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In ovo manipulation of Nile crocodile embryos: egg windowing and potential dental research applications

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ABSTRACT Crocodilians exhibit continuous tooth replacement (i.e., polyphyodonty) and have been identified as suitable models for tooth regeneration research due to the similarity in dental cavity and tooth anatomy between these creatures and humans. Various studies reporting *in ovo* bird embryo manipulation exist, but such reports for reptiles are virtually non-existent. Egg windowing enables direct access to oviparous vertebrate embryos and is therefore an important component of *in ovo* embryo manipulation experiments. The aim of the present study was to window Nile crocodile, *Crocodylus niloticus* eggs and assess the potential of direct manipulations, targeting the pharyngeal region where the maxilla and mandible originates. *Crocodylus niloticus* eggs were successfully windowed, and a limited number of individuals survived the entire gestation period. The 1st trimester of gestation was the most sensitive, and 96.78% of the mortalities occurred within this period. Our data indicate the suitable window for embryo manipulation targeting the mandibular arch and maxillary process, without a risk of damaging the chorioallantoic membrane (CAM) (which may be fatal), was between day six and eight after laying for embryos incubated at 31°C. This data will be of use for future embryo-based experiments related to jaw and tooth development in crocodiles as well as human tooth regeneration research.

KEY WORDS: *Crocodilian embryo, mandibular arch, odontogenesis, development*

Introduction

The application of oviparous vertebrate embryos during early development is challenging because access during the initial stages (I.e., zygote, morula, blastocyst) is not possible unless the mother is sacrificed. However, these animals offer the advantage of *in ovo* manipulation and continuous monitoring at later developmental stages without the need to sacrifice adult animals. Not surprisingly, avian embryos are commonly used as developmental biology models (Williams *et al*., 2018), as well as in clinical (Merckx *et al*., 2020) and applied research such as toxicology (Stark and Ross, 2019) and tissue engineering (Merckx *et al*., 2020).

Crocodilians exhibit continuous tooth renewal (polyphyodonty). The dental laminae of diphyodonts (including humans) correspond anatomically to those of crocodilians, but undergo apoptosis and fragments after a single replacement event (Richman and Handrigan, 2011; Tsai *et al*., 2016; Whitlock and Richman, 2013). These archosaurs therefore hold promise for bioprospecting and applied dental bioengineering for regenerative therapy in humans. Freshly laid fertilized Nile crocodile, *Crocodylus niloticus*, eggs can be obtained relatively easily from commercial farms, making the species a promising candidate to develop further as model for developmental biology and applied research.

Various reports describe protocols for egg windowing and subsequent embryo manipulation experiments in birds (E.g. Blank *et al*., 2007; Morin *et al*., 2017). Reptiles, including turtles and snakes, have also been applied as models for *in ovo* and *ex ovo* experiments, although to a lesser extent (Nomura *et al*., 2015). For example, methods describing turtle embryo electroporation (Moustakas-Verho *et al*., 2019), the harvest of early-stage snake and turtle from eggs (Matsubara *et al*., 2016) and the *ex vivo* culture of turtle and gecko neural progenitor cells (Yamashita *et al*., 2017) have been published. However, reports describing methods for crocodilian embryo *ex ovo* or *in ovo* manipulation are limited, and although mention is made of egg windowing and semi-shell less culture of alligator embryos, a detailed description of the pro-

Abbreviations used in this paper: AO, Aorta; CAM, Chorioallantoic membrane; DA, Dorsal aorta; MA, Mandibular arch; MXP, Maxillary process; NT, Neural tube; VA, Vitellin artery.

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cedure is not given (Ferguson, 1981; Ferguson, 1985). There is a need to assess the potential application of crocodilian embryos for basic and applied research, and a description of a procedure for egg windowing and subsequent embryo incubation will be an important step forward in this regard.

