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REVIEW

Assessing the evidence of 'infertile' sea turtle eggs

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ABSTRACT: There is increasing concern about feminization of sea turtle populations resulting from female-biased production of hatchlings due to climate change and selective loss of males from other anthropogenic drivers. Extreme female-biased breeding populations would reduce the likelihood of successful mating and potentially result in high rates of infertile eggs. Infertile eggs are those in which none of the events between sperm penetration of the ovum and syngamy have occurred. Distinguishing between fertile and infertile eggs is challenging, especially in field conditions, and researchers often have relied on physical evidence gathered from unhatched eggs at the end of the incubation period, which likely have experienced tissue decomposition. We argue that infertility in sea turtle eggs can be demonstrated only by the absence of holes caused by sperm penetration of the inner perivitelline membrane; sperm bound between the inner and outer perivitelline membranes; nuclei in the blastodisc; embryonic tissue or membranes in egg contents; and/or the characteristic white spot on the egg exterior. Unhatched eggs can be examined at the end of the incubation period, but we recommend that studies specifically investigating infertility examine at least 20 oviposited eggs each from clutches laid by at least 20 different turtles at the peak of the nesting season.

KEY WORDS: Sea turtle · Egg · Embryo · Fertile · Infertile · Fertilization · Feminization

1. INTRODUCTION

The number of live hatchlings produced from sea turtle eggs is commonly recorded by most sea turtle nesting beach monitoring projects and used to estimate hatching success of a clutch, defined as the number of hatchlings produced, divided by the number of yolked eggs deposited (Miller 1999). Hatching success is often used to assess various conservation actions, including nest relocation, predator control, and beach nourishment (e.g. Eckert & Eckert 1990, Steinitz et al. 1998, Engeman et al. 2003, García et al. 2003, Abd Mutalib & Fadzly 2015). In addition, examination of dead embryos found within unhatched eggs that remain in the nest cavity after the live hatchlings emerge can provide insight into when embryonic development stopped and thus provide temporal clues of the possible causes for embryonic mortality (e.g. tidal inundation, excessive rainfall, rough handling of eggs during relocation; Ragotzkie 1959, Abella et al. 2007, Hilterman & Goverse 2007). Recently, Miller et al. (2017) published a generalized key to the stages of embryonic development of sea turtles, to facilitate the proper categorization of embryos that did not finish development. However, the early stages of embryonic development are challenging to recognize in the field, especially if eggs are investigated several weeks after embryonic mortality, which is usually the case for sea turtle nests that are inventoried after hatchling emergence.

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In earlier publications of sea turtle biology and management, unhatched eggs without visible signs of embryonic development were categorized as infertile (e.g. Ragotzkie 1959, Balasingam 1967, Hughes & Mentis 1967, Hughes et al. 1967, Schulz 1975, Fowler 1979, Kraemer & Bell 1980, Stancyk et al. 1980, Chan et al. 1985, Hewavisenthi 1994, Peters et al. 1994). Fertility rates in leatherback turtles *Dermochelys coriacea* were assumed to be lower than in other sea turtle species (Balasingam 1967, Schulz 1975, Chan et al. 1985, Chan 1991, Chan & Liew 1996), leading to the recommendation that egg fertility in this species be assessed before purchase and protection in hatcheries in Malaysia for incubation (Chan 1989). More recently, speculation about future extreme female-biased production of hatchlings, due to climate change and temperature-dependent sex determination, has led to predictions of future lack of males in populations, and a concurrent increased rate of infertility in sea turtle eggs (Mrosovsky & Godfrey 1995, Lovich 1996, Binckley et al. 1998, Glen & Mrosovsky 2004, Laloë et al. 2014, Jensen et al. 2018). Extreme female-biased breeding populations could reduce the likelihood of successful mating (Bell et al. 2004, Abella et al. 2017) and potentially contribute to high rates of infertile eggs at specific rookeries (Booth & Dunstan 2018) or in specific populations (Chan & Liew 1996). Concern has also been expressed about the implications of reduced genetic variation in sea turtle populations due to environmental degradation and habitat loss (González-Garza et al. 2015), and the loss of males due to selective fishing practices (Abella et al. 2017), conservation actions (Mrosovsky & Godfrey 1995), and use in traditional medicines (Martins et al. 2015) for future fertility rates of eggs.

