Distinguishing Between Fertilisation Failure and Early Embryo Death in Failed Sea Turtle Eggs

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Introduction

Concern about feminisation of sea turtle populations as the result of climate change, increased nest temperatures, and skewed hatchling sex ratios has raised the possibility of there being an insufficient number of males available for successful breeding (Abella et al., 2017; Bell et al., 2003), which has the potential to result in high rates of unfertilised eggs (Booth & Dunstan, 2018; Chan & Liew, 1996). Environmental degradation and habitat loss leading to reduced genetic variation (González-Garza et al., 2015), and the loss of males via selective fishing practices (Abella et al., 2017), conservation actions (Mrosovsky & Godfrey, 1995), and use in traditional medicines (Martins et al., 2015), could also result in lower fertilisation rates of eggs in the future.

Excavation of sea turtle eggs at the end of the incubation period offers the opportunity for research and monitoring programmes to calculate hatching success and emergence success (see Chapter X). Unhatched eggs can be opened to determine the stage of development that was reached before embryo mortality (see Chapter Y). During this process, eggs that do not contain any obvious signs of embryo development are often assumed to be “infertile”. However, this assumption is likely to be incorrect, because there is no definitive evidence for unfertilised eggs being laid by sea turtles. Current evidence suggests that eggs which fail to develop are more likely to have experienced early embryo death (EED) during the beginning of incubation (Bell et al., 2003) or in the oviduct or soon after oviposition (Lavigne et al., 2025; see also Hemmings & Evans, 2020, for equivalent data in birds).

Unfertilised eggs, often referred to as “infertile” eggs, are those in which the biological events leading up to fusion of the sperm and ovum pronuclei (the process known as syngamy) did not occur. Syngamy happens after sperm have penetrated the ovum and migrated to the germinal disc, so it is possible for an egg to remain unfertilised even if sperm have reached and entered the ovum (Assersohn et al., 2021). Therefore, assessment of egg infertility should be based on an absence of evidence of fertilisation and embryo development. Unfertilised eggs should not be confused with eggs in which early embryo death has occurred, but these two fates can often be difficult to distinguish when eggs show no sign of embryo development. Decomposition, desiccation, insect infestation, and microbial invasion of unhatched eggs can hide signs of embryos that died in the early stages of development due to environmental conditions, congenital condition, egg disturbance, or inappropriate handling (Abella et al., 2017). Typically, the scientific literature on unhatched sea turtle eggs has used the term “infertile” to refer to eggs that show no signs of development, or sometimes even all eggs that fail to hatch (Phillott & Godfrey, 2020; Lavigne et al., 2025). This adds further confusion and limits our ability to fully understand the drivers of fertilisation failure and embryonic death in sea turtles. This chapter describes techniques to help researchers and management personnel determine if an egg was fertilised, or not, when examined at oviposition, during incubation, or at the end of the incubation period.

Methods of assessing fertilisation status of eggs

Traditional macroscopic methods

Until recently (pre-2024), studies that attempted to assess fertilisation status of sea turtle eggs used a range of different macroscopic methods. The three most common macroscopic methods used in the past, along with their strengths and weaknesses, are as follows:

A. Visual assessment of a white spot on the eggshell surface

The yolk of sea turtle (and other reptile) eggs has a lower density than the surrounding albumen and rises to the top of the egg after oviposition. This is different to bird eggs, in which chalazae (strands of albumenous matter) tether the yolk and developing bird embryo in the centre of the egg. In contrast, rather than being central, the sea turtle embryo is positioned next to the shell membrane and water is drawn from the albumen to form sub-embryonic fluid, so that the vitelline membrane adheres to the shell membrane on the inner surface of the eggshell (Ewert, 1985). These processes occur within ~1 day of oviposition and create a chalk-white spot visible on the outer eggshell at the top of the egg (Blanck & Sawyer, 1981; Figure 1). Often referred to as “chalking” of the egg, the white spot on the egg exterior grows progressively in size as the area of adherence of the vitelline membrane to the shell membrane increases over time, until the entire eggshell appears chalk-white (Miller, 1985).

