# Breeding Focus 2021 - Improving Reproduction

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# Contents

# Preface

“Breeding Focus 2021 – Improving reproduction” is the fourth workshop in the series. The Breeding Focus series was developed to provide an opportunity for exchange between industry and research across a number of agricultural industry sectors. With this goal in mind, workshops have included presentations across multiple agriculturally relevant animal species to take participants outside their area of expertise and encourage them to think outside the box. Reproduction is a main driver for profitability and genetic gain. We will discuss existing knowledge, identify gaps and explore genetic and management strategies to improve reproduction further in multiple species.

Successful reproduction is a complex characteristic comprising the formation of reproductive cells, successful mating and fertilisation, embryonic and fetal growth and eventually a successful birthing event. In livestock species, reproduction traits have mostly low heritabilities, which makes it challenging to improve reproduction as part of a multiple trait breeding objective. The complexity arises not just from the cascade of processes required to result in successful reproduction, but the relevant traits are different in males and females and they are influenced through health and fitness, nutrition, climate and other environmental and management factors.

Challenges to the improvement of reproduction can vary widely for different species. For less domesticated species such as abalone, the ability to produce and reproduce the animals in captivity presents a major challenge. In bees, reproduction has not been given great attention and little research has been undertaken to understand the underlying genetics of drone and queen reproduction. However, in all industries reproduction is recognised as the basis for genetic and economic gain. It directly influences the selection intensity that can be applied. It also determines how many animals are not required for replacement and can be sold. In all industries, irrespective of the challenge, cost-effective and easy to measure phenotypes of reasonable heritability are central. New technologies and approaches enable the development of novel phenotypes for genetic improvement which will be combined with a growing amount of genomic data in livestock species and together these developments provide new and exciting opportunities to improve reproduction further.

We would like to thank everyone who has contributed to this event for their time and effort: the authors for their contributions to the book and presentations, the reviewers who all readily agreed to critique the manuscripts. We would like to express a special thanks to Kathy Dobos for her contributions into the organisation of this workshop and the publication. Thank you!

Susanne Hermesch and Sonja Dominik

Armidale, May 2021

# Saltwater crocodile *(Crocodylus porosus)* embryo survival: risk factors

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## Abstract

Saltwater crocodiles (*Crocodylus porosus*) lay clutches of eggs deposited into nests constructed of various quantities of vegetation, mud, soil and so on. If left in the nest, few embryos survive so producers instead collect the eggs and incubate them artificially to improve the hatch rate. After hatching, the crocodiles are grown to produce skins for the luxury leather trade. Despite artificial incubation, substantial production losses from embryo mortality still occur. Significant production gains could be made from understanding, and avoiding, the risk factors that affect embryonic mortality. Herein, we provide an overview of saltwater crocodile reproduction and current industry practices with a focus on the identified risk factors that contribute to reproductive failure. Further, the consequence of high nest embryonic mortality on post-hatch performance of the surviving embryos has not yet been fully explored. However, it is recommended that these two life stages should not be viewed independently but rather as a continuum to provide some predictability for producers to increase their production efficiency.

## Introduction

The foundation of the Northern Territory's crocodile industry is based on the sustainable, regulated harvest of wild crocodile eggs (Webb, 2020). The resultant hatchlings are raised for the production of skins for the luxury leather trade under the Code of Practice on the Humane Treatment of Wild and Farmed Australian Crocodiles (NRMMC, 2009; Finger *et al.*, 2015; Isberg 2016; Isberg *et al.* 2018). Royalties are paid to landowners, primarily traditional owners, for each egg collected as an economic incentive to protect these apex predators, their habitats and species that live alongside them (Webb, 2020). Along with a well implemented and consistent monitoring program, this "sustainable use" conservation initiative has seen the complete (100%) recovery of the Northern Territory *Crocodylus porosus* population from approximately 3,000 adults in the early 1970's to nowadays around 150,000 (Webb, 2020). The international crocodilian skin trade is tightly regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the Parties to CITES have continually supported the Northern Territory's approach to crocodile conservation (Webb, 2020).