However, the definition and best practice/s for assessment of 'infertile' sea turtle eggs have never been established. By definition, infertile eggs should include only those in which events between sperm penetration of the ovum and syngamy (fusion of the sperm and ovum pronuclei) have never occurred (see Birkhead et al. 2008). Assessment of egg infertility should therefore be based on evidence indicating the absence of fertilization or any embryonic development.

The few focused studies on egg fertility by experienced researchers using appropriate methods found that all eggs examined shortly after laying contained embryos (Table 1). Less experienced researchers may incorrectly assume that eggs are infertile if embryonic mortality occurred early in development and tissue had decomposed by the time failed eggs are examined at hatchling emergence (Blanck & Sawyer 1981, Wyneken et al. 1988, Bell et al. 2004). Overall, the current methods used to assess the occurrence of infertile sea turtle eggs require a critical review based on an understanding of the process of fertilization and indicators of early embryonic development.

2. FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT AND MORTALITY

At ovulation, each follicle comprises material which becomes the egg yolk and a blastodisc (or germinal disc) upon which sits the germinal vesicle representing the oocyte nucleus (simply illustrated by Olszanska & Stepinska 2008). Fertilization occurs in the anterior glandular region (magnum) of the oviduct (Solomon & Baird 1979) with spermatazoa received during copulation and stored in sperm-storage tubules (Gist & Jones 1989). Assuming post-fertilization changes in sea turtle follicles are similar to those of other amniotes, sperm penetration of the inner perivitelline membrane (PVM), which encompasses the follicle, over the blastodisc triggers formation of the outer PVM. The new membrane prevents polyspermy but binds sperm in contact with the follicle between the inner and outer PVMs (Birkhead et al. 1994). Embryonic development commences immediately after fertilization. Cleavage of the blastodisc begins within hours of fertilization, followed by blastulation and gastrulation. Embryonic development is arrested in mid-gastrulation (Stage 6; ap-

Table 1. Studies finding evidence of >98% fertility rates in sea turtle eggs, based on detailed examination of egg contents and white spot development

Source	Sea turtle	Number of eggs examined
Limpus et al. (1984)	Leatherback	2 eggs each from 5 clutches at oviposition
Wyneken et al. (1988)	Loggerhead	2 eggs each from 10 clutches at 16–18 h post oviposition
Miller et al. (2003)	Loggerhead	'Hundreds' of eggs at oviposition
Rafferty et al. (2013)	Green	35 eggs from 2 clutches
Rafferty & Reina (2014)	Green	97 eggs from 3 clutches
Williamson et al. (2017a)	Green	40 eggs from 6 clutches
Williamson et al. (2017b)	Olive ridley	303 eggs from 6 clutches

proximately Day 9 after ovulation) until oviposition (Miller 1985). This arrest is maintained by hypoxic conditions in the oviduct (Rafferty et al. 2013). Developmental arrest is broken within 12–16 h of oviposition (Williamson et al. 2017a) by exposure to atmospheric oxygen levels (Rafferty et al. 2013); embryonic development recommences and completes gastrulation followed by neurulation, organogenesis, and growth, with complementary formation of the extra-embryonic membranes, adhesion of the vitelline and shell membranes, and vitelline circulation, until pipping at Stage 31 (Miller 1985). Stages of pre- and post-ovipositional embryonic development in sea turtles have been previously described and reviewed (Blanck & Sawyer 1981, Crastz 1982, Miller 1985, Kaska & Downie 1999, Miller et al. 2017); stages and % development time (%DT, percentage of incubation period from oviposition to pipping for eggs incubated at 29°C) described in this paper all refer to those based on Miller (1985) and Miller et al. (2017).