Visual assessment of eggs for a white spot can be used to determine fertilisation status, although this can be challenging to do with *in situ* clutches, because rotation of eggs during handling between from <12 hours to 14 days after oviposition can cause embryo death

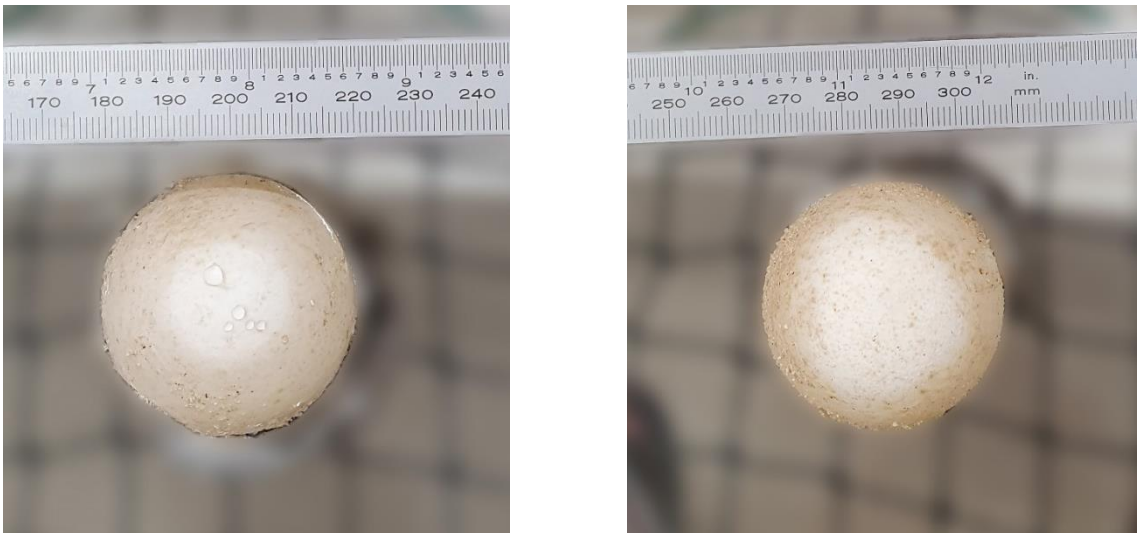

Figure 1. Chalking or white spot formation as seen on the top half of a green sea turtle (*Chelonia mydas*) egg 46 hours (left) and 83 hours (right) after oviposition. Image credit: David Adams.

(Limpus et al.,1979; Parmenter, 1980; Williamson et al., 2017). If a white spot is observed within 5 days of oviposition, the egg can be confidently categorised as fertilised (Blanck & Sawyer, 1981). However, the time taken for appearance of the white spot varies (see Phillott & Godfrey, 2020) and it can disappear within ~44 hours of embryonic death (Phillott & Parmenter, 2007). In addition, the chalk-white colour may be invisible on fertilised unhatched eggs at the end of the incubation period (Phillott & Parmenter, 2007; Sahoo et al., 2009). Hence, fertilised eggs without a white spot may be incorrectly classified as unfertilised and require further inspection (Phillott & Godfrey, 2020).

B. Candling

Candling involves shining a bright light into the egg, allowing the observer to view the egg contents and monitor embryo development without opening the egg (Dovč *et al.*, 2021; Figure 2). Provided that the egg is handled with care, it is a generally safe procedure for developing embryo. The appearance of the contents through the shell changes over time, with albumen changing from thicker and cloudy to thinner and clearer during incubation. The extra-embryonic membranes appear first, followed by blood vessels then the embryo. The white spot appears darker than parts of the eggshell, which are still translucent (see Ewert, 1985). Candling is a quick, non-invasive, and useful method but, as with white spot assessment, it is not completely reliable. Abella et al. (2017) identified embryo development as early as 24 hours post-oviposition via candling, but it remained unclear whether eggs that did not show obvious development were unfertilised. Indeed, Dovč et al. (2021) showed that some eggs categorised as unfertilised by candling during the incubation period had been fertilised, but the embryo had died early in development. Hence, eggs that do not show obvious signs of embryo development should be candled again a few days later and potentially monitored for any changes in eggshell appearance from chalk white to opaque that could indicate embryo death (Phillott & Parmenter, 2007). Some of the methods described below can be applied if the egg fails to hatch.