In the wild, the majority of *C. porosus* embryos die due to flooding, unfavourable nest temperatures and predation (Webb *et al.*, 1983b; references within Isberg 2020). In fact, Webb *et al.* (1983b) reported that only 31.4% of embryos survive to hatch in the wild. To counter this, some farms also maintain breeding adults. This allows earlier collection of the eggs post-oviposition and artificial incubation to overcome many of the environmental variables known to detriment embryo survival leading to increased average hatch rates of 72% ± 21% (mean ± SD; Isberg *et al.*, 2005a). Despite the benefits of artificial incubation, significant embryonic losses still occur. The development of interventions to enhance reproductive success, and/or predict the odds of post-hatch survivability based on *in ovo* performance, would provide significant productivity gains for producers. Only a few studies have begun to explore the risk factors influencing reproductive success but to date, pre- and post-hatch performance have been considered as separate life stages. Herein we present an overview of Australian saltwater crocodile reproduction, current industry practices and a summary of known risk factors that influence both pre- and post-hatch survival. Errors are reported ± 1 standard deviation unless other stated.

## Saltwater crocodile reproduction

In northern Australia, *C. porosus* nest primarily during the wet season between late October and April (Richardson *et al.* 2002). A female lays only one clutch of hard, elliptical shaped eggs in a nesting season, although the odd incidence of double clutching in a season has been observed (pers. obs.). The eggs are laid during a single laying event, normally at night or early in the morning, into a previously constructed mound nest constructed mainly of vegetation, mud, dirt, etc (references within Webb *et al.,* 1983b). The average wild clutch size has been reported to be 49.98 ± 11.43 eggs (range 2-78 eggs; Webb and Cooper-Preston, 1989) and was similar in one reported captive population (43.36 ± 9.40 eggs; range 23-68; Isberg *et al.*, 2005a). But not all eggs laid in a clutch produce a viable hatchling.

Comparable levels (7.2%) of infertile eggs have been reported between wild (Webb *et al.*, 1983b) and captive clutches (data from Isberg *et al.,* 2005a). From the captive clutches, there was no correlation between the number of infertile eggs and clutch size ($r = 0.02$; Figure 1) so these fertilisation failures are unlikely to be a function of spermatozoa shortage in larger clutches unless mating frequency or copulation interruption were contributing factors. Crocodilians are known to be promiscuous and multiple paternity is now well established in all crocodilian species, in both wild and communal settings (references within Isberg 2020) but the number of times a dam needs to mate to ensure maximal fertilisation is not yet understood.

At the time of oviposition, fertile eggs contain embryos at the 10-20 somite stage of development, contained within a free-moving vitelline membrane along with sub-embryonic fluid and egg yolk (Webb *et al.*, 1987). The purpose of the free-moving vitelline membrane allows the egg contents to orientate so the embryo is at the uppermost surface irrespective of orientation at egg laying. At this point, crocodile eggs are translucent but the process of embryo attachment has begun. If eggs are to be removed from the nest, ideally they should be collected as soon as possible after lay and marked on the upper surface to indicate its "upright" position (Isberg *et* *al*., 2005a; NRMMC , 2009). Egg rotation of early stage embryos after attachment will result in embryo death (Ferguson 1985) although the likelihood of this risk is reduced in later stage embryos (Webb *et al.* 1987). Fertile eggs can be identified either by "candling" to observe the presence of sub-embryonic fluid or after 24 hours as an opaque spot develops corresponding to the embryo attachment site. As embryo development continues, the egg opacity also develops in a characteristic pattern providing a rudimentary embryo aging mechanism when the nest is not collected on the day of lay to predict hatching date (Webb *et al.,* 1983a). Obviously, it is not economically feasible to collect wild nests every day so the process of marking the upright position and care in transportation is even more essential.

