Visualization of Perivitelline Membrane-bound Spermatozoa and Embryonic Cells in Nonviable Leatherback (*Dermochelys coriacea*) and Loggerhead (*Caretta caretta*) Eggs

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Sea turtle eggs that fail to hatch can provide valuable insight into the timing and conditions under which embryonic mortality occurs. Additionally, the study of unhatched, nonviable eggs can improve our understanding of the role of infertility in egg failure. Nest monitoring programs that estimate infertility by observing eggs during nest excavations at the end of incubation are likely to overestimate infertility due to the difficulty of identifying signs of early embryonic development (*e.g.*, egg chalking, embryos, blood islands, embryonic tissues, or germinal disc development) (Parmenter 1980; Blanck & Sawyer 1981; Limpus *et al.* 1984; Wyneken *et al.* 1988; Bell *et al.* 2003; Phillott & Godfrey 2020). In earlier studies, if none of these were found, an egg would be designated as infertile (Bell *et al.* 2003). Recognizing the difference between infertility and early embryonic death is important when studying egg failure, as they have different causes and implications for management and conservation.

The necropsy of unhatched sea turtle eggs typically includes both an external and internal examination. Externally, eggs can be examined for signs of fungal infection, predation, and egg chalking (the whitening of the eggshell). Indications of egg chalking have been used as a sign of fertility, because only fertile eggs will chalk (Blanck & Sawyer 1981). However, not all fertile eggs will appear chalked, because the embryo could die before the chalking process begins or the chalking could fade after embryo death (Miller 1985; Phillott & Parmenter 2007). Internally, eggs are examined for any signs of embryonic development (an embryo, blood islands, other embryonic tissues, or germinal disc development). If an embryo is found, it can be staged based on its morphological characteristics (Miller 1985; Miller *et al.* 2017). However, it is often difficult to stage an embryo or even identify if an embryo is present, because the egg contents continue to incubate in warm conditions until the clutch hatches and the nest is excavated for inventory of the contents (Miller 1996). During the time between egg failure and nest inventory, the egg contents significantly degrade (Bell *et al.* 2003). If an embryo dies early in development, it can be difficult or impossible to identify any clear signs of an embryo with the naked eye (Parmenter 1980; Wyneken *et al*. 1988). Therefore, microscopic examination of unhatched eggs is necessary to more accurately classify an egg as fertile or infertile (Parmenter 1980; Phillott & Godfrey 2020).

Previous studies that investigated sea turtle fertility have included excavating *in situ* eggs early in incubation to look for egg chalking (Abella *et al.* 2017), monitoring egg chalking in *ex situ* conditions (Rafferty & Reina 2014; Williamson *et al.* 2017a,b; Booth *et al.* 2021) and necropsying eggs if no chalking was detected (Bell *et al*. 2003), and sacrificing eggs that are collected freshly after oviposition to look for embryos (Limpus *et al.*, 1984; Wyneken *et al.* 1988; Miller *et al.* 2003). This last technique is lethal, and the other approaches are invasive as they require working with eggs that have not degraded in natural nests. Thus, there is a need for methods of fertility assessment that are nonlethal and can be used despite egg degradation.

Fluorescent microscopic approaches for assessing fertilization have been described in birds (Birkhead *et al*. 2008; Croyle *et al*. 2012; 2015), crocodiles (Augustine 2017), and non-marine chelonians (Croyle *et al*. 2016) but have yet to be validated in sea turtles. Here we test this method in unhatched, nonviable eggs from two sea turtle species, the loggerhead (*Caretta caretta*) (n=12 eggs) and leatherback (*Dermochelys coriacea*) (n=43 eggs). Eggs were either collected at or shortly after oviposition and incubated in controlled conditions or were collected from natural nests at the end of incubation during nest inventory. Eggs incubated in controlled conditions were monitored daily for signs of embryonic development (i.e., egg chalking or vascular formation visible during candling). If these were not found within 14 days, eggs were removed from the incubators and necropsied. Unhatched, nonviable eggs from natural nests were collected during routine nest excavations at the end of incubation (58-80 days) and necropsied. During egg necropsy, perivitelline membranes (PVMs) were collected for analysis using fluorescent microscopy.