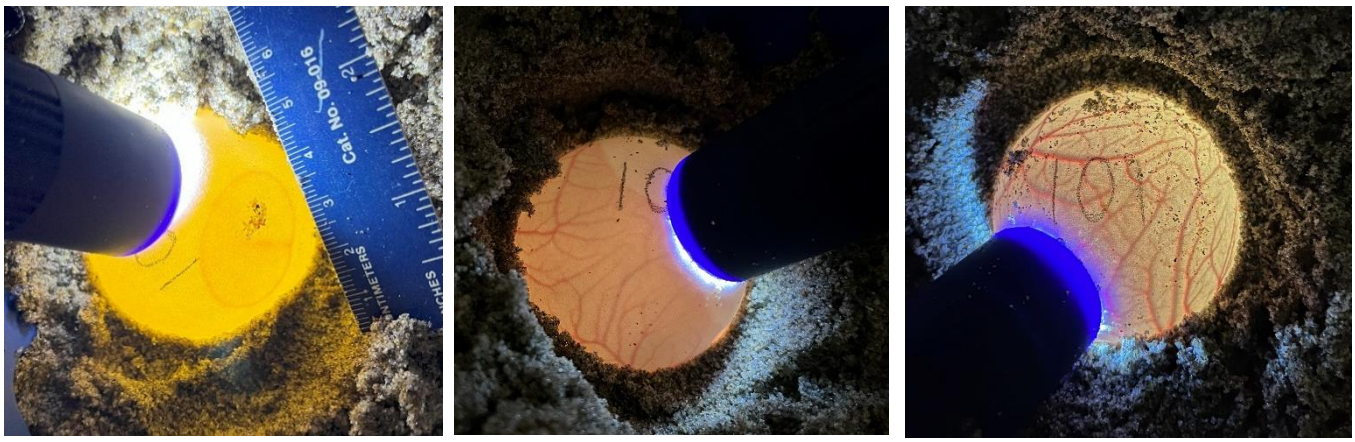

Figure 2. A developing leatherback sea turtle (*Dermochelys coriacea*) egg being candled at day 14 (left), day 39 (middle), and day 54 (right). Image credit: Emily Turla.

C. Visual assessment of egg contents

The only way to assess the fertilisation status of eggs at the beginning of incubation is by opening the egg and visually assessing the germinal disc (e.g. Gárriz et al., 2020). However, the invasive nature of this method means it is not permitted in many situations, and the contents of unhatched eggs may not be examined until the majority of hatchlings have emerged from the nest. Unhatched eggs remaining in the nest can be opened and their contents examined for the presence of blood, blood vessels or embryonic tissues as evidence of fertility (Figure 3). Rarely, ovarian blood spots may be mistaken for embryonic blood islands (Miller et al., 2017; Dovč *et al.*, 2021), leading to unfertilised eggs being incorrectly classified as fertilised. Eggs that do not contain any obvious embryonic tissues should be categorised as “undeveloped”, and not as unfertilised, if there are no further investigations (Phillott & Godfrey, 2020).

Figure 3. A fertilised egg showing a developed green sea turtle (*Chelonia mydas*) embryo (left) and an early-stage hawksbill sea turtle (*Eretmochelys imbricata*) embryo (middle and right). Image credit: Alessia Lavigne.

New microscopic methods

In all the traditional macroscopic methods described above, it can be challenging to differentiate early embryo death from fertilisation failure in undeveloped eggs. Birkhead et al. (2008) developed microscopy-based methods capable of discriminating between fertilisation failure and early embryo death in bird eggs during and after incubation from both captive and wild species. Hemmings et al. (2012) successfully tested these methods on eggs deemed 'unfertilised' after incubation from five endangered species of wild and captive birds. Fertilisation status was accurately confirmed by dissecting the yolk and removing the surrounding perivitelline membrane (PVM) and germinal disc and staining the tissues with a fluorescent DNA marker that stains the nuclei of sperm heads and embryonic cells. When examined under a fluorescence microscope, a fertilised egg experiencing early embryo death would display the presence of embryonic cell nuclei in the germinal disc and sperm in the PVM. An egg would be classified as unfertilised if no cell nuclei were observed. This technique presents a promising avenue for accurately determining fertilisation events in unhatched Testudines eggs where macroscopic methods cannot. This method was recommended by Phillott & Godfrey (2020) for analysing the fertilisation status of sea turtle eggs and has now been validated for sea turtles (Lavigne et al., 2025; Turla & Wyneken, 2024) and other Testudines (Lavigne et al., 2025). The protocol is described in detail below.

