EMBRYONIC DEVELOPMENT OF THE PIG-NOSED TURTLE, *CARETTOCHELYS INSCULPTA*, UNDER CONSTANT AND FLUCTUATING TEMPERATURE REGIMES.

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A thesis submitted in partial fulfilment of the requirements for the degree of honours in Applied Science.

June 1998

Declaration

This thesis is my original work and has not been submitted, in whole or in part, for a degree at this or any other university. Nor does it contain, to the best of my knowledge and belief, any material published or written by another person, except as acknowledged in the text.

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ACKNOWLEDGEMENTS

I sincerely thank the many people who were instrumental to my successful completion of this study.

Firstly, I thank my supervisor, A/Prof. Arthur Georges, for always making time to help me, and for his guidance, encouragement, support and assistance throughout the study.

I thank Mike Ewert for his advice on design aspects of the depth experiments, early in the study. I thank Jeanne Young and Sean Doody for providing advice and assistance throughout the study and particularly in the field. Thanks also to Sean for allowing me to work with nests from his study area. Sean Doody, Peter West and Mick Welch also deserve a big thank you for repeatedly tripping down hundreds of kilometres of the Daly River, to collect the eggs used in the study.

I thank the many volunteers with the “pig-nosed turtle project” who also provided assistance with my study. In particular, thanks to Ben Kropp, for sacrificing much sleep to take opaque patch measurements; and Laura Snedden, Mick Welch and fellow honours student, Enzo Guarino for help with candling work and the depth experiments.

Many thanks also to the people of the Douglas Daly region, NT, for their support and hospitality. In particular, Bill and Eileen Doyle, for providing accommodation in the field and the proprietors of Douglas Daly Park – Ros and John Lucas, and Trevor and Lisa Dunn, for their support. In addition, I thank the Douglas Daly Research Farm (Department of Primary Industries and Fisheries, NT) for providing facilities for the incubators and laboratory work.

I am eternally grateful for Dr Ken Thomas for photographing embryos and also for his advice and encouragement. Thanks also to Sue Nichols and BJ for their advice and assistance with graphical software.

I thank Ross Cunningham, from the Australian National University and David Judge, from the Applied Ecology Research Group, for their statistical advice.

Thanks also to Mike Palmer-Allen, of the University of Canberra, for technical support. I am also grateful to **Denise Byrne** (of AERG) for her administrative support and general advice and encouragement.

I sincerely thank my friends and family for their support and encouragement. In particular, I thank Beck Tailby, Laura Snedden, the Vera family and Enzo Guarino, for his support and encouragement through some of the more trying times in the NT.

Thanks also to my mother, Betty Beggs, for proof-reading the thesis, always being there for me and putting up with me !

My biggest thanks go to Reg Vera, for advice, encouragement, assistance, support, and most of all – for his belief in me.

Lastly, I thank my late father, Bob Beggs, for giving me a thirst for knowledge. Dad, I dedicate this work to you.

This study, as part of a larger project, was predominantly funded by a large Australian Research Council grant awarded to A/Prof. Arthur Georges.

ABSTRACT

This study examined the embryonic development of *Carettochelys insculpta*, under constant and fluctuating temperature regimes, involving artificial incubation and natural nests. The study aimed to enhance our understanding of *C. insculpta* embryology and develop methods of ageing embryos from both candling attributes and embryo characteristics. Research was also undertaken to establish the relationships between soil depth, temperature regime and rates of embryonic development. Our ability to model development using incubation temperatures was also evaluated.

Two highly effective methods of ageing embryos were developed in this study, during constant temperature incubation experiments at 30°C. Qualitative observations and measurements of candling attributes were found to be useful for ageing *C. insculpta* embryos, particularly during the early to mid stages of development (i.e. all but between 2.5 and 8 days, and after 52 days of incubation). The detailed candling ageing system developed during this study is, to date, the only non-destructive method available for ageing reptilian embryos. Ageing was also possible from the direct examination of embryos, using a developmental series based on that of *Chelydra serpentina*, provided morphological differences between the species were taken into account. Strong predictive relationships between embryonic size, age and stage as valuable tools for ageing. A method for adjusting the 30^{o}C ageing work for the effect of incubation temperature on development is presented. Relationships between candling attributes, embryo morphometrics, embryonic stage and age established during the study are therefore applicable to eggs and embryos from a range of incubation environments.

Variation in thermal regime with soil depth was demonstrated during this study. They differed mainly in the magnitude of daily fluctuations and timing of maximum and minimum temperature. However, mean temperatures were generally similar at all depths. The observed variations in thermal regime did not cause developmental asynchrony in *C. insculpta* embryos. This challenges current accepted hypotheses regarding variations in thermal regime and developmental asynchrony.

Lastly, this study demonstrated that *C. insculpta* embryonic development in field nests and artificial cyclic incubation experiments could be satisfactorily predicted from incubation temperatures using a linear development model. However, the model predictions were most accurate when the time of maximum development was considered to occur at stage 24, and when thermal conditions were not too hot (i.e less than 36°C).

In short, the findings of this study have made an important contribution to the descriptive developmental embryology of the pig-nosed turtle, *Carettochelys insculpta*. This research has also contributed to reptilian embryology, in general, through the development of a non-destructive ageing technique. Furthermore, this study has improved our understanding of and ability to model the thermal influences on reptilian embryonic development, under constant and fluctuating temperatures.

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CHAPTER ONE

1. Introduction

1.1 Background and aims

The embryology of reptiles, especially turtles, has stimulated research for over 150 years (Ewert 1985). However, relatively few species have been studied in detail and the physical factors which influence development, and particularly how these act in the natural incubation environment, remain poorly understood.

Early embryological studies, (e.g. Rathke 1848; Agassiz 1857; Parker 1880; Peter 1904, Reese 1915) were generally descriptive and focused on developmental anatomy. Later works included detailed descriptions of normal embryonic stages (e.g. crocodilians: Ferguson 1985; squamates: Hubert 1985; turtles: Yntema 1968 and Miller 1985a) and laboratory experiments on embryology, immunology and physiological ecology (e.g. Yntema 1960; Fitch and Fitch 1967; Borysenko 1969; reviewed by Packard *et al.* 1977 and Tracy 1982). Recent studies have become more focused on the environmental conditions influencing embryonic development and have involved field experiments or the monitoring of natural nests (e.g. iguanas: Muth 1980; lizards: Shine and Harlow 1996; turtles: Burger 1976; Pieau 1982; Bull 1985; Lesham and Dmi'el 1986; Cagle *et al.* 1993).

A number of physical factors have been shown to influence embryonic development in reptiles and the most important of these are temperature, hydric conditions and gas exchange (Gettinger *et al.* 1984; Packard and Packard 1984; Ewert 1985; Miller 1985b; Maloney *et al.* 1990). Temperature can greatly influence a variety of developmental outcomes, many of which have been linked to fitness. These include rate of development and incubation duration (Vinegar 1973; Yntema 1978; Webb *et al.* 1987a; Viets *et al.* 1993), hatchling size (Janzen 1993a; Bobyn and Brooks 1994; Phillips and Packard 1994; Allsteadt and Lang 1995), hatchling sex (Bull 1980; Ewert and Nelson 1991; Vogt and Flores-Villela 1992; Viets *et al.* 1994), amount of residual yolk (Allsteadt and Lang 1995), colouration (Murray *et al.* 1990; Etchberger *et al.* 1993) and post-hatchling growth, metabolism and behaviour (Joanen *et al.* 1987; Lang 1987; Burger 1991; Van Damme *et al.* 1992; Janzen 1993b; Shine and Harlow 1996). Most of

this work has been conducted under constant conditions in the laboratory. Some research has been done with cyclic incubators (e.g. Paukstis *et al.* 1984; Packard *et al.* 1991; Georges *et al.* 1994; Shine and Harlow 1996) and in the field (e.g. Bull 1985; Schwarzkopf and Brooks 1985; Congdon *et al.* 1987; Georges 1992; Castilla and Swallow 1996). Consequently, the effect of temperature on development in natural nests is less well understood, especially for shallow-nesting species for which nest temperatures are likely to be highly variable.

The egg stage is the most vulnerable period in the life cycle of many oviparous reptiles (Burger 1976; Plummer 1976; Tinkle *et al.* 1981). A sound knowledge of the influence of the thermal incubation environment on embryogenesis may be critical for understanding life history strategies and distributions (Muth 1980; Gutzke *et al.* 1987), and the for successful conservation and management of oviparous reptiles.

This study examined the development of *Carettochelys insculpta* embryos under both constant and fluctuating temperature regimes. The work was undertaken during the *C. insculpta* nesting season, from July to December 1997. Through a series of experiments involving artificial incubations and natural nests, the study aimed to enhance our knowledge of *C. insculpta* embryology and improve our understanding of and ability to model thermal influences on embryonic development.

Specific research objectives were:

1. To develop a non-destructive method of ageing *C. insculpta* embryos through the identification and description of specific embryonic characteristics, based on the examination of candled eggs incubated at constant temperatures;

2. To assess the developmental stages of *C. insculpta* embryos through the identification and description of specific embryonic characteristics, based on the direct examination of embryos incubated at constant temperatures;

3. To establish the relationships between soil depth, temperature regime and development of *C. insculpta* embryos;

4. To establish the implications of these relationships for current hypotheses on the effects of fluctuating temperatures and thermal gradients on reptilian embryonic development and developmental asynchrony; and

5. To evaluate the predictive ability of a linear embryonic development-incubation temperature model, in natural nests and controlled cyclic temperature incubation experiments.

1.2 Significance of the aims

1.2.1 Reptilian embryonic series and methods of ageing

A standard series of embryonic development for a species is the primary basis for the organisation of information in embryological studies (Yntema 1968; Miller 1985b). Furthermore, it provides a standardised framework which permits consistency of staging between different authors; a basis for comparison of morphological development between embryos from different incubation conditions or from different groups; and a method for the prediction of incubation time elapsed in the field and expected hatch date (in conjunction with information about the incubation environment) (Miller 1985b; Ferguson 1987, Deeming and Ferguson 1991).

Embryonic staging is primarily based on the description of sequential changes in morphology and each stage is usually defined by the presence or absence of a suite of characters (Miller 1985b; Deeming and Ferguson 1991). As mentioned in Section 1.1, early studies on the normal stages of development for reptile embryos began last century with work on turtles (e.g. Agassiz 1857; Parker 1880; Hoffman 1890). More recently, standard embryonic series have been completed for all reptile groups (e.g. crocodilians: Ferguson 1985; lizards: Dufaure and Hubert 1961; Hubert 1985; snakes: Hubert and Dufaure 1968; Raynaud 1961; Hubert 1985; tuataras: Moffat 1985; turtles: Yntema 1968; Mahmoud *et al.* 1973; Miller 1985a). Some additional work has been published to serve as methods of ageing reptilian embryos as opposed to comprehensive standard series (e.g. crocodilians: Magnusson and Taylor 1980; Webb *et al.* 1983a; 1983b; snakes: Zehr 1962).

Miller (1985b) critically reviewed these works and concluded that there were shortcomings in many cases. In most studies the series described were stages of development based on qualitative external morphological changes. Only the crocodilian work (Magnusson and Taylor 1980; Ferguson 1985; Webb *et al.* 1983a; 1983b) utilised embryo morphometrics for staging. Miller (1985b) recommended that normal series should not be based on solely on morphological descriptions, but also age and size of embryos. He also stressed that the following additional information should be provided: estimates of variation in embryo morphometrics; duration of stages; illustrations to supplement descriptions; and detailed conditions of incubation (temperature, hydric

conditions including the type of substrate, water potential and contact between eggs and substrate, and gas exchange) (Miller 1985b). Many studies did not report this additional information and in at least one case, incubation conditions were not strictly controlled (e.g. Zehr 1962) (Miller 1985b). Despite these deficiencies, the developmental series and ageing work have been useful for further research. One study in particular, defining the normal stages of development for *Chelydra serpentina* (Ynetma 1968), has become the general reference for most turtle embryology studies (Ewert 1985). This is despite the fact that all eggs were incubated at a temperature (20°C) at which hatching did not actually occur (Yntema 1968, Miller 1985b).

Almost all the staging work published to date is based on the direct observation or measurement of embryos and hence requires eggs to be opened and animals killed. Few studies have reported information on egg characteristics visible during candling. Some work has been published on the spread of the opaque white patch with time (e.g. Webb *et al.* 1983a; 1983b; Thompson 1985; Webb *et al.* 1986). This phenomenon, in which a small opaque white patch develops on the egg soon after laying and enlarges to completely cover the egg by the time of hatching, has been reported for a number of reptile species (e.g. crocodilians: Ferguson 1982; Webb *et al.* 1983a; 1983b; turtles: Einem 1956; Ewert 1979; 1985; Miller 1985; Thompson 1985; Chan 1989). Opaque patches are indicative of normal embryonic development, as they are not present on infertile eggs or eggs in which early embryonic death has occurred (Blanck and Sawyer 1981; Webb *et al.* 1983a; Webb *et al.* 1983b; Ewert 1985; Thompson 1985). The spread of the opaque patch has been found to be associated with embryonic development and it forms as a result of the eggshell drying to facilitate gas exchange to the developing embryo (Thompson 1985; Webb *et al.* 1987b; Whitehead 1987; Deeming and Thompson 1991). As incubation proceeds the opaque band or patch increases in size in line with the expansion of the chorioallantoic membrane (Deeming and Thompson 1991). Webb *et al.* (1983a; 1983b), Webb *et al.* (1986) and Thompson (1985) presented data on the relative size of the opaque patch (patch size scaled by egg size) as a function of incubation time, but only Webb *et al.* included staging information.

Another “egg” characteristic associated with embryonic development is the formation of the area of extraembryonic vitelline circulation, which is visible during candling (Ewert 1985). The vitelline circulatory system first appears as blood islands which can be seen as reddish-pink spots towards the top of the egg during candling

(Ewert 1985). With further development, it appears as a small, nearly circular red-bordered pink disc (a “haemodisc”), with the elongate, many-somite embryo near the centre (Ewert 1985; Ewert and Wilson 1996). In addition, anecdotal reports suggest the outline of the allantois and various embryonic features can be distinguished at certain stages of development, during candling (M. Ewert, pers. comm.; J. Young, pers. comm.). Ewert (1985) used candling attributes to establish a chronology of developmental stages (based on Yntema 1968), for 37 turtle species, from six families. This work documented five stages based on the appearance of blood islands (stage 5), the haemodisc (stage 8+), eye pigmentation (stage 12), body pigmentation (stage 20) and the time to hatching (stage 26). However, despite the clear association between developmental stages and the appearance of the haemodisc and other embryonic features, no other studies have reported such characters as a part of a method of ageing embryos or normal series of development.

Developmental rates are strongly influenced by temperature, yet many attributes are related more closely to the proportion of development that has transpired than to chronological time. An embryological series provides an ordinal scale for developmental time that has important applications. In particular, staging is critical for research on reptile species which have temperature dependent sex determination (TSD). The thermosensitive period for sex determination (when sex is irreversibly influenced by temperature) occurs between specific stages, usually in the middle third to half, of development (Yntema 1979; Bull and Vogt 1981; Webb *et al.* 1987a). Therefore the ability to determine developmental stage is crucial for laboratory research aimed at pinpointing this period. Similarly, the capacity to stage embryos has applications in the field as it provides a method for assessing the extent of development in natural nests and, whether or not sex has been determined. Another important application of embryological series in the field, is for the estimation of lay and hatching dates and in some cases ageing wild embryos is the only method available for quantifying the timing of nesting (Webb *et al.* 1983b).

Unfortunately, the only accurate methods of ageing are based on direct examination of embryos, yet in many cases, especially with research on species of high conservation status such as marine turtles (IUCN 1990), it is desirable to minimise embryo mortality. Clearly, a method of ageing embryos – one which is both reliable and non-destructive

such as one based on candling attributes, is needed. The present study aims to provide such a method.

1.2.2 Thermal gradients in reptile nests

Temperature can have a profound influence on many aspects of embryonic development for oviparous reptiles. As mentioned in Section 1.1, the relationship between temperature and embryonic development in natural nests is, at present, poorly understood. It is likely to be more complex than that which has been established under constant laboratory conditions, especially for species which construct shallow nests in which temperatures may vary considerably with depth, diel cycles and seasonal trends (e.g. *Carettochelys insculpta*: Georges 1992). The issue of thermal gradients associated with depth, and the potential effect these may have on the rate embryonic development, has generated some interesting hypotheses.

Thermal gradients within nests provide the potential for embryos in the same nest to develop at different rates and therefore hatch at different times (Thompson 1988), yet synchronous hatching has many advantages (Whitehead and Seymour 1990). Successful escape from the nest may be achieved through the combined effort of many hatchlings (Carr and Hirth 1961). Predators may be saturated by the simultaneous emergence of the hatchlings, so increasing the proportion that avoids predation (Carr 1967). Early emergence of some hatchlings may attract predators to the nest by disturbance at the surface or by exposing egg and membrane residues (Congdon *et al.* 1983). Furthermore, early emergence of some hatchlings may promote growth of bacteria and other pathogens that is deleterious to the remaining eggs. Though data are few, simultaneous hatching appears to be common. These advantages have presumably led to selection for mechanisms that provide individual embryos with the capacity to vary the time of hatching, in response to some external stimulus, and so ensure synchronous hatching of eggs within a single clutch.

One mechanism of ensuring synchronous hatching in physiological. Thompson (1989) investigated patterns of metabolism in embryonic reptiles to explain how synchronous emergence was achieved in nests which were likely to have developmental asynchrony. Thompson (1989) noted that there were three basic patterns of rate of

oxygen consumption, VO_2 – exponential, sigmoid and peaked (Figure 1.1). Snake embryos showed the exponential pattern, with metabolism increasing throughout incubation (Dmi'el 1970; Black *et al.* 1984). Most reptile species showed the sigmoid pattern, with metabolism reaching a maximum late in development (Ackerman 1981a; Gettinger *et al.* 1984; Packard and Packard 1988; Vleck and Hoyt 1991). However, some species showed the peaked pattern in which there is a late-term metabolic depression and/or embryonic aestivation (e.g. crocodilians: *Crocodylus johnstoni* - Whitehead 1987; *Alligator mississipensis* - Thompson 1989; turtles: *Carettochelys insculpta* - Webb *et al.* 1986; *Emydura macquarii* - Thompson 1989; *Chelodina longicollis* - Beynon 1991). Thompson (1989) noted that the exponential and sigmoid patterns in reptiles were similar to those exhibited by altricial and precocial birds, respectively (Vleck and Hoyt 1991). Also, the peaked pattern was similar to that found in the large, precocial ratites, where the decline in VO_2 late in incubation has been interpreted as a resting stage to facilitate synchronous hatching (Hoyt *et al.* 1978; Vleck *et al.* 1980; Cannon *et al.* 1986).