The 1st pharyngeal arch, also known as the mandibular arch, develops into the lower jaw (Lee *et al.*, 2004). Genome edits performed in the embryonic mandibular arch during early development will theoretically be present throughout the lower jaw once formed. The genomes of cells differentiating into tooth families and successional laminae can therefore be altered. Ferguson (1985) characterised the developmental stages of the American alligator, *Alligator mississippiensis*. Other studies featuring the Saltwater crocodile, *Crocodylus porosus* and the broad snouted caiman, *Caiman latirostris* provide further accounts of the embryonic development of crocodilians (Iungman *et al.*, 2008; Webb *et al.*, 1983). Collectively, these aforementioned studies suggest that crocodilian embryos are at a more advanced stage of development at the time of laying than birds (Hamburger and Hamilton, 1951). Moreover, the anatomy and composition of crocodilian eggs differs from that of birds (Brown *et al.*, 2019; Ferguson, 1985). A direct application of egg windowing methodologies described for chickens may require modification to be successful in crocodilians.

The chorioallantoic membrane (CAM) is an extraembryonic membrane with a respiratory and ion transport function. The CAM therefore contains an extensive network of blood vessels. Minor damage to the CAM can be fatal to an embryo. The CAM develops and expands rapidly during the early gestation period, eventually encapsulating the majority of the egg contents (Patten, 1920). The expanding CAM interferes with experimental manipulation of later stage embryos due to increased risk of blood vessel damage, and the temporal development of this membrane needs to be assessed prior to experimentation. The development of the CAM and subsequent influence on accessibility to the pharyngeal region of crocodilian embryos is yet to be described.

The aim of the present study was to contribute to the development of *C. niloticus* embryos as a model for applied and basic research. The objectives were: (1) to describe the egg windowing procedure and assess the success thereof for subsequent *in ovo* embryo manipulation experiments; (2) determine the appropriate developmental stage for manipulations (e.g., microinjections) targeting the mandibular arch and maxillary process accounting for CAM expansion.

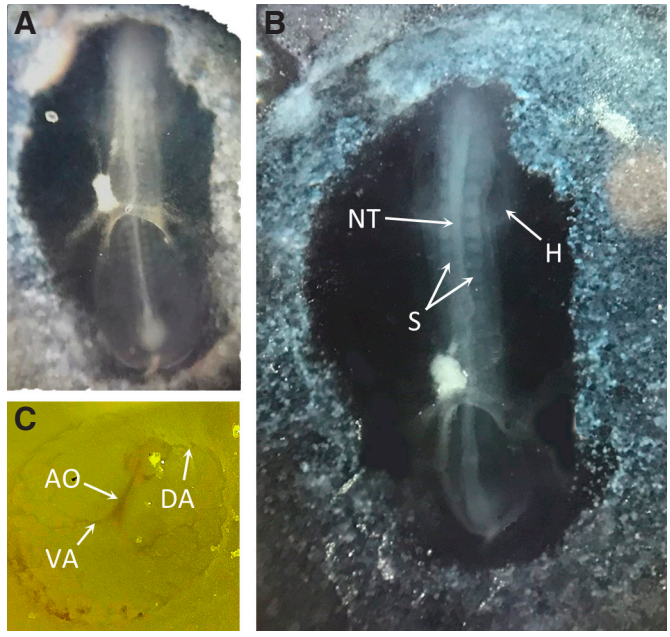


Fig. 1. Early development of *Crocodylus niloticus* embryo. *Developing* C. niloticus *embryo photographed on the first* **(A)** *and second day after laying* **(B-C)**. *India ink was injected beneath the embryos for enhanced visualization* **(A-B)**. *S: Somites; NT: Neural tube; H: Heart; AO: Aorta; VA: Vitellin artery; DA: Dorsal aorta.*

Results

Embryos were predominantly at Ferguson (1985) Stage 1 when windowing took place – the morning of on-farm egg collection, during which the first branchial arches are visible, the heart in the form of an S-shaped tube, and cranial flexure is yet to commence. The primitive streak and blastopore are clearly discernible at the said stage (Fig. 1A). Ferguson (1985) Stage 1 corresponds relatively to chick embryo Hamburger and Hamilton (HH) (1951) Stage 10. Extensive blood vessels and islands were present on the blastoderm at day two after laying, and the heart and dorsal aorta were functional (Fig. 1C).

