Final report for the IUCN-SSC Crocodile Specialist Group Student Research Assistance Scheme

Why are crocodiles unable to sustain embryonic development in utero?

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Abstract

Captive crocodilians that are withheld from appropriate nesting sites will either lay eggs in the water, or delay oviposition until a site has been provided. This delay will often result in developmental limitations or an increase in embryonic mortality, and would suggest that unlike many other aquatic reptiles, crocodilians lack the capacity to arrest embryonic development prior to oviposition. We have previously shown hypoxia extends developmental arrest in turtles, hence we hypothesise that hypoxia would also extend development arrest if it were present in crocodilians. We examined how extended exposure to hypoxic environments during incubation altered crocodilian development and mortality. We also tested whether increased oxygen availability during incubation altered hatching success. Freshly laid saltwater crocodile (*Crocodylus porosus)* eggs (*N*=83) were incubated in one of five treatments; control (normoxia; 21% O_2), 3-day hypoxia (1% O_2), 6-day hypoxia (1% O_2), 3-day hyperoxia (42% O_2), and 6-day hyperoxia (42% O_2). There was a significant effect of treatment on survival of embryos through to hatching ($p<0.001$). The hypoxic treatments experienced almost no hatching success (6.7% and 0% survival for the 3- and 6- day treatments respectively), while the hyperoxic and control treatments experienced normal to high hatching success rates (86.6%, 100% and 64.2% for the control, 3- and 6- day hyperoxic treatments respectively). Our results provide the first experimental confirmation that, unlike turtles, crocodiles do not exhibit preovipositional arrest. An absence of embryonic arrest is of ecological significance as crocodilians therefore lack an ability to avoid adverse environmental conditions through extended retention of eggs and delayed nesting.

Introduction

No crocodilians or turtles sustain embryonic development past early stages of development in utero and instead are oviparous (egg laying). This indicates that there is a physiological constraint on further development *in utero* (Rafferty *et al.* 2013). Rafferty et al. (2013) provided evidence that the delivery of oxygen to the embryo is a significant constraint limiting the degree of development possible in turtles. It is remains untested as to whether oxygen is similarly significant for crocodilians.

A greater understanding of the processes that limit embryonic development is necessary to develop effective conservation strategies. There is the potential to reveal mechanisms responsible for high rates of reproductive failure due to embryonic death in particular species. Further research into the role of oxygen supply during development may implicate low oxygen levels with embryonic death in reptiles. For example if it can be shown that increasing oxygen supply to eggs in a laboratory incubator prevents embryonic mortality, survival of eggs and hatchling production can be maximised. Increasing the number of hatchlings produced per egg collected is a major objective of any crocodile “sustainable use” operation. However a significant number of eggs collected by wild-harvest operations will

fail to hatch. Furthermore, there is an even higher amount of mortality in eggs collected from captive females as opposed to wild females.

Wink *et al.* (1990) found a correlation between pore density of the shell of alligator eggs and the rate of embryonic mortality. Alligator eggs with lower pore density were less likely to develop in entirety to hatching, suggesting that limited gas exchange is preventing sufficient metabolism. Low oxygen concentrations have been shown to restrict embryonic development in various reptile species. However, there has been no previous investigation of whether increases in the oxygen concentration eggs are exposed to will positively impact developmental success of eggs. Significant reductions in cost and improved outcomes for breeders could be realised through increasing hatching success rates. This would apply to both sustainable use and conservation hatcheries.

In this study we aimed to test how exposure to hypoxic environments extended during incubation altered crocodilian development and mortality. We also aimed to test whether increased oxygen availability during incubation altered hatching success.

Methods

Egg Collection

Captive saltwater crocodiles (*Crocodylus porosus*) at Crocodylus Park (Northern Territory, Australia) were monitored visually from a distance (10-20 m) or via a CCTV camera for any signs of nesting behaviour at least every hour (24 hrs a day) from 7th of January till 21st of February, 2014. Once nesting behaviour was displayed by a female, visual checks from a distance (10-20 m) were undertaken at least every 20 minutes for signs of oviposition. Eggs were collected during or immediately after oviposition from three captive females (See Figures in Supplementary Material). Eggs were quickly candled to determine if they were fertile, with any infertile eggs being discarded.

Effects of Oxygen on Embryonic Development

A series of treatments were devised to test how changes in partial pressure of oxygen (PO_2) effect embryonic development in saltwater crocodiles. Eggs (*N*=83) from each clutch (*N*=3) were randomly distributed between five PO_2 treatments after oviposition (Table 1). One egg from each clutch (*N*=3) was sacrificed and staged at the time of egg collection to determine approximate embryonic age (Webb *et al.* 1983; Ferguson 1985). Preference was given to an egg from each clutch that had suffered mechanical damage from the mother. The remainder of the eggs (*N*=80) were immediately transferred into airtight Perspex containers (Resi-PLex Plastics, North Geelong, Australia). The eggs were placed on a wire mesh allowing them to sit above approximately 10 mL of water at the base of each box.