On examination of turtle nesting habits, Thompson (1989) found that the shallow-nesting species generally exhibit the peaked pattern and the deep-nesting species show the sigmoid pattern. He proposed that, as marine turtles nests experienced relatively invariable thermal conditions (Bustard 1972; Chan and Liew 1995), they were unlikely to experience developmental asynchrony. In contrast, he further suggested that:

"…clutches...incubated in shallow nests are influenced by daily temperature fluctuations such that the top eggs experience significantly warmer temperatures than the bottom eggs…This must cause considerable variation in developmental rates, and therefore incubation times." (Thompson 1989; p 251)

The presumed link between differing thermal regimes for top and bottom eggs and developmental asynchrony is a primary focus of the work presented in this thesis.

Thompson (1989) proposed that developmental asynchrony was overcome by a "catch-up" period towards the end of incubation. Hatching could occur at any time

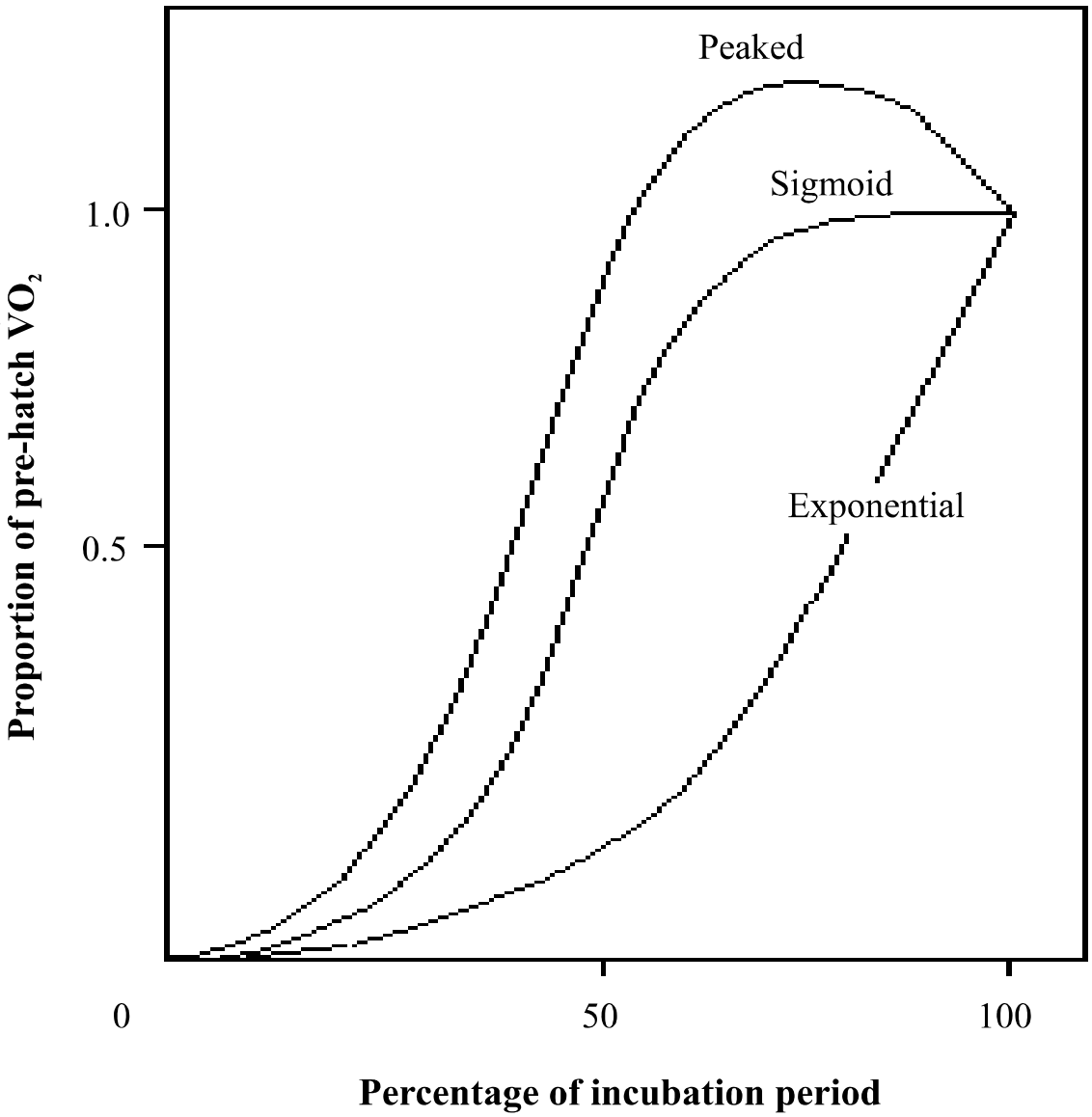


Figure 1.1. Patterns of rates of oxygen consumption $(\dot{V}O_2)$ observed during incubation of embryonic birds and reptiles (after Thompson 1989 and Beynon 1991).

during the catch-up period, in response to a specific stimulus, thus facilitating simultaneous hatching (Thompson 1989).

An alternative hypothesis regarding the effect of thermal gradients within nests on embryonic development has been formulated by Georges (in prep.). According to this hypothesis, developmental asynchrony is not expected to occur if thermal regimes at differing depths vary in the magnitude of daily fluctuations rather than in mean temperatures, provided the temperature remains above developmental zero at all times, at all depths (Georges, in prep.)

Thermal gradients have been reported for many shallow-nesting reptile species (e.g. iguanas - Muth 1980; crocodilians - Whitehead and Seymour 1990; turtles - Burger 1976; Wilhoft *et al.* 1983; Thompson 1988; Palmer-Allen *et al.* 1991; Georges 1992). but their mode of action and implications for overall developmental outcomes have not been examined closely. In particular, principles established by soil physics indicate that there may be only slight gradients in mean daily temperature at depths similar to that of shallow turtle nests (Carson and Moses 1963; Marshall and Holmes 1979; Hanks 1992). Also, the phase of the temperature cycle alters with depth (Hanks 1992) so that at any one time there may be pronounced temperature differences between the top and bottom of a nest, but temperatures may cycle about roughly the same mean, regardless of depth (Carson and Moses 1963; Hanks 1992). Most of the studies which report thermal gradients in reptile nests did not record detailed thermal regimes, but instead relied on only a few "spot" temperatures taken each day (e.g. *Chelydra serpentina*: Packard *et al.* 1985; *Emydura macquarii*: Thompson 1988; *Dermochelys coriacea*: Chan and Liew 1995). The protocols used in these studies may not provide accurate information regarding the thermal regimes within the nests.

No studies have specifically investigated the issue of thermal gradients and reptilian embryonic development. Hence, support for the notion of developmental asynchrony resulting from thermal gradients in nests is not yet firmly founded, and nor are the hypotheses of Thompson (1989) or Georges (in prep.) in either theory or practice. If thermal gradients do not cause appreciable asynchrony, alternative explanations for the metabolic patterns examined by Thompson (1989) should be considered. The present study aimed to determine the differences in thermal regime associated with depth and the effect of any differences on embryonic development. The study also considered the

evolutionary advantages of the peaked pattern of oxygen consumption and late embryonic aestivation.

1.2.3 Modelling reptilian embryonic development

The above discussion has highlighted the complexity of the relationship between temperature and developmental outcomes. A poor understanding of the relationship between temperature and embryonic development makes modelling development difficult, although the ability to predict certain developmental outcomes may be desirable. For example, concern has grown recently over major environmental changes such as climatic change resulting from the human-induced greenhouse effect and the possible effects such changes may have other organisms. Even moderate shifts in climate (2-3°C) have the potential to alter the incubation environment and hence affect developmental outcomes such as sex ratios, for those species which have temperature-dependent sex determination (TSD) (Mrosovsky *et al.* 1984).

There has been little work done on modelling reptilian embryonic development, although other poikilotherms, namely insects, have received considerable attention (e.g. Eubank *et al.* 1973; Hagstrum and Milliken 1988; Hagstrum and Milliken 1991; Liu *et al.* 1995). For reptiles, the developmental modelling work has stemmed primarily from desire predict sex ratios in the field. Early work revealed that mean nest temperature alone was a poor indicator of hatchling sex ratios (Pieau 1982) and that incorporating variance in temperature proved more successful (Bull 1985). The influence of temperature fluctuations on sex determination over and above that of mean temperature was thought to result from the accelerated development that occurs at temperatures above the mean compared to that which occurs below the mean (Bull and Vogt 1981; Pieau 1982; Mrosovksy *et al.* 1984; Bull 1985) but this idea remained untested until formulated in a more precise form by Georges (1989; 1994). Recent experiments of Georges *et al.* (1994) demonstrate conclusively that it is proportion of development that occurs at a temperature, not duration of exposure, which is best able to predict hatchling sex ratios under fluctuating temperatures.

The critical period for sex determination occurs during the middle third to half of incubation (Yntema 1979; Bull and Vogt 1981; Yntema and Mrosovsky 1982; Webb *et*

al. 1987a). The ability to model development and hence make predictions about the incubation period and middle third of development hinges on understanding the relationship between incubation temperatures and embryonic development. The model of Georges (1989; 1994) is based on knowledge of the relationship between temperature and developmental rate, obtained under controlled laboratory conditions for a range of constant temperatures. Developmental parameters used in the model are: the time to complete development at a particular reference temperature; the relationship between temperature and developmental rate; and the lower limit of temperature which supports embryonic development (T_0) (Georges 1989; 1994). The model works by taking a detailed temperature trace from the incubation environment (e.g. a nest) and calculating the associated developmental rate at the time of each temperature reading (Georges 1989; 1994). The model then calculates the cumulative proportional development and constant temperature equivalent for each day (Georges 1989; 1994). Sex ratios can then be estimated by examining the CTEs during the sex determining period (e.g. between 33% and 66% proportional development, for the middle third) (Georges 1989; 1994).

The model has proven useful in predicting hatchling sex ratios in turtles (Georges *et al.* 1994) but the fundamental prediction of proportional development has not been tested. In addition, an underlying assumption of the model is the linear relationship between developmental rate and temperature (Georges 1989; 1994). Linear relationships between developmental rate and temperature have been demonstrated for reptilian embryos (e.g. *Caretta caretta*: Georges *et al.* 1994; *Carettochelys insculpta*: Georges unpublished data) (Fig 1.2), however this has not been investigated for many species or over a wide range of thermal incubation conditions. The present study aimed to test the ability of this model to predict proportional development and, in particular, assess the suitability of a model based on a linear relationship between developmental rate and temperature.

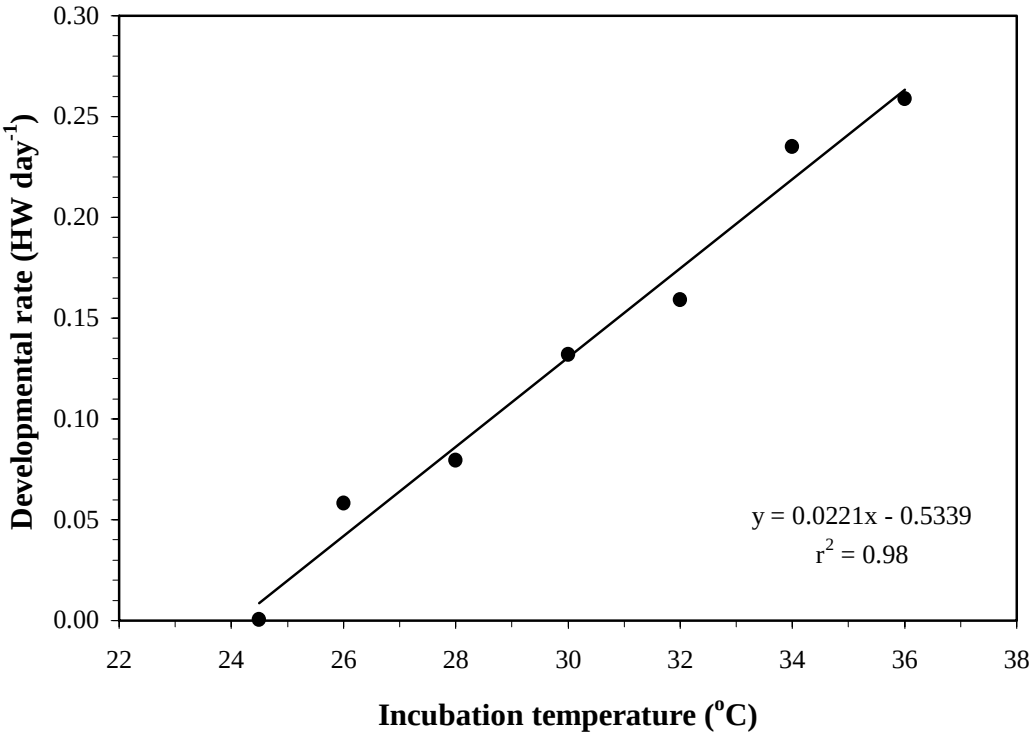


Figure 1.2. Embryonic developmental rate of *Carettochelys insculpta* as a function of incubation temperature (after Georges, unpublished data). HW = embryo head width (mm).

1.2.4 Conclusions

There is a vast body of literature concerning reptilian embryology and many issues have been well researched. However, more study is needed in both basic embryology and work involving the effects of temperature on embryonic development.

Embryonic development has been studied in each reptile group but comprehensive developmental series have been established for relatively few species. Although sometimes fruitful, it is generally difficult to compare embryos of one species to the developmental series of another, especially late in development. In addition, most of the published work on stages of reptilian embryonic development is based on descriptive embryo morphology with little including morphometrics or candling features. All of this work requires direct examination of embryos and hence the opening of eggs and killing of animals. Clearly more work is needed to further existing work on species, and to provide basic information for more species. Importantly, non-intrusive and therefore non-lethal methods of ageing reptilian embryos should be developed, especially if embryological research on threatened species is to continue. The present study aimed to development such a method.

Research to date has established that various physical factors and temperature, in particular, can have profound effects on developmental outcomes of reptilian embryos. However, much work has been laboratory based and the relationship between temperature and development in natural nests remains poorly understood. Some studies have suggested that thermal gradients may exist in some reptile nests and that these may cause developmental asynchrony. The effects of thermal gradients are not clearly understood and there is little evidence to support either of the two conflicting hypotheses regarding such effects. The present study aimed to address this issue and by doing so will further the general understanding of the influences of the thermal environment on developmental outcomes in natural reptile nests.

The ability to model embryonic developmental outcomes may provide an insight into the potential effects broad scale environmental changes such global warming may have on reptiles. However, little work on modelling has been published, although Georges (1989; 1994) has developed a model which has proven useful in predicting

hatchling sex ratios. The present study aimed to perform further tests of this model's ability to predict proportional development from incubation temperature.

CHAPTER TWO

2. Materials and Methods

2.1 General methods

Although the study was divided into three main sections of work, there were a number of procedures and materials common to each section. These are described in this section.

2.1.1 Study area

The study area was on the Daly River in the Northern Territory of Australia. The study site was an 18km stretch of the river between the inflow of Jinduckin Creek (14°37'S, 131°47'E) and approximately 4km downstream of the inflow of Cattle Creek (14°48'S, 131°39'E). All natural nests and field experiments were located within this site, however, eggs for experimental work were collected from outside the study site as far upstream as Claravale Crossing and downstream to the Daly River Mission Crossing. The river flows continuously throughout the year. The river banks were generally sand or sandy loam, covered in dense vegetation and steeply sloping to a height of about 20m. Exposed areas of clean, fine sand found on bends of the river, at junctions of small drainage gullies with the river, or behind fallen trees or other debris were suitable nesting habitat in the study area (Webb *et al.* 1986). The climate is typical of the wet-dry tropics of northern Australia and has the following: mean monthly rainfall is less than 7mm from May to September and 284mm in February; mean relative humidity is 32% in August and 73% in February; mean monthly maximum air temperature ranges from 30.9°C in June to 36.8°C in October characteristics (as described by Georges 1992).

2.1.2 Study animal

Carettochelys insculpta is large, freshwater turtle that inhabits permanent water in the major rivers of the Northern Territory, Australia, and southern rivers of Papua New

Guinea (Georges and Rose 1993). This species is the sole surviving member of the Carettochelydidae, a family of turtles widely distributed during the Tertiary (Georges and Rose 1993). *C. insculpta* is cryptodirous and feeds on a variety of plant and animal material including riparian vegetation, crustaceans, molluscs and aquatic plants (Georges and Kennett 1989). In Australia, nesting occurs between mid July and early November and there is evidence of multiple clutching (Georges and Kennett 1989, A. Georges and J. S. Doody, pers. comm.). *C. insculpta* nests in clean, fine sand adjacent to water where it lays seven to 20 hard-shelled, almost spherical eggs in shallow nests (Webb *et al.* 1986, Georges and Rose 1993). Hatchling sex of *C. insculpta* is determined by incubation temperature, with females and males being produced from constant incubation temperatures above and below 32.0°C, respectively (Georges, unpublished data). Incubation period also varies with temperature. At 30°C the species takes 64 to 74 days to reach the point of yolk internalisation after which it enters an embryonic aestivation within the egg (Webb *et al.* 1986). This period, during which metabolic rate decreases and growth ceases, can last up to 50 days. Hatching is stimulated by inundation. Early wet season rains and/or flooding are thought to stimulate emergence in natural nests (Webb *et al.* 1986).

C. insculpta nests are subject to predation by monitor lizards and humans, but potentially the most serious threat to the species (in Australia) is habitat degradation caused by various human activities and feral water buffalo (Georges and Rose 1993). Although locally abundant, *C. insculpta* is geographically restricted and for this reason, along with its taxonomic distinctiveness, it is of conservation concern. It has an Action Plan Rating of 1 (known threatened and in need of specific conservation measures) (IUCN 1989).