65.08% of the eggs sourced for the experiment were fertilized (Fig. 2A). Egg windowing without damaging the vitellin membrane was achieved during 76.98% of the attempts (Fig. 2B). Of the eggs

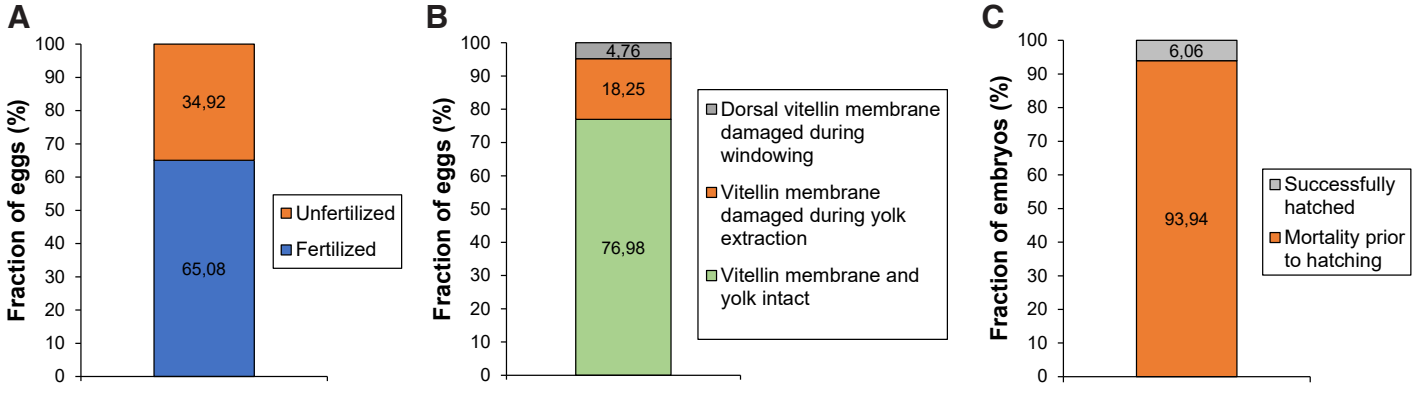


Fig. 2. Fertilization and windowing success. *Indicating the fraction of* Crocodylus niloticus *eggs sourced for the experiment which were fertilized* **(A)**, *as well as the success rates for egg windowing* **(B)** *and full-term development* **(C)**.