Distinguishing between infertility and early embryonic death is challenging (Miller 1997). Failed eggs may be incorrectly categorized as 'infertile' if mortality occurs before the embryo is visible upon inspection by eye. Intra-oviducal embryonic mortality can occur during gastrulation or developmental arrest, and potentially at higher rates if eggs are retained in the oviduct during a long inter-clutch interval (Rafferty et al. 2011, Booth & Dunstan 2018; but also see Rings et al. 2015, Williamson et al. 2019). Embryos at this time are developed to Stages 1–6 (Miller 1985) and <2 mm in length (Miller 1985, Miller et al. 2017). Post-ovipositional early embryonic mortality, due to unsuitable temperature, moisture, or respiratory gas availability in the nest environment (Ragotzkie 1959, Foley et al. 2006, Howard et al. 2014, Bézy et al. 2015) or inappropriate movement while handling during sensitive periods (Limpus et al. 1979, Parmenter 1980, Chan et al. 1985), may also be difficult to discern before signs of embryonic development become visible to the unaided eye at approximately Stages 13–16 and an embryo length of ~10 mm (Days 4–9 after oviposition) (Leslie et al. 1996, Ralph et al. 2005).

3. STUDIES ASSESSING 'INFERTILITY'

To date, many studies on the occurrence of infertility in sea turtle eggs have used the absence of a white spot on the egg exterior, and/or tissues representing embryonic development in the contents of failed eggs during their assessment.

3.1. White spot on the egg exterior

Soon after oviposition, the embryo becomes positioned at the top of the egg, adjacent to the shell membrane. The difference in specific gravities of yolk and albumen in the sea turtle egg and lack of chalazae (strands of albuminous material) result in the yolk rising through the albumen to a position at the top of the egg after oviposition (Blanck & Sawyer 1981). As observed in crocodile eggs, the yolk is also likely to rotate within the albumen due to a density gradient created by formation of sub-embryonic fluid (see Webb et al. 1987). This positioning of the embryo during the first day of post-ovipositional development and further dehydration of the albumen as more sub-embryonic fluid is formed allows the vitelline membrane to adhere to the shell membrane and results in formation of a white spot on the shell exterior (Blanck & Sawyer 1981, Webb et al. 1987). Membrane adherence likely changes the optical and structural properties of the eggshell as dehydration occurs, altering its appearance from translucent to opaque white and facilitating respiratory gas exchange (see Phillott & Parmenter 2001, 2007). As the area of adherence increases progressively over time with embryonic development, the white spot on the exterior grows in size, until the entire eggshell is colored chalk-white (Miller 1985; our Table 2)

The absence of the characteristic white spot soon after oviposition has been used as an indication of infertility (e.g. Whitmore & Dutton 1985, Chan 1989, Bell et al. 2004, Abella et al. 2007, 2017). However, the timing of inspection may influence its visibility, as there is evidence of variation in the duration between oviposition and white spot appearance (Table 2). Examining eggs too early may result in incorrect assessment. For example, Abella et al. (2017) assessed fertility rates in clutches of loggerhead turtle *Caretta caretta* in the days after oviposition and again after hatchling emergence; estimates of fertility were lower in 17% of clutches (n = 29) assessed using the white spot at 96 h after oviposition than estimates using embryo and tissue fragments in unhatched eggs after hatchling emergence.

3.2. Embryonic tissue in egg contents

Studies designed to carefully investigate fertility in sea turtle eggs have found embryos in 100% of eggs dissected at oviposition and at 16–18 h after oviposition (Table 1). However, early-stage embryos can be challenging to find under field conditions and/or by

Table 2. Temporal variation in appearance of the characteristic 'white spot' on the sea turtle egg exterior and complete 'chalking' as the white spot encompasses the entire egg, among species and regional management units (RMU; as defined by Wallace et al. 2010). (–) details not reported

