

**Effect of variation in incubation temperature and exposure to  
extreme temperature on the hatchability of Nile crocodile  
(*Crocodylus niloticus*) eggs**

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## Declaration of originality

I, Mahlasinyane Mofokeng, do hereby declare that this thesis, which I hereby submit for the degree of Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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## Abstract

In contrast to older experimental systems which typically incubated eggs at constant temperature, recent wide availability of inexpensive programmable microcontrollers has enabled the development of incubators capable of simulating circadian temperature variation. The present study aimed to compare the effect of two sinusoidally fluctuating temperature regimens ( $31\pm 3$  °C and  $31\pm 6$  °C) with a constant temperature regimen (31 °C) on egg hatchability of the Nile crocodile, *Crocodylus niloticus*. Towards the end of the incubation period, an electronic malfunction introduced extreme temperatures (cumulative exposure of eggs to 47 hours of extreme temperatures, with a range of 16.3 to 45.4 °C) which confounded the results. A conditional logistic regression model was used with hatching outcome as the response variable, and incubation regimen and extreme temperature exposure group as the predictor variables. Exposure to extreme temperatures had a severe negative effect on hatchability compared with eggs incubated at a constant temperature of 31 °C ( $P < 0.001$ ). An interesting trend ( $P = 0.12$ ) was that both fluctuating temperature groups had a positive association with hatchability when compared to the constant-temperature group. The hypothesis that fluctuating temperatures may be positively associated with measures of *C. niloticus* embryo and hatchling health warrants further investigation after addressing the technical challenges identified during the current study.

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## List of abbreviations

CITES — convention on the international trade in endangered species of wild fauna and flora

HSP — heat shock protein

HTL — high thermal limit

Kg — kilogram

kPa — kilopascal

LTL — low thermal limit

ODR — optimal developmental range

OTR — optimal temperature range

SD — secure digital card

SVL — snout-vent length

TL — total length

TSD — temperature-dependent sex determination

## Chapter 1. Introduction

An organism may react to changes in its environment by altering its morphology, physiology, or behaviour—such responses are most pronounced during early life, introduce sources of phenotypic variation, and are known as ‘developmental plasticity’ (West-Eberhard, 2003, While et al., 2018). Poikilothermic, oviparous reptiles are a convenient choice for studying the effect of environmental conditions on developmental plasticity (Ackerman and Lott, 2004), since eggs may be incubated under controlled conditions and resulting outcomes carefully measured. In wild reptiles, substantial variability exists in temperature and moisture content between nests (Shine et al., 1997a) despite the moderating influence of maternal nest site selection (Elphick and Shine, 1998), including in those species that demonstrate parental care (Shine et al., 1997b). This may have downstream consequences for the organism’s fitness in the short- and long term (Andrews et al., 2000, Warner and Shine, 2008). Most studies into the effect of incubation temperature on reptile hatchlings have utilised constant-temperature incubation systems (Georges et al., 2005). However, such systems are not representative of natural conditions. Solar radiation varies in a sinusoidal pattern (Bowden et al., 2014, Les et al., 2009) and is not maintained at a constant level as has been modelled in many prior research studies involving reptile eggs, such as those by Hutton (1987) and Maciejewski (2006) on the Nile crocodile, *Crocodylus niloticus*. Eggs of *C. niloticus* buried in shallower nest depths are exposed to a higher mean temperature than eggs in the deeper depths (Hutton, 1987); and more deeply situated nests also have less diurnal temperature fluctuation than shallower nests (Booth, 2006). These differences could conceivably affect phenotype, and indeed it has been found that artificial incubation under diurnally fluctuating temperature conditions may result in complex differences in hatchling phenotype, when compared to exposure to constant temperatures during incubation (Les et al., 2007).

*Crocodylus niloticus* is an African aquatic predator and a commercially important farmed reptile (Carruthers, 2008). Incubation temperature has been shown to affect the rate of embryonic development, hatchling size, hatchability, as well as hatchling sex in all known crocodile species including *C. niloticus* (Hutton, 1987). However, there are no reports describing the effect of fluctuating artificial incubation temperature in these species. Given its economic and ecological importance, a better understanding of factors affecting *C. niloticus* hatchling health would be of benefit to zoologists and crocodile farmers. The recent availability of affordable, programmable microcontrollers has allowed for the easier development of experimental fluctuating-temperature incubators as described by Greenspan et al. (2016). This manuscript describes the use of a purpose-designed, programmable egg incubator in the investigation of the effect of two fluctuating-temperature incubation regimens on hatchability of *C. niloticus* eggs, relative to a constant-temperature incubation regimen. A review of pertinent literature is provided, followed by a description of the conception and design of the study, a discussion and conclusion of the findings. Issues encountered during the execution of the study are described, and strategies to mitigate risk in future studies are proposed.

## 1.1 Research questions and hypotheses

### **Research question:**

Is there a statistically significant difference in hatching rate between groups of eggs from the same clutch incubated under three different incubation regimens: two sinusoidal diurnally varying temperature regimens of  $31\pm 3$  °C and  $31\pm 6$  °C, and, as a control, a constant temperature regimen of 31 °C.

**H<sub>0</sub>:** No statistical difference in hatchability exists between eggs incubated at either or both  $31\pm 3$  °C and  $31\pm 6$  °C, relative to eggs incubated at a constant temperature of 31 °C.

**H<sub>A</sub>:** A statistical difference in hatchability exists between eggs incubated at either or both  $31 \pm 3$  °C and  $31 \pm 6$  °C, relative to eggs incubated at a constant temperature of 31 °C.

## Chapter 2. Literature review

### 2.1 The Nile crocodile in the wild and captivity

Four crocodile species are found in Africa—the Nile crocodile (*Crocodylus niloticus*) (the largest, most abundant species), two species of slender-snouted crocodile (*Mecistops cataphractus* and *M. leptorhynchus*), and the dwarf crocodile (*Osteolaemus tetraspis*) (Revol, 1995). These species are not only important apex predators in wild ecosystems (Pooley, 1982) but, particularly in the case of *C. niloticus*, may be economically important species farmed for their meat and skin (Carruthers, 2008). *Crocodylus niloticus* is an apex predator which may aid in maintaining healthy aquatic ecosystems by preying on predatory fish such as barbel, which eat the small fry of other species (Bourquin, 2008). These animals may also assist in keeping waterways clean by scavenging on rotting carcasses (Guggisberg, 1972, Pooley, 1982). Furthermore, *C. niloticus* may make water holes and furrows in swampland (Martin, 2007), creating habitats for crustaceans and arthropods (Mazzotti et al., 2009). In addition to preying on aquatic species, *C. niloticus* are ambush predators of large terrestrial mammals (including livestock and humans), which may result in conflict at the human-animal interface (Pooley, 2016). Since crocodiles may attack people and livestock outside protected areas, communities may protect their lives and livelihoods against this perceived threat by destroying crocodile nests and eggs wherever they encounter them (McGregor, 2005). During the first half of the 20<sup>th</sup> century, crocodiles were considered vermin, and landowners were granted essentially unlimited hunting permissions (Cott and Pooley, 1972). From 1950 to 1970, many crocodile populations sharply declined due to heavy hunting as well as habitat destruction (Revol, 1995, Tosun, 2013). As the ecological and economic role of *C. niloticus* was gradually recognized, the significance of such population declines was noted, leading in 1975 to the listing of these

organisms as CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix I species (Hoffman et al., 2000, Tosun, 2013).