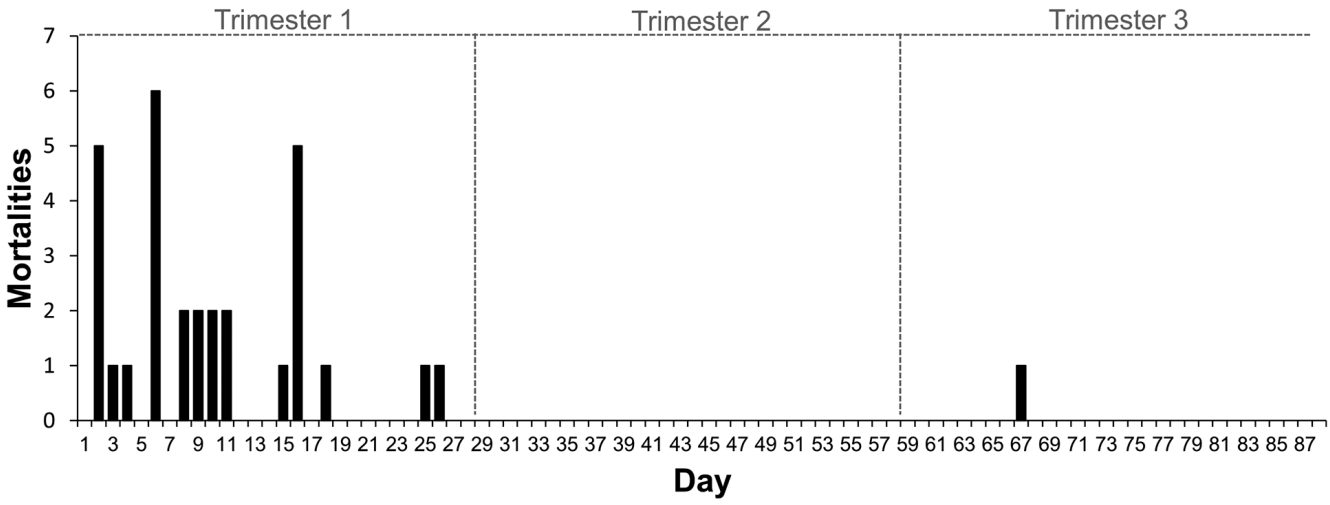
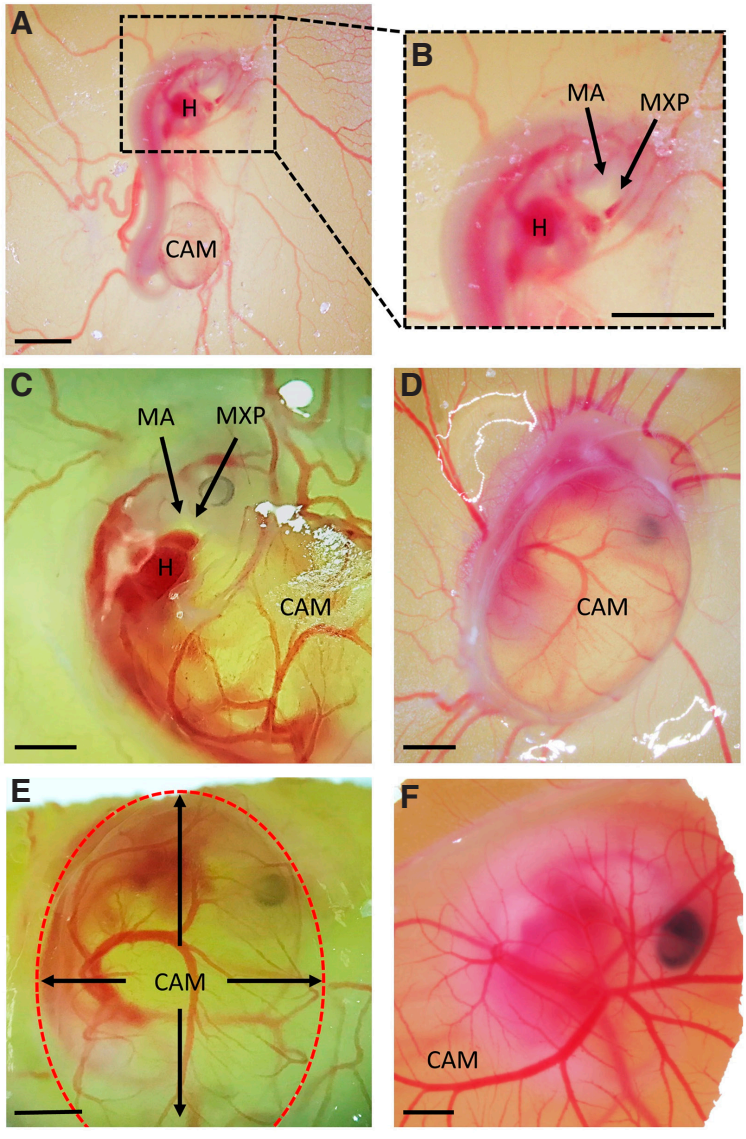


Fig. 3. Embryo mortalities over time. *Temporal distribution of* Crocodylus niloticus *embryo mortalities associated with egg windowing across an 87-day incubation period.*



in which windowing were attempted, the dorsal vitellin membranes of 4.67% were damaged during the incision procedure, whereas the lateral region of the vitellin membranes of 18.25% were damaged during albumin extraction (Fig. 2B). The eggs in which the dorsal membranes were damaged had already developed to the stage where embryos attach to the dorsal shell membrane. None of the embryos with damaged vitellin membranes survived. Two cases were observed where the embryo attached to the Parafilm used to cover the cavity in the egg. Attempts to remove the Parafilm was fatal to the embryos.

Crocodilian embryos are expected to be between Ferguson Stage 18 and 19 at the end of the 1st trimester based on data for *A. mississippiensis, Crocodylus johnsoni*, *C. latirostris* and *C. porosus* (Ferguson 1985; Iungman *et al.,* 2008) and correspond relatively to HH Stage 31 (chick embryo) at the time. Twenty-eight (90.3%) of embryo mortalities occurred during the 1st trimester of incubation, none in the second, with a single mortality on day 67 (Fig. 3). Two (6%) of the embryos survived the full gestation period and hatched successfully (Fig. 2C). The gestation periods were 78 and 87 days respectively. Both hatchlings were assisted to exit the eggs by enlarging the egg window – performed when excessive calling by the embryos was observed. The 78-day-old hatchling showed signs of premature delivery including sluggish movements and a bloated abdominal region due to the size of the yolk body, whereas the 87-day-old hatchling was apparently in good health and the abdomen shape was normal.