The approach described here is a modification of the methods of Assersohn *et al*. (2021) and Birkhead *et al*. (2008). PVMs are collected from unhatched eggs and stained with Hoechst 33342, a fluorescent nucleic acid stain that binds to DNA. PVMs are then examined microscopically for the presence of spermatozoa and/or embryonic cells. The collection of PVMs requires tissue forceps, dissection scissors, petri dishes, and 1% phosphate buffered saline (PBS). The eggs were gently washed in water to remove external debris, and then either held in the hand or placed on a stable surface, such as a petri dish with a small ring of clay to prevent rolling. If the original orientation of the egg from its position in the nest is known, keep the egg oriented the same way with the top facing up. The eggshell was cut away beginning in the upper 1/3 of the egg, with care taken to not puncture the yolk. The cut eggshell was removed, and the yolk (with the intact PVM) was separated from the albumen. Next, the PVM was grasped with tissue forceps and an opening was cut in order to evacuate the yolk. The remaining yolk was rinsed from the PVM using 1% PBS, and the PVM was excised from the rest of the egg (Fig. 1). The PVM then was placed as flat as possible on a microscope slide and stained with working solution (1µg/mL) Hoechst 33342 (Thermo Scientific™), using enough to ensure that the entire sample was covered. The slides were covered with a cover slip and left in the dark (in a closed drawer or box) to incubate for at least 10 minutes. The slides were then examined under a compound microscope with the appropriate fluorescent attachments for Hoechst 33342 (maximal excitation at 350 nanometers and maximal emission at 461 nanometers). For this study, an Olympus BX50 compound microscope with a Chroma 69002 ET DAPI/FITC/Texas Red® filter

