but it is important to note that egg examinations require access to a lab with a fluorescence microscope, which may not be possible for many turtle and tortoise conservation entities. Developing methods that use less specialized equipment and ideally can be carried out in situ is therefore an important future direction. However, there has been at least one recent report of the methods being used successfully on sea turtle eggs in another laboratory (Turla & Wyneken, 2024), which demonstrates the utility of the method and shows it may be easily adopted by other researchers in the future.

We recommend that future research combines accurate data on fertilization failure and embryo mortality rates, generated using the approach we have described, with data on breeding conditions (e.g. nest site temperature), conservation interventions (e.g. nest relocations) and other potential drivers of reproductive failure such as pollutants and disease exposure, to monitor the impact of environmental change on early reproductive processes. We also anticipate that this approach will be applicable to other reptile groups, such as has been shown for crocodiles (Augustine, 2017), allowing scope to investigate similarities across birds and reptiles. The methods outlined here may therefore equip researchers and conservationists working across broad taxonomic groups with a tool to inform conservation and breeding management in the face of global change.

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Author contributions

AL and NH conceived the ideas, designed the methodology and led the writing of the paper. AL carried out the literature searches, laboratory work, data collection and analysis. RB, CT and AZ were pivotal for acquiring samples, and alongside NS has all been involved in the revision of drafts. All authors gave final approval for publication.

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Supporting information

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

Data S1. Depiction of the literature search and screening process to filter English studies that have claimed to investigate both Chelonian/Testudines hatching success and fertility of unhatched eggs, following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher *et al*., 2009; O'Dea *et al*., 2021).

Material S2. Turtle and tortoise hatching success studies investigating the fertilisation status of unhatched eggs that have met the criteria of the systematic review.

Material S3. Number and proportion of papers on turtle and tortoise (Order Testudines) hatching success (A) that assessed egg fertility. (B) categorised by their chosen methods of determining egg fertility.

Material S4. The description and limitations of methods for assessing fertilisation status of unhatched turtle and tortoise eggs, used by studies that met our systematic review criteria.

Material S5. Two 200× images of fungal/bacterial DNA found in the perivitelline membrane of a Spiny turtle (*Heosemys spinosa*) egg stained with Hoechest 3342.

Material S6. An unusual Hawksbill turtle (*Eretmochelys imbricata*) egg clutch from Cousin Island, Seychelles that had several anomalies.