Table 1 Allocation of eggs amongst experimental treatments and the number opened for staging.

Treatment	Eggs	Eggs opened (day postoviposition)	Oxygen concentration & duration of treatment
Control (normoxia-6)	18*	2 (day 3 and 6)	Normal atmospheric oxygen levels, 6 days
Treatment one (hyperoxia-3)	16	1 (day 3)	~ 42% oxygen, 3 days
Treatment two (hyperoxia-6)	15	1 (day 6)	~ 42% oxygen, 6 days
Treatment three (hypoxia-3)	16	1 (day 3)	~ 1% oxygen, 3 days
Treatment four (hypoxia-6)	15	1 (day 6)	~ 1% oxygen, 6 days

*One additional egg was removed from the control treatment for analyses as it contained two embryos.

The experimental gasses were created using pure nitrogen for the hypoxia treatment and 42% O_2 in nitrogen for the hyperoxia treatment (Air Liquide, Australia). Each respective gas was humidified by bubbling it through a chamber filled with water prior to administering the gas to each container using the inflow and outflow valves. Gas was administered for three minutes at a flow rate of eight litres per minute for each container. Ambient air was circulated through each container in the control treatment for three minutes. The PO_2 of gas leaving the outflow valve of each box was monitored using an oxygen sensor (Analytical Industries, Pomona, CA) and a data collection device (Pasco, Roseville, CA). The containers were then sealed and placed in an incubator. The amount of time between oviposition of the first egg in each clutch and the placement of all eggs in respective treatments was approximately one hour. The temperature and humidity throughout the entire incubation period remained at 32 ± 0.2°C and 100% respectively. The Perspex boxes were re-gassed approximately every 24 hours over the treatment period (3 or 6 days).

Eggs were checked twice daily (morning and afternoon) for presence of an opaque white spot, which is the first visual sign of embryonic development. At the completion of the experimental treatments one egg from each treatment was sacrificed and staged to determine embryonic age. An extra egg was also sacrificed from the control treatment at 3 days post oviposition, so that embryonic age at 3 and 6 days post oviposition could be recorded for all treatments. All remaining eggs (N=73) were then placed into plastic trays. After formation of a white spot was recorded for every egg in the clutch, eggs were monitored every few days. Any eggs that were deemed to have died (identified by the appearance of fungus or discolouration) were removed and staged to assess embryonic age at death. Towards the end of the 80 day incubation period, eggs were separated by a tray divider, and checked twice daily (morning and afternoon) for any newly emerged hatchlings. Hatchlings were housed individually in plastic trays in the incubator until 2 days post hatching, allowing time for absorption of any excess yolk. After this two-day period hatchlings had there fitness and morphometrics measured before being distributed into the raising pens at the farm.

Hatchling Success and Fitness

Hatching success was calculated for each treatment as the proportion of hatched eggs out of the total number of eggs (not including eggs that were used for staging, *N*=6). The total length of each hatchling was measured using a ruler (± 1 mm). Measurements of the snout vent length (SVL), head width, limb lengths, belly width, and yolk scar width, were then recorded for each hatchling using a digital dial caliper (± 0.01 mm). Hatchling mass was measured using a digital scale (± 0.1 g). Hatchlings were allowed to re-acclimate to 32 ± 0.2°C for at least 5 minutes before being subjected to running and swimming tests. The running ability of each hatchling was assessed using a 3 m PVC guttering pipe lined with moist sand (15 cm wide). The swimming ability of each hatchling was assessed using a 5.1 m PVC guttering pipe (15 cm wide) filled with water (10 cm deep). Timing (sec) of a hatchling commenced as soon as they began moving at one end of the pipe and concluded once it reached the other end. Presence of the researcher at the starting point of the track encouraged a unidirectional response by the hatchling towards the opposite end. Each test was repeated three times, with an interval of at least ten minutes between each test. All tests were conducted at an air and water temperature of 32 ± 0.2°C.

Staging Dead Embryos

All dead and sacrificed eggs were preserved for staging by injecting approximately 4 mL of Formalin into the centre of the egg and then placing the whole egg in a specimen jar filled with formalin. Preserved eggs were carefully dissected following the methodology described by Webb et al. (1983) using a compound microscope mounted with a camera (Leica Microsystems Pty Ltd, North Ryde, Australia). Embryos were removed and photographed before again being preserved in formalin. Young embryos (less than 10 days old) were later stained and further examined under a microscope to determine the precise number of somites. The number of somites is positively correlated to the age of the embryo and fits to a regression formula derived by Webb *et al.* (1987). Using this formula (adjusted for growth at 32°C) approximate age of the embryo was calculated.

Data Analysis

Day of white spot formation and hatching, with respect to time of oviposition, was calculated. One egg from the control treatment did not hatch and was found to have twin embryos inside, because of this it was excluded from all analyses.