In South Africa, populations continue to decline due to industrialization and habitat destruction (Ashton, 2010). For example, construction of the wall of the Loskop Dam in Mpumalanga substantially reduced the dam's water quality (Botha et al., 2011), and destruction of nesting sites in the Ndumo Game Reserve in Kwazulu-Natal led to a decline in reproduction rate (Calverley and Downs, 2014). Further, many wild *C. niloticus* populations in Southern Africa are subject to poaching and uncontrolled harvesting (Calverley and Downs, 2014).

Since crocodile-derived products command economic value, crocodiles are raised commercially. An important distinction exists between crocodile farming, where captive females lay eggs on-farm which are collected and incubated, and crocodile ranching, where eggs laid in wild nests are collected and incubated on-farm (Khosa et al., 2012). In South Africa, crocodile farming started in the 1960s (Dzoma et al., 2008, Hoffman et al., 2000). In Zimbabwe, crocodile ranching started around a similar period but involved the collection of eggs or hatchlings from wild populations, and the rearing of the hatchlings in captivity until slaughter or release in the wild (Khosa et al., 2012). Meat and skins are the principal products of crocodile farming or ranching operations, but other body parts such as feet, scales and bones are also sold (Revol, 1995). *Crocodylus niloticus* are typically slaughtered at 1.2 to 1.5 m long, or at two to three years of age (Hutton and Webb, 1990). Belly skins of *C. niloticus* rank second in value to those of the estuarine crocodile, *Crocodylus porosus* insofar as desirability for leather goods is concerned (Revol, 1995, Fergusson, 2010, Tosun, 2013). In 2019, the number of *C. niloticus* skins exported from South Africa was 68 820, an 80% increase from the previous year (Caldwell, 2021). Crocodile meat is considered healthy for human consumption due to its unsaturated lipid fatty acid profile and low sodium content. In South Africa, the meat is exported, sold to local restaurants, or used raw on-farm for feeding mature crocodiles

(Hoffman et al., 2000). During the years 2017 to 2019, *C. niloticus* and the Siamese freshwater crocodile, *Crocodylus siamensis*, were the two major species sold as crocodile meat in the Far East (Caldwell, 2021). In addition to human consumption of crocodile-derived products, crocodiles may serve a role as tourist attractions in zoos and national parks (Tosun, 2013).

Given these clear economic and conservation-associated motives, a better understanding of *C. niloticus* nesting physiology and hatchling development may contribute to more effective captive hatchling production, easing the burden on wild populations. Additionally, an improved understanding of factors affecting wild hatchling health under natural nesting conditions may be of importance to conservationists and zoologists interested in the study of wild populations. The present chapter aims to provide a summary of previously researched factors shown to affect embryo health *in ovo* and post-hatchling health, culminating in an emphasis on environmental temperature as a central regulating influence on egg hatchability.

## 2.2 Factors influencing embryo development and hatchling health

### 2.2.1 Initial egg mass

Among oviparous species in general, eggs may vary in mass between (Les et al., 2009) as well as within species (Andrewartha et al., 2010, Brown et al., 2019, Webb and Cooper-Preston, 1989). Furthermore, variation in the mass of eggs may occur both within as well as between clutches of the same individual (Maciejewski, 2006, Piña et al., 2007, Webb and Cooper-Preston, 1989).

Findings have differed regarding the influence of egg mass on duration of incubation period. In the skink species, *Oligosoma suteri*, Hare et al. (2002) did not find a significant impact of the initial egg mass on the incubation period, whereas Andrewartha et al. (2010) and Les et al. (2007) found that the incubation period increased with a corresponding increase in the initial

egg mass of the prickly gecko, *Heteronotia binoei* and the red eared slider turtle, *Trachemys scripta*, respectively. Initial egg mass has been shown in certain species to affect embryo survival—extremely heavy and light eggs of the rock partridge, *Alectoris graeca* were associated with poorer hatching success than those of average mass (Kirikci et al., 2004). However, Nygård et al. (2019) reported that the initial egg size of *S. typhle* did not affect embryo survival and in the snapping turtle, *Chelydra serpentina*, initial egg size did not influence survival of the hatchling (Bobynd and Brooks, 1994).

Regarding the influence of initial egg mass on offspring size, the egg mass at laying of *C. porosus* explained 81.3% variation in the hatchling mass, 38.6% variation in the head length, and 40.3% variation in the snout-vent length or SVL (Webb and Cooper-Preston, 1989). Similarly, prior research in other species reported an increase in the hatchling mass with an increase in the initial egg mass of the turtle species *Apalone mutica*, *Chrysemys picta*, as well as *T. scripta* (Ashmore and Janzen (2003), Janzen and Morjan (2002), Les et al. (2007)). The total length (TL) and snout-vent length SVL of *H. binoei* was shown to increase with an increase in the initial egg mass (Andrewartha et al., 2010). Similarly, larger eggs of emu (*Dromaius novaehollandiae*) produced larger and heavier hatchlings (Dzialowski and Sotherland 2004), and a strong, linear, positive relationship was reported between the estimated volume of *C. niloticus* eggs and the mass of their resulting hatchlings (Brown et al., 2019). Assuming that a larger hatchling is a fitter, stronger hatchling with higher survival rate, this data suggests that across species, a larger, heavier egg may be the first step to improving the likelihood of offspring survival.

### 2.2.2 Gas and moisture concentration

As embryos consume oxygen, they release carbon dioxide into the nest (Booth and Dunstan, 2018). When eggs of the loggerhead (*Caretta caretta*) and green sea turtles (*Chelonia mydas*) were incubated at 27, 30, 33, and 34 °C temperatures with gas mixtures of 21% O<sub>2</sub> 0% CO<sub>2</sub>

(control), 17% O<sub>2</sub> 4% CO<sub>2</sub>, 14% O<sub>2</sub> 7% CO<sub>2</sub>, and 10% O<sub>2</sub> 11% CO<sub>2</sub> applied for 5.5 days; the incubation period was prolonged from incubations at high CO<sub>2</sub> levels (10–15%) than from incubations at low CO<sub>2</sub> levels (0–5%) (Booth et al., 2020). By contrast, Williamson et al. (2017) found that the incubation period of *C. porosus* embryos was not significantly affected by oxygen concentration at 32 °C.

Olive ridley sea turtle (*Lepidochelys olivacea*) eggs from nests with higher bacterial loads had lower hatching success, suspected to be due to a reduction in oxygen concentration associated with higher microbial activity (Bézy et al., 2015). The hatching success rates of *C. porosus* eggs at 1% O<sub>2</sub> for 3 and 6 days of incubation were 6.7 and 0%, respectively; at 42% O<sub>2</sub> were 100% (at 3-days incubation) and 64.2% (at 6-days incubation), and at 21% O<sub>2</sub> (control) was 86.6% (Williamson et al., 2017). Contrastingly, the incubation temperature and O<sub>2</sub>-CO<sub>2</sub> gas mixtures did not have a significant impact on the early stage embryo mortality in both *C. caretta* and *C. mydas* embryos (Booth et al., 2020). These findings suggest that sea turtles have evolved a mechanism of withstanding prolonged periods of embryonic hypoxia.

