A method for the collection of early-stage sea turtle embryos

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ABSTRACT: Early-stage turtle embryos, immediately after oviposition, are very small (<5 mm diameter), hindering research on the initial period of embryonic development. For example, assessing whether turtle eggs had been fertilized and contained a viable embryo at oviposition, especially under field conditions, is complicated by the microscopic size of embryos that may have died at an early stage of development. Further, little is known about the molecular pathways that promote and regulate early developmental processes in turtles, such as pre-ovipositional embryonic arrest. To enable further investigation of the processes critical to early embryonic development in turtle species, a reliable method is required for extraction of early-stage embryos from the egg. Therefore, our aim was to develop a novel and reproducible method for extracting early-stage sea turtle embryos. Herein, we describe the technique for extracting *Chelonia myda*s embryos before and after white spot formation. Once the embryos were collected, the total RNA of 10 embryos was extracted to validate the method. The total RNA concentration was above 5 ng μl^{-1} and the RNA integrity number varied between 7.0 and 10.0, which is considered acceptable for further RNA-sequencing analyses. This extraction technique could be employed when investigating fertilization rates of turtle nests and for further investigation of the molecular biology of embryonic development in turtles. Furthermore, the technique should be adaptable to other turtle species or any oviparous species with similar eggs.

KEY WORDS: Egg · Embryo collection · Embryonic development · Extraction · Sea turtle

1. INTRODUCTION

There are many interesting unanswered questions regarding the embryonic development of freshwater and sea turtles. For example, we remain largely ignorant of the molecular pathways that drive early developmental processes. Further, there are certain processes that are apparently unique to this taxon, like pre-ovipositional embryonic arrest (Ewert 1985, Miller 1985, Andrew 2000, Williamson et al. 2017b) driven by oviductal hypoxia (Rafferty et al. 2013), which occurs in turtles but not in other reptiles, such as crocodiles (Williamson et al. 2017a). To enable detailed investigation of these and other processes critical to early embryonic development in turtle spe-

cies, a reliable method is required for extraction of an early-stage turtle embryo from the egg.

There are only a few published turtle embryology studies, these mainly being detailed descriptions of the morphological changes that occur during embryonic development of various sea turtle species (Mitsukuri 1894, Ewert 1985, Miller 1985, Miller et al. 2017). These studies have mainly focused on descriptions of the morphology of embryonic stages or detection of embryo presence/absence. There has been little investigation of the molecular processes occurring at the early stages of turtle development. Investigation of molecular and developmental processes requires removal of the embryo from the egg, but there are no published descriptions of methods for extraction of

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early-stage embryos for molecular analyses. Further, biologists and conservationists sometimes assess rates of fertilization (i.e. whether eggs have been fertilized and a viable embryo formed) in clutches of turtle eggs (Blanck & Sawyer 1981, Whitmore & Dutton 1985, Bell et al. 2004, Rafferty et al. 2011). However, the ability to detect the difference between an unfertilized egg and an embryo that died at an early stage of development is complicated by the small size of these embryos. This problem is exacerbated if the failed egg is examined at the conclusion of the normal incubation period, when an undeveloped (i.e. little or no embryonic development post-oviposition) or unfertilized (i.e. no formation of an embryo) egg (Miller 1985) has potentially decomposed in the nest for many weeks.

Within the first stages of their development (until stage 16 according to Miller 1985), sea turtle embryos, such as those of the green sea turtle *Chelonia mydas*, are relatively small (<5 mm diameter) compared with the size of the egg (~41–44 mm diameter) (Miller 1985, 1997). Hence, they are difficult to collect. However, collecting the embryo, in a manner that preserves the cellular integrity, is a necessary first step for physical, genomic, proteomic or other molecular examination. Therefore, the aim of our study was to develop and validate a simple and reliable method for extracting early-stage sea turtle embryos, thus facilitating further analysis of the molecular biology or histology of embryonic development in turtles. Here, we describe in detail methods for extracting *C. myda*s embryos before and after the formation of the 'white spot', that appears as a result of the fusion of the eggshell membranes with the vitelline embryonic membrane (Webb et al. 1987). This white spot is typically used as an externally visible indicator that the embryo has broken developmental arrest after oviposition (Rafferty & Reina 2012), but the white spot's presence does not guarantee that the embryo remained viable throughout the full period of incubation (Williamson et al. 2019). Embryos harvested using our technique can be used for histological or molecular studies, and the procedure should be easily adaptable to other turtle species or any oviparous species with similar embryonic and egg structural characteristics.