C. insculpta was chosen for the present study for three main reasons:

1. basic embryological information, and in particular methods of ageing *C. insculpta,* was required to supplement concurrent research on temperature dependent sex determination (TSD) in the species;

2. the species is shallow-nesting and exhibits delayed hatching (and the peaked VO_2 pattern), therefore *C. insculpta* was a suitable species to work on to assess the effects of soil depth on thermal regime and embryonic development; and

3. *C. insculpta* is the only Australian freshwater species with TSD and is therefore of particular interest for modelling development.

2.1.3 Location of nests and egg collection

Sands beds along the river were inspected for evidence of *C. insculpta* nesting (distinctive tracks and depressions in the sand) during the 1997 nesting season (late July to early October). The sand was probed with a fine steel rod to locate nests (Georges 1992). Eggs were removed from each nest and the uppermost surface was marked by pencil to preserve original orientation in order to reduce the risk of rotation induced mortality (Limpus *et al.* 1979; Parmenter 1980). Eggs were numbered and either placed in an insulated plastic tub filled with moist vermiculite and transported by boat and 4WD vehicle to the field laboratory, or processed further (see Section 2.4.1) and returned to the nest chamber to complete incubation naturally. Extreme care was exercised during further handling of eggs in order to reduce the risk of movement induced mortality (Limpus *et al.* 197; Parmenter 1980).

2.1.4 Egg processing, candling and artificial incubation

For all eggs, maximum and minimum egg diameter was measured (to the nearest 0.1mm) and the fertility/developmental status was assessed. Infertile eggs were designated as those without an opaque white patch (after 24 hours) (Thompson 1985), though it is recognised that they may have been fertile but died at a very early stage of development. Fertile eggs were candled using a fibreoptic lamp in a dimly lit room to evaluate the extent of embryonic development. Eggs were deemed “fresh” if embryonic development was estimated to be less than stage 8+ (Yntema 1968), on the basis that blood islands were visible but a distinct haemodisc was not, during candling (Ewert 1985). Artificially incubated eggs were buried in moist vermiculite (four parts water to three parts vermiculite, by weight) in plastic containers with lids. The initial mass of each container of eggs was recorded (to the nearest gram) before they were placed in incubators. The exact protocol of artificial incubation differed between constant and cyclic temperature experiments and further details are provided in the relevant sections

(2.2.1 and 2.4.2). However, in all instances eggs were placed in the designated incubators within three days of collection or shipment.

Two types of refrigerated incubators were used in the study: Thermoline Model RI-170 incubators, for constant temperature experiments (at the Douglas Daly Research Farm, NT), and programmable incubators (Model LM550R with microprocessor control; Clayson Laboratory Equipment, PO Box 5401, Brendale QLD 4500) for cyclic experiments (at the University of Canberra, ACT). Water trays were placed in the bottom of each incubator to maintain a high but unmeasured humidity. The moisture level within plastic containers was preserved by weighing them at regular intervals and restoring each container to the initial mass by adding water. Temperature within incubators was monitored by either mercury thermometers (to the nearest 0.2°C, in constant temperature incubators) or temperature probes (to the nearest 0.1°C, in cyclic temperature incubators). Temperatures inside the constant temperature incubators remained within 0.5°C of the nominated temperature throughout the study.

2.1.5 Killing, measurement and preservation of embryos and hatchlings

Embryos were killed by chilling the eggs (to less than 5°C) for a minimum of 36 hours prior to opening. Hatchlings and late stage embryos were killed by intracranial injection of an overdose of anaesthetic (minimum 0.001mL Nembutal per gram body weight). The embryos were separated from the yolk and extra-embryonic membranes, blotted dry and weighed on a balance (to the nearest 0.1g). Hatchlings were cleaned of sand or vermiculite, blotted dry and weighed on a balance (to the nearest 0.1g) within twelve hours of hatching. All animals were preserved in 10% buffered formalin. Embryonic stage was determined using the developmental series for *Chelydra serpentina* described by Yntema (1968) and all further references to embryonic stage refer to this system. Maximum head width (including the optic capsules) of embryos was measured after preservation with a graduated eyepiece under a stereo microscope (embryos up to stage 20) or with callipers (embryos older than stage 20). Hatchling head widths were measured before preservation with callipers (all measurements to nearest 0.1mm). The head widths of all embryos and hatchlings were scaled by mean egg diameter and expressed as a head to width ratio (HWR).

2.1.6 Temperature recording and processing

Probes (PT100 4-wire RTD thermistors; Industrial Pyrometers Australia Pty Ltd, 9/183 McCredie Road, Smithfield NSW 2164) attached to a multichannel data taker (model Datataker 500; Data Electronics Australia Pty Ltd, 7 Seismic Court, Rowville VIC 3178) were used to record temperatures in the field experiments, natural nests and cyclic temperature incubators. The probes were calibrated over the range of approximately 5°C to 50°C in a water bath against a mercury thermometer certified as accurate to 0.1°C by the National Association of Testing Agencies (NATA). Probes were placed in the desired location in a field experiment, natural nest or cyclic incubator and temperatures were recorded at ten or fifteen minute intervals. Temperature data were downloaded at regular intervals using a personal computer. Erroneous data (such as temperatures recorded whilst an incubator door or nest was opened, or periods of probe malfunction) were identified and truncated. A time series analysis (TSA) program developed by A. Georges was used to replace missing values with estimates obtained by cross-correlation with complete traces (see Appendix 1 for explanatory notes).

2.1.7 General statistical procedures

All statistical procedures undertaken in the study generally followed Zar (1984) and Sokal and Rohlf (1995) and were performed using the statistical analysis system SAS® (SAS Institute 1995). Means are presented with standard errors unless otherwise specified.

2.2 Development of techniques for ageing embryos

Ageing characteristics for *Carettochelys insculpta* embryos were based on candling and embryonic observations, measurements, descriptions and illustrations.

2.2.1 Incubation of eggs, candling procedures and embryo description

Early ageing characteristics of *C. insculpta* eggs were investigated by monitoring the development of the opaque white patch (Thompson 1985). Four viable eggs were obtained from a gravid female (confirmed by x-ray) by injection of oxytocin (Ewert and Legler 1978). The eggs were measured and placed in 30°C and 34°C constant temperature incubators (two eggs at each temperature). Eggs were examined and the opaque patch was measured (to the nearest 0.5mm) at approximately two hour intervals for the first 36 hours, approximately twelve hour intervals from 36 to 60 hours, and thereafter every one to two days until the eggs were ten days old. Figure 2.1 shows how opaque patch measurements were taken. The opaque patch measurements were included with the features measured during candling, although the patch was visible without candling. Collectively, these features are hereafter referred to as “candling features”.

The ageing characteristics of older embryos (more than approximately two days old) were investigated by candling and direct examination of embryos at various stages of development. A total of 56 fresh eggs from 16 clutches (including the eggs used for the opaque patch development incubation) were artificially incubated at 30°C. One clutch (nine eggs) was incubated specifically to obtain information for ageing, and the remaining clutches (excluding the opaque patch eggs) were from a concurrent TSD switch experiment headed by Jeanne Young. Eggs were candled every second day. Qualitative observations and the following measurements were recorded: haemodisc diameter, width of the allantois and embryo crown-rump and carapace length (measurements recorded with callipers to nearest 0.5mm). In addition, haemodisc measurements from eggs incubated at 28°C, 30°C, 32°C and 34°C, recorded by A. Georges during an experiment in 1996, at the University of Canberra, ACT (Georges, unpublished data), were included to assess the effect of temperature on the rate of growth of the haemodisc. The eggs used in this experiment were “fresh” and collected and incubated in the same way as described in Sections 2.1.3 and 2.1.4 (A. Georges

pers. comm.). One to three eggs were incubated at each temperature (eight eggs in total, from three clutches) and five haemodisc measurements were made (at nine, 12, 15, 18 and 28 days after collection) (A. Georges, pers. comm.). Figure 2.1 shows how the haemodisc, allantois, embryo crown-rump and carapace length were measured during candling. No measurements were taken if the feature was not clearly visible (e.g. because of embryo orientation or excessive movement) in which case only qualitative notes were recorded. All candling measurements (including opaque patch) were scaled by egg diameter and expressed as an index. The stage associated with candling features and measurements was determined by the direct examination of embryos killed in the course of the work described hereafter.

Embryonic stage was estimated from candling observations and a table derived from the preliminary work of Webb *et al.* (1986) (see Appendix 2) and embryos were killed to obtain at least one specimen at each stage of development from stages 12 to 26. Once killed, embryos were preserved, measured and external morphological features were described in detail. No extraembryonic characteristics (such as blood vessels or membranes) were described. Particular attention was paid to the development of features unique to *C. insculpta*. Where possible, multiple specimens from the same stage were compared in order to establish the variability in characteristics at each stage. Specimens deemed typical for each stage were selected for illustration by photography.

2.2.2 Statistical procedures

Relationships between developmental characteristics (candling and embryonic measurements), age and stage were investigated by regression analyses. The analyses were undertaken on both the raw and $\log_{10}$ transformed data, and involved linear and polynomial regression with the strongest relationships presented (based on the goodness of fit of the regression line to the data, significance of the regression and coefficient of determination, r^2) (Sokal and Rohlf 1995). In cases where multiple measurements were recorded (e.g. multiple measurements of eggs of the same age), regression analyses were applied to means but weighted by sample size (i.e. number of eggs measured). Also, as embryonic stage was measured on an ordinal scale, significance was not tested during the regression analyses involving stage (Sokal and Rohlf 1995); the relationships were simply

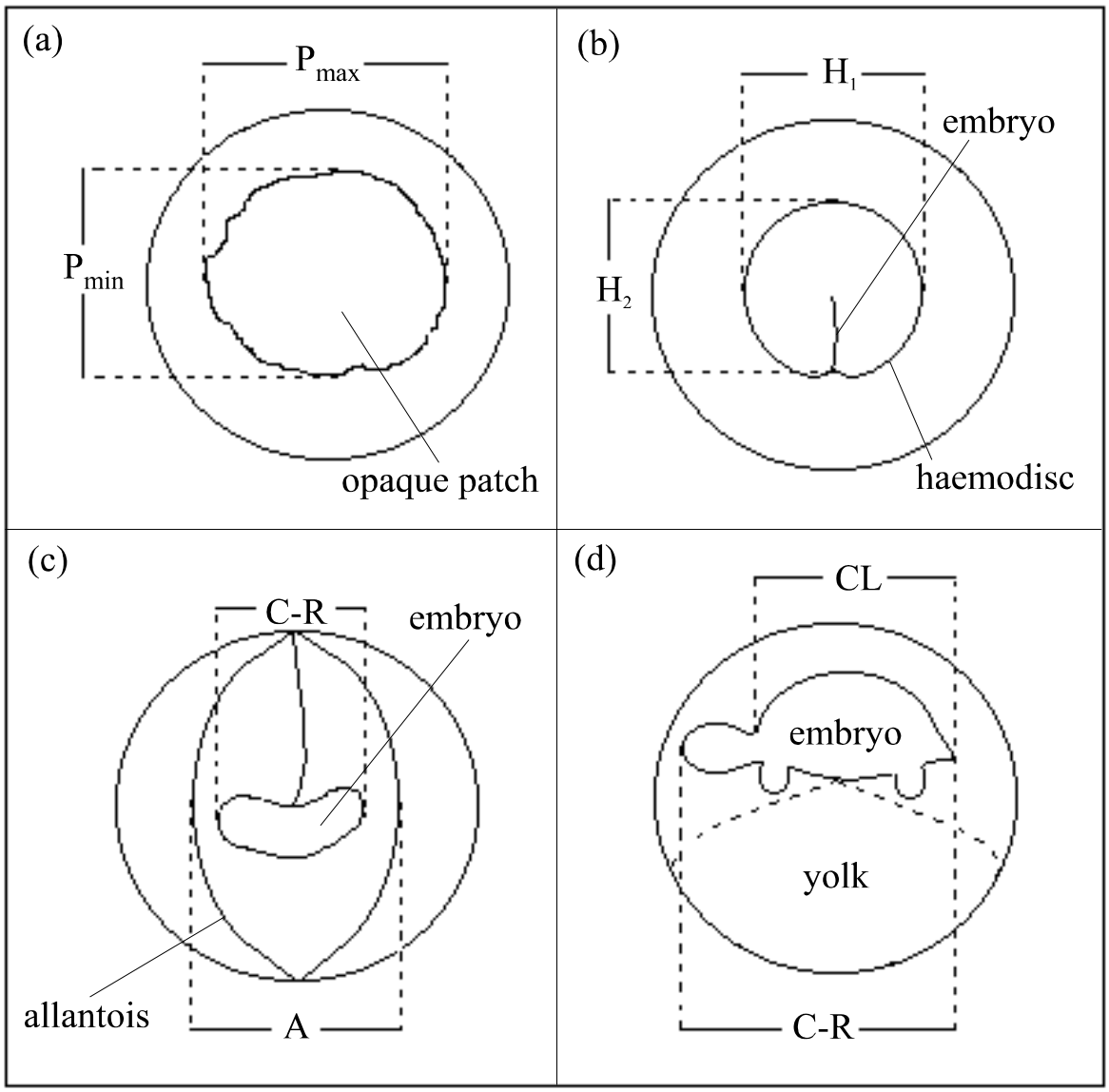


Figure 2.1. Schematic diagram illustrating the measurements recorded during the candling of *Carettochelys insculpta* eggs: average of P_{min} and P_{max} for the opaque patch; average of H_1 and H_2 for the haemodisc; A for the width of the allantois; and C-R and CL for the embryo crown-rump length and carapace length measurements, respectively. All measurements were straight line measurements and hence did not account for the curvature of the egg shell. Eggs were viewed dorsally for (a), (b) and (c), and laterally for (d).

descriptive. An analysis of covariance (ANCOVA) was performed on the opaque patch and haemodisc data to assess the effect of temperature on the relationship between index size and time (Sokal and Rohlf 1995). The relationship between HWR and embryonic stage obtained from the analysis was tested using other measured embryos of known stage (i.e. those obtained from other parts of the study and not incubated at 30°C).

2.2.3 Correction of 30°C ageing work for different incubation temperatures

In order for ageing work undertaken at 30°C to be applicable to embryos from a range of incubation temperatures, corrections for temperature were calculated, based on the work of Webb *et al.* (1983a; 1983b; 1986). Webb *et al.* produced correction factors – "developmental rate coefficients" (DRC), which represented the development time for a range of temperatures relative to the development time at a reference temperature (30°C). In the present study, DRC were calculated using the relationship between developmental rate and temperature (established by Georges, unpublished data) and were expressed as a function of development at 30°C.

2.3 Thermal gradients and *Carettochelys insculpta* embryonic development

The relationship between soil depth, temperature and embryonic development was investigated in three stages:

1. an evaluation of thermal conditions at various soil depths;
2. a pilot "depth" experiment designed to provide preliminary data on the effects of soil depth on temperature and embryonic development; and
3. a final "depth" experiment (designed after an assessment of the results from the pilot experiment) designed to provide comprehensive information on the relationship between soil depth, temperature and embryonic development.

2.3.1 Thermal gradients in the soil profile

Temperature probes were placed at 0cm, 10cm, 20cm, 30cm and 50cm below the surface and in vertical alignment, on a sand bank in the study area. Temperatures were recorded at fifteen minute intervals between the end of April and early December 1997. The data were downloaded regularly then processed, as described in Section 2.1.6. The TSA program (see Appendix 1) was used to obtain the average diurnal thermal regime at each depth and these were assessed graphically. In addition, the thermal regimes at each depth were characterised by calculating summary statistics (mean, standard deviation, minimum and maximum) for the eight month period. The relationship between these characteristics and depth was evaluated graphically and by linear and log linear regression analyses. Time lag, defined as the time between maximum temperatures at the surface and at other depths, was also investigated graphically and by regression analyses (linear, log linear and polynomial).

2.3.2 Pilot depth experiment

2.3.2.1 Experimental design and procedures

A preliminary assessment of the effects of soil depth on thermal regime and embryonic development was undertaken by way of a pilot depth experiment which ran from August 13 to October 21. A total of 80 eggs, from ten clutches were used in the experiment and all clutches were at early but variable stages of development (none more developed than stage 8+), as verified by candling. Eggs were measured and buried on a beach in the study site in rows of sixteen at five depths, in the arrangement shown in Figure 2.2. The location chosen for the experiment was used for nesting by *Carettochelys insculpta.* A large sheet of chicken wire was placed over the arrangement, buried just below the surface of the sand and secured with pegs to protect the eggs from predation by monitor lizards. Temperature probes were placed in between two eggs of each row and readings were taken every fifteen minutes. Temperature data were downloaded regularly and preliminary analyses of the data were undertaken about two weeks after the start of the experiment. Also, at around this time, the experiment was relocated to an alternative (more moist) beach as sand at the initial location was found to

be extremely dry and therefore likely to cause excessive water loss from eggs and potentially high embryo mortality. Twice through experimental incubation, two eggs were removed from each depth, candled and opened and the embryos measured. A third egg was opened if there appeared to be substantial developmental differences between the first two eggs. The remaining eggs were left to continue development until approximately one week prior to the earliest estimated hatching date. At this time, all eggs were removed and placed in plastic containers (as described in Section 2.1.4), incubated at 34°C and monitored every second day by candling. Eggs from different depths and clutches were provided with the stimulus to hatch by submergence in water each second day (Webb *et al.* 1986) and the successful hatch date was recorded. For each turtle, incubation period was calculated from the time between the estimated lay date and hatch date.

2.3.2.2 Data analyses

The complete temperature data for the field experiment were firstly processed as described in Section 2.1.6 and differences in the thermal regimes as a function of depth were investigated by comparison of daily means and variances. The data were in the form of time series, that is, autocorrelated. Because successive observations are not independent (Chatfield 1989), conventional statistical analyses such as Analysis of Variance (ANOVA) were not appropriate. On the advice of a professional consultant statistician (R. Cunningham, pers. comm.), significance of trends in the temperature data were ascertained as follows. Mean daily temperatures were assessed in relation to depth by paired comparisons of the difference in mean daily temperature for each depth with each other depth. In this procedure, temperature traces in each comparison were firstly aligned to remove the effect of time lag, using the TSA program; then the difference in mean daily temperature was calculated (shallowest minus deepest); and lastly temperature differences were graphed as standard box-plots showing means, two standard errors (as the approximate 95% confidence interval of the mean) and range (Sokal and Rohlf 1995). For each depth comparison, if the standard error box did not cross the zero vertical reference line, the mean difference (in mean daily temperatures) was significantly different from zero ($\alpha \cong 0.05$) indicating a significant difference in mean daily temperature between the depths compared (R. Cunningham, pers. comm.).