Source	White spot formation	Complete chalking of egg	Turtle	RMU	Incubation location	Incubation temperature (°C)
Caldwell (1959)	2 d	10–12 d	Loggerhead	–	–	–
Blanck & Sawyer (1981)	<1 d	–	Loggerhead	NW ATL	Laboratory	28 ± 2 SD
Yntema & Mrosovsky (1982)	2 d	–	Loggerhead	NW ATL	Long-distance transport to laboratory	–
Chan (1986)	4–5 d	20 d	Leatherback	–	Styrofoam boxes	28–29
Chan (1989)	4–5 d	–	Leatherback	W PAC	Hatchery	–
Marquez-M (1994)	<1 d	7 d	Kemp's ridley	NW ATL	*In situ*	–
Kaska & Downie (1999)	<1 d	–	Green, Loggerhead	MED, MED	*In situ*	–
Hewavisenthi & Parmenter (2000)	3 d	–	Flatback	SW PAC	Laboratory	29.5
Abella et al. (2007)	>24 h	–	Loggerhead	NE/NW ATL	*In situ*	–
Mrosovsky et al. (2009)	<27 h		Hawksbill	W ATL	Long-distance transport to laboratory	–
Rafferty et al. (2013)	1–2 d	–	Green	SW PAC	Long-distance transport to laboratory	28
Rafferty & Reina (2014)	1–2 d	–	Green	SW PAC	Long-distance transport to laboratory	24, 28, 32
Abella et al. (2017)	>24 h	–	Loggerhead	NE/NW ATL	Laboratory	26–30
Williamson et al. (2017a)	1–2 d	–	Green	SW PAC	Laboratory	28
Williamson et al. (2017b)	1–2 d	–	Olive ridley	E PAC	Laboratory	28
Williamson et al. (2019)	1 d	–	Olive ridley	E PAC	Laboratory	28

novice researchers (Wyneken et al. 1988, Kaska & Downie 1999, Bell et al. 2004). For example, Blanck & Sawyer (1981) examined 5 eggs each from 10 loggerhead turtle clutches, fixed within 2 h of oviposition, and were unable to find any embryos; they assumed that embryos were too small to discern at this stage. Even at 2–3 d post oviposition, the length of the embryonic disc measured only a few millimeters and was unpigmented (Crastz 1982, Miller 1985, Kaska & Downie 1999, Miller et al. 2017). Extra-embryonic membranes, which may appear as tissue fragments in failed eggs, form only ~36 h post oviposition (Blanck & Sawyer 1981). Blood islands and the first blood cells form at Stage 11 (5.7 ± 0.5%DT; ~3–4 d post oviposition) and vitelline circulation commences at Stage 13–14 (7.6–8.6 ± 0.5%DT; ~4–7 d post oviposition) (Yntema 1968, Ewert 1985, Miller 1985, Miller et al. 2017), which is why blood may not be visible to the unaided eye until after 4 d of development (Leslie et al. 1996, Ralph et al. 2005). In the absence of macroscopic signs of an embryo, blood or other tissues (and/or a white spot), failed fertilized eggs have been categorized incorrectly as infertile (e.g. Whitmore & Dutton 1985, Bell et al. 2004, 2010), due to insufficiently close examination.

4. BEST PRACTICES AND POTENTIAL TECHNIQUES FOR ASSESSING INFERTILITY IN SEA TURTLE EGGS

There is a broad need to better understand factors, including the potential for infertility, that influence sea turtle hatchling production (Hamann et al. 2010, Rees et al. 2016). The best practices and potential techniques for assessing evidence of infertility in sea turtle populations and distinguishing between infertile eggs and those experiencing intra-oviducal or early embryonic mortality are described in Sections 4.1–4.3; the order of presentation is based on both rigor of outcomes and ease of application depending on available resources. We did not find any relevant studies that used methods for extraction and amplification of haploid DNA to distinguish between the germinal vesicle on an unfertilized follicle and the haploid cells comprising the blastodisc in a fertilized egg (see Arnold et al. 2003). If available, such a test would likely be more successful when applied to freshly oviposited eggs.

Bell et al. (2004) could not identify embryos or tissues in a small proportion of eggs that developed white spots but later failed to hatch, and Abella et al. (2017) estimated lower rates of fertility using pres-

ence/absence of the white spot in the first few days of incubation than in the same clutch using embryo and tissue fragments in unhatched eggs at the end of the incubation period. Therefore, we recommend a combination of techniques be used and that tests finding no indications of fertilization and/or embryonic development in unhatched eggs at the end of the incubation period which have undergone decomposition be repeated using freshly oviposited eggs. This is especially important if the research objective is to quantify infertility rates.