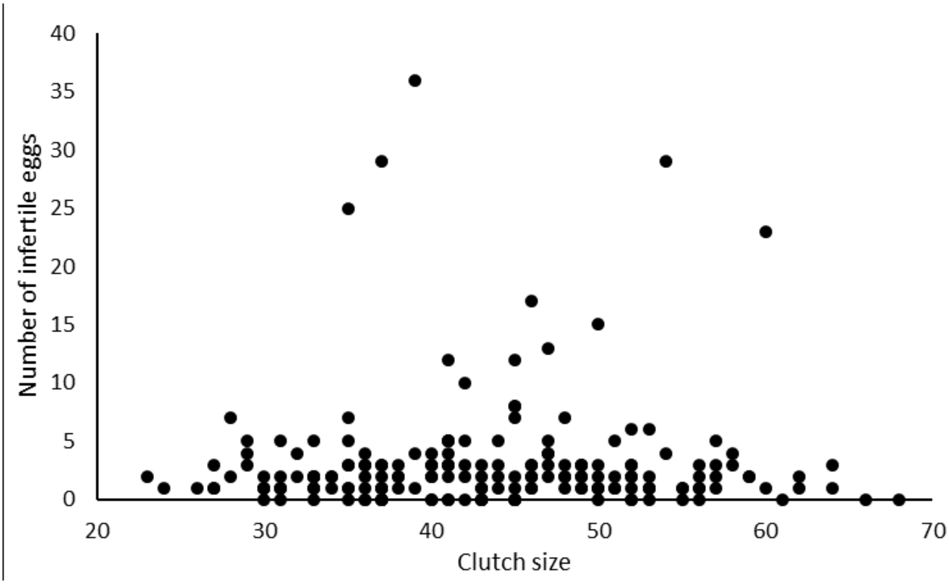

*Figure 1. Scatterplot of number of infertile eggs and clutch size (total of egg in a clutch)*

## *Incubation conditions*

Outside of flooding, the main cause of embryo mortality in wild eggs is unfavourable nest temperatures (Webb *et al.*, 1983b). Incubation temperature in the nest is determined by the type and proportion of the vegetation and it's associated heat of decomposition as well as nest position in relation to sun exposure, incidence of rain and, at later developmental stages, the metabolic heat produced by the embryo itself (Webb and Cooper-Preston, 1989). Temperature gradients of 1-3°C have been noted between the top and bottom eggs within nests (Webb *et al.,* 1977), with a mean temperature of 31.65 ± 1.94°C ($n$ = 246 wild nests; Webb and Cooper-Preston, 1989).

Incubation temperature affects the rate of embryo development (Webb *et al*., 1983a) and has direct implications on embryo mortality, the number of live hatchlings, the proportion exhibiting congenital defects as well as post-hatching growth and survival rates (Webb and Cooper-Preston, 1989 and references within). For example, at 32°C, embryo development is 1.26 times the development rate at 30°C (Richardson *et al.*, 2002) which translates to average incubation times of 80 and 101 days, respectively. Furthermore, crocodilians lack sex chromosomes and instead sex is determined by temperature-dependent sex determination (Lang and Andrews, 1994) At 32°C, Lang and Andrews (1994) determined 86% males were produced compared to either 31°C (16%) or 33°C (17%). There are no production time advantages to producing male crocodiles compared to females (Isberg *et al.*, 2005b), but instead crocodile producers incubate eggs at 32°C as this is the incubation temperature with the highest embryo survival (Richardson *et al.,* 2002).

Maintaining humidity between 95-100% is also a critical environmental parameter, particularly for eggs with cracks, as they otherwise become dehydrated and die. Under natural conditions, rainfall would regulate humidity within the egg chamber of the nest. Females have also been observed urinating on the nest which has been proposed to increase humidity and aid vegetative decomposition (Webb *et al.*, 1983b). In artificial incubators, humidity can be maintained by having areas of free water, misters and, as required, manually spraying water on the eggs.