Lower oxygen concentration limits embryonic metabolism and may result in reduced hatchling mass (Crossley II et al., 2017, Liang et al., 2015). At a 30 °C incubation temperature, heavier American alligator (*Alligator mississippiensis*) embryos were produced when incubated at a 21% O<sub>2</sub> concentration than at a 10% O<sub>2</sub> concentration (Crossley II et al., 2017). There was no significant relationship between oxygen concentration (21% control, 1% and 42%) and TL ( $P = 0.29$ ), SVL ( $P = 0.21$ ), head width ( $P = 0.37$ ), and hatchling mass ( $P = 0.22$ ) of *C. porosus* at 32 °C incubation temperature (Williamson et al., 2017). Booth et al. (2020) reported the production of smaller hatchlings of *C. caretta* and *C. mydas* with larger residual yolks from incubations at high CO<sub>2</sub> levels (10–15%) than from low CO<sub>2</sub> levels (0–5%) at 27, 30, 33, and 34 °C. This two contrasting findings suggest that hatchling size is likely influenced by multiple

factors and that simple manipulation of one variable may not necessarily have a direct linear positive or negative effect on the outcome.

The rigidity, thickness, and lower permeability to water of crocodile eggshells and shell membranes make embryo survival and hatching less dependent on environmental water than other reptilian species, such as snakes and lizards (Packard et al., 1982). However, excess moisture, as may be encountered during total immersion, has been conclusively shown to result in complete clutch failures (Kofron, 1989, Pooley, 1969). At 75 and 100% air moisture content and air temperature of  $27.8 \pm 3.7$  °C, the incubation period of *C. caretta* eggs was longer than in the 0, 25, and 50% moisture content groups (McGehee, 1990). Similarly, the incubation period of the common snapping turtle (*C. picta*) incubated at a range of 20 to 30.5 °C was longest in wet (-150 kPa), longer in intermediate (-825 kPa), and shortest in dry (-1500 kPa) substrates (Packard and DeMarco, 1991). This finding was confirmed by Miller (1993), who incubated eggs of *C. serpentina* at 29 °C and reported that the eggs from a dry substrate (-850 kPa) hatched about 4 days earlier than those from a wet substrate (-150 kPa).

McGehee (1990) reported that the hatching success of *C. caretta* eggs incubated at  $27.8 \pm 3.7$  °C was highest at 25% moisture content than other evaluated moisture contents (0, 50, 75, and 100%). The hatching success rates of *C. picta* eggs incubated at 20 to 30.5 °C were ~90% at -150 kPa, ~50% at -825 kPa, and ~25% at -1500 kPa (Packard and DeMarco, 1991).

Miller (1993) reported the production of heavier and longer *Chelydra serpentina* hatchlings from an incubation temperature of 29 °C in a wet substrate (-150 kPa) than from a dry substrate (-850 kPa). Low nest moisture content (e.g., -1500 kPa) was associated with increased blood viscosity in *Chrysemys picta* embryos, which was believed to play a role in the reduced size at hatching (Packard and DeMarco, 1991). These findings demonstrate that there is likely a species-specific optimal environmental moisture range, and that incubation duration is affected by suboptimal hydric conditions. Perhaps shortening of incubation length, if accompanied by

faster maturation of the foetus, may occur as an adaptation to allow greater survival under dry conditions: a mobile hatchling can seek water, whereas an immobile foetus within an egg cannot.

### 2.2.3 Temperature

During the first half of embryo development, squamate (snake and lizard) embryos are confined in the maternal oviducts, allowing the dam to regulate temperature by behaviour, whereas non-squamate (such as turtle or crocodilian) embryos are contained within eggs buried in soil and exposed to outside thermal variation (Shine, 1985). Reptile nests under natural conditions may be subject to varying diurnal amplitudes in temperature as well as thermal spikes (Booth, 2006, Hall and Warner, 2018, Hutton, 1987). Booth (2006) reported that reptile eggs buried deeper in the earth experienced more constant and lower temperatures, whereas eggs at shallower depths experienced more fluctuating and higher temperatures. Female *C. niloticus* in the Flag Boshielo Dam, Mpumalanga, laid eggs in holes in the ground at an average depth of 27 cm (Botha, 2006); and those in the Okavango Delta, Botswana, were laid at an average depth of 24.5 cm (Maciejewski, 2006). At such shallow depths, *C. niloticus* eggs were shown to experience markedly fluctuating temperatures (Hutton, 1987). Shade may also influence nest temperature: shaded nesting sites of *C. niloticus* eggs had a soil temperature of 5 to 6 °C cooler than those in sun-exposed nesting sites (Leslie and Spotila, 2001). Fully shaded nesting sites of the hawksbill turtle, *Eretmochelys imbricata*, with nest temperature 28.9 °C, had a lower hatching success than those with full sun exposure and a nest temperature of 29.6 °C (Gane et al. 2020).

Extreme temperatures may be detrimental to embryo development—in crocodiles, the first half of incubation has been shown to be particularly sensitive to such effects (Webb et al., 1987). In wild nests, temperature spikes may substantially exceed mean nest temperature, resulting in embryonic stress (Sanger et al., 2018, Pörtner et al., 2017). Such heat stress may induce

morphological (Sanger et al., 2018) and physiological (Hall and Warner, 2019) abnormalities of embryo development. Sanger et al. (2018) reported that brown anole, *Anolis sagrei* embryos incubated at an extreme temperature of 36 °C were smaller than those incubated at more typical temperatures of 27, 30, and 33 °C. The day after thermal spikes of 40 to 45 °C on fertile eggs of *A. sagrei* and the Puerto Rican crested anole, *Anolis cristatellus*, 97% of the embryos had no heart rate when measured using a non-invasive egg monitoring system (Buddy® Digital Egg Monitor, Avitronics, United Kingdom) (Hall and Warner, 2019).

Some embryos have been reported to attempt to moderate the effect of thermal extremes through behavioral (Zhao et al., 2013) or biochemical thermoregulation (Bentley et al., 2017). Du et al. (2011) reported that embryos of the Chinese softshell turtle, *Pelodiscus sinensis* were to some extent able to move within the egg, and in their research, the embryos followed a source of heat in both a vertical and a lateral direction. Similarly, eggs of the Chinese three-keeled pond turtle, *Chinemys reevesii* were subjected to different patterns of heating on their sides and ends using heating mats. Embryos heated at 26 °C from the sides remained near the midpoint of eggs, while those heated from the sides at 29 and 30 °C moved towards the heat source and those heated at 33 °C moved away from the heat source (Zhao et al., 2013). Eggs of the Chinese alligator, *Alligator sinensis* were incubated at 28, 30, or 38 °C for about 25% of the total incubation period, then randomly assigned to groups which were incubated at 30 °C (control) and 33.5 °C (treatment) using heating mats. Embryos incubated at 30 °C stayed near the center of the egg, whereas the embryos incubated at 33.5 °C reportedly moved towards the heat source (Li et al., 2014). In contrast to the findings of the above researchers, other researchers found no evidence of behavioral thermoregulation. Eggs of *C. serpentina* exposed to large (8.6 °C) and small (1 °C) thermal gradients, showed no statistically significant difference in embryo orientation, thereby providing no evidence for embryonic behavioural thermoregulation in this species (Telemeco et al., 2016). In support of these findings, Cordero

et al. (2018) found that the internal egg space of *P. sinensis* quickly declined as the embryo grew and that the embryo's muscles at the time had not yet developed for coordinated movements such as behavioural thermoregulation. Should the latter researchers' findings be true and reptile embryos cannot behaviourally thermoregulate, then they would thus have to endure extreme nest temperatures during embryogenesis or else employ some evolved form of biochemical defence against thermal extremes.