D. Detection of sperm penetration of the ovum and early embryonic development

The following protocol can be used on fresh, frozen-and-then-thawed, or formalin-preserved eggs that show no visible embryonic development when the contents are examined. The protocol has been used successfully on eggs that have been examined at the end of the incubation period and have undergone decomposition (Lavigne et al., 2025).

Materials required:

- Dissecting kit, including fine forceps x 2, dissecting scissors, hair loop*
- 3-4 petri dishes
- Automated pipettes (e.g. Gilson, P20 and P200)
- Correctly sized pipette tips

- Microscope slides
- Glass coverslips
- Phosphate buffered saline solution**
- Hoechst 33342 fluorescent DNA stain (can be purchased from most scientific suppliers e.g., Sigma-Aldrich, Thermo-Fisher Scientific).
- Eppendorf tubes / screw-tip microcentrifuge tubes

*The hair loop is a custom-made instrument, consisting of a strand of human hair, fashioned into a small loop and taped to either a cocktail stick or pipette tip to allow easy manipulation. The hair loop can be easily handled via the stick/tip, and the hair loop acts as a gentle, flexible brush to remove excess yolk and other debris from the delicate PVM.

**Phosphate buffered saline solution (PBS) can either be purchased from a scientific supplier in ready-made sachets or tablets to be added to distilled water, or it can be made from its base ingredients using the recipe below. The latter is more cost-effective if you intend to use a lot of PBS over a long period of time, but the former is more convenient for small projects and remote working.

Phosphate buffered saline, 2 litres solution:

- 2.3g di-sodium hydrogen orthophosphate
- 16g sodium chloride
- 0.4g potassium chloride
- 0.4g potassium di-hydrogen orthophosphate
- 2 litres distilled water

The above ingredients are available to purchase from most scientific suppliers in powder form. Weigh out all ingredients using a digital scale and add to distilled water. Mix well until all salts have dissolved. Autoclave if sterility is required. Store in refrigerator at 1-5ºC.

Equipment required:

- Stereomicroscope (dissecting microscope) with gooseneck lighting
- Compound light microscope with the following: a fluorescence light source, BP 340-380 excitation filter and LP 425 suppression filter, and darkfield filter
- Refrigerator for storing Hoechst stain and egg samples prior to examination

Methods:

1. Prepare the Hoechst fluorescent DNA stain solution

In a sterile Eppendorf tube (or similar) dilute 1µl of concentrated Hoechst stain in 10µl of distilled water to make a stock solution. In a separate sterile vessel, dilute 1µl of stock solution in 100µl of phosphate buffered saline solution to make the working solution. Always keep both stock and working solutions refrigerated and away from light (e.g., by covering the containers with aluminium foil). The concentration of the working solution can be adjusted

higher or lower according to your requirements, because samples can vary in staining efficacy. It is recommended that Hoechst stain solutions are tested prior to use on important samples, e.g., on a fresh cheek cell swab from the mouth, or other known source of cells/DNA.

Health and safety note: Hoechst dye is a known mutagen and contact with skin should be avoided by using appropriate personal and protective equipment (PPE). Ensure you read all the relevant safety documentation supplied with the product.

2. Open the egg

Use scissors to cut through the shell being careful not to penetrate the egg contents too deeply. Once opened, empty the egg contents into a petri dish. If examining a formalin-preserved egg, carefully drain off any excess formalin in a fume hood or well-ventilated area and dispose of the formalin appropriately before placing the egg contents into a petri dish. Submerge the egg in phosphate buffered saline solution.

Health and safety note: Formalin is an irritant and may be carcinogenic. Contact with skin and inhalation of fumes should be avoided. Please ensure you read all the relevant safety documentation supplied with the product.

3. Locate the germinal disc

Fertilisation and embryogenesis occur at the germinal disc. It is therefore important to locate the germinal disc to look for embryonic cells. The germinal disc appears as a white disc on the yolk surface (Figure 4). However, locating the germinal disc in the eggs of sea turtles and other Testudines is often very difficult when the egg is not fresh. If the germinal disc is not visible, it may be hidden under layers of albumen; if this is the case, proceed to the following step. The appearance of the germinal disc can also vary, depending on whether the egg has been fertilised and, if so, how much development has taken place. The integrity of the germinal disc can also deteriorate over time, making it difficult to identify/extract.