4.1. Examination of egg contents for embryonic development

Parmenter (1980) challenged the assumption of infertility unless microscopic examination failed to find any evidence of embryonic development. The procedures below are suitable for fresh eggs at oviposition or unhatched eggs at the end of the incubation period. However, examination of failed eggs for indications of embryonic development would be most successful if conducted soon after embryonic death, as even dead, 3 wk old embryos have been known to disintegrate by the time of nest excavation at 9 wk (Wyneken et al. 1988).

The turtle egg can be opened by hand or with fine scissors and examined for signs of an embryo, blood, or other tissue as evidence of fertilization. If none are visible macroscopically, the albumen should be discarded and the yolk placed in a watch glass or petri dish with the blastodisc or embryo (appearance described below) upwards for easiest examination. The blastodisc or embryo in a recently oviposited egg will be at a variable location on the yolk. Once the egg contents become re-organized by specific gravity after oviposition, the blastodisc or embryo will be located at the top of the yolk and immediately adjacent to the shell membrane; rotation should be minimized while removing eggs from the nest and the egg opened at a location other than its north pole. If decomposition has occurred between embryonic mortality and egg examination, and the yolk has become mixed with the albumen, the egg contents should be placed in a petri dish with a small volume of phosphate-buffered saline and carefully sorted.

In unfertilized eggs, the blastodisc will appear as a dense white spot alone and may seem granular due to the presence of abundant vacuoles. In fertilized eggs at the blastoderm stage, the blastodisc has undergone cleavage (see Stages 1–5 in Miller 1985 for descriptions), and the resulting blastoderm can be seen as a central, pale *area pellucida* surrounded by the dense, white ring of the *area opaca*. The one cell thick *area pellucida* is formed as cells in the center of the blastoderm are shed and die; the embryonic shield, the area which will undergo further differentiation during embryonic development, may be visible at its posterior location within the *area pellucida*. The *area opaca* represents the area of the blastoderm that has not shed its deeper cells. (For guiding images and potential variations in appearance as described in the freshwater turtle and avian literature, see Nayar 1958, Ewert 1985, Bakst et al. 1998, Onbaşilar et al. 2006, Birkhead et al. 2008.) In fertilized eggs where development has proceeded to a gastrula or later stage, embryo appearance will be as described by Miller et al. (2017). A dissecting microscope or hand lens (minimum magnification power 10×, with illumination via lamp or handheld light as appropriate; Miller et al. 2017) may be required to see the blastodisc, blastula, gastrula, or small embryo. Mounting tissue on a slide (Birkhead et al. 2008) and/or a biological stain (equal parts methylene blue and 70% ethanol; Bell et al. 2004) can also aid examination.

4.2. Examination of egg exterior for the white spot

Monitoring for appearance of the characteristic white spot in the first days of incubation may be used as a complementary assessment to examination of egg contents for embryonic tissue. Visual examination for a white spot can be conducted on eggs incubated *in situ*, in a relocated location elsewhere on the beach or a hatchery, or under appropriate laboratory conditions. It should not be used as the sole technique if the research objective is to specifically quantify infertility, due to the likelihood of underestimating fertility, as eggs that fail to develop a white spot will include those that were not fertilized plus those that were fertilized but experienced intra-oviducal death or early post-ovipositional death (Miller 1985). Bell et al. (2004), Ralph et al. (2005), and Rafferty et al. (2011), but not Eckert & Eckert (1990), found that the majority of embryonic mortality occurred at Stage 6 (intra-oviducal arrested development to oviposition) or earlier, before the embryo had likely attached to the eggshell membrane and resulted in the formation of a white spot. Researchers should also be aware that the white spot can fade within ~44 h of embryonic death (Phillott & Parmenter 2007) and the chalk-white coloration may not appear on both viable (Sahoo et al. 2009) and non-viable (Phillott & Parmenter 2007) unhatched eggs at the

end of the incubation period. Thus, for eggs inspected within 1–5 d after oviposition, presence of the white spot definitively indicates that an egg was fertilized (Blanck & Sawyer 1981, Miller 1985), but an egg without a white spot cannot be definitively categorized as infertile and requires further inspection.