Maintenance of a high resting body temperature ( $\sim 32$  °C) by females of the montane lizard, *Bassiana duperreyi* was shown to result in embryos adapting to warm temperatures both pre- and post-oviposition through phenotypic plasticity (Telemeco et al., 2010). An increased mean temperature ( $28 \pm 3$  °C) as well as heat wave treatments ( $26 \pm 3$  °C for 15 days,  $31 \pm 3$  °C for 10 days, and then  $28 \pm 3$  °C until hatching) were shown to shorten the incubation period in two sympatric lizards, *Phrynocephalus przewalskii* (that occupies open microhabitats) and *Eremias argus* (that prefers closed microhabitats), relative to control treatment ( $26 \pm 3$  °C). Furthermore, hatching success of *E. argus* was reduced both by increased mean temperature as well as exposure to heat waves, while that of *P. przewalskii* was not (Ma et al., 2018). Battles and Kolbe (2019) reported that the reduction of vegetation and an increase in heat-retaining surfaces (such as tar and concrete in an urban environment) resulted in an increase in mean temperature. It follows then that mean nest temperatures of oviparous reptiles found in urban areas are also higher than those in non-urban habitats (Hall and Warner, 2018). Incubation of eggs of *A. sagrei* and *A. cristatellus* from forest habitats (temperature variance of 26 to 27 °C) and urban habitats (temperature variance of 26.5 to 33.9 °C), resulted in shorter incubation periods in both species when found in urban habitats (Tiatragul et al., 2017).

In *T. scripta*, a turtle species with temperature-dependent sex determination (TSD), a slight increase in temperature in the middle third of embryogenesis produced more females than males (Carter et al., 2018). The incubation period of *A. sagrei* eggs was prolonged by an hourly

exposure to a heat spike of 39 °C on the day of oviposition: this delay in development was hypothesised to be due to cellular damage caused by the heat shock, forcing the embryos to undergo cellular repair prior to resuming normal development (Sanger et al., 2018).

Exposure of embryos to short-term, extreme temperature fluctuations resulted in acute heat stress, which further resulted in cardiac arrest or oxygen limitation (Angilletta et al., 2013, Hall and Sun, 2021, Smith et al., 2015), and embryo survival declines once a species-specific upper thermal limit is reached (Massey and Hutchings, 2021): for example, in the olive ridley turtle, *L. olivacea*, hatching success was higher from mean nest temperatures below 34 °C than from those above 34 °C (Maulany et al., 2012). Although knowledge of crocodile thermal limits is limited, some prior work has indicated a reduction of greater than 50% in *A. mississippiensis* hatching success when incubation is performed at temperatures above 34 °C (Ferguson and Joanen, 1982, Lang and Andrews, 1994). Low temperatures may also have profound effects on hatchability: incubation at temperatures below 28 °C resulted in poor hatching success (Ferguson and Joanen, 1982).

Biochemical mechanisms employed by embryonic reptiles in adjusting to heat stress are a current topic of much research. Mitochondrial respiration and metabolic enzyme activity of the turtle *P. sinensis*, was shown to increase at 32 °C relative to 24 and 28 °C incubations, indicating that the embryo was making use of excess metabolic energy to accelerate its biochemical processes (Sun et al., 2015). *Caretta caretta* embryos exposed to a 3-hour period of incubation at 36 °C, had greater expression of heat shock protein (Hsp) genes than those not exposed to the thermal stress; and genes responsible for cell formation, development, and biogenesis were less abundant in the treatment group than in the control group (Bentley et al., 2017). These findings suggest that fundamental biochemical changes occur in heat-stressed embryos which have evolved to ameliorate the effects of exposure to high temperatures during incubation.

Given that temperature has been shown to increase the rate of biochemical reactions and metabolic rate, Les et al. (2009) investigated the relationship between temperature and embryonic developmental rate of *C. picta* and *T. scripta*, identifying upper and lower thermal limits as well as a temperature range optimal for development (Figure 1). Eggs of both species were incubated at constant (23 °C and 31 °C) and fluctuating (23±3 °C and 31±3 °C) temperatures. These researchers demonstrated that the incubation period was shortest and hatching success was best at 23±3 °C.

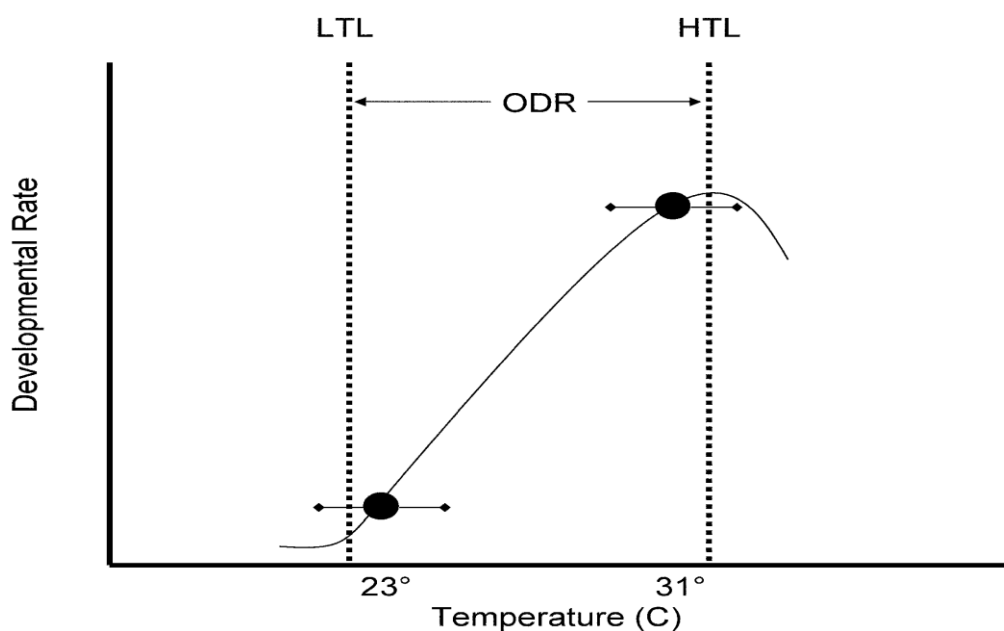


Figure 1: Schematic from Les et al. (2009) representing the relationship between embryonic developmental rate and incubation temperature.