Other gaseous exchange also needs to be considered. In a nest, oxygen consumption, and carbon dioxide output, gradually increases as the embryos develop, peaking well before hatching (Grigg *et al.*, 2010). Interestingly the amount of decaying vegetation in the nesting material adds to the oxygen demand of the nest as a whole. Under artificial incubation, eggs are incubated in trays without nesting medium thus removing this demand on oxygen. At 30°C, Grigg *et al.* (2010) determined the peak oxygen consumption to be 0.074 ± 0.005 ml $O_2$/gram egg/hour for *C. porosus* eggs. Grigg *et al.* (2010) also reported that embryo oxygen consumption increased with increasing incubation temperature so, by extrapolation, the oxygen consumption at 32°C is approximately 0.1 ml $O_2$/gram egg/hour.

Assuming an average egg weight of 109.2 ± 14.97 grams (Webb and Cooper-Preston, 1989), this equates to approximately 11 ml $O_2$/egg/hour or 545 ml $O_2$/clutch/hour assuming an average of 50 eggs in the clutch (Webb and Cooper-Preston, 1989). On farm, further consideration should be given to the number of clutches, at different embryonic stages, often within a single incubator. Using a rough approximation of the oxygen consumption curve in Grigg *et al.* (2010), and assuming 10 clutches of 50 eggs are laid on the first of each month between October and March all hatching at day 80, Figure 2 shows the oxygen requirements if all eggs are incubated within the same incubator.

Opposite to oxygen consumption, a concern for staff safety is the amount of carbon dioxide produced as a product of metabolism when working in the confinements of an incubator. However, if air turnover in the incubator is sufficient to meet embryo requirements, carbon dioxide levels will also be offset.

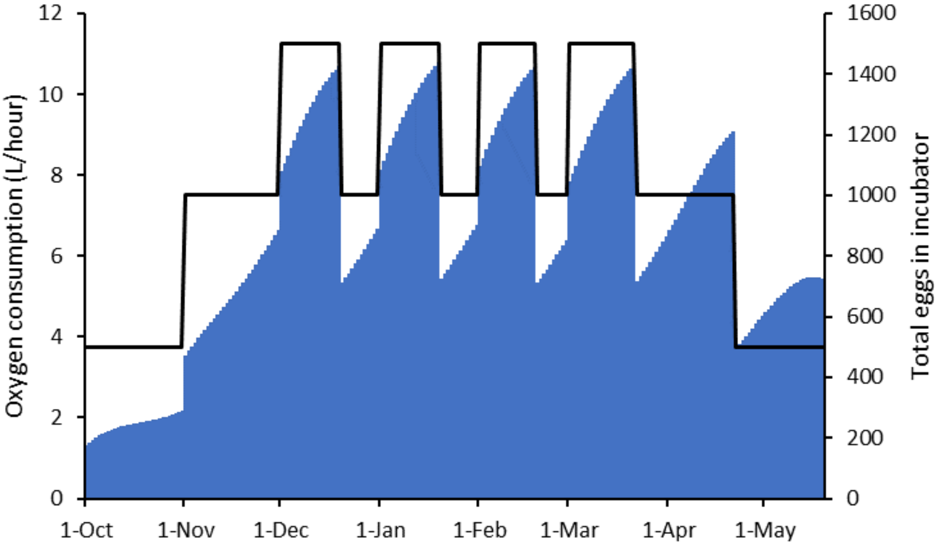

*Figure 2. Simulation of oxygen requirements (under curve area) using an approximation of oxygen consumption from Grigg et al. (2010), and assuming 10 clutches of 50 eggs are laid on the first of each month between October and March, all hatching at day 80 with no embryonic death. The number of eggs in the incubator at any point in time is shown by the line*

## Embryo survival

Despite the comparative environmental-control of artificial incubation, embryo mortality still occurs. After excluding the infertile eggs from the data used in Isberg *et al.* (2005a), the average number of fertilised eggs in each clutch was 40.23 ± 10.56 with a hatch rate of 72% ± 21% or, in other words, an average of 31.25 ± 11.45 hatchlings per clutch. Isberg *et al.* (2005a) reported the number of live hatchlings to be influenced by egg size, parental pen design, collection age as well as genetic variation but there was no variation between the collection years.