4.3. Candling to visualize the embryo and extra-embryonic membranes

At oviposition, the sea turtle embryo (Stage 6; Miller et al. 2017) may be visible during candling, but extra-embryonic membranes (including the chorioallantois) are not present at this time (Miller 1985), so the suitability of this technique in oviposited eggs requires validation. Abella et al. (2017) observed signs of embryonic development by candling at 24 h post oviposition, although it is unclear if eggs in which structures were not found by candling showed other signs of embryonic development. If candling is used on recently oviposited eggs, negative findings should be followed by examination of the egg exterior for a white spot at the appropriate time or of egg contents for signs of embryonic development as described in Sections 4.1 and 4.2.

4.4. Indications of sperm penetration of the ovum and syngamy

Egg fertilization comprises a sequence of events, including sperm penetration of the inner perivitelline membrane and fusion of the haploid sperm and ovum pronuclei (syngamy) to form a diploid nucleus (see Birkhead et al. 2008). The techniques described look for indications of successful events in fertilization and, while also independently applicable, should be used in sea turtle populations for which examination of the egg contents aided by microscopy has consistently failed to locate embryonic or other tissue before conclusions about the frequency of infertile eggs are made.

4.4.1. Holes in the PVM

In birds, sperm penetration of the inner PVM over the blastodisc creates visible holes (Bramwell et al. 1995, Birkhead et al. 2008) which, even though the interval between fertilization and oviposition is longer, may be visible in sea turtle eggs. Holes were visible in unhatched tree sparrow eggs after 25 d of incubation and up to 15 d of cool storage (Birkhead et al. 2008), so the technique also shows promise for turtle eggs with no visible signs of development that are examined during or at the end of the incubation period. The technique required a piece of the blastodisc be removed from the yolk, and the inner PVM to be isolated and examined using light microscopy (see Bramwell et al. 1995, Birkhead et al. 2008).

4.4.2. PVM-bound sperm

Tests for evidence of sperm trapped in the PVMs have been described for birds (Birkhead et al. 1994, 2008), freshwater turtles (Croyle et al. 2016), and crocodiles (Augustine 2017) and may also have applications for sea turtles. Croyle et al. (2016) used a preparation of the PVMs followed by staining with the nucleic acid dye Hoechst 33342 and fluorescence microscopy to detect PVM-bound sperm in eggs from 12 freshwater turtle species; see Birkhead et al. (2008) for further explanation of the methods. The sperm-staining test was successfully used on eggs which were maintained under different incubation and storage conditions. However, the dye will also stain fungal and bacterial DNA (Croyle et al. 2016), which can lead to a false positive for fertilization, so care must be taken when distinguishing between sperm heads and microbes in contaminated and infected eggs.

PVM-bound sperm in the radiated tortoise *Astrochelys radiata*, African spurred tortoise *Centrochelys sulcata*, and leopard tortoise *Stigmochelys pardalis* were also detected by mitochondrial DNA amplification and sequencing (technique and primers further described by Croyle et al. 2016). With appropriate primers, this technique should also be successful with sea turtle samples.

Further research on techniques to detect PVM-bound sperm is needed in order to evaluate their utility for examining unhatched sea turtle eggs that have been recovered from nests at the end of the incubation period. As an additional note, the absence or low numbers of PVM-bound sperm in freshly oviposited eggs collected from a representative sample of females in a population is potentially the most reliable indicator of limited numbers of breeding males currently available.

4.4.3. Detection of nuclei in the blastodisc

The presence of numerous cell nuclei in the blastodisc indicates successful fertilization and cleavage.

A piece of the blastodisc may be removed from the yolk and PVMs (see Gupta & Bakst 1993, Birkhead et al. 2008) and then stained with Hoechst dye (see Birkhead et al. 2008; given as 'Hoechst 33324' in that study, but believed to be Hoechst 33342) and examined using fluorescence microscopy. Nuclei will be stained bright blue and can be quantified in fertilized eggs; the absence of nuclei indicates that the egg was not fertilized (Birkhead et al. 2008). Nuclei were detected in the blastodisc of fertilized but unhatched tree sparrow eggs after 25 d of incubation and up to 15 d of cool storage (Birkhead et al. 2008), so the technique also shows promise for failed turtle eggs examined during or at the end of the incubation period.