Temperature variability was assessed in relation to depth simply by the examination of box-plots of daily temperature standard deviation versus depth.

An assessment of embryonic development in relation to depth was based on embryonic size (HWR) rather than developmental rate because this was unable to be calculated (as eggs from the first and second openings were not from the same clutches). The effect of depth on embryonic development was analysed separately for each opening by a mixed model ANOVA without replication (Sokal and Rohlf 1995).

2.3.3 Final depth experiment

2.3.3.1 Experimental design and procedures

Preliminary results obtained from the pilot depth experiment were used to develop the protocol for the final experiment. The fundamentals of the final experiment were similar to the pilot in the sense that eggs were incubated at different depths and detailed temperature data and embryonic development data (obtained twice through incubation) was obtained for each depth. However, care was taken to ensure the eggs used in the final experiment were at the same early stage of development. Also, the design incorporated fewer eggs (at least six per depth c.f. sixteen) and a narrower range of depths (12cm, 17cm, 22cm and 30cm) (Fig. 2.3). In addition, the arrangement of eggs was such that eggs from the same clutch were removed on both opening occasions hence facilitating the direct calculation of developmental rate at each depth. As with the pilot experiment two or three eggs from each depth were removed and opened, twice through development, and the remaining few eggs at each depth were left to continue incubation. These remaining eggs were dealt with in the same way as those in the pilot experiment, except all hatchlings were released. Replication in the final experiment was achieved by setting up the arrangement shown in Figure 2.3 twice, such that two independent (simultaneous) experiments were run (from September 16 to November 10).

2.3.3.2 Data analyses

Temperature data were analysed in the same way as described for the pilot depth experiment (Section 2.3.2.2).

Embryonic developmental rate for each clutch, at each depth was calculated as follows:

$$\text{Developmental rate (HWR day}^{-1}\text{)} = \frac{HWR_2 - HWR_1}{\Delta t}$$

where:

HWR_2 = HWR at 2nd opening

HWR_1 = HWR at 1st opening

Δt = time (number of days between openings).

As with the pilot experiment, the effect of depth on developmental rate was analysed by a mixed model ANOVA, but with replication.

2.4 Testing the Linear Development Model

The linear developmental model was tested in two ways: in the field, using natural nests; and in the laboratory, using eggs incubated under cyclic temperature regimes. The following sections detail the methods of testing the model for each of these approaches.

2.4.1 Natural incubation of eggs in field nests

Fresh nests in the study site were located and the eggs of each were removed (with depth to the top of each egg recorded before removal), as described in Section 2.1.3. The lay date of each nest was estimated by evaluating the freshness of the crawl, size of the opaque patch on eggs, and from any other information available (e.g. the turtle was observed nesting). Errors associated with the estimation of lay date were recorded as follows: zero days, if the turtle was observed nesting; zero to one day, if the nest was

found from a new crawl; one to two days, if the nest was found from a crawl one to two days old; and two to three days, if the lay date was estimated from the extent of opaque patching (based on information from the ageing work – see Section 2.2.1) and the nest was found from a crawl more than two days old. Maximum and minimum diameters of eggs were measured by callipers (to the nearest 0.02mm) before they were replaced in as close to the original arrangement as possible. Probes were placed underneath the deepest egg, in the core of the nest and on top of the uppermost egg (Fig. 2.4), and temperatures were recorded every fifteen minutes. Chicken wire was placed over each nest to protect the eggs from predation by monitor lizards, as described in Section 2.3.2.1.

At approximately two-thirds through incubation, two eggs from the centre of the nest were removed and opened. The embryos from these eggs were then killed, preserved and measured. Close to the estimated hatching date, the remaining eggs from each nest were removed, partially buried in moist sand in individual plastic containers, transported to the field camp and then left to hatch. Eggs were opened when, despite being left until well past the expected hatch date, they failed to hatch naturally. The hatch or open date was recorded and incubation period was calculated as the time between hatch or open date and the estimated lay date. Hatchlings were measured and the proportional development of the two embryos killed during incubation, per nest was calculated as follows:

$$\% \text{ development} = \frac{HWR_{emb}}{HWR_{hat}} * 100\ \%$$

where:

HWR_{emb} = HWR of embryo

HWR_{hat} = mean hatchling HWR for each nest.

2.4.2 Artificial incubation of eggs in cyclic thermal regimes

Seven fresh clutches were collected and air freighted to the University of Canberra, ACT. At least four viable eggs from each clutch were allocated to each of seven cyclic thermal incubation regimes shown in Table 2.1. The incubators were programmed to

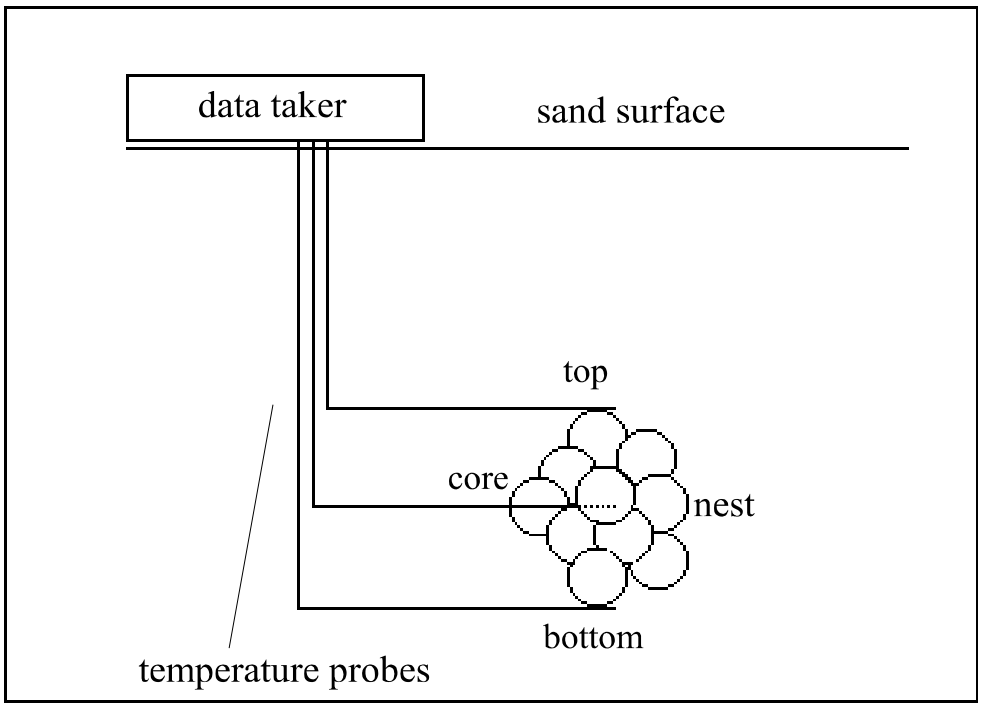


Figure 2.4. Schematic diagram illustrating the positions of temperature probes in a natural *Carettochelys insculpta* nest. The depth of the core probe was calculated by averaging the depths of the top and bottom probes.

Table 2.1. Thermal regimes used in the cyclic incubation experiments to test the Linear Developmental Model.

Regime number	Thermal regime (°C) (mean ± fluctuation)
1	24 ± 8
2	25 ± 7
3	26 ± 6
4	28 ± 4
5	29 ± 5
6	30 ± 6
7	30.5 ± 6.5

adjust temperature to each of six values at a set time each day resulting in the temperature following a sinusoidal regime. Eggs were placed in circular plastic buckets (inside diameter 21.2cm, inside depth 18.3cm), filled with moist vermiculite and loose fitting lids were set in place. Georges *et al.* (1994) performed similar cyclic temperature incubation experiments and the arrangement of eggs within buckets for the present study was based on their methods. Eggs were placed in a circular arrangement within their
containers such that they were an equal distance from each other and the edge of the bucket. Buckets were then placed in the centre of the designated incubator. By this arrangement, each egg was identically position with respect to the proximal sources and sinks of thermal energy generated by fluctuating temperatures in the incubators (Georges *et al.* 1994). A temperature probe was positioned immediately adjacent to the top of one egg in each incubator and readings were taken every ten minutes. Moisture levels in the buckets were maintained as described in Section 2.1.4.

Twice through incubation, two eggs from each regime were opened and the embryos were killed, preserved and measured. A third egg was opened if there appeared to be substantial developmental differences between the first two eggs. Developmental rate (proportional development per day) was calculated as follows:

$$\text{Developmental rate } (\%\text{HWR day}^{-1}) = \frac{HWR_2 - HWR_1}{HWR_{hat}} \div \Delta t * 100\ \%$$

where:

HWR_2 = mean HWR at 2nd opening

HWR_1 = mean HWR at 1st opening

Δt = time (number of days between openings)

HWR_{hat} = "population" hatchling HWR
mean = 0.3382 (standard error = 0.0009),
minimum = 0.3021, maximum = 0.3620
(based on 130 hatchlings from the entire study).

The estimates of "population" hatchling HWR were used in the calculation of developmental rate because no embryos from the clutches used in these experiments were left to develop to full term, and therefore no estimates of hatchling HWR from

these clutches were available. Developmental rate was calculated using the mean, minimum and maximum hatchling HWR estimates and reported accordingly (i.e. as means and ranges).

2.4.3 Analyses of temperature data and testing the Linear Development Model

The temperature data recorded from natural nests were processed as described in Section 2.1.6 and complete temperature traces from each nest were entered into the model. Temperatures recorded inside the cyclic incubators were examined to assess the extent of deviation from the nominal thermal regimes and the actual regimes were summarised and presented. At each cyclic regime, the proportion of development occurring on each day was expected to be the same because the thermal regimes were essentially the same each day (c.f. natural nests, which were subject to other influences). Therefore, representative subsets of the temperature data (i.e. periods containing no erroneous temperatures), rather than complete traces recorded in the cyclic incubators, were entered into the model.

The model is based on: the time to complete development at a particular reference temperature; the relationship between temperature and developmental rate (established from incubations at a range of constant temperatures); and the lower limit of temperature which supports development (T_0) (Georges 1989; 1994) (see Section 1.3.3). In this study, the average time for *C. insculpta* embryos to complete development (reach stage 26) at 30°C (established during the ageing work), the relationship between developmental rate and temperature, and T_0 (≅ 24.5°C) established Georges (unpublished data), were used in the model.

For each nest or cyclic regime, the model produced estimates of the cumulative proportional amount of development occurring on each day, based on the thermal conditions experienced. For the field nests, the proportional development predicted by the model was compared directly with the proportional development observed in nests, on the days eggs were opened. Prediction of the time to complete development was also evaluated by comparing the observed hatch or opening dates with the expected date for 100% development. For the cyclic incubation experiments, the predicted proportional development was converted to a developmental rate (% development day^{-1}) as follows:

$$\text{Developmental rate (\% development day}^{-1}\text{)} = \frac{\%DEV_2 - \%DEV_1}{\Delta t}$$

where:

$\%DEV_2$ = predicted % development on the final day of the period used as a thermal regime subset

$\%DEV_1$ = predicted % development on the first day of the period used as a thermal regime subset

Δt = time (number of days in the period used as a thermal regime subset).

Comparisons were then made between the observed (%HWR day^{-1}) and predicted (% development day^{-1}) developmental rates for each regime in the cyclic temperature incubation experiments.

The relationship (agreement) between observed (mean of the two embryos) and expected embryonic development was assessed by linear regression analyses with the line forced through the origin (i.e. the intercept was set at zero). On advice from a professional statistician, regression analyses involving the natural nests were inversely weighted by the error in estimated lay date (see Section 2.4.1), such that data from nests with higher errors contributed less to the analyses (A. Georges, pers. comm.). Weightings were assigned as follows: error = 0 days, weighting = 1; error = 0 – 1 days, weighting = 0.9; error = 1 – 2 days, weighting = 0.7; error = 2 – 3 days, weighting = 0.5 (A. Georges, pers. comm.). No weightings were used with the data from the cyclic temperature incubation experiments. The differences between slopes of the resultant regression lines and a slope of one were compared using standard Student's *t*-tests (Sokal and Rohlf 1995), and significant results indicated a significant difference between observed and expected embryonic development.

CHAPTER THREE

3. Results

3.1 Techniques for ageing embryos

Two approaches – candling and direct embryo examination, were investigated as methods for ageing *Carettochelys insculpta* embryos and both were found to be highly effective. Candling observations and measurements proved to be especially useful during the early to mid stages of development. Embryo characteristics and measurements also provided useful information for ageing *C. insculpta* embryos throughout the period of incubation assessed (mid to late development). Further details on the two ageing techniques are reported below.

3.1.1 Candling observations and measurements

3.1.1.1 Qualitative candling observations

Various features were visible during candling and many of these were associated with specific embryonic stages (Table 3.1). The opaque patch was the first characteristic observed, appearing about eighteen hours after laying. Development of blood islands and the haemodisc followed and these were visible when eggs were around seven to ten days old (stages 5 to 8) The outline of the allantois was visible by about day fifteen (stage 13) The embryo itself was visible from about ten days onwards, and the eye (pigmentation), limbs and carapace and were distinguishable by approximately 14, 18 and 25 days (stages 12, 14 and 17), respectively. Embryonic pigmentation was first visible on the tail at about 36 days (stage 20). Limb movement was apparent from about 32 days (stage 19) onwards and a “swimming” action was observed around day 41 (stage 21). Embryonic orientation changed during development. Early in development, the embryo was oriented such that it was laterally facing the yolk, but from 36 days (stage 20) onwards, the embryo was ventrally facing the yolk. Late in development, from approximately 52 days (stage 23) onwards, no specific embryonic characteristics were visible and overall size and evidence of yolk were the only features described.

Table 3.1. Qualitative features of normal development for *Carettochelys insculpta* embryos, based on candling eggs at 30°C. Further details on the development of quantitative attributes are given in Figures 3.1 to 3.5 and Table 3.2. Stage was verified by direct examination of embryos. Note: the thermosensitive period for *C. insculpta* is from stage 15 to 22.

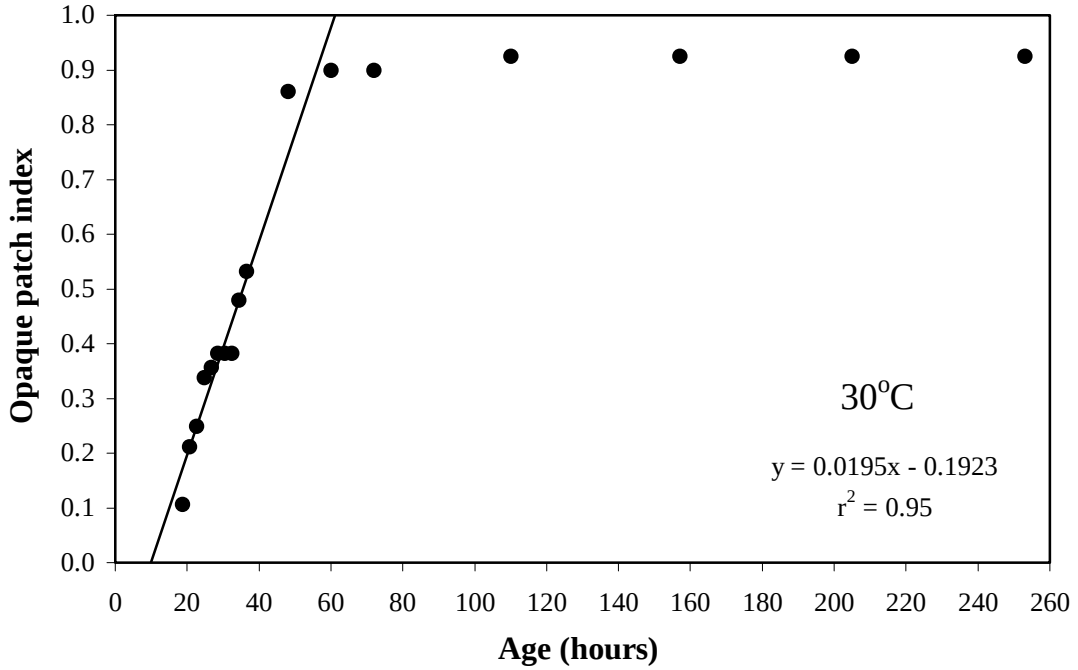
Stage	Age (days)	Candling notes
.	< 7	Horizontal layers visible when viewed laterally (embyronic fluids at the top and yolk at the bottom of eggs). A small opaque white patch is visible on the dorsal surface of the egg.
5 - 6*	7 - 8	A blood island is visible as a small pale pink spot on the dorsal surface of the egg.
7 - 8*	8 - 10	The blood island has developed further and now appears as a measureable haemodisc. The embryo appears as a partial "cleavage" (straight) line running radially across the haemodisc.
9 - 11*	10 - 14	The embryo appears curved and thicker laterally as development progresses. The embryo moves independently of the haemodisc when the egg is jiggled.
12	14 - 15	The head of the embryo is distinguishable and hence the anterior and posterior ends of the embryo can be differentiated and a crown-rump measurement is possible. The embryo is oriented with the dorso-ventral and anterior-posterior axes in the horizontal plane. The eye of the embryo may be visible as a dark spot.
13	15 - 18	The outline of the allantois is visible and its width is measureable.
14	18 - 20	Limb buds are distinguishable and the haemodisc is as large as the egg diameter.
15	20 - 22	*no new observations*
16	22 - 25	*no new observations*
17	25 - 29	The embryo carapace is distinguishable and its length is measureable.
18	29 - 32	*no new observations*
19	32 - 36	Limbs are moving and the width of the allantois is about equal to the egg diameter.
20	36 - 41	Embryo orientation has changed with its ventral side facing the yolk. The tail is visible and it appears pigmented.
21	41 - 46	A swimming action is observable and hind limb pigmentation is evident.
22	46 - 52	Early in this stage, fore limb pigmentation is evident. Late in this stage, most of the embryo appears pigmented and crown-rump length exceeds egg diameter and can no longer be measured reliably.
23	52 - 59	The embryo occupies approximately or just less than half the volume of the egg.
24	59 - 66	The embryo occupies between 50 and 70 % of the volume of the egg.
25	66 - 77	The embryo occupies about 70 - 80 % of the volume of the egg and the rest of the space remains yellowish (yolk is not fully internalised).
26	≥ 77	The embryo occupies more than 80 % of the volume of the egg and the remaining space appears whitish (yolk is fully internalised). Embryo is full term.