2. MATERIALS AND METHODS

2.1. Procedure

Green turtle eggs were collected from ovipositing females during October 2018, at Heron Island, Great Barrier Reef, Australia (23° 26′ 18.71″ S, 151° 54′ 30.23″ E).

Once the eggs were collected, they were transported in plastic bags a short distance to the laboratory (less than a 1 km or 15 min walk). The eggs were settled for at least 2 min, avoiding any rotation and/or unnecessary movement. This allows the yolk and embryo to move towards the top of the egg, enabling extraction. Starting from 12 h after oviposition, embryos break from pre-ovipositional arrest (Williamson et al. 2017b). This renders the embryos susceptible to movement-induced mortality because the embryo becomes attached to the eggshell (Limpus et al. 1979).

Embryos were removed from some eggs within approximately 2 h of collection (n = 5), while other eggs were incubated in sand at 26 to 28°C for up to 36 h before extraction of the embryo (n = 5).

2.1.1. Extraction of the embryo

The technique for collection of the embryo differs according to the stage of its development. Specifically, the method for collection of embryos before formation of the white spot differs from that after white spot development. The white spot (Fig. 1) appears as a result of fusion of the eggshell membranes with the vitelline embryonic membrane, which migrates to the upper pole or animal pole (AP) of the egg after oviposition, and the drying of the shell (Thompson 1985, Webb et al. 1987). The presence of the white spot requires a modification of the technique as described below.

2.1.2. Extraction of the embryo before white spot formation

The following steps are portrayed in Video S1 at www.int-res.com/articles/suppl/n042p059_supp/.

Step 1: If the embryo samples are to be used for genomic or gene expression studies, ensure all instruments and laboratory surfaces are cleaned with 70% v/v ethanol and RNase-free solutions. Use only RNase-free materials.

Step 2: The egg surface should be cleaned from adhering sand and debris using dry soft tissue paper or, if sand is strongly adhered to the eggshell, a moist tissue may be used. Avoid rotating the egg during handling and cleaning. Then, to facilitate handling, the egg should be placed in a petri dish and stabilized with a handmade foil ring (or something similar) in the petri dish. In this position, the AP, where the embryo is located, will be at the upper surface of

Fig. 1. Green sea turtle (*Chelonia mydas*) eggs 36 h post-oviposition, incubating in sand. Black arrow: a white spot that developed after oviposition and represents the first external sign of the resumption of active development of the embryo. The other eggs in the photo have not yet developed a white spot. The average diameter of *C. mydas* eggs is 41.2 ± 1.19 mm (Limpus et al. 1984). Scale bar = 10 mm

the egg, and the lower pole or vegetal pole (VP), with the yolk, at the bottom. Carefully place the petri dish containing the egg under a dissection microscope and illuminate.

Step 3: To open the egg, use a small pair of dissecting scissors to cut the eggshell around the upper section, approximately 1 cm down from the AP (Fig. 2). Once this shell section is removed using fine forceps, the embryo will be visible as a small round disc floating in the yolk and covered with several layers of albumen (Fig. 3). To remove the albumen, small dissecting scissors can be used to cut the layers of albumen, which can then be removed using scientific grade tissues (e.g. Kimwipes, Kimberley Clarke). Lay

the tissue on the albumen surface, lift the tissue and adhering albumen and then cut through the albumen as close to the embryo as possible. This process should be repeated several times until the layer attached to the embryo is reached.