ODR = optimal developmental range, LTL = low thermal limit, HTL = high thermal limit

Raynal et al. (2022) in a recent broad reptile meta-analysis, reported that in general, fluctuating incubation temperatures result in shorter incubation periods than constant temperatures. In support of this, it was found previously that when eggs of the smooth softshell turtle, *A. mutica* were incubated at a mean constant temperature of 30.5 °C and diel temperature fluctuations of ±2 and ±4 °C around the same mean temperature; shorter and longer incubation periods were produced from the ±2 and ±4 °C temperature fluctuations, respectively, than from the constant

temperature (Ashmore and Janzen, 2003). Other researchers found that *C. picta* and *T. scripta* produced a longer incubation period at a fluctuating temperature of  $28.5 \pm 3$  °C than at a constant temperature of 28.5 °C (Les et al., 2009). Furthermore, Andrewartha et al. (2010) reported a shorter incubation period from the incubation of *H. binoei* eggs at 32 °C, than at temperature fluctuations of  $\pm 3$  and  $\pm 5$  °C ( $P < 0.001$ ).

Previous studies performed on reptile nests incubated under natural conditions have reported a significant impact of temperature on embryo survival (Ferguson and Joanen, 1982, Hutton, 1987, Webb and Cooper-Preston, 1989), whereas others did not find any significant impact (Maciejewski, 2006, Piña et al., 2003, Piña et al., 2007). Maciejewski (2006) reported that variation in incubation temperature (from 30 to 34 °C) only explained a 2% variation in the hatching success of *C. niloticus* eggs in the Okavango Delta, Botswana. Extreme incubation temperatures have been reported to reduce the hatching success of *C. niloticus* and *A. mississippiensis* embryos (Ferguson and Joanen, 1982, Hutton, 1987, Lang and Andrews, 1994). A summary of prior research on incubation temperature on embryo survival is provided in Table 1.

Table 1: The impact of constant temperature incubations on embryo survival of *Alligator mississippiensis* and *Crocodylus niloticus*

| Species                           | Temperature | Hatching Rate | References                            |
|-----------------------------------|-------------|---------------|---------------------------------------|
| <i>Alligator mississippiensis</i> | 26 °C       | 20%           | (Ferguson and Joanen, 1982)           |
| <i>Alligator mississippiensis</i> | 36 °C       | 14%           | (Louisiana swamps, USA)               |
| <i>Crocodylus niloticus</i>       | 28 °C       | 79%           | (Hutton, 1987) (Lake Ngezi, Zimbabwe) |
| <i>Crocodylus niloticus</i>       | 31 °C       | 83%           |                                       |
| <i>Crocodylus niloticus</i>       | 34 °C       | 69%           |                                       |
| <i>Alligator mississippiensis</i> | 28 °C       | <10%          | (Lang and Andrews, 1994)              |
| <i>Alligator mississippiensis</i> | 34 °C       | 87%           | (Rockefeller Wildlife Refuge, USA)    |
| <i>Alligator mississippiensis</i> | 34.5 °C     | 29%           |                                       |
| <i>Alligator mississippiensis</i> | 35 °C       | 11%           |                                       |

At an incubation temperature of 34 °C, nine out of eleven *C. porosus* embryos died, while temperatures between 29 to 30 °C, and 31 to 32 °C produced the highest hatching success rates (Webb and Cooper-Preston, 1989). The incubation temperature (28, 30, 32, and 34 °C) of *A. mississippiensis* eggs did not have a significant impact on the hatching success (Ferguson and Joanen, 1982). Similarly, the embryo survival of the mugger crocodile, *Crocodylus palustris* was not significantly affected by incubation temperatures between 28 and 33 °C (Lang et al., 1989). At 29, 31, and 33 °C, the survival of the broad-snouted caiman, *Caiman latirostris* embryos were also not significantly affected by temperature (Piña et al., 2003). These findings indicate the need for further investigation on the impact of temperature on the hatching rate of crocodiles, since knowledge of their thermal limits is unknown.

A reptile meta-analysis by Raynal et al. (2022) reported that at cool and warm mean temperatures, fluctuating temperatures result in lower and higher embryonic mortalities than constant temperatures, respectively. This was evident when considering that the lower mean temperature ( $23\pm 3$  °C) fluctuation increased the hatching success of both *C. picta* and *T. scripta* than the higher mean temperature ( $31\pm 3$  °C) fluctuation (Les et al., 2009).

Temperature fluctuations in the wild are not constant, but can also increase or decline in their amplitudes (Hutton, 1987). Extreme increases in the amplitude of temperature fluctuations can be more lethal to developing embryos than moderate amplitudes: it has been found that the hatching success of *H. binoei* was lowest at  $32\pm 9$  °C than at  $32\pm 3$  and  $32\pm 5$  °C (Andrewartha et al., 2010).

## 2.3 Summary

In general, as temperature increases, the incubation period decreases (Andrewartha et al., 2010, Les et al., 2009, Maciejewski, 2006, Webb and Cooper-Preston, 1989). As initial egg mass increases, the hatchling size increases (Andrewartha et al., 2010, Ashmore and Janzen, 2003, Janzen and Morjan, 2002, Les et al., 2007, Webb and Cooper-Preston, 1989). Extreme temperatures decline the hatchability of most reptiles in embryogenesis (Andrewartha et al., 2010, Hall and Warner, 2018, Hutton, 1987). Most studies indicate that there is a significant variation in the hatchability of reptiles between constant and fluctuating temperature incubation regimens (Andrewartha et al., 2010, Ashmore and Janzen, 2003, Les et al., 2007, Les et al., 2009). However, such knowledge is lacking in crocodiles.

Embryos can tolerate thermal extremes through either behavioural or biochemical thermoregulation (Bentley et al., 2017, Zhao et al., 2013), although the evidence for embryonic behavioural thermoregulation is debated (Telemeco et al., 2016). Within limits, embryos may use excess energy available at high incubation temperatures to speed up their biochemical

processes (Sun et al., 2015), or express heat shock protein genes (Bentley et al., 2017) which may allow them to temporarily withstand extremes of temperature, albeit at the possible expense of embryo or hatchling health. Research studies that compare the effect of constant and fluctuating incubation temperature regimes on hatchability and measures of hatchling health have not been performed in *C. niloticus*, a species of economic and ecological importance on the African continent. Improving *C. niloticus* hatchling yield and hatchling health would have conservation and economic benefits. Knowledge of the effect of various incubation regimens on hatchability would add to the existing body of literature in reptiles. Therefore, as a pilot project, an evaluation of egg hatchability under different incubation regimens was designed to test an artificial incubation system and identify areas where project design could be improved.

## Chapter 3. Materials and methods

### 3.1 Ethical approval

This project was approved by the Animal Ethics Committee of the University of Pretoria (REC090-21).

### 3.2 Study area

This study was conducted at the Inyoni Estate, a commercial crocodile farm in North West province, South Africa (25.7401° S, 27.8592° E). On this farm, eggs are laid by adult females, collected by farm staff and then incubated, buried in vermiculite, at constant temperature in a large walk-in incubator at 31 °C. Hatchlings are raised to slaughter size, slaughtered on-farm and the meat and skins are processed for export or domestic trade. Nesting alcoves are provided for gravid females, and a sandy substrate is provided to allow for nest-digging (Figure 2). Nesting areas are exposed to sunlight. There is shade at times and some nests are in the proximity of trees. Shaded areas are provided for the adult crocodiles.