* No embryos were killed at these stages therefore the stages shown are estimates only.

3.1.1.2 Candling measurements

In general, there were strong significant relationships between the developmental indices calculated from candling measurements, and time. Each index monotonically increased with time (Figs 3.1 to 3.5), except embryo crown-rump (ECR) length, which initially decreased (Fig. 3.4). The opaque patch index increased linearly with time up to about 60 hours then remained approximately constant at about 0.95 of egg diameter, for eggs incubated at both 30°C and 34°C (30°C: $F = 189.3$, $df = 1, 10$, $P < 0.001$, $r^2 = 0.95$; 34°C: $F = 166.8$, $df = 1, 10$, $P < 0.001$, $r^2 = 0.94$) (Fig. 3.1). Relationships between the haemodisc index ($F = 425.2$, $df = 2, 10$, $P < 0.001$, $r^2 = 0.99$), ECR index ($F = 349.1$, $df = 2, 18$, $P < 0.001$, $r^2 = 0.97$) and embryo carapace length (ECL) index ($F = 230.0$, $df = 2, 16$, $P < 0.001$, $r^2 = 0.97$), and time were curvilinear (Figs 3.2, 3.4 and 3.5). The allantois index was related to time linearly but this relationship was notably weaker than those of the other candling indices ($F = 42.9$, $df = 1, 14$, $P < 0.001$, $r^2 = 0.75$) (Fig. 3.3).

As with any measurement, candling measurements were subject to error, exacerbated because certain features were sometimes difficult to distinguish. Such errors were generally minimised by restricting measurements to periods when features were well defined. Nevertheless, the allantois was consistently more difficult to distinguish than the other features. Of all the candling indices, the allantois index was least strongly related to time and the higher degree of error probably accounts for this weak relationship. Another potential source of error in candling measurements arises because two dimensional measurements were made from a three dimensional objects (e.g. the egg, the haemodisc, the embryo). Certain candling features were more likely to be affected by this problem than others. For example, the haemodisc was close to the surface of the egg and therefore measurements were not likely to be severely affected by the error caused by the vertical distance between the haemodisc and the callipers. On the other hand, the position of the embryo in relation to the egg shell was more variable and measurements were likely to be affected by the error associated with the vertical distance to the callipers. An embryo situated close to the centre of the egg would be subjected to greater distortion than an embryo positioned close to the egg shell.



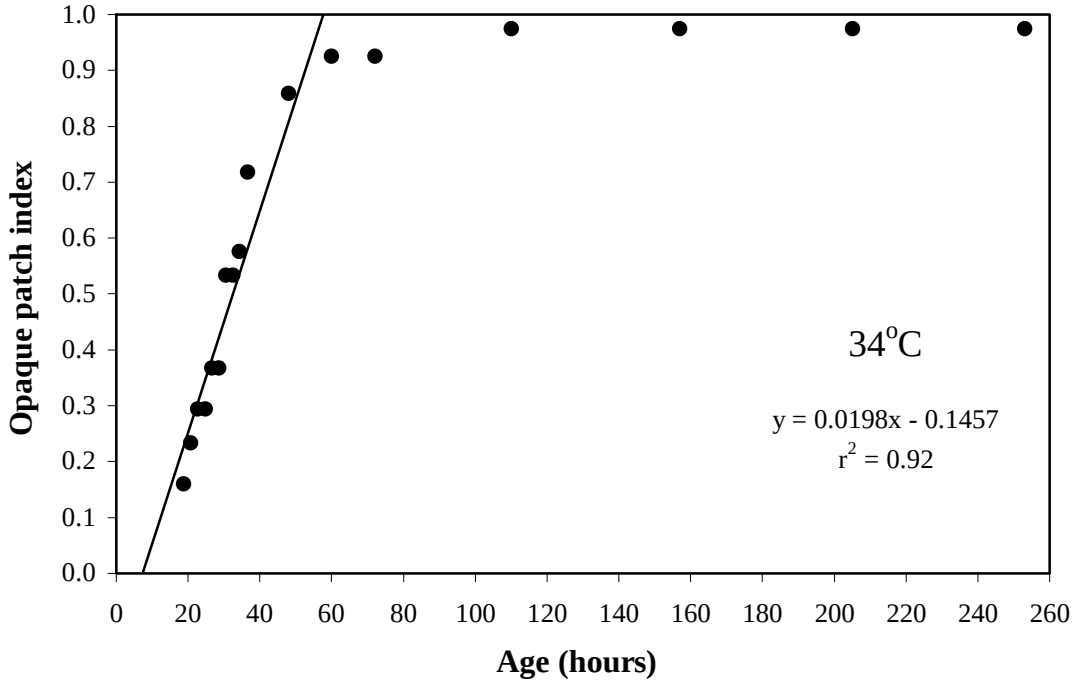


Figure 3.1. Relationship between opaque patch index and age for *Carettochelys insculpta* eggs incubated at 30°C and 34°C. The regression equations and r^2 are presented for each incubation treatment. Data points are means. Note: data from 72 hours onwards were excluded from the regression; regression was weighted for sample size (n = 2).

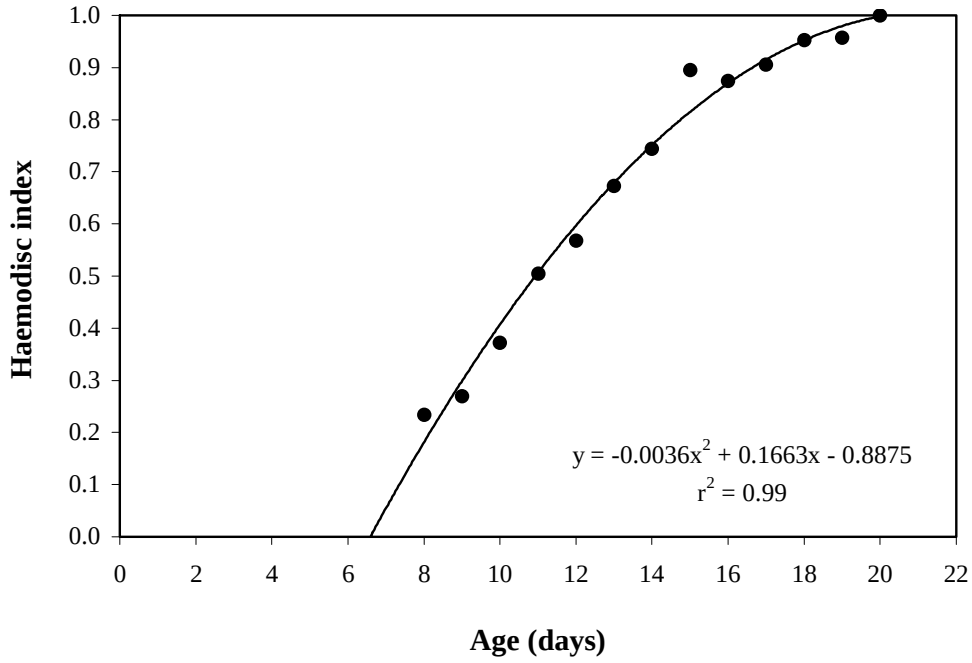


Figure 3.2. Relationship between the haemodisc index and age for *Carettochelys insculpta* eggs incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: regression was weighted for sample size (n = 1 - 33).

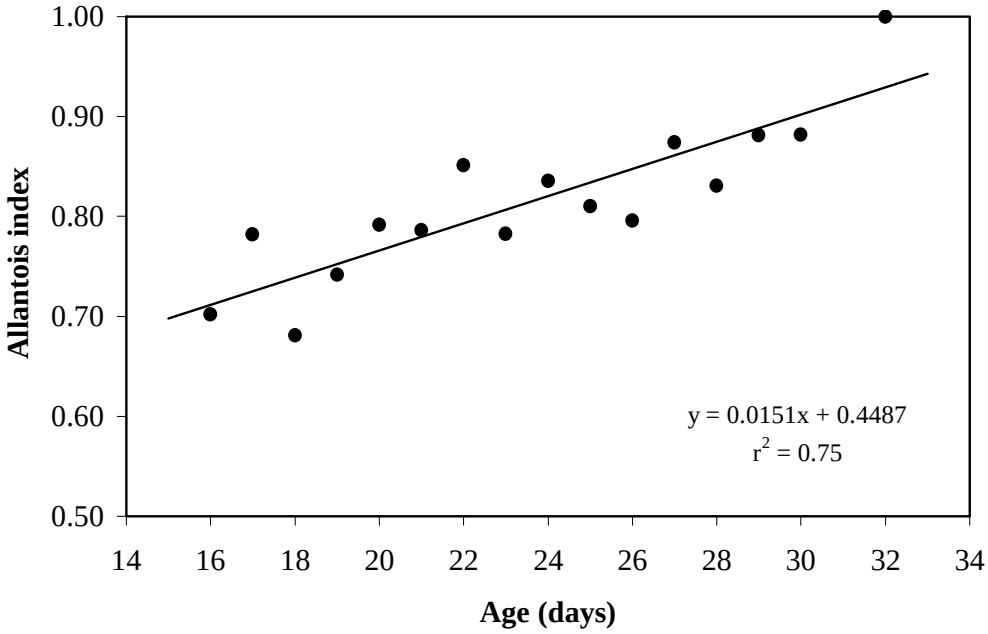


Figure 3.3. Relationship between the allantois index and age for *Carettochelys insculpta* eggs incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: regression was weighted for sample size (n = 1 - 12).

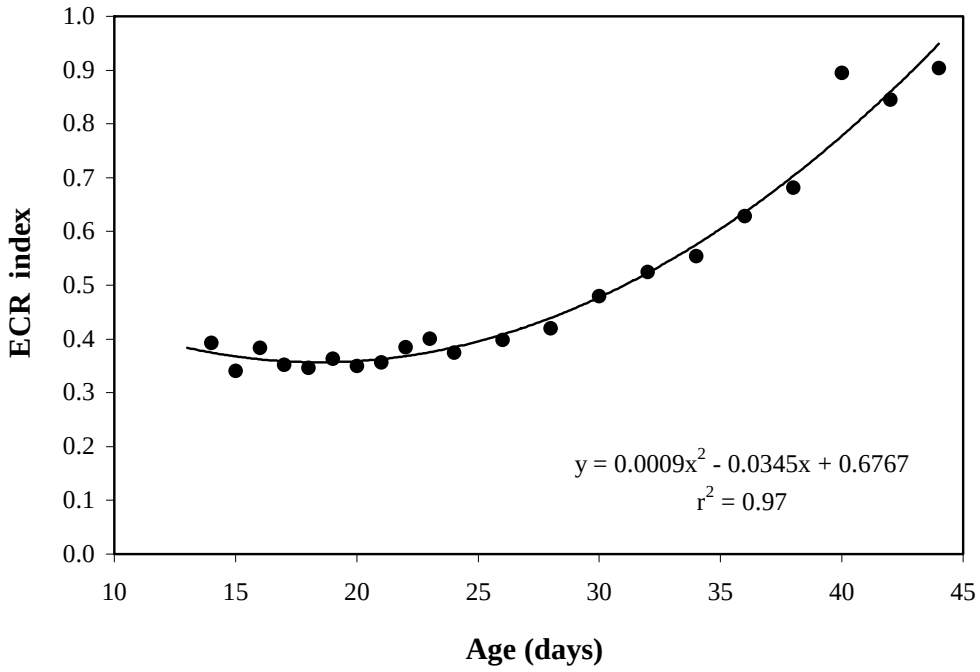


Figure 3.4. Relationship between the embryo crown-rump (ECR) index and age for *Carettochelys insculpta* eggs incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: regression was weighted for sample size (n = 1 - 16).

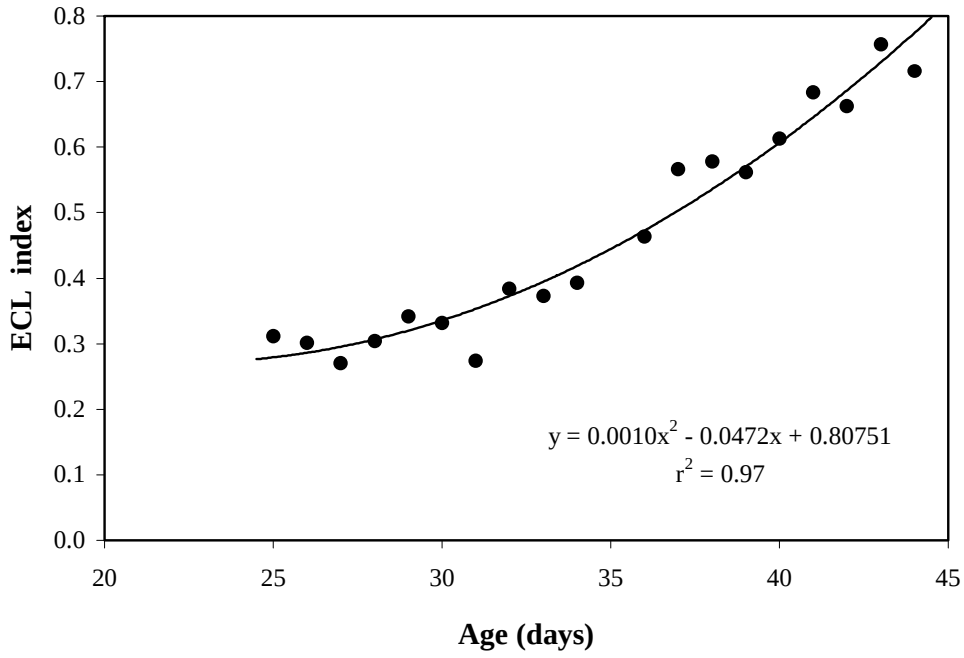


Figure 3.5. Relationship between the embryo carapace length (ECL) index and age for *Carettochelys insculpta* eggs incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: regression was weighted for sample size (n = 1 - 13).

Table 3.2 shows the range of opaque patch, haemodisc, ECR and ECL indices expected throughout development for eggs incubated at 30°C, based on the regression equations. The allantois index was not included in Table 3.2 because of its weak predictive relationship with time ($r^2 = 0.75$) (Fig. 3.3). Collectively, the candling indices in Table 3.2 covered most of the incubation time, with the exception of two periods: 2.5 to 8 days (around stage 6), and greater than 52 days (stage 23 onwards).

The effect of incubation temperature was assessed only for the development of the opaque patch and haemodisc. Incubation temperature had no effect on the rate of increase in the opaque patch index (ANCOVA: $F = 0.12$, $df = 1, 43$, $P = 0.41$) (Fig. 3.1). On the other hand, incubation temperature had a significant positive effect on the rate of increase in the haemodisc index (ANCOVA: $F = 12.8$, $df = 3, 42$, $P < 0.001$) (Fig. 3.6). Typically, haemodisc index after a given time period of incubation was greater at higher temperatures (Fig. 3.6), however the magnitude of this difference varied with time (i.e. there was a significant interaction between temperature and time: $F = 13.7$, $df = 3, 42$, $P < 0.001$). In general, eggs incubated at the warmest temperatures (32°C and 34°C) had a rate of increase of the haemodisc index that was similar, but notable faster than for eggs incubated at 28°C (Fig 3.6). The haemodisc index reached its maximum by fifteen days, for eggs incubated at 32°C and 34°C, compared with 32 days, for eggs incubated at 28°C (Fig 3.6).

3.1.2 Embryonic characteristics and measurements

Carettochelys insculpta embryos from stage 12 to 26 were described and measured (Table 3.3, Figs 3.7 to 3.13). Descriptions from the embryonic series for *Chelydra serpentina* (Yntema 1968) formed the basis of the *Carettochelys insculpta* descriptions. Only minor differences between *Carettochelys insculpta* and *Chelydra serpentina* embryos were observed for stages 12 to 17, and these were mainly in the development of the limbs and carapace (Table 3.3 and Figs 3.7 to 3.8). However, *Carettochelys insculpta* embryos of stage 18 or older differed in many ways from *Chelydra serpentina*, especially in the development of the digital plates, pigmentation and cranial features (Table 3.3 and Figs 3.9 to 3.13). In comparison to *Chelydra serpentina*, *Carettochelys insculpta* embryos had only two claws on each limb, generally paler (grey) pigmentation,

Table 3.2. Quantitative measurements of normal development for *Carettochelys insculpta* embryos based on candling eggs incubated at 30°C. The range of each index for each age range was predicted using the equations obtained from regression analyses. For explanation of measurements refer to section 2.2.1, in materials and methods. Stage was verified by direct examination of embryos. Note: the thermosensitive period for *C. insculpta* is from stage 15 to 22.

Stage	Age	Opaque patch index	Haemodisc index	Embryo crown-rump length (ECR) index	Embryo carapace length (ECL) index
	0 - 18 h	≤ 0.16			
.	18 - 24 h	0.16 - 0.28	.	.	.
.	24 - 30 h	0.28 - 0.39	.	.	.
.	30 - 36 h	0.39 - 0.51	.	.	.
.	36 - 42 h	0.51 - 0.63	.	.	.
.	42 - 48 h	0.63 - 0.74	.	.	.
.	48 - 60 h	0.74 - 0.98	.	.	.
≤ 6*	2.5 - 8 d	≥ 0.98	.	.	.
7 - 8*	8 - 10 d	.	0.21 - 0.42	.	.
9 - 11*	10 - 14 d	.	0.42 - 0.74	.	.
12	14 - 15 d	.	0.74 - 0.80	0.37 - 0.36	.
13	15 - 18 d	.	0.80 - 0.94	0.36 - 0.35	.
14	18 - 20 d	.	0.94 - 1.00	0.35	.
15	20 - 22 d	.	≥ 1.00	0.35	.
16	22 - 25 d	.	.	0.35 - 0.38	.
17	25 - 29 d	.	.	0.38 - 0.43	0.25 - 0.28
18	29 - 32 d	.	.	0.43 - 0.49	0.28 - 0.32
19	32 - 36 d	.	.	0.49 - 0.60	0.32 - 0.40
20	36 - 41 d	.	.	0.60 - 0.78	0.40 - 0.55
21	41 - 46 d	.	.	0.78 - 0.99	0.55 - 0.75
22	46 - 52 d	.	.	≥ 0.99	≥ 0.75
23	52 - 59 d	.	.	.	
24	59 - 66 d	.	.	.	.
25	66 - 77 d	.	.	.	.
26	≥ 77 d	.	.	.	.