Step 4: The embryo is ready for extraction when there are no remaining layers of albumen (i.e. albumen no longer adheres to the tissue). Carefully aspirate the embryo into a 3 ml plastic Pasteur pipette (e.g. Falcon, Corning) by applying gentle suction, and use fine scissors to cut the embryonic disc with the embryo from the vitelline membrane. This step should be done as quickly as possible to ensure that the embryo is drawn inside the pipette while at the same time avoiding the extraction of excess albumen and/or yolk. For best results, the end of the pipette should be cut so that its diameter is slightly larger than that of the embryonic disc. When the embryo is inside the plastic Pasteur pipette (Fig. 4), place it in an Eppendorf tube or other receptacle for storage/preservation, as appropriate, for subsequent analysis.

2.1.3. Extraction of the embryo after white spot formation

The following steps are portrayed in Video S2.
Step 1: As described in Step 1, Section 2.1.2.
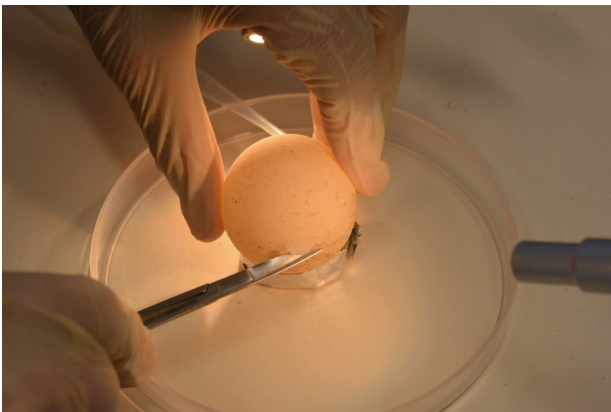Step 2: As described in Step 2, Section 2.1.2. How-

Fig. 2. Green sea turtle (*Chelonia mydas*) egg within 120 min of oviposition. The upper section of the egg is being cut along its circumference to expose the embryo at the animal pole (upper section) for extraction

Fig. 3. Green sea turtle (*Chelonia mydas*) egg without the upper shell section and before the albumen has been removed (approx. 2 h after oviposition; Stage 6, Miller 1985). The embryo disk (black arrow) can be seen floating on the albumen
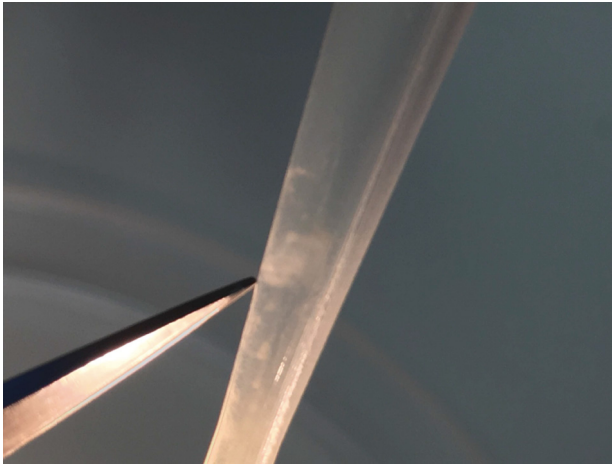
Fig. 4. Green sea turtle (*Chelonia mydas*) embryo before white spot formation, extracted with a plastic Pasteur pipette. Location of the embryo is indicated by the tip of the scissors

ever, also ensure that the white spot remains facing upwards. It is very important to avoid unnecessary movement of the egg during these preparatory procedures to avoid displacement of the embryo and/or rupturing of membranes within the egg. If membranes rupture, the embryo will become difficult or impossible to find.

Step 3: Cut a circle within the opaque white spot on the eggshell (Fig. 1), following closely inside the circumference of the white spot, with a small pair of dissecting scissors. The incision must be sufficiently deep (approx. 3 mm) to cut through the membranes below the white spot that are attached to the embryo. Once this section of the shell is completely separated from the rest of the eggshell, identify the position of the embryo on the undersurface of the removed eggshell. The embryo is usually situated in the center of the white spot. To collect the embryo, use a sterile scalpel/surgical blade (e.g. Swann Morton) to detach the embryo and the surrounding membrane from the shell. To remove the embryo, use fine forceps to grasp it from its periphery. Place the embryo in an Eppendorf tube or other receptacle for later analysis.