4.5. Other techniques

Recently described techniques for distinguishing between fertilized and unfertilized bird eggs at oviposition include measuring gas exchange rates using a customized microtesting examination platform (see Wang et al. 2017) and hyperspectral imaging technology (Zhu et al. 2015). These techniques have yet to be used in additional studies and taxa, so their potential application for turtle eggs is unknown; however, we believe that the other techniques described above will be more easily applied in sea turtle research in the future, due to increased availability of equipment and/or potential application in the field.

5. RECOMMENDATIONS

The window between initial fertilization and the end of successful egg incubation in sea turtles often spans >50 d, and the conditions in the nest cavity are highly conducive to tissue degradation of early stage embryos that have died. This greatly limits the ability of researchers to accurately determine whether unhatched sea turtle eggs are indeed fertilized. Thus, we believe that researchers must assume a greater burden of proof before designating unhatched eggs without signs of egg development as infertile. There is no evidence to date of depensation occurring in sea turtle populations (Bell et al. 2010, Wright et al. 2012, Abella-Perez et al. 2016), so such a burden of proof is especially important if rates of 'infertility' are used as justification for local conservation action, including removal of 'infertile' eggs from the nest (Abella et al. 2007) or manipulation of the nest environment via shading, watering, or other means to increase production of male hatchlings (Hill et al. 2015, Jourdan & Fuentes 2015).

The gold standard for assessing rates of fertility is to evaluate freshly oviposited eggs for the presence of embryonic development (e.g. Wyneken et al. 1988). Given the lack of baseline information on fertility rates of sea turtle eggs, we recommend dedicated studies be undertaken to document rates of fertility of various rookeries and regional management units. Given that the outcome for each egg is binary (fertilized or unfertilized), having sufficient power to detect significant differences in average fertility rates across clutches with >100 eggs requires that at least 20 eggs per clutch are examined to generate a fertility ratio (Spotila et al. 1983, Mrosovsky et al. 2009). To derive a simple fertility estimate of a nesting population, we recommend examining 20 eggs from 20 clutches laid by different females during an 8 d period (to avoid collecting eggs laid by the same female) at the peak of the nesting season. More sampling will be needed to examine potential changes in fertility rates across the season, both in individual females and in the population as a whole. Rigorous sampling should also be applied to nesting populations demonstrating hatching success in undisturbed nests that is below the normal range (e.g. <60% for loggerheads, Dodd 1988; <70% for green turtles *Chelonia mydas*, Hirth 1997) and cannot be attributed to factors other than infertility. However, given the ethical and permitting constraints of sacrificing viable eggs for this type of study, researchers must determine what the most appropriate sampling regime is for their study population.

In addition, when describing and categorizing eggs at the end of the incubation period, we recommend the terms 'fertile' and 'infertile' and the related forms for each be used with the strictest of accuracy according to their definition to ensure naïve researchers and conservationists do not try to address issues that have not been conclusively demonstrated, let alone quantified. Parmenter (1980) recommended that the term 'undeveloped' be applied to eggs that show no macroscopic signs of development at the end of the incubation period and to avoid using the term 'infertile' (as inappropriately applied by Hughes & Mentis 1967, Hughes et al. 1967, Simon 1975, Simon et al. 1975, Peters et al. 1994, Hitchins et al. 2004, Bell et al. 2010, Önder & Candan 2016, Abella et al. 2017). Furthermore, we recommend that hatching and/or emergence success not be used as a proxy for fertility. These measures are more appropriately used as proxies for reproductive success, with the understanding that many environmental factors affect the

hatching rate of incubating sea turtle eggs (Miller et al. 2003). While fertility rates and hatching/emergence success values may be related, they should be assessed separately.

Currently, the lack of information on fertility in sea turtle eggs hampers deeper understanding of the potential impacts of climate change on sea turtle populations. We encourage validating the techniques suggested above that have yet to be trialed with sea turtle eggs, and conducting appropriately designed studies to quantify rates of fertility and infertility in different sea turtle populations.

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