The CAM was already prominent on day five after laying, expanding rapidly, and covered the entire embryo on day ten (Fig. 4). The 1st pharyngeal arch (I.e. mandibular arch) is located proximal of the pericardial region within the subcephalic pocket (Fig. 4). The pharyngeal region was exposed until day

Fig. 4. Embryo and chorioallantoic membrane development. Crocodylus niloticus *embryos photographed on day 6* **(A)**, *day 8* **(C)**, *day 9* **(D)**, *day 10* **(E)** *and day 13* **(F)**. *The expansion of the chorioallantoic membrane (CAM) over time is indicated. CAM: chorioallantoic membrane; H: heart; MA: mandibular arch; MXP: maxillary process. Scale bars: 2mm.*

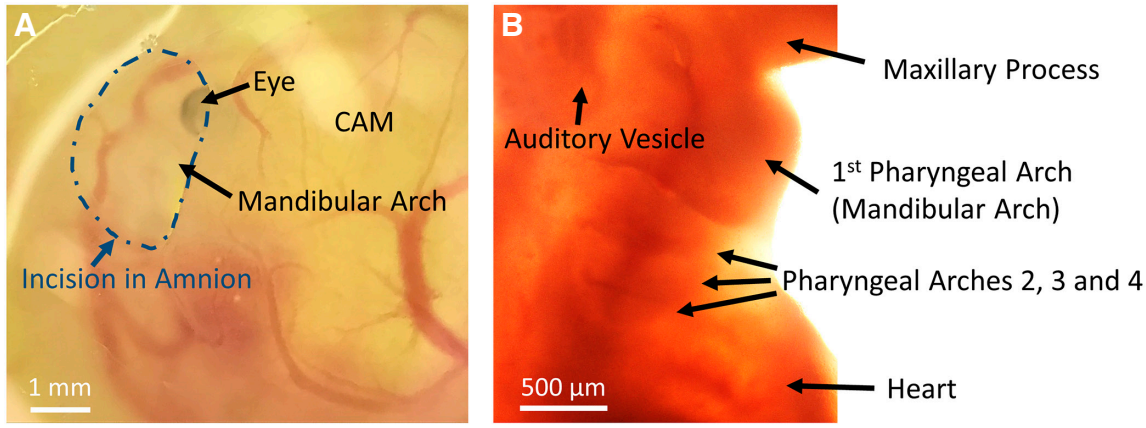


Fig. 5. Amnion incision and pharyngeal arches. **(A)** *Nine-day-old* Crocodylus niloticus *embryo with incision in the amnion enabling access to the pharyngeal region indicated.* **(B)** *The pharyngeal region with the maxillary process and pharyngeal arches indicated. CAM: chorioallantoic membrane; H: heart; MA: mandibular arch; MXP: maxillary process.*

8, after which the CAM expansion partially covered the area (Fig. 4). Incisions, to expose the pharyngeal region, were successfully performed on day 8 after laying (Fig. 5A). The incision was not fatal to the embryo and enabled direct access to the pharyngeal arches and maxillary process for experimental manipulation purposes (Fig. 5B).

Discussion

In this study we show that *in ovo* windowing and full-term gestation is possible for *C. niloticus*, albeit, with a low proportion of survival. The procedure for windowing *C. niloticus* eggs was illustrated, as well as temporal expansion of the CAM and the associated influence on access to the pharyngeal arches and maxillary process.

Captive crocodiles breed successfully in captivity, unlike alligators and caimans (Huchzermeyer, 2003). The eggs utilized in the present experiment were sourced from a commercial crocodile farm. The fertilization success observed corresponded relatively to a previous report for farmed *C. niloticus* (Arukwe *et al.*, 2016). Although candling can be used to determine whether a crocodilian egg is fertilized, banding only initiates after the embryo has attached to the dorsal shell membrane and therefore 24h after laying (Ferguson, 1985; Iungman *et al.*, 2008; Webb *et al.*, 1983). A proportion of unfertilized eggs should be expected in batches sourced from *C. niloticus* farms and needs to be accounted for during the initial planning of experiments.