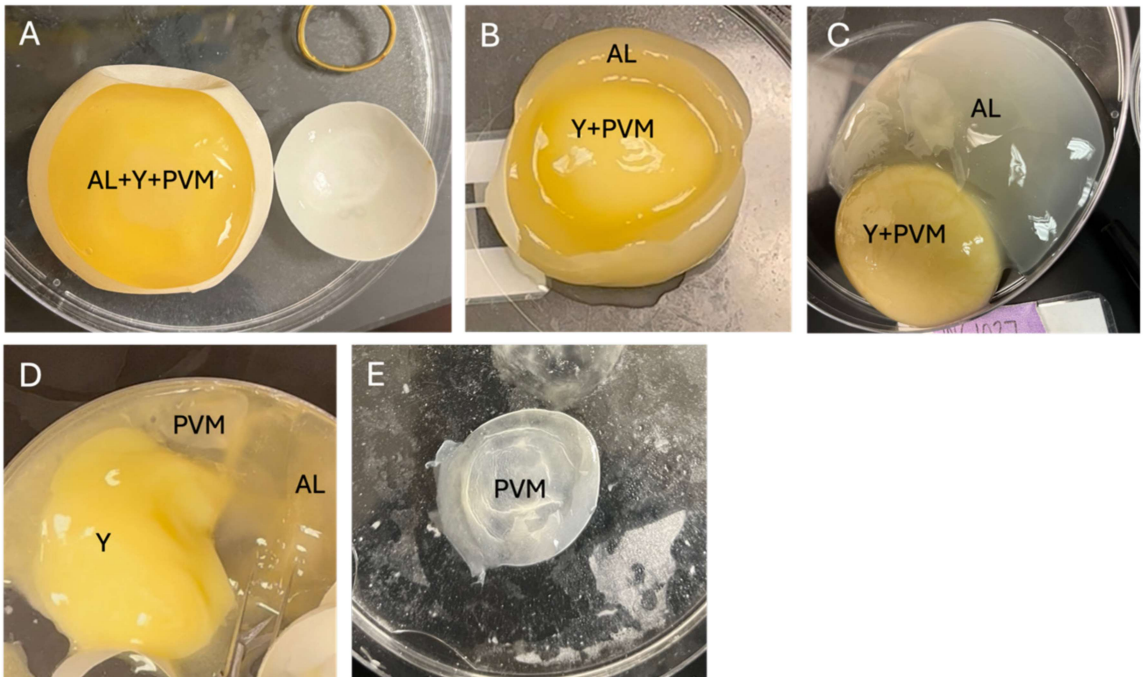

Figure 1. Egg necropsy process for perivitelline membrane collection. (AL=albumen, Y=yolk, PVM=perivitelline membrane). A: Eggshell top is removed to expose the egg contents. B and C: Yolk and PVM are separated from albumen. D: The yolk is separated from the PVM. E: A PVM separated from yolk and albumen after rinsing in 1% phosphate buffered saline.

Species	Incubation condition (days of incubation)	Egg storage condition prior to necropsy (storage time in days)	PVM storage condition prior to examination (storage time in days)	Number of eggs
Leatherback	Natural nest (58-59)	Necropsied same day as collection	70% ethanol (7-163)	3
Leatherback	Natural nest (59-80)	Refrigerated at 4°C (1-2)	10% buffered formalin (148-287)	7
Leatherback	Natural nest (59-80)	Refrigerated at 4°C (1-6)	70% ethanol (4-171)	7
Leatherback	Natural nest (71)	Frozen at -18°C (169)	70% ethanol (4-259)	4
Leatherback	Natural nest (78)	Frozen at -18°C (48)	10% buffered formalin (290)	1
Leatherback	Incubator (15)	Necropsied same day as collection	No fixation, examined same day as necropsy	2
Leatherback	Incubator (15-19)	Refrigerated at 4°C (1-2)	No fixation, examined same day as necropsy	2
Leatherback	Incubator (14)	Refrigerated at 4°C (12)	10% buffered formalin (1)	2
Leatherback	Incubator (12)	Frozen at -18°C (315)	10% buffered formalin (9)	2
Loggerhead	Incubator (16)	Frozen at -18°C (238)	No fixation, examined same day as necropsy	1
Loggerhead	Incubator (17-24)	Frozen at -18°C (238-276)	10% buffered formalin (1-12)	4
Loggerhead	Incubator (16-17)	Frozen at -18°C (130-141)	70% ethanol (4-96)	5

Table 1. Description of egg and perivitelline membrane (PVM) storage conditions in which embryonic cells were visualized within the PVM. Eggs from the "Incubator" incubation condition were in controlled temperatures (28, 30, or 32°C).