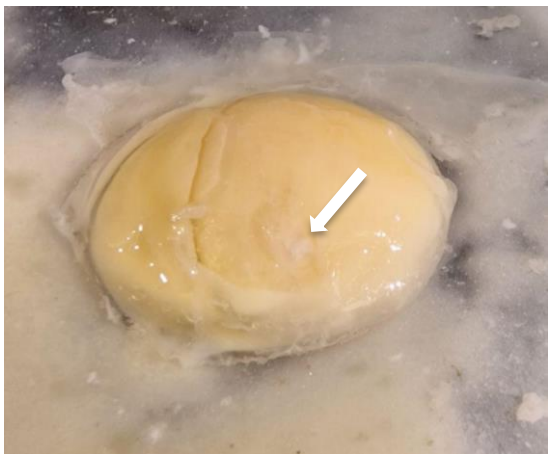

Figure 4. The germinal disc as seen in a green sea turtle (*Chelonia mydas*) egg. Image credit: Alessia Lavigne.

4. Remove outer layers of albumen

There may be a thick layer of albumen surrounding the yolk. Using scissors and forceps, carefully cut sections of the layers, ideally without piercing the yolk whilst peeling back and removing the layers of the albumen (Figure 5). Continue removing the layers until you have reached a final thin membrane enveloping the yolk which, if punctured, will expose the contents of the yolk - this is the PVM. Extra information on how to continue if the germinal disc still cannot be located, or if the egg is very degraded, is provided at the end of the next step.

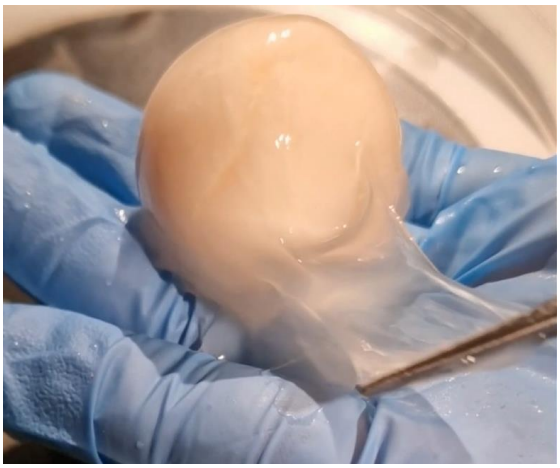

Figure 5. Removing the layers of albumen surrounding the yolk in a hawksbill sea turtle (*Eretmochelys imbricata*) egg. Photo credit: Alessia Lavigne.

5. Extract the germinal disc

If the yolk is intact and the germinal disc can be seen, use fine dissecting scissors to cut out a section of the PVM (which holds the yolk together) overlaying the germinal disc. This can be done by cutting a square section of the PVM overlaying the germinal disc. Working quickly, take hold of the edge of the PVM with the forceps and gently peel it away from the yolk to expose the germinal disc. Place the section of the PVM into a petri dish with PBS and place to one side (working with the extracted germinal disc and PVM in a separate petri dish helps to keep a clearer view of the germinal disc), and then slide the hair-loop under the germinal disc, lifting it away from the yolk and place into a petri dish with PBS. Using a stereomicroscope (dissecting microscope), carefully remove excess yolk material from the germinal disc using the hair loop, leaving only white matter. Use a pipette to transfer the germinal disc onto a microscope slide – we recommend using an automated P20 pipette (e.g., Gilson) set at 10µl for Step 6.

As mentioned above, sea turtle eggs may (a) not have a clearly visible germinal disc, or (b) have suffered from degradation to the point that the yolk and PVM are no longer intact, making it difficult to locate the germinal disc. If the yolk is intact, use fine dissecting scissors to cut all the PVM (which holds the yolk together) into flat, squared sections (recommended surface area of >0.5cm^2) and then examine them systematically under the microscope once you reach step 8. If the yolk has broken down, dilute the egg contents with plenty of phosphate buffered saline solution and use forceps to fish out as many pieces of PVM as possible for inspection.

Once you have located as much PVM material as possible, move onto step 6.

6. Prepare the PVM section(s)

Using the hair loop, gently clean any yolk material that is adhering to the PVM section(s) that you have cut out or retrieved. Use the hair loop again to place the PVM section(s) onto the microscope slide and smooth out gently using the hair loop and/or forceps until the section(s) lie flat (Figure 6).