* No embryos were killed at these stages therefore the stages shown are estimates only.

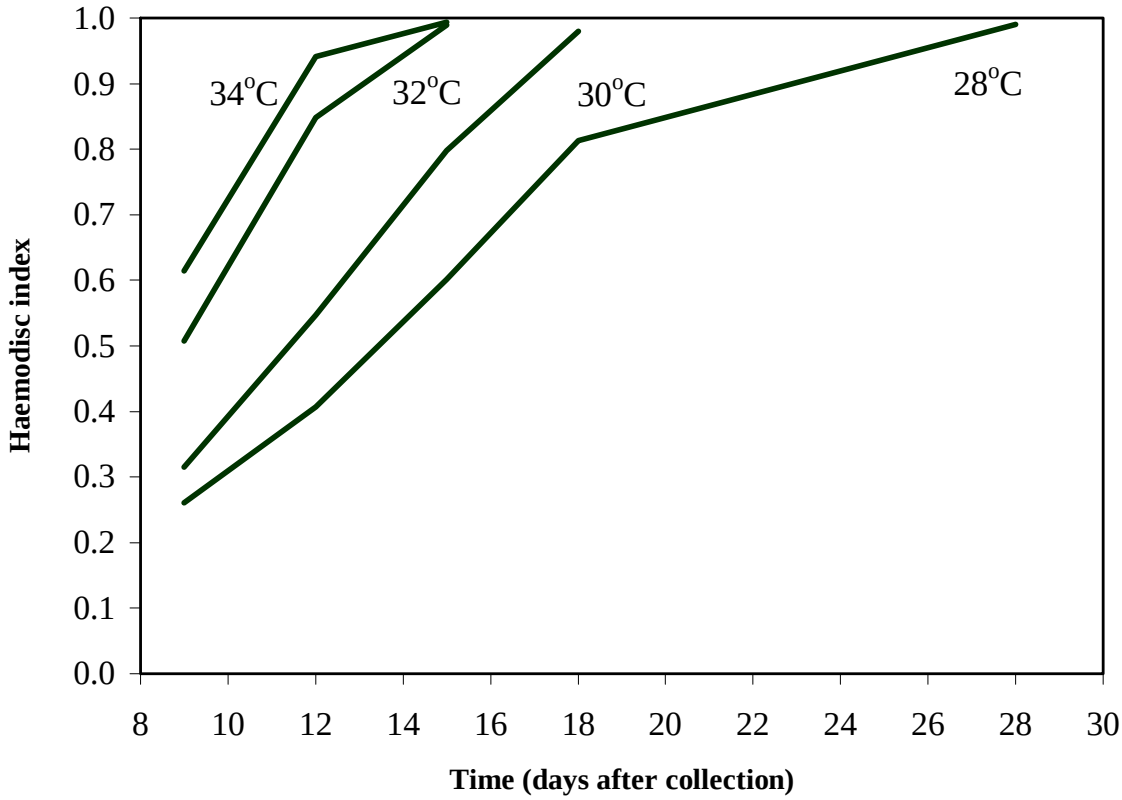


Figure 3.6. Haemodisc index versus time for *Carettochelys insculpta* eggs incubated at 28°C, 30°C, 32°C and 34°C. Data points are means; sample size: n = 1 - 3.

slower eyelid development and a fleshy proboscis (Table 3.3 and Figs 3.9 to 3.13). Another notable difference between the species was in the carapace, as *Carettochelys insculpta* embryos had a soft, skin-covered carapace compared with the rigid scutes which are described for *Chelydra serpentina* (Figs 3.9 to 3.13). Taking into account these morphological differences, *Carettochelys insculpta* embryos could be successfully staged using a developmental series based on that of Yntema (1968).

Embryonic size, age and stage were found to be closely related (Figs 3.14 to 3.16). Embryonic size (head width as a ratio of egg diameter – HWR) increased linearly with time, up to about 60 days (stage 24), when it reached a maximum of about 0.33 ($F = 150.5$, $df = 1, 15$, $P < 0.001$, $r^2 = 0.91$) (Fig. 3.14). From 60 days onwards, HWR remained approximately constant at its maximum (Fig. 3.14). By coincidence, stage being ordinal only, was found to increase linearly with HWR, up to the maximum HWR (about 0.33), after which stage continued to 26 ($r^2 = 0.95$) (Fig. 3.8). The relationship between stage and age could be approximated by a log-linear relationship ($r^2 = 0.97$) (Fig. 3.16).

Embryonic size, expressed as absolute (HWR) and relative size (%HWR – of maximum HWR), was calculated from the HWR-age regression model for stages 12 to 26 and included in the table of embryo descriptions (Table 3.3).

3.1.3 Correction of 30°C ageing work for different incubation temperatures

In the present study, ageing was primarily based on morphological features and the association between candling or embryo attributes and particular stages would be the same regardless of incubation temperature. Consequently, the main adjustment required to render the 30°C ageing work applicable to a range of incubation temperatures was correction for development time.

The relationship between developmental rate and temperature was used to calculate developmental rate coefficients (DRC) in which developmental rate for a range of temperatures was expressed as a function of developmental rate at 30°C (i.e. DRC for 30°C = 1; DRC for < 30°C = < 1; DRC for > 30°C = > 1). Developmental rates were

Figure 3.7. Lateral (right) views of *Carettochelys insculpta* embryos for stages 12, 13, 14 and 15.

Figure 3.8. Lateral (right) views of the whole embryo and dorsal views of the right forelimb of *Carettochelys insculpta* embryos for stages 16 and 17.

Figure 3.9. Lateral (right) views of the whole embryo, anterior views of the head and dorsal views of the right forelimb of *Carettochelys insculpta* embryos for stages 18 and 19.

Figure 3.10. Lateral (right) view of the whole embryo, anterior/ventral view of the head and anterior view of the right forelimb of *Carettochelys insculpta* embryos for stage 20; and dorsal view of the whole embryo, lateral (right) view of the head and dorsal view of the right forelimb of *Carettochelys insculpta* embryos for stage 21.

Figure 3.11. Lateral (right) views of the whole embryo and head, and dorsal views of the right forelimb of *Carettochelys insculpta* embryos for stages 22 and 23.

Figure 3.12. Lateral (right) views of the whole embryo and head, and anterior/dorsal views of the right forelimb of *Carettochelys insculpta* embryos for stages 24 and 25.

Figure 3.13. Lateral (right) view of the whole hatchling and head, and dorsal view of the right forelimb of a *Carettochelys insculpta* hatchling (stage 26).

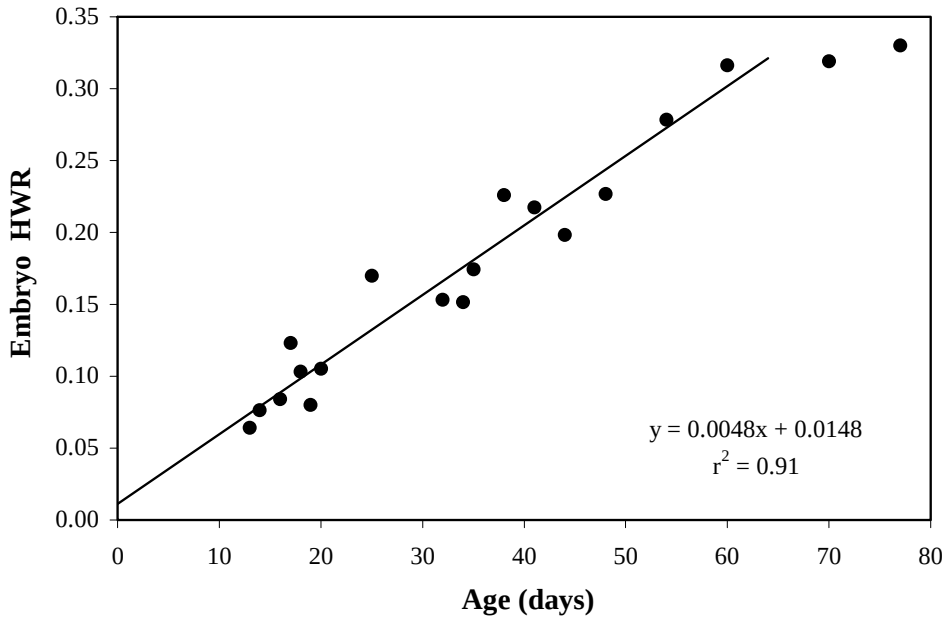


Figure 3.14. Relationship between head width ratio (HWR) and age for *Carettochelys insculpta* embryos incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: embryos at 70 and 77 days of age were excluded from the regression; regression was weighted for sample size (n = 1 - 3).

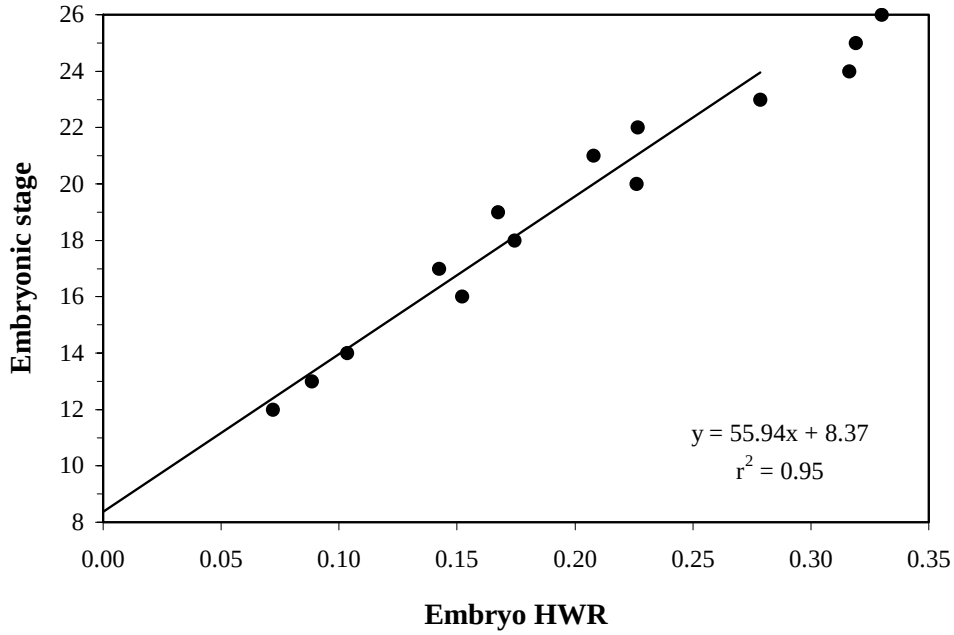


Figure 3.15. Relationship between embryonic stage and head width ratio (HWR) for *Carettochelys insculpta* embryos incubated at 30°C. The regression equation and r^2 are presented. Note: embryos at stage 24 to 26 were excluded from the regression.

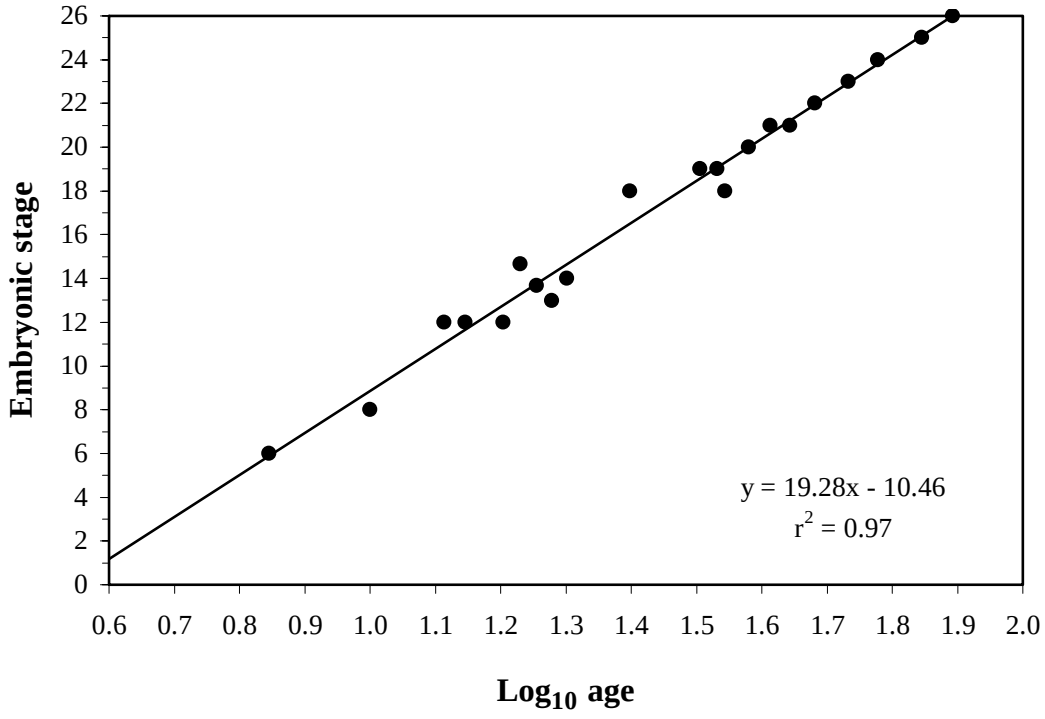


Figure 3.16. Relationship between embryonic stage and age (log10-transformed) for *Carettochelys insculpta* embryos incubated at 30°C. The regression equation and r^2 are presented. Data points are means. Note: regression was weighted for sample size (n = 1 - 3).

estimated from the predictive relationship between developmental rate and incubation temperature derived by Georges (unpublished data) (see Fig. 1.2, Section 1.3.3) for a range of temperatures. DRC were calculated as follows:

DRC (T °C) = developmental rate (T °C) ÷ developmental rate (30°C).

Developmental rates and DRC for a selection of incubation temperatures are shown in Table 3.4. The development time (age) for embryos incubated at 30°C could therefore be corrected to be applicable to a range of incubation temperatures using the DRC, as follows:

Age (T °C) = Age (30°C) ÷ DRC.

Table 3.5 shows the corrected timing of developmental stages for a range of incubation temperatures.

3.2 Thermal gradients and *Carettochelys insculpta* embryonic development

3.2.1 Thermal gradients in the soil profile

The average temperature regime for a 24 hour period, based on the temperatures recorded from April to November 1997, at various soil depths is shown in Figure 3.17. The thermal regime at the surface at the site of the soil profile was characterised by warmer (as high as 45.2°C), but also cooler (as low as 20.2°C) temperatures, than those recorded at the other depths (Fig. 3.17). Temperatures were therefore highly variable at the surface (range = 25°C) but this variability was damped with increasing depth. Temperature varied by only 0.2°C at 50cm (Fig. 3.17). There were significant log-linear relationships between standard deviation and depth ($F = 118.4$, $df = 1, 3$, $P < 0.005$, $r^2 = 0.97$), maximum temperature (°C above mean) and depth ($F = 82.4$, $df = 1, 3$, $P < 0.005$, $r^2 = 0.96$) and minimum temperature (°C below mean) and depth ($F = 205.9$, $df = 1, 3$, $P < 0.005$, $r^2 = 0.99$) (Fig. 3.18). A substantial time lag was also evident. The time

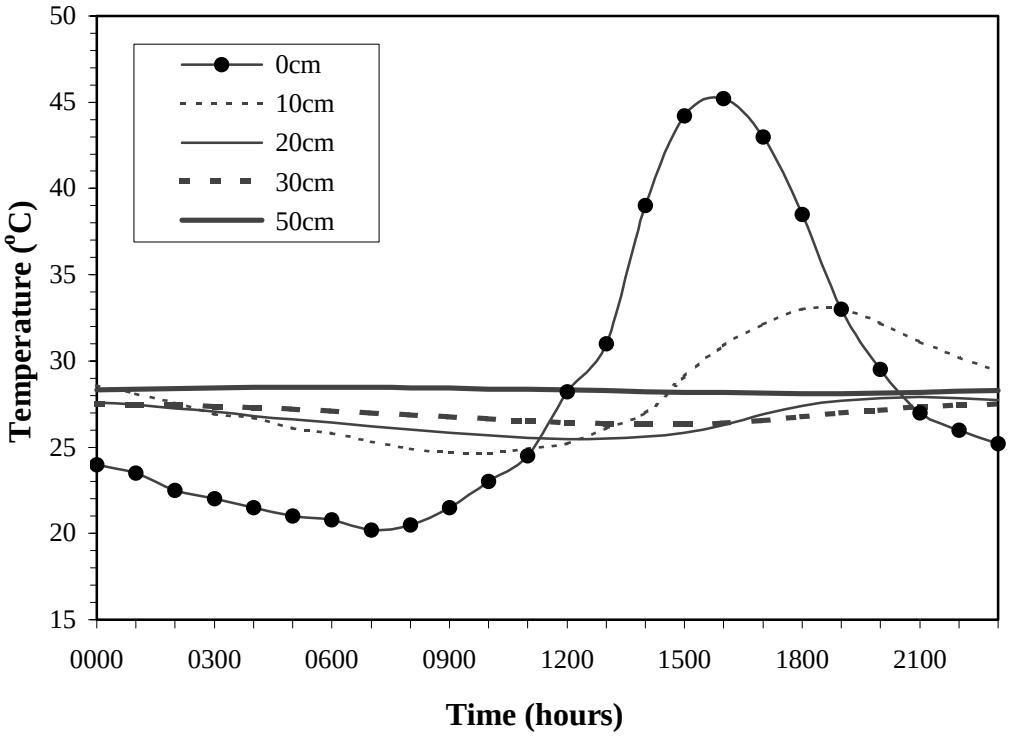


Figure 3.17. The average temperature cycle over 24 hours at five soil depths (recorded from April to November 1997).