### *Egg dimensions as a proxy of female size*

Since the majority of breeding crocodiles on farms are wild sourced, their age cannot be determined but there is a well-established allometric relationship between female size, clutch size and egg dimensions (Webb *et al.,* 1983b; Thorbjarnarson 1996). Egg dimensions, that is egg length and width, can be easily measured with digital callipers and thus used as proxies to adjust for female age. From Isberg *et al.* (2005b), both egg length and egg width were significant predictors of clutch size (number of eggs), viability and number of hatchlings. For each trait, egg length decreased slightly as the number of eggs or hatchlings increased whereas egg width increased with each additional egg or hatchling (Table 1). Functionally, this implies that to increase the number of eggs able to be accommodated within the oviduct, the eggs are slightly shorter to fit more units into the length but, to compensate, they are wider to accommodate the developing embryo.

*Table 1. Regression coefficients (estimate ± SEM) for egg length and egg width from multivariable models (from Isberg et al., 2005a)*

| Trait | Egg length (mm) | Egg width (mm) |
|---|---|---|
| Clutch size (total number of eggs) | -1.41 ± 0.29 | 2.01 ± 0.41 |
| Number of viable eggs | -1.09 ± 0.36 | 2.37 ± 0.52 |
| Number of live hatchlings | -1.03 ± 0.38 | 1.81 ± 0.57 |

## *Breeding pen design*

There were two types of breeding enclosures reported in Isberg *et al.* (2005a) with the crocodiles randomly allocated between them. The “B-pens” (144m$^2$) were designed to accommodate one male and one female. There were two small ponds of water, up to 1m deep, to allow refuge for both crocodiles. By contrast, the “UB-pens” (600-800m$^2$) had one male and either one or two females. The UB-pens were considered to better mimic the natural environment with deeper water (up to 5 m) and adequate basking banks allowing the animals to regulate their own body temperature. Further, the water depth may have allowed better copulation as crocodiles mate in the water with the male entangling himself around the female whereas the shallow water within the B-pen may have hampered this activity.

As a result, the UB-pens cumulatively produced better reproductive statistics including 4.97 more eggs in each clutch of superior viability which resulted in an average of 8.36 additional hatchlings per clutch. As discussed above, the number of infertile eggs and overall clutch size are not correlated ($r = 0.02$; Figure 1) so if fertilisation failure is a function of mating frequency, copulation interruption or inability to copulate aptly, pen design considerations such as water depth could explain this result.

## *Collection age*

The majority of clutches (142 of 190 nests) reported in Isberg *et al.* (2005a) were collected on the day of oviposition (day 0). This was apparent for two reasons. Firstly, the eggs were still covered in the mucous that facilitated their oviductal passage. Secondly, the eggs were still transparent with no spot yet formed on the upper surface where the embryo has begun to attach as described above. For the remaining nests that had collection age recorded, 35 nests were collected on days other than day 0. Figure 3 shows the impact of delayed egg collection after oviposition on the number of live hatchlings using day 0 as a comparison. Clutches collected on day 1, 3 and 10 were not significantly ($p > 0.05$) detrimented compared to those collected on the day of lay. However, clutches collected on day 2 (-8.40 ± 6.60; $p = 0.01$) and day 5 (-42.46

± 6.53; $p = 0.001$) had significantly fewer hatchlings. Whilst these are not survival analysis hazard ratios and the number of nests collected >day 0 were limited, these estimates could begin to approximate critical stages of embryo attachment and development that, if disturbed, increase the chance of embryo death.