2.2. Technique validation

Five embryos were extracted as described above in Section 2.1.2 (before white spot formation) and another 5 embryos as described in Section 2.1.3 (after white spot formation). Immediately after extraction, the embryos were flash frozen in liquid nitrogen and stored at −80°C. To verify that viable embryo tissue could be obtained using the method described in this study, RNA was isolated with a RNeasy Micro Isolation Kit (Qiagen), following the manufacturer's protocol. Briefly, each sample was homogenized in QIAzol lysis buffer and incubated for 5 min at room temperature. Chloroform was then added to the homogenate; this was followed by centrifugation at $12\,000 \times g$ at 4°C for 15 min. Next, the aqueous layer was mixed with 1.5 volumes of absolute ethanol and washed through an RNeasy Mini Spin Column. Buffers RWT and RPE (both provided in the kit) and 80% ethanol were then sequentially added to wash the column. Finally, 12 µl of RNase-free distilled water was used to elute the RNA from the column. RNA samples were stored at −80°C until further analysis.

The concentration and purity of RNA obtained from embryos was assessed using a Qubit Fluorometric Quantification (Invitrogen). Total RNA quality was determined by examination of RNA size distribution on RNA Nano LabChips (Agilent Technologies) processed on an Agilent 2100 Bioanalyzer (Agilent Technologies), using the total RNA electrophoresis program to generate an RNA integrity number (RIN), an algorithm for assigning integrity values to RNA measurements that quantifies the fragmentation of RNA samples (Schroeder et al. 2006). The maximum RIN score is 10.0 and samples with RIN values between 7.0 and 10.0 are considered viable for RNA-sequencing analyses (Jahn et al. 2008).

2.3. Statistical analyses and data presentation

All statistical analyses were performed using GraphPad Prism Software (version 8.0) and data are expressed as mean ± standard error of the mean (SEM). Normality was assessed using a Shapiro-Wilks test. Mean RNA concentration between groups and mean RIN scores between groups were compared using Student's unpaired *t*-test. Two-tailed p-values < 0.01 were considered statistically significant.

3. RESULTS

We successfully extracted RNA from all collected embryos (Table 1). Total RNA concentration across all 10 samples ranged from 5.0 to 51.6 ng µl^{-1}, with a mean ± SEM of 20.65 ± 4.71 ng µl^{-1}. The mean RIN

Table 1. RNA concentration and RNA integrity number (RIN) for green sea turtle (*Chelonia mydas*) embryos collected before (Samples 1–5) and after (Samples 6–10) formation of the white spot, using the methods described in the current study

Sample no.	RNA concentration (ng μl^{-1})	RIN
1	6.50	9.6
2	26.70	9.4
3	15.05	9.5
4	32.35	8.8
5	6.20	9.4
6	24.05	7.2
7	10.40	7.2
8	51.60	7.6
9	28.60	7.6
10	5.00	7.0

score was 8.33 ± 0.35. RIN values greater than 7.0 indicate little contamination with degradation products, so such samples are considered viable for RNA-sequencing analyses (Jahn et al. 2008).

Mean total RNA concentration for Samples 1 to 5 (embryos collected before white spot formation; 17.36 ± 5.29 ng μl^{-1}) did not differ significantly from that for Samples 6 to 10 (embryos collected after white spot formation; 23.93 ± 8.15 ng μl^{-1}; $p > 0.05$, *t*-test). The RNA concentration obtained for each embryo sampled (Table 1) was sufficient to permit reproducible PCR assays (PCR, RT-PCR, qPCR or RT-qPCR) and meaningful gene expression data.

There was a significant difference in the mean RIN score between embryos extracted prior to white spot formation (Samples 1 to 5; 9.34 ± 0.14) and those extracted after white spot formation (Samples 6 to 10; 7.32 ± 0.12; $p < 0.01$). Nevertheless, the RIN score of embryos from all eggs sampled was equal to or above 7.00 (Table 1), the threshold considered viable for RNA-sequencing analyses (Jahn et al. 2008).