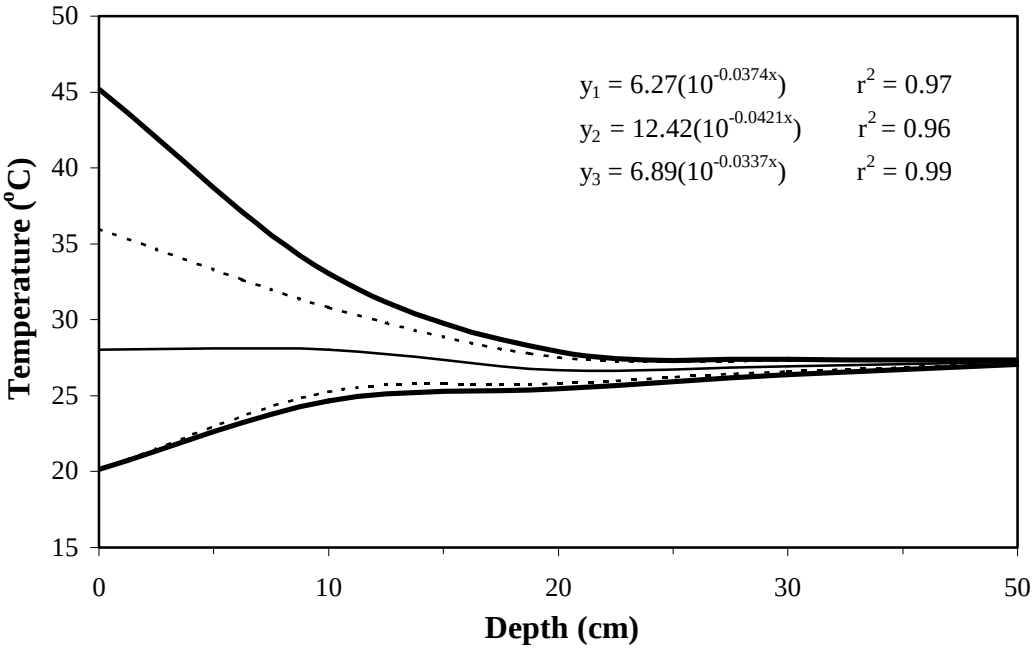


Figure 3.18. Relationship between general temperature characteristics and soil depth. The mean (solid line), standard deviation (dashed lines) and range (bold lines) are presented. Regression equations and r^2 are presented for the following relationships: standard deviation (y_1), maximum temperature (^oC above mean) (y_2) and minimum temperature (^oC below mean) (y_3), versus depth (x).

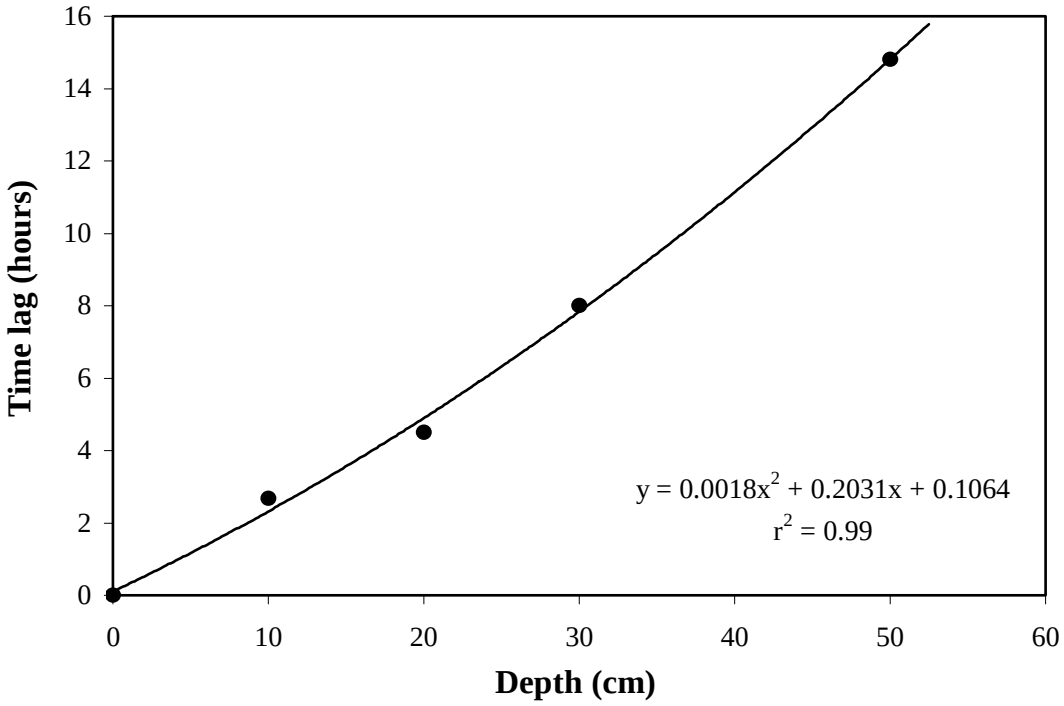


Figure 3.19. Relationship between temperature time lag and depth. Time lag is the difference between times of maximum temperature at the surface and other depths.

of maximum temperature occurred progressively later in the day at greater depths (Fig. 3.18). The relationship between time lag and depth was curvilinear ($F = 413.1$, $df = 2, 2$, $P < 0.005$, $r^2 = 0.99$) (Fig. 3.19). These log-linear relationships and lags were consistent with expectations based on models of thermal diffusion through homogenous soils (Carson and Moses 1963; Hanks 1992). While general estimates of daily variation in temperature decreased dramatically with depth, mean temperature varied little with depth (Figs 3.17 and 3.18). Minor variation in mean temperature with depth was also consistent with models of thermal diffusion through soils (Carson and Moses 1963; Hanks 1992) and will be shown to have important consequences for the interpretation of the effects of thermal gradients on embryonic development.

3.2.2 Thermal gradients and embryonic development

3.2.2.1 Pilot depth experiment

Temperature regimes were generally found to be dissimilar across the five depths of the pilot depth experiment (Fig. 3.20). Comparisons among depths revealed that daily mean temperatures were significantly different in all cases and daily mean temperatures were generally warmer closer to the surface (Fig. 3.21). However, the average difference in daily mean temperature between depths was small, no more than 0.8°C (Fig. 3.21). The maximum difference in mean daily temperature, between the shallowest and deepest probes (12cm and 44cm) was only 2.6°C (Fig. 3.21).

In contrast, there was a marked difference between the depths in the magnitude of the daily range of temperatures experienced, with temperatures fluctuating about 4°C and less than 0.5°C at 12cm and 44cm, respectively (Fig. 3.22). Daily standard deviation was 2.19°C, on average at 12cm (range: 1.51 to 3.65°C), whereas at 44cm, average standard deviation was only 0.14°C (range: 0.08 to 0.25°C) (Fig. 3.22). The overall thermal regimes at 36cm and 44cm were quite similar (average difference in daily mean temperature 0.1°C; average daily temperature standard deviation 0.27°C and 0.14°C, at 36cm and 44cm, respectively) (Figs 3.21 and 3.22).

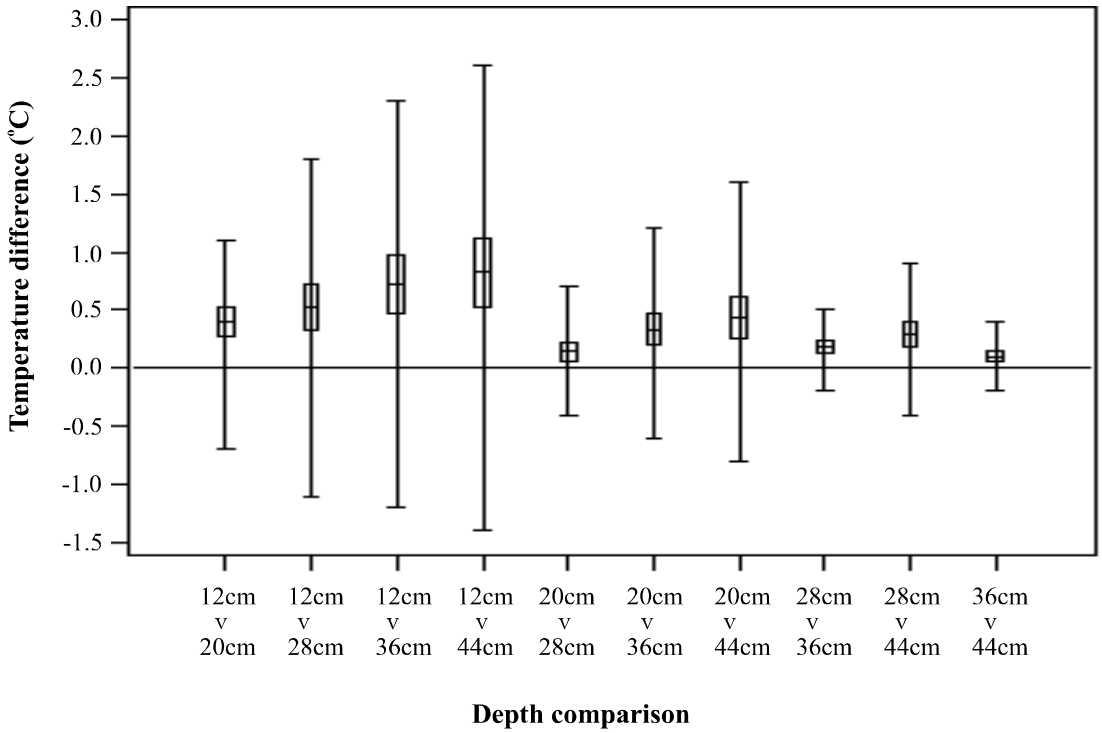


Figure 3.21. Difference in daily mean temperature between each depth of the pilot depth experiment, throughout incubation. Vertical bars represent the range, boxes represent two standard errors (~ 95% confidence interval) of the mean (horizontal lines).

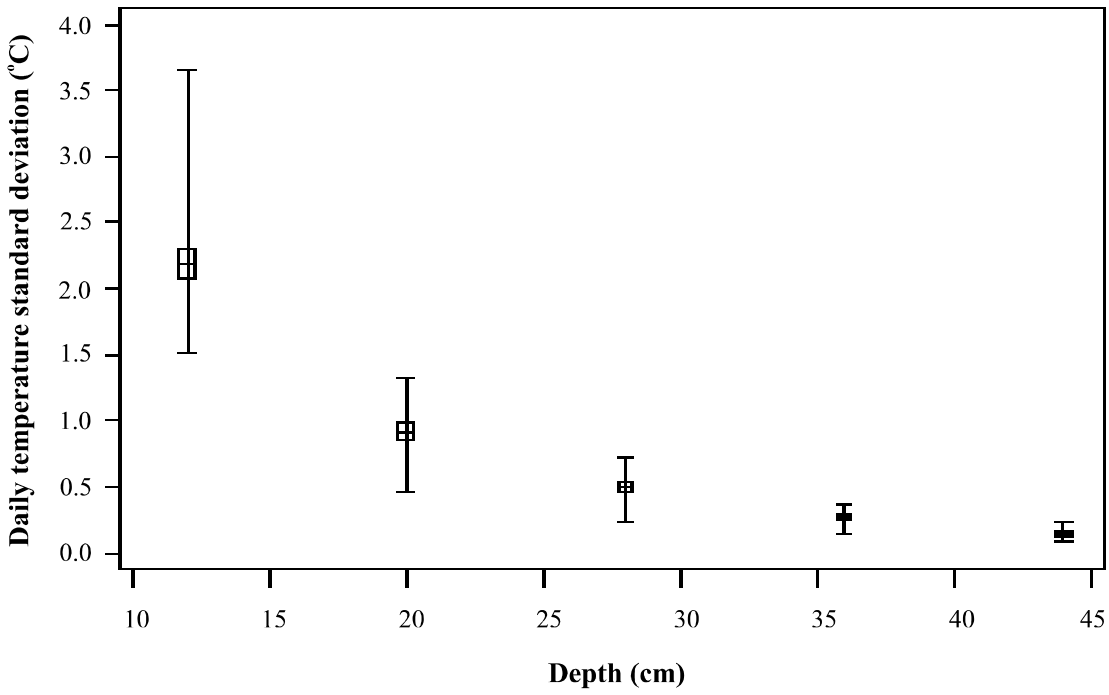


Figure 3.22. Daily temperature standard deviation at each depth of the pilot depth experiment, throughout incubation. Vertical bars represent the range, boxes represent two standard errors (~ 95% confidence interval) of the mean (horizontal lines).

Rate of embryonic development appeared to be unaffected by the differences in thermal regime associated with depth. Embryo size (HWR), which was used as the index of development, was not significantly different between depths on either opening occasion (1^{st} opening: $F = 1.5$, $df = 4, 6$, $P = 0.32$; 2^{nd} opening: $F = 1.3$, $df = 4, 6$, $P = 0.37$) (Fig. 3.23). However, there were significant differences in embryonic size between clutches (1^{st} opening: $F = 16.9$, $df = 2, 6$, $P < 0.01$; 2^{nd} opening: $F= 45.3$, $df = 2, 6$, $P < 0.001$) (Fig. 3.23). The difference between clutches was expected because, at the start of the experiment, eggs were at various stages of early development. Furthermore, depth had no effect on incubation period as eggs from the same clutch hatched on the same day, regardless of incubation depth (incubation period was 69 to 82 days for the remaining 41 eggs from nine different clutches). Mortality was low and similar at all depths (one deaths at 12cm, three deaths at 20cm and 28cm, and two deaths at 36cm and 44cm).

3.2.2.2 Final depth experiments

Despite differences in the arrangement of eggs between the pilot and the final depth experiments (eggs were incubated across a narrower range of depths in the final experiments), the overall trend in thermal characteristics was similar – thermal regimes varied with depth (Fig. 3.24). As for the pilot study, multiple comparisons between depths showed that daily mean temperatures were significantly different in all cases and daily mean temperatures were generally warmer closer to the surface (Fig. 3.25). The average difference in daily mean temperature between depths in Experiment 1 was small, as in the pilot experiment, no more than 0.8°C (Fig. 3.25). In Experiment 2, however, the average difference in daily mean temperatures between depths was slightly more pronounced and was as high as 1.7°C (Fig. 3.25). Also in Experiment 2, daily mean temperatures appeared to warmer at 30cm than at 22cm, as indicated by the negative differences obtained during this comparison (Fig. 3.25). As the two experiments were situated in close proximity, had similar localised geographical attributes (i.e. aspect, slope, height and distance from the water) and were run simultaneously, the most plausible explanation for this anomaly was temperature probe malfunction. However, the probes were calibrated at both the start and end of the study – and no such deviations were detected. The data for all probes were therefore included in the analysis. As in the

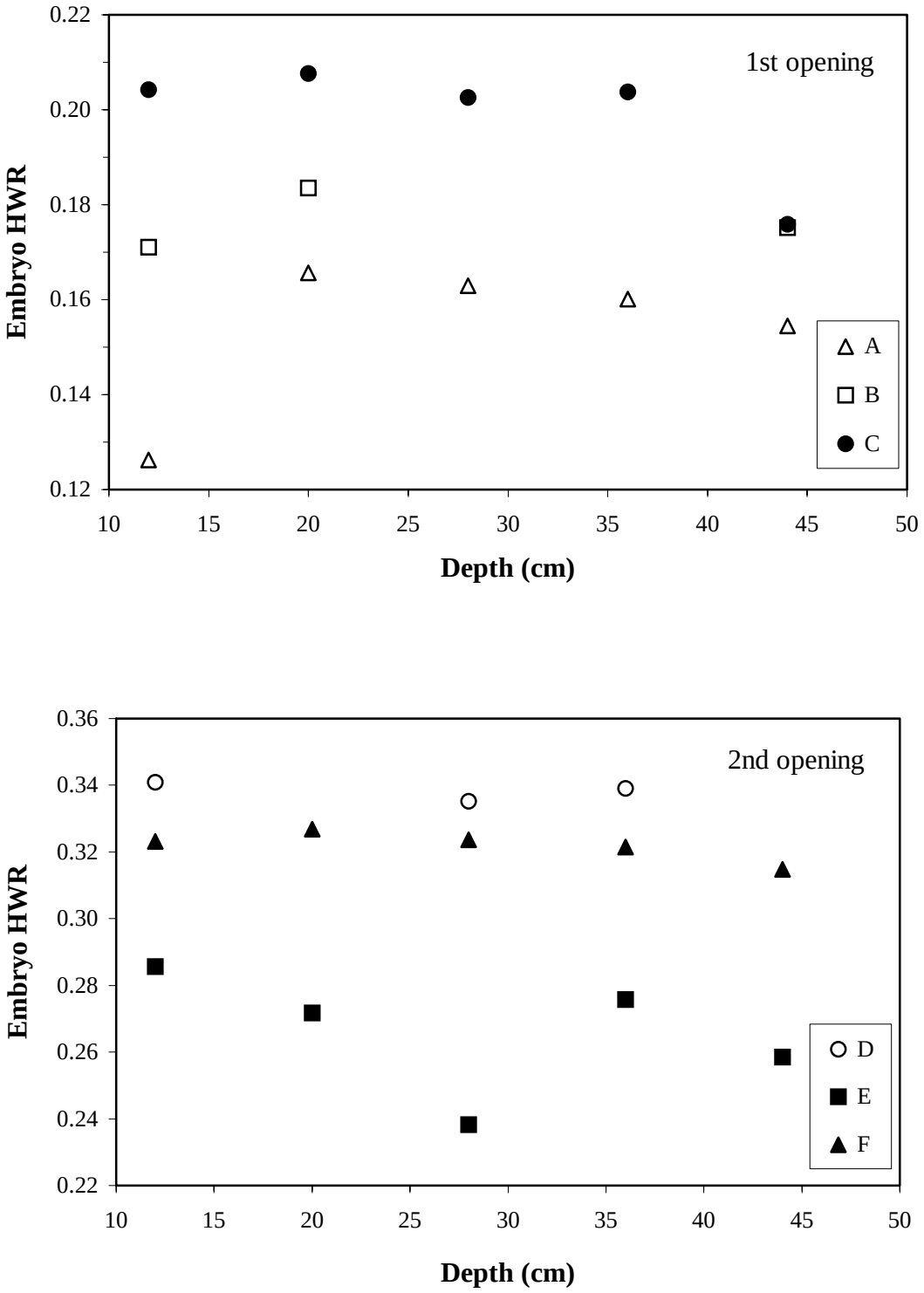
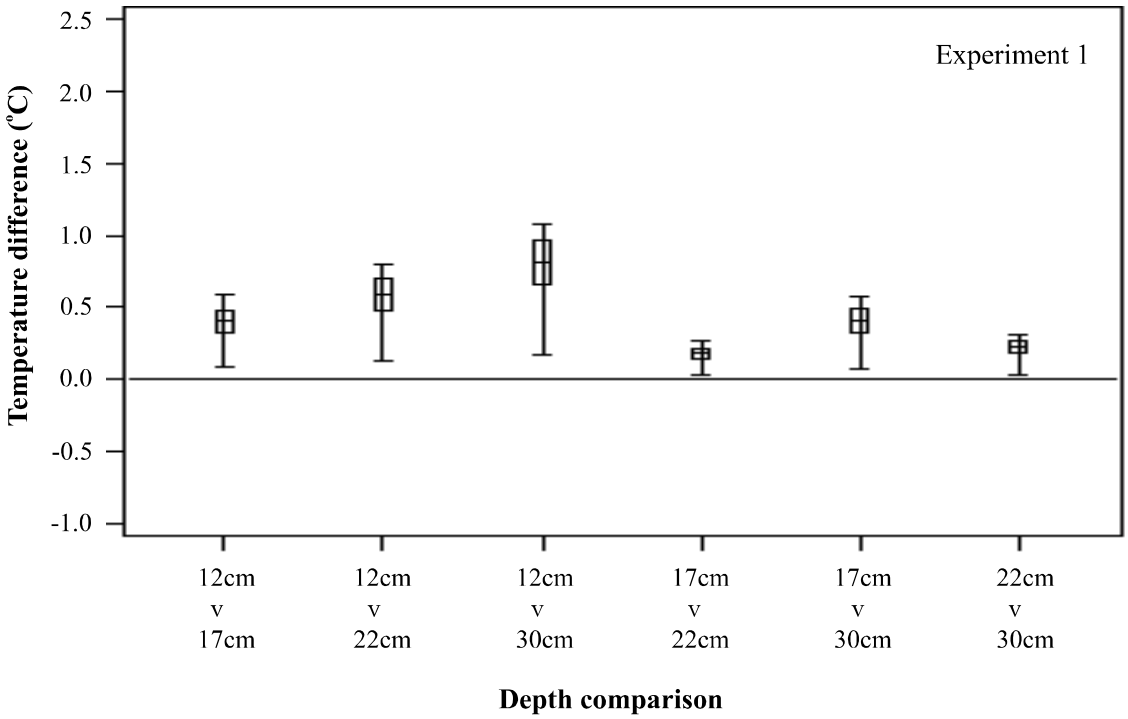


Figure 3.23. Embryo head width ratio (HWR) as an index of development for *Carettochelys insculpta* embryos incubated at five depths during the pilot depth experiment. Eggs from clutches A, B and C, and D, E, and F were opened on the first and second opening dates, respectively.