Figure 2: Aerial image of crocodile enclosure at Inyoni Estate, North West Province.

### 3.3 Study material

*Crocodylus niloticus* eggs (n=306, nine eggs from each of 34 clutches, mean mass 108.1 g) were collected and placed in experimental incubators between the 2<sup>nd</sup> and the 21<sup>st</sup> of October 2021. Farm staff were solely responsible for egg collection from the nesting sites within 24h of laying. After a week's incubation on-farm (31 °C), eggs with an opaque band across their lesser diameter were defined as fertile and were selected for inclusion in the study. Selected eggs were cleaned using dry paper towels and weighed using a Mettler PM1200 digital scale. Eggs were identified with a permanent marker to indicate both the clutch and egg numbers (Figure 3). Once marked, eggs were placed within plastic netting to ensure that hatchlings could be captured at hatching and traced to their egg of origin (Figure 4).



Figure 3: *Crocodylus niloticus* eggs marked with a permanent marker.



Figure 4: Hatching *Crocodylus niloticus* hatchling trapped inside a net.

A large, air-conditioned plywood box with an internal volume of 1.5 m<sup>3</sup>, mounted on a vehicle trailer, was used as the incubator unit (Figure 5). The air-conditioner was intended to maintain the internal temperature of the box at around 22 °C throughout summer. A solar backup power system was present as a contingency to power outages.



Figure 5: Incubator unit loaded on a trailer for transportation and fitted with an air-conditioner.

Twelve polystyrene boxes (incubators), each of 0.04 m<sup>3</sup> internal volume were placed inside the larger plywood box (Figure 6). Each incubator was numbered and heated individually with a 220v reptile-heating pad controlled by an Arduino microcontroller with a solid-state relay (Figure 7 and Figure 8). The temperature in the large plywood box, each incubator, and the outside environment was continuously measured using MCP9080 temperature sensors (Adafruit Industries, New York City, New York, USA). The data were then logged automatically on a secure digital (SD) card connected through a breakout board to the Arduino Mega microcontroller (Arduino, Monza, Italy), with a data string logged approximately every three minutes. Data were logged in comma-separated variable (.csv) format to allow for compatibility with Microsoft Excel.

Heating pads were covered with moist vermiculite to maintain a relative humidity of around 100%. Once a week, water was poured onto the vermiculite to replace moisture lost through evaporation. Inside each incubator, a wire rack was used to suspend the eggs above the moist vermiculite (Figure 7).



Figure 6: Incubator boxes (from left to right columns:  $31\pm 3$  °C,  $31\pm 6$  °C, and 31 °C). Incubator box at bottom centre was removed at the time the picture was taken.



Figure 7: An opened incubator box containing a heating pad, overlaid with moist vermiculite. A temperature sensor is attached to the wire rack.



Figure 8: An Arduino microcontroller and associated recording and relay hardware enclosed in a Tupperware to prevent any undesirable contact, but air circulation is provided by the fitted air-conditioner in Fig. 5 above.

### 3.4 Incubation regimens

Three eggs from each of the 34 clutches were randomly selected and assigned to an incubation group: a constant (control) group at 31 °C (Figure 9), a sinusoidally fluctuating group at  $31 \pm 3$  °C, and a sinusoidally fluctuating group at  $31 \pm 6$  °C (Figure 10).

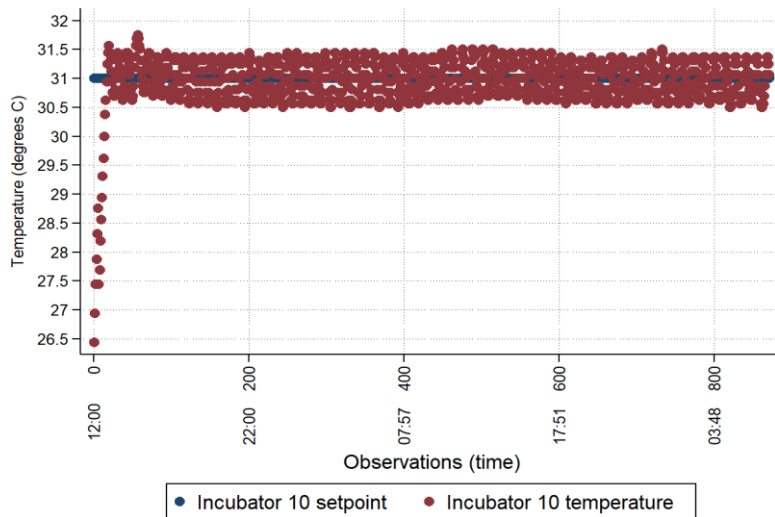


Figure 9: Plot of temperature set point and actual temperature against time (constant temperature incubator) over a 42 hour period.

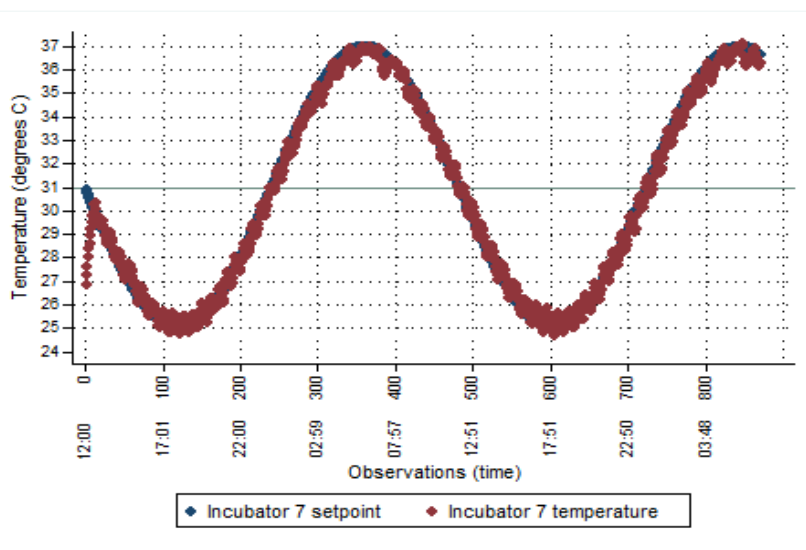


Figure 10: Plot of incubator set point and actual temperature against time ( $31 \pm 6$  °C fluctuating temperature treatment group) over a 42 hour period.

### 3.5 Incubator failure

Incubators functioned as intended from the 2<sup>nd</sup> of October 2021, until a system error occurred on the 9<sup>th</sup> of December 2021. The cause of these issues remains unknown but is suspected to be due to repeated episodes of mains electricity failure. As described, temperature regulation

was by computer-controlled relays that switched heating pads on or off to maintain a specific temperature set point. During each period of system failure, some incubator heating pads were suspended in the ‘off’ state, others in the ‘on’ state. As a result, some incubators experienced prolonged periods of undesirably low or high temperatures. The first system failure occurred on the 9<sup>th</sup> of December 2021 at 18:51:37. This issue was detected and fixed only on the 11<sup>th</sup> of December 2021 at 09:06:12, resulting in 38 hours of heating (five incubators) or chilling (seven incubators). The second system failure occurred on the 3<sup>rd</sup> of January 2022 at 00:59:30 and was restored on the 4<sup>th</sup> of January 2022 at 10:00, resulting in nine hours of heating (nine incubators) or chilling (three incubators). These two undesired system failures radically altered the course of the study and are summarised in Table 2. Temperatures shown in Table 2 were electronically recorded immediately after restarting the computer control system, and are roughly indicative of the temperature extremes to which the eggs were exposed: as low as 16.3 °C and as high as 45.4 °C. Each pattern of extreme temperatures (Low-Low, Low-High and High-High) was assigned a code (1, 2 or 3) (Table 2).