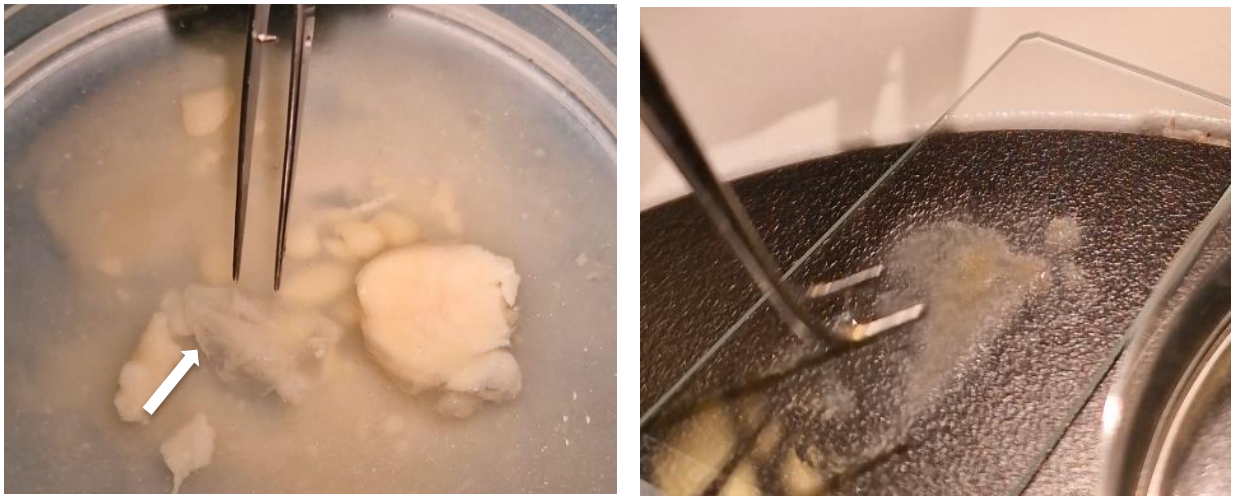

Figure 6. A large section of the perivitelline membrane from a hawksbill sea turtle (*Eretmochelys imbricata*) egg (left) among yolk and albumen, in a petri dish with PBS. A sample of perivitelline membrane being prepared on a microscope slide (right). Image credit: Alessia Lavigne.

7. Stain the samples

Using an automated P20 pipette (e.g., Gilson), add sufficient Hoechst working solution (typically around 10µl) to cover the germinal disc and PVM samples on the slide. Cover with a glass coverslip and place somewhere dark (e.g., drawer or box) at room temperature for 10 minutes to allow the dye to penetrate the sample.

8. Search for cells

Using a compound light microscope with a fluorescence light source and darkfield filter enabled, search the microscope slide systematically using 200X or 250X magnification. Cells containing DNA that are stained with Hoechst dye will fluoresce bright blue. Hence, the heads (which contain the cell nucleus with the DNA) of any sperm cells that reached the germinal disc will stain bright blue; their tails will not stain brightly because they do not contain DNA, but they can be seen by using a brightfield light source with darkfield filter (Figure 7A). If fertilisation occurred (i.e., a sperm pronucleus fused with the female pronucleus and embryogenesis started), embryonic cells will be located within the germinal disc. They may appear as small clusters of cells (Figure 7B) or entire tissue layers (Figure 7C). Embryonic cells can often also be found adhering to the PVM that overlaid the germinal disc. Embryonic cell nuclei can be easily differentiated from sperm heads by their morphology: sperm nuclei are elongate and thin, usually with a visible tail emerging (Figure 7A), whereas embryonic nuclei are round, and usually found aggregated with other embryonic cells (Figure 7B-D).