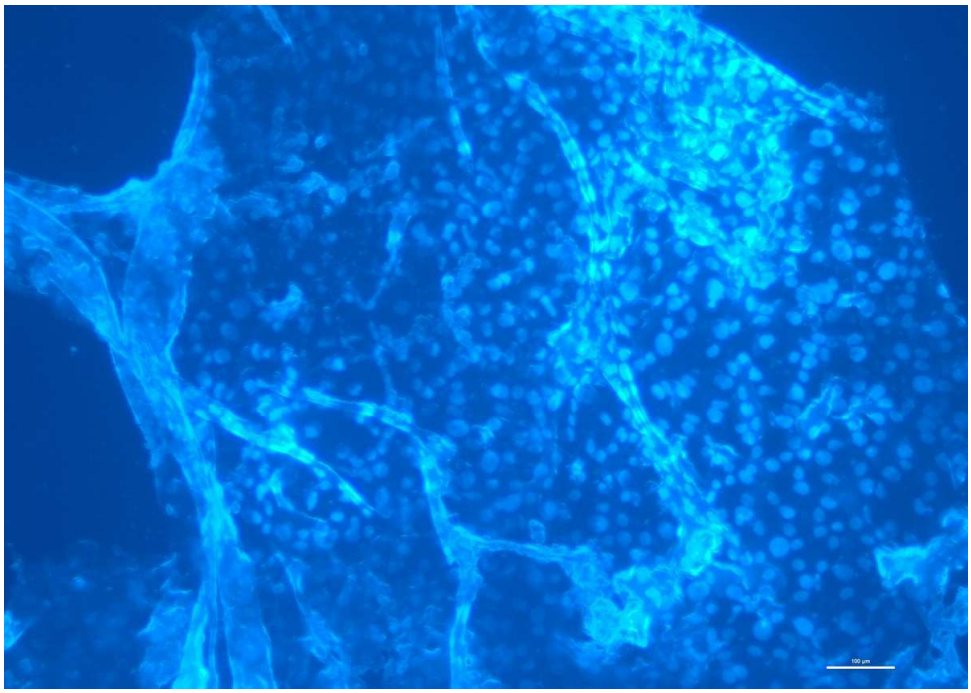

Figure 2. Embryonic cells stained with Hoechst 33342 nucleic acid stain as light blue on a perivitelline membrane from a loggerhead egg (100x magnification). Lighter lines of material in this figure are folds in the perivitelline membrane. Scale bar = 100µm.

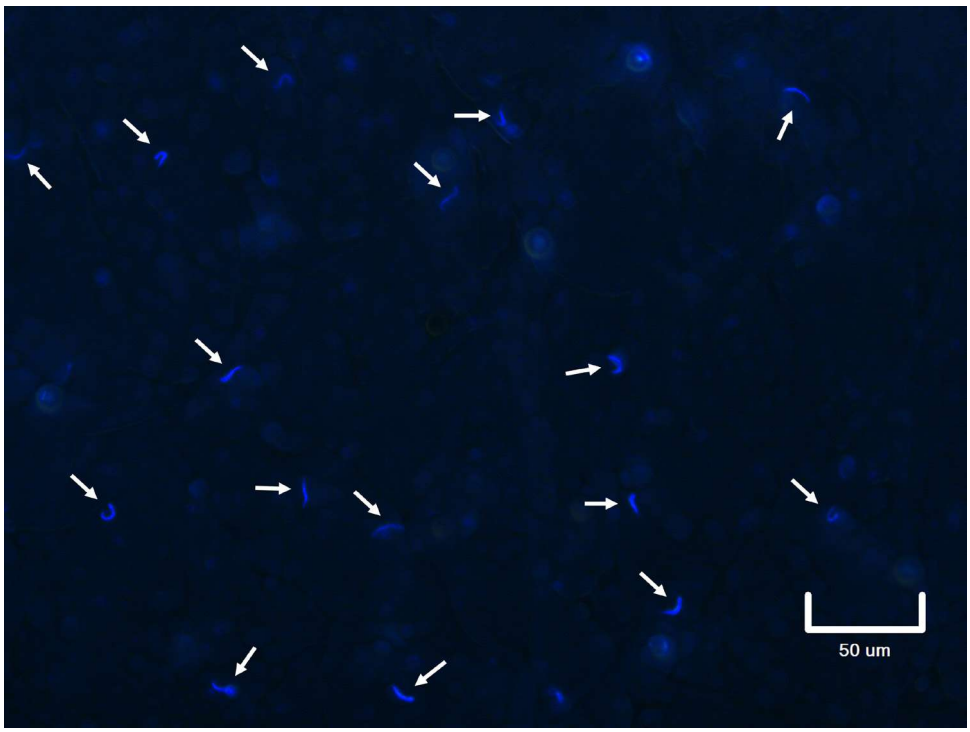

Figure 3. Sperm heads stained with Hoechst 33342 nucleic acid stain on a perivitelline membrane from a leatherback egg (200x magnification). Arrows point to fluorescing sperm heads. Scale bar = 50µm.