The developmental stages of *C. niloticus* embryos may vary substantially among clutches collected on a particular day, even though all the eggs were laid in the same 24h period. The variation may be due to the exact time of day a particular clutch was laid, because on-farm egg collection occurs in the early morning hours irrespective of when a female visited the nest. Moreover, the embryos of stressed females may be further developed at the time of laying (Ferguson, 1985). Variation in embryonic stage among members of a clutch at the time of laying remains undescribed, although anecdotal observations of such intra-clutch variation for *C. niloticus* exist (J Nöthling, unpublished data). 4.76% of the eggs that were windowed in the present investigation were too advanced in development and embryos were already attached to the shell membrane. Windowing therefore damaged the vitellin membrane. Rapid windowing after egg collection on a farm is therefore required to reduce failed attempts.

Egg windowing followed by full term development was successfully performed in American alligators (Ferguson, 1981), indicating the potential of *in ovo* manipulation of crocodilian embryos. Ferguson (1981) however did not describe a detailed protocol of the procedure. The windowing methodology described for chicken experiments were generally applicable, but slight adjustments are needed to improve success rates due to difference in egg anatomy between birds and crocodilians. Albumin is extracted from the pole of an egg (using a large needle, e.g. 18G) during the windowing procedure, causing a void to form on the dorsal surface of the egg contents. The consequent cavity allows the circular incision (I.e. window) to be made without damaging the vitellin membrane. Nile crocodile eggs have a low albumin volume in proportion to

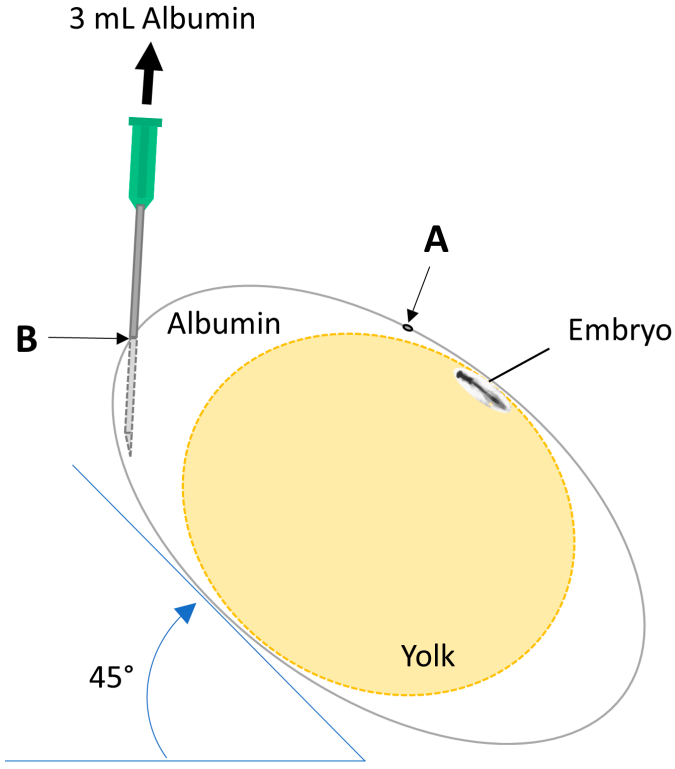


Fig. 6. Albumin extraction and shell penetration. *Illustration indicating the* Crocodylus niloticus *egg during the windowing procedure with the positions of shell rupture for windowing* **(A)** *and albumin extraction* **(B)** *indicated.*

yolk in comparison with chickens. In particular, the ratio of yolk mass to albumin mass in *C. niloticus* eggs approximates 1:1, yolk: albumin (Brown *et al.*, 2019), whereas chicken eggs typically have a yolk: albumin ratio of 1:2 (Ho *et al.*, 2011; Silversides and Budgell, 2004). The large yolk mass within crocodile eggs relative to albumin content increases the challenge of extracting albumin without damaging the vitellin membrane, which may explain the relatively high proportion of eggs damaged in the present study (I.e. 18.25%). Moreover, crocodilian egg albumin is more viscous than that of birds (Ferguson, 1985), and extraction using a needle is therefore more challenging. None of the embryos from eggs in which the vitellin membrane was pierced survived, indicating the importance of successful albumin extraction during egg windowing. The majority of embryo mortalities occurred during the 1stt trimester of incubation, suggesting that this early window of development is the most sensitive (Fig. 3). Survival past the 1stt trimester is therefore a reasonable indicator of successful hatching.