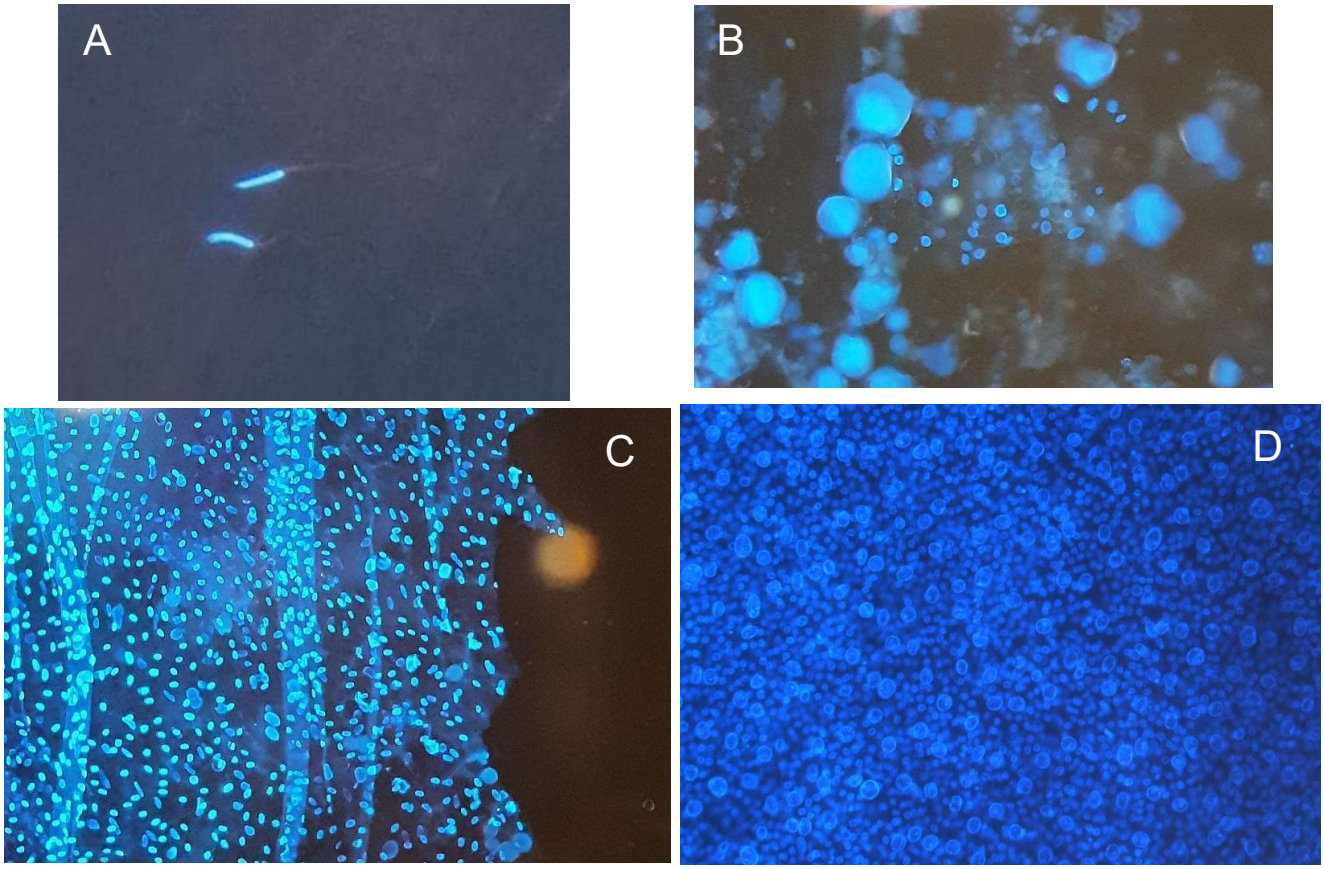

Figure 7. Stained nuclei of (A top left) perivitelline membrane-bound sperm in the egg of a green sea turtle (*Chelonia mydas*) at 400X magnification; (B top right) Stained nuclei of a green sea turtle embryo at 200X magnification; (C and D bottom) Embryonic nuclei of a hawksbill sea turtle (*Eretmochelys imbricata*) at 100X magnification. Please note that morphology (i.e., form, shape and structure) and not size of the cells are the determining factor for identification of embryonic cells, as size is likely to vary across several factors such as species and developmental stage, Image credit: Alessia Lavigne.