Results

There was a significant effect of treatment on survival of embryos through to hatching ($p<0.001$). The hypoxic treatments experienced almost no hatching success, while the hyperoxic and control treatments experienced normal to high hatching success rates (Table 1). However all eggs formed white spots regardless of treatment (Table 1). Although the time taken to form a white spot varied between treatment (Figure 1).

The remaining results from this study are still being analysed and will be written up for submission to a peer reviewed journal in the coming 12 months.

Table 1. Summary of the effects of various oxygen levels on incubation of saltwater crocodile eggs (*N*=82*).

	Normoxia (21% O_2) control	Hyperoxia, (42% 0_2) 3-day treatment	Hyperoxia, (42% 0_2) 6-day treatment	Hypoxia, (1% 0_2) 3-day treatment	Hypoxia, (1% 0_2) 6-day treatment
Eggs in treatment (*n*)	17	16	15	16	15
Eggs sacrificed for staging	2	1	1	1	1
No. (%) of remaining eggs forming white spot	15 (100)	15 (100)	14 (100)	15 (100)	14 (100)
Mean days taken for white spot formation (± S.D.)	1.2 ± 0.4	1.3 ± 0.5	1.0 ± 0	3.1 ± 1.7	5.1 ± 3.1
No. (%) of remaining eggs to hatch	13 (86.6)	15 (100)	9 (64.2)	1 (6.7)	0 (0)
Mean days taken to hatch (± S.D.)	81.5 ± 0.9	81.3 ± 0.7	81.8 ± 1.7	82	-
Mean embryonic stage of dead embryos (Early, Mid or Late)	Mid	N/A	Mid	Early	Early

*****Includes three eggs sacrificed at the start of incubation period (One per clutch)

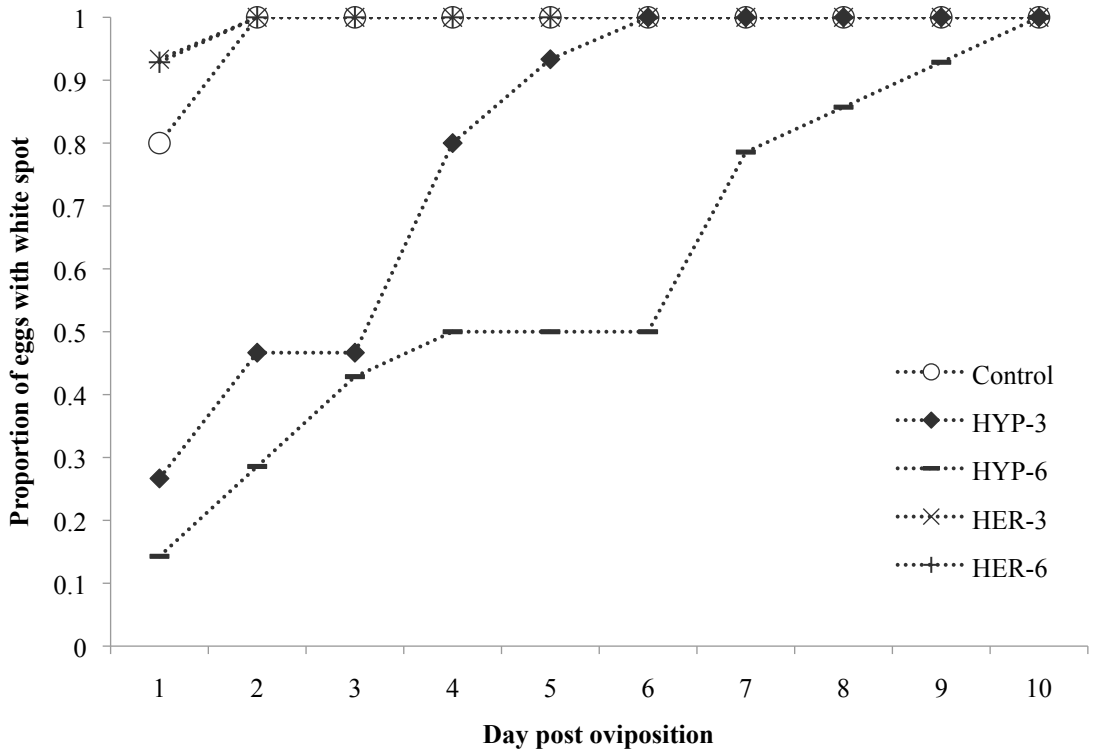


Figure 1. Time taken (days) for formation of white spot on eggs incubated in one of five different oxygen treatments; normoxia (Control; *n* = 15), hypoxia 3-day treatment (HYP-3; *n* = 15), hypoxia 6-day treatment (HYP-6; *n* = 14), hyperoxia 3-day treatment (HER-3; *n* = 15), and hyperoxia 6-day treatment (HER-6; *n* = 14).

Conclusions

Our results thus far provide the first experimental confirmation that, unlike turtles, crocodiles do not exhibit preovipositional arrest. An absence of embryonic arrest is of ecological significance as crocodilians therefore lack an ability to avoid adverse environmental conditions through extended retention of eggs and delayed nesting.

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