The surface of crocodilian egg shells are irregular, and the typical approach of closing windows with polypropylene tape (Blank *et al.*, 2007; Lu *et al.*, 2017) is not suitable; Parafilm is preferred to prevent detachment. Effective sealing of eggs will reduce the risk of infection and dehydration.

An incision in the amnion was not fatal to embryos and enabled direct access to the pharyngeal region. Such an intervention will enable physical manipulation of the pharyngeal arch or maxillary process, or microinjection for transgenesis experiments. The present data indicate a suitable window for mandibular arch or maxillary process manipulation from day seven to nine after laying, after which such a procedure will be hampered by the CAM, if the eggs are incubated at 31°C. The CAM can be considered a respiratory organ and therefore contains a high number of blood vessels. Damage to the CAM and blood vessels will likely be fatal to the embryo. Spurlin and Lwigale (2013) described a method in chicken embryos to change the anatomy of the CAM allowing access to the embryo at later stages of development. The method entails the dissection of extraembryonic membranes on embryonic day 5. The Spurlin and Lwigale (2013) method is yet to be tested in crocodilians and may allow access to embryos beyond the age shown to be suitable (i.e. 6 to 9 days after laying) in the present study.

Germ-line gene-editing is challenging in oviparous vertebrates because access to the zygote and early stages of development is not possible. Germ-line edits have, however, successfully been performed by targeting migrating primordial germ cells (PGCs) whilst present in the dorsal aorta of stage HH14-16 chicken embryos (Tyack *et al.*, 2013), or *ex vivo* followed by re injection into the bloodstream of surrogate embryos (Woodcock *et al.*, 2017). It is yet to be determined whether crocodilian PGCs migrate in a similar way to avian PGCs. Assuming crocodilian PGCs behave in similar fashion to chicken PGCs, the present data show that the dorsal aorta is clearly discernable and can be targeted on day two after hatching in eggs incubated at 31°C (Fig. 1C).