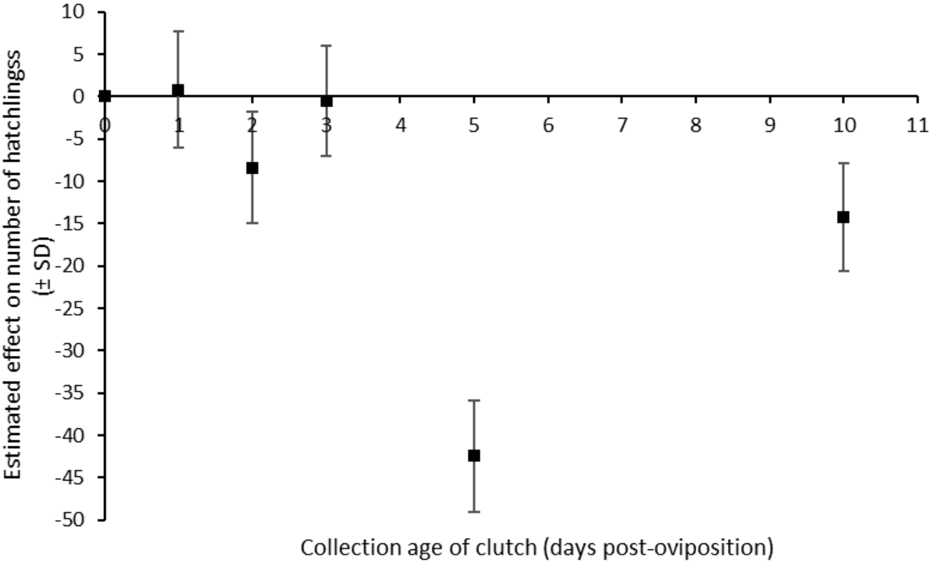

*Figure 3. Estimated effect of the number of days between oviposition and egg collection on the number of hatchlings produced from each clutch (from Isberg et al. 2005a)*

## *Genetic effects*

Since there are no generational reproductive data available for saltwater crocodiles, heritability could not be estimated from the data in Isberg *et al.* (2005a). Instead, repeatability was estimated and sets an upper limit for heritability (Falconer & Mackay 1996). The repeatability for the number of hatchlings was 0.34 ± 0.02 (± SEM; Isberg *et al.,* 2005a) revealing considerable variation to improve reproductive success in future crocodile breeding programs.

Using repeatability as the genetic parameter estimate, crocodile breeding values were estimated for each breeding pair that was evaluated (Figure 4). Referring back to the type of crocodile breeding pen design, 11 of the 13 breeding pairs from the UB-pens (UB prefix on Figure 4 x-axis) were in the top 50% of the breeding herd for reproductive output, ie number of hatchlings. The suffix of "-1" and "-2" indicates either female 1 or 2, respectively. Thus, the UB-pens not only reproductively outperform the B-pens, but also have the additional advantage of being able to estimate individual breeding values for each adult crocodile rather than as a pair. Being able to distinguish if reproductive failures are related to the male or a particular female allows more informative replacement decisions to be made by the crocodilian producer and is highly encouraged for future crocodile breeding program set-ups. However, having too many females can also become problematic as the nests need to be traced to a female, unless parentage determination using genetic tools is employed, allowing accurate breeding values to be estimated (Isberg 2020). As a suggested optimum, three to four females per male would be ideal as long as there is adequate space allowance for each female to display nest fidelity and thus accurate parentage assignment (see Isberg 2020).