4. DISCUSSION

We describe a reliable method for the extraction of turtle embryos, before and after white spot formation. This method lays the foundation for further studies of early stages of embryonic development in turtles and could be applied in investigations that require molecular and/or histological analysis. Further, our method represents a useful technique for field biologists and conservationists to assess clutch fertilization rates (i.e. the proportion of eggs fertilized by sperm) and determine whether embryos have formed but died prior to the appearance of the white

spot, or to undertake genetic analyses of early stage embryos, which can help to inform management strategies for sea turtle populations. Early-stage embryonic death may be a significant but cryptic factor in poor hatching success of some turtle nests (Rafferty et al. 2011) that deserves further investigation.

We showed that RNA of sufficient quantity and quality can be obtained using the method described. Therefore, it is possible to utilize RNA sequencing techniques or other molecular methods that require RNA after extracting embryos using the techniques we have described. The use of molecular techniques in sea turtle research has recently increased (Bentley et al. 2017, Hernández-Fernández 2017, Komoroske et al. 2017). Our current method should allow the use of such techniques to be extended to early-stage embryos.

There was no significant difference in total RNA concentration obtained for embryos collected before or after white spot formation. Moreover, the concentration obtained from each of the 10 embryos was sufficient to permit PCR assays to generate meaningful gene expression data. However, there was a significant difference in RIN score between the 2 groups of embryos. This may be because, after white spot formation, embryos attached to the inner eggshell membrane and therefore the proportion of non-embryonic tissue in the sample was likely greater than that in samples of freshly laid embryos. Minimizing the amount of non-embryonic tissue collected with the embryo should improve purity and integrity. This could potentially be achieved by minimizing the amount of albumen aspirated with embryos collected before white spot formation, and by minimizing the non-embryonic membranous tissue (like inner eggshell membrane) collected with embryos after white spot formation. However, the RIN score for all samples was above 7.00, which is considered sufficient for viable RNA-sequencing analyses (Jahn et al. 2008). It is important to highlight that the assessment of RNA integrity is a critical step for successful PCR or RNA-seq analyses.

To the best of our knowledge, the extraction of early-stage embryos of sea turtle species has not previously been documented in detail. We are aware of only 2 relevant previous reports. In 1894, Mitsukuri described extraction of the embryo of *Caretta caretta* using a fixative (Mitsukuri 1894). Clearly, such an approach would not be suitable for generating material for genetic analysis. More recently, Matsubara et al. (2016) described how to isolate and harvest early-stage embryos of the soft-shelled freshwater turtle *Pelodiscus sinensis*, prior to the pharyngula stage

(Stage 7; Ewert 1985). The technique they used is comparable to the one we describe for green sea turtle embryos after white spot formation. However, we believe our approach is both easier to perform and superior to that described by Matsubara and colleagues, because it minimizes the potential for contamination of the sample. We performed the extraction of the embryo by cutting the eggshell and attached membranes inside the boundaries of the white spot. This step allows extraction of the embryo without yolk contaminating and/or covering the sample. Furthermore, we describe a method for embryo extraction prior to white spot formation, allowing the extraction of embryos at the earliest stage of development after oviposition.

Our suction technique for lifting the embryo from the egg prior to white spot formation may impact the structural integrity of the embryo. Whilst we did not specifically assess this, our visual observation at the time of collection was that embryos usually appeared intact after extraction. However, if an embryo is being collected specifically for histological examination, this method of collection should be undertaken using the least suction necessary to lift the embryo, in order to minimize the risk of damage.

To perform the technique described in the current study, one important consideration is to avoid unnecessary movement of the egg. In particular, rotation should be strictly avoided from 12 h after oviposition. When it is necessary to move freshly laid eggs (<12 h after oviposition) considerable distances for the extraction of the embryo, embryonic development can be paused by maintaining eggs in hypoxia (Williamson et al. 2017c) or cooling them down to temperatures between 7 and 10°C (Miller & Limpus 1983, Harry & Limpus 1989).