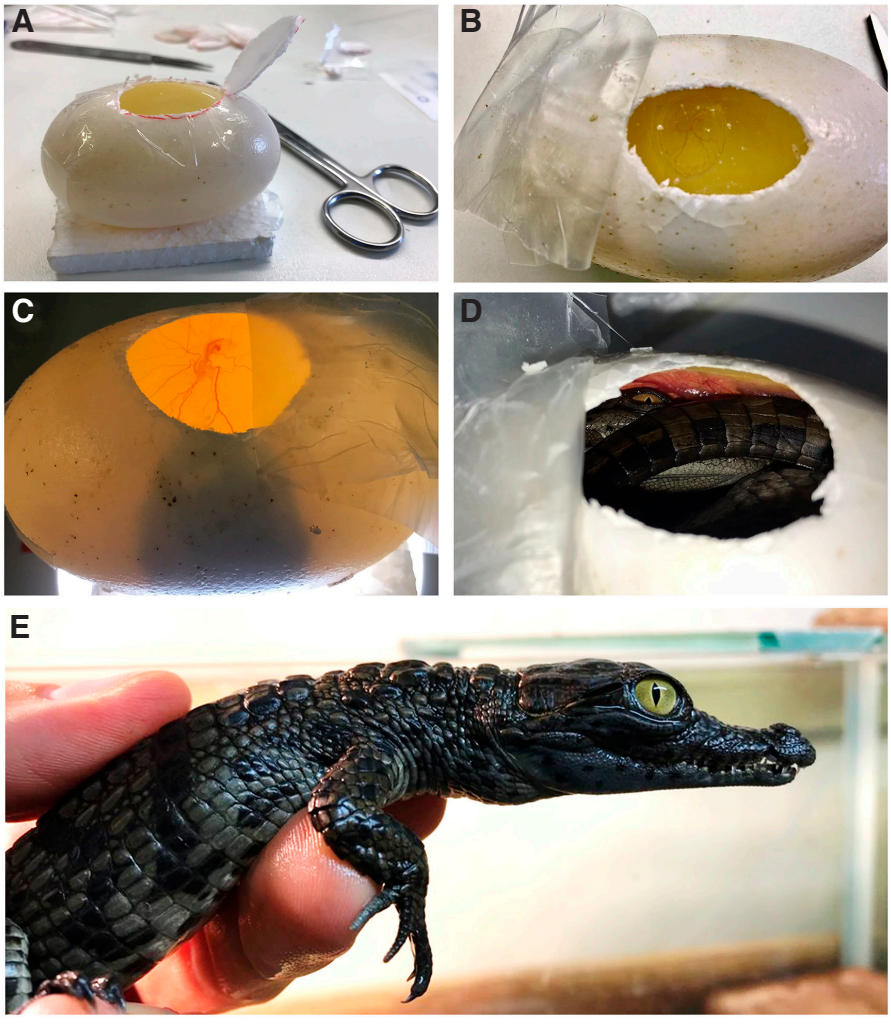


Fig. 7. Windowing procedure and development over time. *Images illustrating the windowing procedure* **(A)**, *consequent development of the embryo* **(B-D)** *and* Crocodylus niloticus *hatchling* **(E)**.

Conclusions

The windowing of crocodilian eggs is apparently more challenging than chicken eggs, even though the eggs are larger in size. The brittle and hard eggshell, larger yolk size, highly viscous albumin, extended (70+ days) incubation period compared to the 21-day chicken incubation, contribute to the difficulty to successfully window crocodilian eggs. Nonetheless, in this study we show that windowing and full-term gestation of *C. niloticus* is possible. The present data indicate that the 1stt trimester is the most sensitive and survival past this window will likely be maintained until hatching. The CAM develops rapidly during the initial seven days after laying and access to the pharyngeal region is hampered from day 9 after laying. The suitable window for experiments involving embryonic manipulations targeting the mandibular arch and maxillary process is between day 6 and 8 after laying if eggs are incubated at 31°C.

Materials and Methods

Egg collection

Fertile eggs were collected from the Inyoni Crocodile Farm, Brits, South Africa. The eggs were carefully removed from nests during the early morning hours by farm personnel (in accordance with the on-farm safety protocol) and transported on the day of collection to the laboratory in polystyrene boxes containing dampened vermiculite.

Egg windowing

Egg windowing was performed on the day of collection in a laminar flow cabinet under aseptic conditions following the protocols of Blank *et al.,* (2007) and Lu *et al.,* (2017) with slight modifications. The surfaces of eggs were decontaminated using 70% ethanol and penetrated on the anterior and ventral surfaces with a scalpel blade at positions A and B indicated on figure 6. Approximately 3 ml of albumin was extracted from the anterior opening (B) with an 18-gauge needle and 10 mL syringe pointed downwards with the egg held at a 45° angle (to prevent damaging the yolk or embryo) (Fig. 6). The dorsal opening allows air to move into the egg (during albumin extraction), forming a cavity on the surface of the yolk mass. The anterior opening was subsequently sealed using Parafilm. The contents of crocodile eggs are enclosed by a rigid leather-like egg membrane beneath a brisk hard eggshell. Starting at the dorsal opening (A), a circular cavity (~2 cm diameter) was made through the eggshell and membrane using dissection scissors (Fig. 7). The viability and developmental stage of the embryo was subsequently assessed under a stereo microscope. The window was then covered with Parafilm and the egg placed in a humidified incubator at 31°C and ~95% humidity.

Amnion incision to expose the pharyngeal region

Embryo development was monitored daily to determine the suitable timing and developmental stage for manipulations targeting the mandibular arch, such as microinjection for plasmid delivery. The development of the embryo and the CAM position and coverage of the embryo was described until day 14, when the embryo was completely covered. An incision was made in the amnion of selected embryo to further assess potential access to the maxillary process and mandibular arch, and to determine whether such incision would be fatal.

Ethical clearance

The research was approved by the University of Pretoria Animal Ethics Committee (Reference number: REC054-20) and subject to Section 20 of the South African Animal Diseases Act, 1984 (Permit number: 12/11/1/8 [1622 LH]).

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