Method validation:

Using the methods described above, Lavigne et al. (2025) tested whether they could detect PVM-bound sperm and embryo nuclei in the germinal disc of 113 undeveloped green and hawksbill sea turtle eggs. They determined the fertilisation status of 85% of these undeveloped eggs, including degraded eggs that were collected at the end of their incubation period. Some limitations to the methods were revealed: if the germinal disc cannot be located and significant portions of the PVM are missing, an egg's fertilisation status may never be conclusively identified. If embryonic nuclei are found on the PVM, the egg can be confidently classified as "fertilised" (because it must have been fertilised for the embryo to start developing). However, even if sperm cells are found on the PVM, an absence of embryonic nuclei means there is a chance none of the sperm that reached the egg were able to fertilise. It must therefore be classified as 'inconclusive'.

Similarly, it may not be possible to determine fertilisation status for eggs that have suffered from excessive microbial (fungal or bacterial) growth on the PVM. The cells of fungi/bacteria will also be stained by Hoechst, but because they have a different size and morphology to embryonic cells, they can be easily identified. If infections are at low enough concentrations, detection of sperm and/or embryonic nuclei may still be possible. However, depending on the quantity of such contaminants, the surface of the PVM can be completely obscured under the microscope.

It is worth noting that the method used to preserve egg samples may influence the ease and success of dissections. For example, Lavigne et al., (2025) found that freezing Galapagos Giant tortoise eggs sometimes caused the yolk's surface to turn grey. This made it impossible to identify the germinal disc, but the PVM remained completely intact, and identification of embryonic nuclei was successful. Additionally, Lavigne et al., (2025) found that the PVM of formalin-fixed eggs can be more brittle and difficult to work with than refrigerated or frozen eggs, and the germinal disc may fix to the egg and become inseparable when the egg was formalin-fixed.

Nevertheless, the methods described here can serve as a new tool for monitoring egg fertilisation rates and embryo survival for sea turtles and other Testudines species to a significantly higher degree of accuracy than has been previously achieved. We recommend that future research combines accurate data on fertilisation failure and embryo mortality rates, using the methods outlined above, with data on incubation conditions (e.g., nest site temperature), conservation interventions (e.g., nest relocation), and other potential drivers of egg failure, such as pollutants and disease exposure, to monitor the impact of environmental change on early embryo development.

Assessment of fertilisation failure and EED at the individual vs population level

Breeding populations that have a) a high female-biased operational sex ratio, and/or b) low hatching success in undisturbed nests, that are not experiencing another threat that could cause embryo mortality (e.g., high environmental temperatures, frequent tidal washover) should be investigated further to increase our understanding of factors influencing fertilisation and reproductive output of sea turtles. The focus should be on determining whether low fertilisation rates are occurring at a population level, because it is at this scale that a potentially low number of male turtles is of greatest concern.

Researchers and managers concerned about fertilisation rates of eggs should conduct a focused assessment. Detecting low fertilisation rates at a population level ideally requires examination of 20 eggs, randomly selected at oviposition, from each of 20 clutches of >100 eggs, laid by different females (Phillott & Godfrey, 2020), using the methods described in this chapter or validated methods that emerge in the future. If individual turtles can't be identified by an external flipper tag, PIT tag or similar, then eggs should be collected during an 8-day period at the peak of the nesting season to avoid sampling from clutches laid by the same female. If results in the first year indicate low fertilisation rates of eggs, then the assessment should be repeated for several nesting seasons and at different times during the nesting season to understand the scale of the problem (Phillott & Godfrey, 2020).

Examining eggs at the beginning of the incubation period reduces the risk of samples being too degraded to accurately determine their fertilisation status; however, sampling of potentially viable eggs may not be appropriate for highly threatened populations and will be subject to rigorous permit and licensing procedures. If sampling at oviposition is deemed unfeasible, researchers may instead examine 20 (or as many as possible) undeveloped eggs from 20 clutches of >100 eggs at the end of incubation, after the other eggs in the clutch have hatched and it is clear that the undeveloped eggs are not viable. Clutches examined should have been laid by different nesting turtles, through nest tags based on known female identity or sampling over an 8-day period at the peak of hatchling emergence to avoid sampling from clutches laid by the same female. Again, if results in the first year indicate low fertilisation rates of eggs, then the assessment should be repeated for several nesting seasons and at different times during the nesting season to understand the scale of

the problem (Phillott & Godfrey, 2020). Study of fertilisation failure can require the sacrifice of viable eggs and/or excavation of emerged nests, so researchers and managers will need to decide the best method and most appropriate sampling regime for their population as well as obtain relevant government permits/permissions and ethical clearances as required.

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