The method described in the present study could easily be implemented in the field. For example, the extracted embryo could be flash frozen in liquid nitrogen and stored at −80°C until RNA extraction. But it should also be possible to use this method to generate embryonic tissue for histological and/or immunohistochemical analysis. For these analyses, the sample can be placed in a fixative such as formalin for 12 to 24 h, and then in a preservative solution, such as 70% ethanol, until analysis. Preservation could easily be performed in the field, with the required materials and equipment being sourced from a research station or transported directly to the field.

Reliable estimation of clutch fertilization rate is critical for monitoring sea turtle populations (Glen & Mrosovsky 2004). It is an important concern for conservationists and field biologists, especially in global warming research where there is evidence that increasing temperatures have diminished the production of male hatchlings (Janzen 1994, Bell et al. 2004, Glen & Mrosovsky 2004, Jensen et al. 2018). This bias towards female hatchlings, among other factors, has the potential to influence clutch fertilization rate if it exceeds the mitigating effect of promiscuous sea turtle mating systems. Yet the methods that are commonly used to assess the viability of sea turtle embryos have serious limitations. In the field, it is difficult to discern the early stages of embryonic development and it is also difficult to distinguish between fertile and infertile eggs within sea turtle clutches. A commonly deployed method is to simply observe the presence or absence of a white spot, which develops between 24 to 72 h after oviposition (Blanck & Sawyer 1981). However, the absence of a white spot does not necessarily reflect the absence of an embryo (Williamson et al. 2017b). Consequently, this method may lead to underestimation of the number of fertilized eggs in a clutch (Abella et al. 2017). An alternative method is to wait until hatching and then determinate the proportion of eggs that failed to hatch and/or had no sign of embryonic development. This method, by nature, requires the investigator to wait until the viable eggs have hatched. There is also the problem that eggs with non-viable embryos can decompose in the nest, so discriminating between unfertilized eggs and eggs in which the embryo died early in development may be problematic. Consequently, this approach leads to an even greater underestimation of the number of fertilized eggs in a clutch (Abella et al. 2017). The method for the extraction of early-stage embryos we describe herein allows clutch fertilization rate and viability to be reliably and rapidly assessed and also facilitates investigation of the genetic and morphological determinants of viability.

In summary, we describe reliable methods for extraction of early-stage turtle embryos both before and after formation of the white spot. The techniques we describe should be adaptable to other turtle species or any oviparous species with similar egg structure and characteristics. This method should be a useful tool for studies of embryology and development to improve our understanding of sea turtle biology and conservation.

LITERATURE CITED

Abella E, García-Cerdá RM, Marco A (2017) Estimating the fertilization rate of sea turtle nests: Comparison of two techniques. Basic Appl Herpetol 31:33–44

Andrew RM, Mathies T (2000) Natural history of reptilian development: Constraints on the evolution of viviparity. BioScience 50:227–238

Bell BA, Spotila JR, Paladino FV, Reina RD (2004) Low reproductive success of leatherback turtles, *Dermochelys coriacea*, is due to high embryonic mortality. Biol Conserv 115:131–138

Bentley BP, Haas BJ, Tedeschi JN, Berry O (2017) Loggerhead sea turtle embryos (*Caretta caretta*) regulate expression of stress response and developmental genes when exposed to a biologically realistic heat stress. Mol Ecol 26:2978–2992

Blanck CE, Sawyer RH (1981) Hatchery practices in relation to early embryology of the loggerhead sea turtle, *Caretta caretta* (Linné). J Exp Mar Biol Ecol 49:163–177

Ewert MA (1985) Embryology of turtles. In: Gans C, Billet F, Maderson PFA (eds) Biology of the Reptilia, Vol. 14. Wiley, New York, NY, p 75–268

Glen F, Mrosovsky N (2004) The impact of climate change on sand and nest temperatures at a hawksbill turtle (*Eretmochelys imbricata*) nesting beach. Glob Change Biol 10:2036–2045

Harry JL, Limpus CJ (1989) Low-temperature protection of marine turtle eggs during long-distance relocation. Wildl Res 16:317–320