Table 2: Summary of maximum or minimum extreme temperatures encountered due to incubator control system failure

| Incubator | Clutches | Failure 1 (° C) | Failure 2 (° C) | Failure pattern <sup>1</sup> | Code |
|-----------|----------|-----------------|-----------------|------------------------------|------|
| 1         | 1–8      | 39.3            | 40.0            | High-High                    | 3    |
| 2         | 9–17     | 19.8            | 42.4            | Low-High                     | 1    |
| 3         | 18–26    | 40.4            | 42.1            | High-High                    | 3    |
| 4         | 27–34    | 40.6            | 44.9            | High-High                    | 3    |
| 5         | 1–8      | 18.9            | 37.4            | Low-High                     | 1    |
| 6         | 9–17     | 18.5            | 40.6            | Low-High                     | 1    |
| 7         | 18–26    | 38.4            | 45.4            | High-High                    | 3    |
| 8         | 27–34    | 38.9            | 40.9            | High-High                    | 3    |
| 9         | 1–8      | 17.7            | 16.4            | Low-Low                      | 2    |
| 10        | 9–17     | 16.3            | 17.2            | Low-Low                      | 2    |
| 11        | 18–26    | 16.6            | 42.5            | Low-High                     | 1    |
| 12        | 27–34    | 17.2            | 19.9            | Low-Low                      | 2    |

<sup>1</sup> No High-Low failure pattern occurred

Despite these system failures, the fluctuating temperature treatments maintained their fluctuating pattern, whereas the constant temperature treatment maintained its constant pattern. Therefore, the system failures only brought a change in the magnitude (i.e., Low-Low, Low-High, and High-High) of the temperatures.

### 3.6 Statistical analysis

Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used for data collation and organization, and Stata 14 (College Station, Texas, USA) was used for statistical analysis.

As described previously, 9 eggs from each of 34 clutches were distributed across three artificial incubation regimens within the experimental incubator. After removing 9 eggs from each of these 34 clutches, all banded eggs remaining in each clutch were incubated in an on-farm walk-in incubator at a constant temperature of 31 °C as per normal farm protocol.

Experimental treatment groups (variable name: 'tx') were assigned code 0 for constant 31 °C (on-farm and experimental), code 1 for 31±3 °C, and code 2 for 31±6 °C.

The pattern of accidental extreme-temperature exposure was recorded with the variable name 'extremegrp' and assigned code 0 for the on-farm, constant-temperature incubator (i.e., no extreme temperature exposure), code 1 for a Low-High pattern of temperature exposure, code 2 for a Low-Low pattern of temperature exposure, and code 3 for a High-High pattern of exposure.

Summary statistics were generated that displayed the number of eggs that hatched per exposure group (Low-Low, Low-High, and High-High), as well as the hatching percentage, using the Stata command:

```
collapse (count) egg (sum) hatched, by(extremegrp)
```

Since the experimental incubation treatments (31±0 °C, 31±3 °C, and 31±6 °C) were confounded by exposure to the extreme temperature events (Low-Low, Low-High, and High-High), it was necessary to create new variables for each combination of experimental

incubation treatment and extreme temperature exposure. Six such exposure categories were created (refer to Table 3).

Table 3: Summary of experimental and extreme temperature exposure code groups

| Code | Treatment            |
|------|----------------------|
| 1    | (31±3 °C, High-High) |
| 2    | (31±3 °C, Low-High)  |
| 3    | (31±6 °C, Low-High)  |
| 4    | (31±6 °C, High-High) |
| 5    | (31±0 °C, Low-Low)   |
| 6    | (31±0 °C, Low-High)  |

As was done for the exposure to the extreme temperature groups, summary statistics were generated, displaying the number of eggs that hatched per exposure group (Low-Low, Low-High, and High-High), as well as the hatching percentage, using the Stata command:

```
collapse (count) egg (sum) hatched, by(txgroup)
```

Conditional (fixed-effects) logistic regression was used to assess the effect of coded indicator variables, incubation regimen ('tx') and extreme temperature exposure ('extremegrp') on the discrete outcome variable describing hatching status ('hatched'), where eggs were assigned a 'hatched' value of 1 if they hatched, and a value 0 if they did not. The following STATA command was used:

```
clogit hatched i.tx i.extremegrp, group(clutch)
```

## Chapter 4. Results

When comparing hatching percentages grouped by clutch, it was clear that the on-farm, constant-temperature incubator had superior hatching results to the experimental incubator unit (Figure 11; Table 4).



Figure 11: Per-clutch comparison of hatching rate between an experimental incubator and on-farm incubator. Clutches 12 and 28 had no eggs hatch in the on-farm incubator — all banded eggs from those clutches were removed for incubation in the experimental incubator. Compared to eggs incubated on-farm, the hatching rate was far poorer in all experimentally incubated eggs subjected to the accidental extreme incubation treatments (Table 4).

Table 4: Comparison of hatching rate among extreme temperature exposure groups

| Extreme exposure group             | Eggs exposed | Eggs hatched | Hatching percentage |
|------------------------------------|--------------|--------------|---------------------|
| 0 (on-farm incubator) <sup>1</sup> | 816          | 721          | 88.4                |
| 1 (Low-High)                       | 105          | 33           | 31.4                |
| 2 (Low-Low)                        | 75           | 19           | 25.3                |
| 3 (High-High)                      | 126          | 2            | 1.6                 |

<sup>1</sup>Not exposed to extreme temperature

When the hatching rate per incubation regimen was combined with the confounding exposure to extreme temperature, it was clear that in some exposure groups, very few eggs hatched (Table 5).

Table 5: Hatching rate of experimental combined with extreme exposure groups

| Code | Experimental/extreme exposure group | Eggs exposed | Eggs hatched | Hatching percentage |
|------|-------------------------------------|--------------|--------------|---------------------|
| 1    | (31±3 °C, High-High)                | 75           | 1            | 1.3                 |
| 2    | (31±3 °C, Low-High)                 | 27           | 10           | 37.0                |
| 3    | (31±6 °C, Low-High)                 | 51           | 18           | 35.3                |
| 4    | (31±6 °C, High-High)                | 51           | 1            | 2.0                 |
| 5    | (31±0 °C, Low-Low)                  | 75           | 19           | 25.3                |
| 6    | (31±0 °C, Low-High)                 | 27           | 5            | 18.5                |

Overall, the conditional logistic regression model had a pseudo  $R^2$  value of 0.50, a likelihood-ratio chi-square statistic of 562.84 (5 degrees of freedom) with a p-value less than 0.001. All

three of the extreme temperature exposure patterns had a statistically significant ( $P < 0.001$ ) severe negative effect on hatchability. Fluctuating incubation temperature groups had a statistically non-significant, positive effect on egg hatching when compared to constant temperature incubation (Table 6).