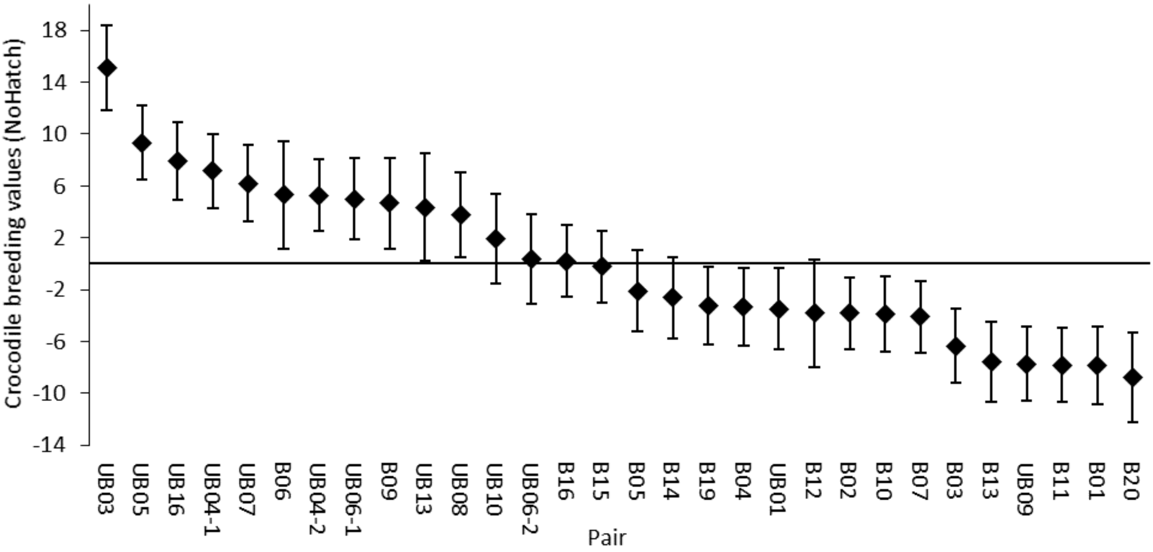

*Figure 4. Estimated crocodile breeding values (CBVs; ± SEM) for the number of hatchlings produced per clutch per year (NoHatch). CBVs were produced from the ASReml multivariate analysis described in Isberg et al. (2005a)*

## *Embryo survival and the relationship with post-hatch performance*

The implication of reproductive success does not end at hatch. Isberg *et al.* (2009) reported that post-hatch survival of hatchlings from wild collected nests were significantly impacted by the number of live hatchlings produced from a clutch. In quantitative terms, for each additional hatchling produced in a clutch, the risk of post-hatch mortality within that clutch decreased by 2.25%. This implies that the embryo mortality experienced by a clutch can be a precursor for continued post-hatch performance. A specific example is the risk of post-hatch mortality from a congenital defect (eg kinked spine, curly tail, large yolk sac, etc) which increased by 5.08% for each hatchling less than the herd average produced. This stands to reason whereby if only a few embryos from a clutch survive to hatch full-term, they have most probably been compromised by the same factors that killed their siblings *in ovo* so their probability of long-term survival has also been compromised.

## *Developing a predictive selection tool for post-hatch performance*

Realising the possibility of a continuum between *in ovo* and post-hatch survival, four years of embryo survival data were collected between 2007 and 2010. Standard hygiene practice in incubators assumes the removal of dead eggs on a regular basis. Dead eggs can be identified by a discoloured appearance or a suspended band development (Richardson *et al*., 2002) relative to the remainder of the clutch. Using the embryo aging system developed by Webb *et al.* (1983a), albeit at 30°C rather than the industry-standard incubation temperature of 32°C, embryo stage can be recorded to allow survival modelling similar to those presented in Isberg *et al*. (2009). Resultant hatchlings were then traced through the production system and their deaths also recorded. Analysis of this combined pre- and post-hatch data is currently underway.

This manuscript outlines a number of factors that influence crocodile mortality, both at the embryonic (pre-hatch) stage as well as post-hatch. The next step is to join these two life stages with the ultimate aim to develop a tool for the crocodile industry to increase reproductive output (embryo survival) through optimised egg collection and incubation strategies as well as set a selection criterion for continued input into those hatchlings with prime chances of survival fitness.

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