Hernández-Fernández J (2017) Data of first *de-novo* transcriptome assembly of a non-model species, hawksbill sea turtle, *Eretmochelys imbricate*, nesting of the Colombian Caribean. Data Brief 15:573–576

Jahn CE, Charkowski AO, Willis DK (2008) Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. J Microbiol Methods 75:318–324

Janzen FJ (1994) Climate change and temperature-dependent sex determination in reptiles. Proc Natl Acad Sci USA 91:7487–7490

Jensen MP, Allen CD, Eguchi T, Bell IP and others (2018) Environmental warming and feminization of one of the largest sea turtle populations in the world. Curr Biol 28:154–159

Komoroske LM, Jensen MP, Stewart KR, Shamblin BM, Dutton PH (2017) Advances in the application of genetics in marine turtle biology and conservation. Front Mar Sci 4:156

Limpus CJ, Baker V, Miller JD (1979) Movement induced mortality of loggerhead eggs. Herpetologica 35:335–338

Limpus CJ, Fleay A, Guinea M (1984) Sea turtles of the

Capricornia Section, Great Barrier Reef Marine Park. In: Ward WT, Saenger P (eds) The Capricornia Section of the Great Barrier Reef: past, present and future. Royal Society of Queensland and Australian Coral Reef Society, Brisbane, p 61–67

Matsubara Y, Kuroiwa A, Suzuki T (2016) Efficient harvesting methods for early-stage snake and turtle embryos. Dev Growth Differ 58:241–249

Miller JD (1985) Embryology of marine turtles. In: Gans C, Billett F, Maderson PFA (eds) Biology of the Reptilia, Vol. 14. Wiley, New York, NY, p 269–328

Miller JD (1997) Reproduction in sea turtles In: Lutz PL, Musick JA (eds) The biology of sea turtles. CRC Press, Boca Raton, FL, p 51–79

Miller JD, Limpus CJ (1983) Method for reducing movement-induced mortality in turtle eggs. Mar Turtle Newsl 26:10–11

Miller JD, Mortimer JA, Limpus CJ (2017) A field key to the developmental stages of marine turtles (Cheloniidae) with notes on the development of *Dermochelys*. Chelonian Conserv Biol 16:111–122

Mitsukuri K (1894) On the process of gastrulation in *Chelonia*. J Colloid Sci 6:227–277

Rafferty AR, Reina RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. Proc Biol Sci 279:2299–2308

Rafferty AR, Santidrian Tomillo P, Spotila JR, Paladino FV, Reina RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). PLOS ONE 6:e21038

Rafferty AR, Evans RG, Scheelings TF, Reina RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. Am Nat 181:245–253

Schroeder A, Mueller O, Stocker S, Salowsky R and others (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3

Thompson MB (1985) Functional significance of the opaque white patch in eggs of *Emydura macquarii*. In: Grigg G, Shine R, Ehmann H (eds) Biology of Australasian frogs and reptiles. Surrey Beatty, Chipping Norton, New South Wales, p 387–395

Webb GJW, Manolis CS, Whitehead PJ, Dempsey K (1987) The possible relationship between embryo orientation opaque banding and the dehydration of albumen in crocodile eggs. Copeia 1987:252–257

Whitmore CP, Dutton PH (1985) Infertility, embryonic mortality and nest-site selection in leatherback and green sea turtles in Suriname. Biol Conserv 34:251–272

Williamson SA, Evans RG, Manolis SC, Webb GJ, Reina RD (2017a) Ecological and evolutionary significance of a lack of capacity for extended developmental arrest in crocodilian eggs. R Soc Open Sci 4:171439

Williamson SA, Evans RG, Reina RD (2017b) When is embryonic arrest broken in turtle eggs? Physiol Biochem Zool 90:523–532

Williamson SA, Evans RG, Robinson NJ, Reina RD (2017c) Hypoxia as a novel method for preventing movement-induced mortality during translocation of turtle eggs. Biol Conserv 216:86–92

Williamson SA, Evans RG, Robinson NJ, Reina RD (2019) Synchronised nesting aggregations are associated with enhanced capacity for extended embryonic arrest in olive ridley sea turtles. Sci Rep 9:9783