Table 6: Summary of logistic regression statistics predicting hatching outcome

| Predictor     | Coefficient (95% CI)   | Odds Ratio (95% CI) | P-value |
|---------------|------------------------|---------------------|---------|
| 31±3 °C group | 1.42 (-0.35 – 3.20)    | 4.15 (0.71 – 24.5)  | 0.12    |
| 31±6 °C group | 1.30 (-0.34 – 2.95)    | 3.68 (0.71 – 19.03) | 0.12    |
| Low-High      | -4.47 (-5.96 – -2.98)  | 0.01 (0.03 – 0.05)  | <0.001  |
| Low-Low       | -3.69 (-4.39 – -2.99)  | 0.02 (0.01 – 0.05)  | <0.001  |
| High-High     | -8.41 (-10.92 – -5.90) | <0.01 (0.00–0.003)  | <0.001  |

In general, eggs exposed to a Low-High and a Low-Low pattern of extreme temperatures had better hatchability than those exposed to a High-High pattern.

## Chapter 5. Discussion

The superior hatching results shown by the on-farm incubator relative to the experimental incubator (Figure 11), are likely the result of extreme temperature exposure in the experimentally incubated groups due to control system malfunction. This was supported by the logistic regression analysis (Table 6). Most evidently, exposure to the Low-Low and High-High patterns of extreme temperature resulted in a dramatic reduction in hatchability, although the Low-High pattern also resulted in diminished hatchability (Table 4).

The first extreme temperature exposure, on the 9<sup>th</sup> of December, was 38 hours in duration, and the second extreme temperature exposure on the 3<sup>rd</sup> of January 2022, was nine hours in duration. Unfortunately, temperatures were not continuously logged during the extreme exposure periods. Had this been done, the cumulative ‘dose’ of exposure to extreme conditions could have been estimated by measuring the approximate area under the temperature curve less than or greater than 31 °C, which would have allowed more meaningful comparison with existing literature in other reptile species. Notwithstanding this missing data, these findings suggest that the duration of the extreme temperature exposure was the major cause of embryo fatalities, and that temporary exposure of reptile embryos to extreme low temperature is less harmful to the developing embryo than temporary exposure to extreme high temperatures. This is in accordance with the finding of Sanger et al. (2018), who found that a short period of exposure of the lizard, *A. sagrei* embryos to an extreme temperature of 39 °C resulted in slightly diminished survival, but that prolonged exposure resulted in a severe reduction in egg hatchability. Hutton (1987) found that the incubation of *C. niloticus* eggs at 34 °C resulted in a lower hatching rate than those incubated at lower temperatures. However, the incubation in Hutton’s study was performed at a constant temperature throughout the incubation period, and

the temperature exposure was not as extreme as that described by Sanger et al. (2018), or notably, the accidental exposure described in the current research study.

Due to the confounding effect of extreme temperature exposures, it was not possible to meaningfully answer the research question or test the hypotheses defined in 1.1 of this manuscript. The three treatment groups were fragmented into smaller sub-groups by the accidental extreme temperature exposure (Table 5), which led to a reduction in group sample size to the point of statistical insignificance. Despite these issues, in the logistic regression model a non-significant ( $P=0.12$ ) trend was noted, whereby eggs incubated at fluctuating temperatures appear to have a positive association with hatchability. It is not possible to comment authoritatively further on this beyond stating that this observed trend lends circumstantial, weak support to the rejection of the null hypothesis stated in 1.1, and that the study should be repeated to either confirm or repudiate this finding.

The incubator functioned as designed for approximately two months prior to malfunction. This suggests that intrinsic hardware or software issues were unlikely to be the cause of the malfunction, but that changes in the external environment, such as the formation of condensation on sensitive electronics, or instability in the mains power supply, were the cause of control system malfunction. When the problem was identified and the system restarted, the incubator again functioned normally for almost a month before the issue recurred. Since it functioned normally between episodes of malfunction, and a relatively long period of extreme temperature exposure appears necessary to cause foetal demise, in future studies the issue could be avoided by regularly manually checking that the system is working. This was not possible when the incubator was located on the crocodile farm, due to its relatively remote location. An improvement on the study design may be to house the incubator at a more accessible location with better Internet connectivity, where it can be directly or remotely monitored.

An interesting finding was that although most foetuses were killed by exposure to high temperatures, there were two clutches containing four eggs that were exposed to the most lethal temperature exposure group (High-High), but which nonetheless survived to hatching. Incubator 2, a  $\pm 3$  °C incubator, contained one of these eggs, while incubator 7, a  $\pm 6$  °C incubator, contained three. Although impossible to prove using the current dataset due to small per-group sample size and low overall survival, it could be the case that a pattern of exposure to a fluctuating incubation temperature regimen induces biochemical changes resulting in an ability to withstand sudden extreme temperatures, as identified by Bentley et al. (2017) in *C. caretta*. Of these four hatchlings, one had a bloated abdomen indicating failure of metabolism after yolk uptake, and one had a severely kinked spine. Prior studies (Sanger et al., 2018) have reported morphological abnormalities in reptiles after thermal stress, with the severity of malformation dependent on timing, duration and severity of thermal stress. Our crocodile foetuses were exposed to thermal stress at approximately two-thirds of the way through their expected incubation period: indeed the many dead and decaying foetuses recovered from nonviable eggs were comparable to those described as around embryonic development day 52 (Peterka et al., 2010). In the crocodilian embryo, this roughly corresponds with Stage 23-25 of the description by Ferguson (1985) for *A. mississippiensis*. During this stage, external pigmentation of the embryo is becoming well-established, a large volume of yolk exists outside the body which serves as a source of nutrition for the developing embryo, and integumentary scales, external genitalia and hooked nails are forming. In addition to these obvious external changes, it seems likely that many externally unseen transformations are occurring that are susceptible to disruptive influences such as thermal stress, which in the current investigation manifested as relatively non-specific musculoskeletal deformation and a failure of ventral midline fusion with full uptake and metabolism of yolk.

The research project described in this manuscript sought to describe the effect of fluctuating incubation temperature regimens on the hatchability of the eggs of a commercially important reptile, with the aim of expanding the literature on thermal biology. Due to unforeseen technical complications, it was not possible to conclusively address the initial research question, and the scope of the study was expanded to a description of the effect of unexpected extreme temperature exposure on crocodile embryos. It was found that *C. niloticus* embryos can withstand short periods of high temperatures, but prolonged exposure is typically fatal, a finding in accordance with that of prior researchers. A repeat of the study with greater oversight during incubation may provide a less ambiguous dataset which will allow the original research question to be addressed. As far as future studies are concerned, if experimental incubation can be successfully performed and yield viable hatchlings, a logical next step is to assess the effect of various incubation regimens on easily measurable external hatchling parameters, such as snout-vent length, total length and hatchling mass. If differences are identified among treatment groups, further in-depth investigation into the underlying pathophysiology may be warranted.

## Chapter 6. References

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