Species	Incubation condition (days of incubation)	Egg storage condition prior to necropsy (storage time in days)	PVM storage condition prior to examination (storage time in days)	Number of eggs
Leatherback	Natural nest (60)	Refrigerated at 4°C (1)	70% ethanol (224)	1
Leatherback	Natural nest (71)	Frozen at -18°C (169)	70% ethanol (4)	1
Leatherback	Incubator (14-15)	Necropsied same day as collection	No fixation, examined same day as necropsy	6
Leatherback	Incubator (14)	Necropsied same day as collection	10% buffered formalin (254-354)	4
Leatherback	Incubator (14-15)	Refrigerated at 4°C (2-12)	No fixation, examined same day as necropsy	7
Leatherback	Incubator (14-15)	Refrigerated at 4°C (6)	10% buffered formalin (1-348)	7
Leatherback	Incubator (14)	Refrigerated at 4°C (6)	70% ethanol (218)	1
Loggerhead	Incubator (22)	Frozen at -18°C (4-23)	10% buffered formalin (1)	2

Table 2. Description of egg and perivitelline membrane (PVM) storage conditions in which spermatozoa were visualized within the PVM. Eggs from the "Incubator" incubation condition were in controlled temperatures (28, 30, or 32°C). Some leatherback eggs (n=11) had PVMs separated into multiple storage conditions.

set was used, and slides were examined at 100x, 200x, and 400x. The fluorescent stain dyes blastodermal embryonic cells (Fig. 2) and/or the heads of spermatozoa (Fig. 3) in the PVMs. An embryo's blastodermal cells will be too numerous to count in the PVM of a fertile egg, while an infertile egg will have a uniform background (Barna *et al*. 2020).

This study is the first to validate this PVM analysis approach to identify fertility in sea turtle eggs. Embryonic cells and spermatozoa were identified in nonviable eggs from various incubation and storage conditions (Tables 1 and 2). Therefore, this method can be used with eggs collected shortly after oviposition, degraded eggs in various post-collection storage conditions, and PVMs stored in fixatives (10% buffered formalin or 70% ethanol) for various amounts of time. Phillott & Godfrey (2020) recommend that studies focused on fertility should use eggs that are collected freshly after oviposition since this approach avoids major degradation. However, such studies are not always possible due to logistic or permitting constraints.

Validation of this method in sea turtles provides a valuable tool for studying egg failure because it clarifies the differences between infertility and early embryonic death in unhatched eggs that show no signs of development, including degraded eggs. A deeper understanding of fertility rates of different nests, individual females, populations, and species can be further developed using this technique. Identifying how much infertility contributes to egg failure may inform further studies on reproductive shortcomings, such as causes for ova to fail to be fertilized and changes in fertility rates over time. Alternatively, if infertility is not a major contributor to egg failure, this suggests that investigations into early embryonic mortality are necessary.

Although an improvement, this method of fertility identification remains limited by the level of degradation of the PVM and the spermatozoa. As they degrade, PVMs become extremely fragile and difficult to recover (Croyle *et al*. 2012), which can limit the number of eggs that can be examined. Additionally, especially in degraded eggs, the absence of spermatozoa could be the result of spermatozoa degradation during incubation, rather than infertility (Croyle *et al*. 2015). It is important to note that if spermatozoa are detected, their presence is not necessarily indicative of the multiple essential processes that must occur to facilitate syngamy (the fusion of two nuclei) (Birkhead & Fletcher 1998; Croyle 2015). Further research is necessary to understand what numbers (or threshold numbers) of spermatozoa are representative of successful fertilization, as this likely varies across species and is usually identified in controlled studies (Birkhead & Fletcher 1998; Liptói *et al*. 2004; Croyle *et al*. 2015). Conservatively, only eggs that are identified to have embryonic cells should be distinguished as fertile when using fluorescent microscopy methods. However, eggs with at least two spermatozoa present on the PVM have been classified as fertile in studies on avian fertility and could be considered as "apparently fertile" (Croyle *et al*. 2015). The presence of spermatozoa indicates successful mating at some point. However, the presence or absence of spermatozoa in sea turtle eggs is not yet a definitive marker for fertility. There is a need for further research on PVM-bound spermatozoa numbers and their relationship to fertility.

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