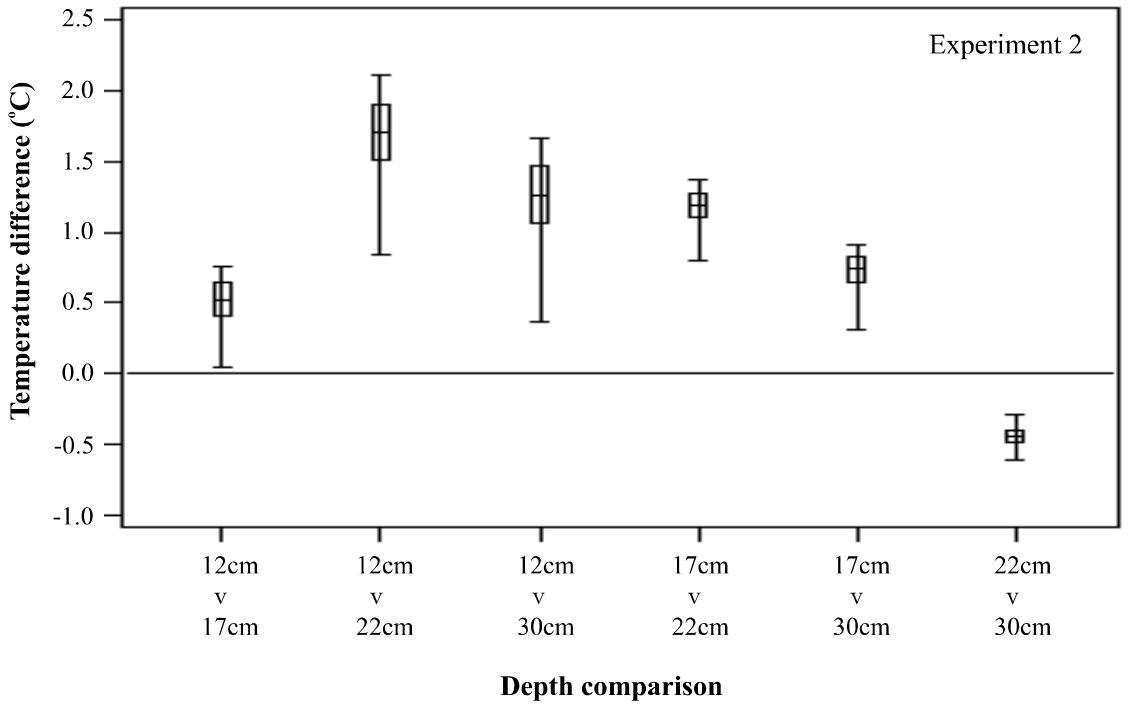
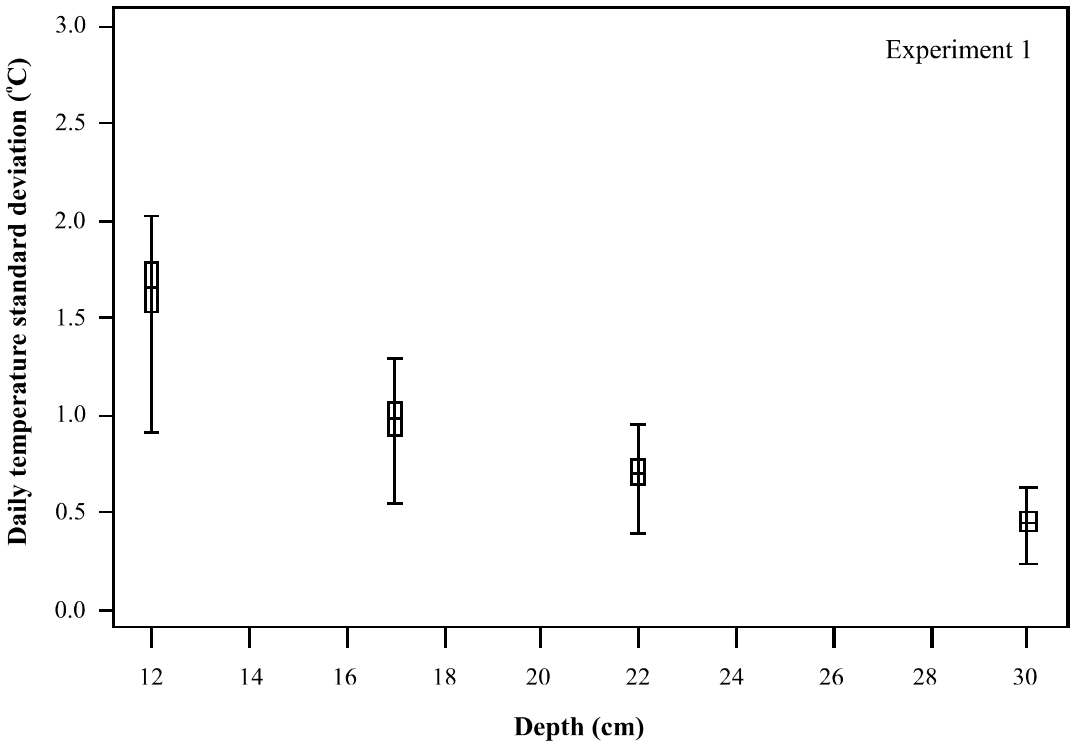


Figure 3.25. Difference in daily mean temperature between each depth of the final depth experiments, between egg opening times. Vertical bars represent the range, boxes represent two standard errors (~ 95% confidence interval) of the mean (horizontal lines).

pilot experiment, maximum differences in mean daily temperature were generally found between the depth extremes and were 1.1°C (12cm versus 30cm) in Experiment 1 and 2.1°C (12cm versus 22cm) in Experiment 2 (Fig. 3.25).

Daily fluctuations in temperature were reduced at greater depths, although the differences in variability were not as pronounced in these experiments as in the pilot – a direct consequence of the lower range of depths in the final experiments (Fig. 3.26). Daily standard deviation at 12cm was 1.65°C on average (range: 0.91°C to 2.02°C), in Experiment 1; and 2.21°C on average (range: 1.10°C to 2.55°C), in Experiment 2 (Fig. 3.26). At 30cm, daily temperature standard deviation was 0.45°C on average (range: 0.24°C to 0.62°C), in Experiment 1; and 0.64°C on average (range: 0.34°C to 0.89°C), in Experiment 2 (Fig. 3.26).

Embryonic developmental rates (HWR day^{-1}) were not affected by the differences in thermal regime associated with depth (Fig. 3.27). Developmental rate appeared to decrease with depth, in Experiment 1, however there was no significant difference in developmental rate between depths in either experiment (Experiment 1: $F = 1.5$, $df = 3, 3$, $P = 0.37$; Experiment 2: $F = 0.2$, $df = 1, 2$, $P = 0.92$) (Fig. 3.27). Also, there was no significant difference in developmental rate between clutches (Experiment 1: $F = 1.6$, $df = 1, 3$, $P = 0.30$; Experiment 2: $F = 0.02$, $df = 1, 2$, $P = 0.90$) (Fig. 3.27). Moreover, the surplus eggs that were left to incubate to completion and tested for hatching response at two day intervals hatched on the same day, regardless of clutch membership or incubation depth (incubation period was 57 days for the remaining 18 eggs from 5 clutches). This result reflected the greater care taken to ensure that clutches were at the same early stage of development at commencement of the final experiments. Embryo mortality in the final experiments was very low and there was no trend associated with depth (Experiment 1: no mortality; Experiment 2: one death at 12cm, 17cm and 30cm, no mortality at 22cm).



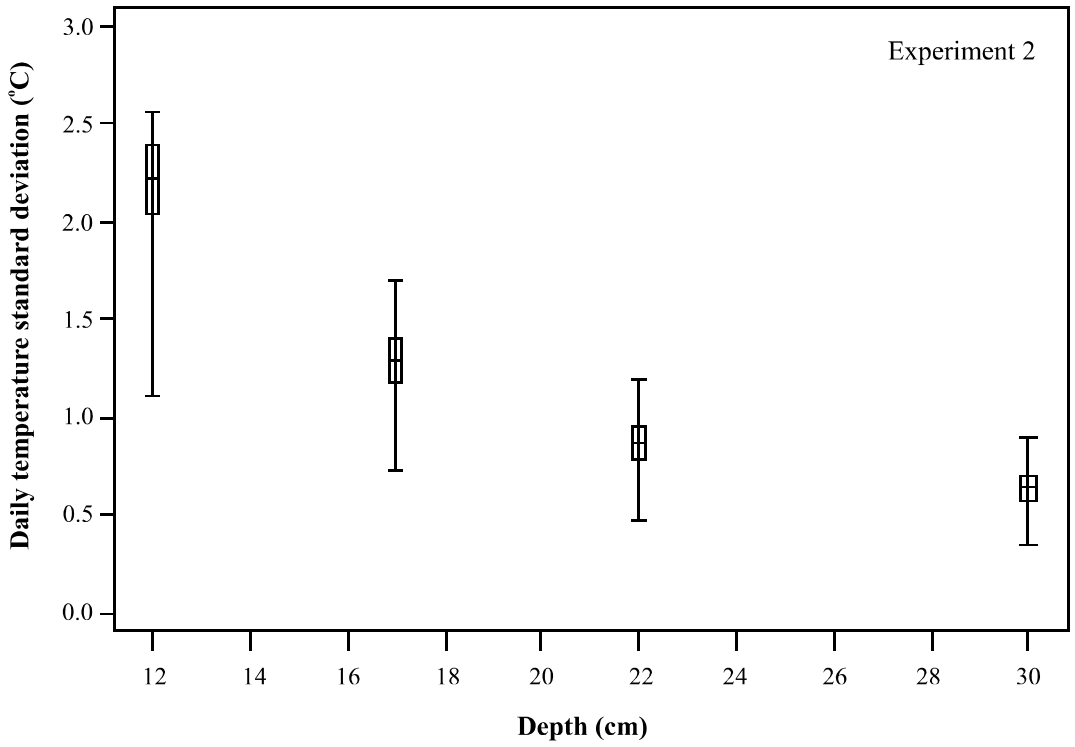


Figure 3.26. Daily temperature standard deviation at each depth of the final depth experiments, between egg opening times. Vertical bars represent the range, boxes represent two standard errors (~ 95% confidence interval) of the mean (horizontal lines).

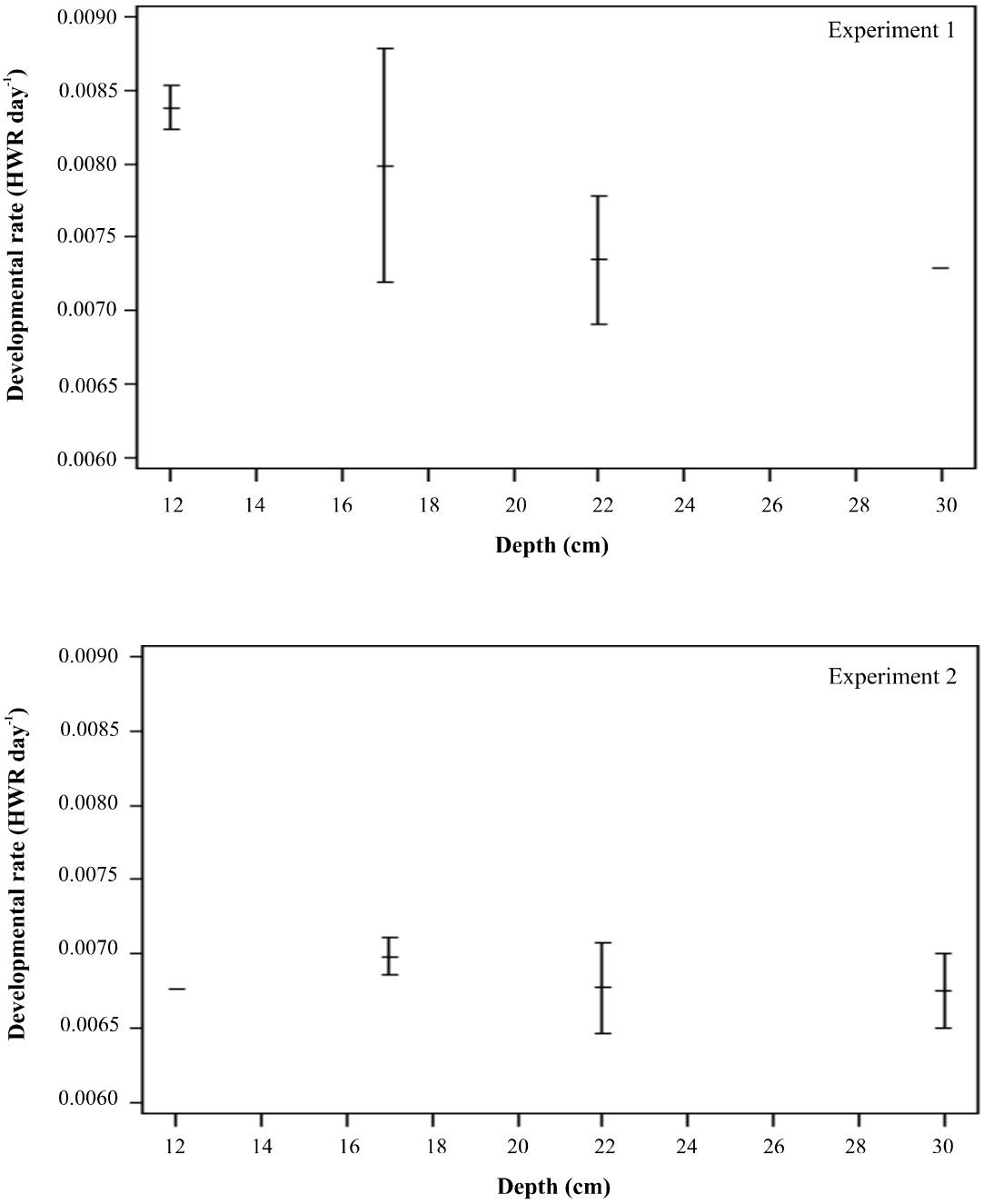


Figure 3.27. Develomental rate for *Carettochelys insculpta* embryos incubated at four depths during the final depth experiments. Devlopmental rate was calculated from the change in embryo head width ratio (HWR) between egg opening dates (Experiment 1 = two clutches; Experiment 2 = three clutches). Vertical bars represent the range and horizontal lines represent the mean.

3.3 Testing the Linear Development Model

3.3.1 Natural incubation of eggs in field nests

A summary of relevant information about the fourteen natural nests used to test the Linear Development Model is provided in Table 3.6. This set of nests included early, mid and late season nests and therefore was subjected to a wide range of natural thermal regimes (Table 3.6). Nests laid early in the season (late July) generally experienced cooler incubation regimes compared with those laid late in the season (late August/early September) (Table 3.6). The variability in thermal incubation regimes associated with the timing of nesting was also evident from the sex ratios produced, that is, the earlier nests were male or mixed whereas late season nests were female (Table 3.6). A complete temperature trace of one nest (Nest 9) was graphed in order to provide an indication of the complete thermal regime experienced by a natural nest (Fig. 3.28). There was no embryo mortality in most nests, however, a high percentage of embryos died in three late season nests (60% to 67%, in Nests 10, 11 and 14) (Table 3.6).

Information about the embryos killed during development and their observed and expected proportional development is shown in Table 3.7. In most cases, two embryos from each nest were killed there was generally little difference in development (HWR or
stage) between embryos from the same nest (Table 3.7), consistent with the findings of the depth experiments. However, it was noted that the extent of development of embryos from Nest 10 was considerably different (18% difference in HWR, 1 stage difference). The smaller embryo (a) was regarded as abnormally underdeveloped (Table 3.7), and excluded from the analyses.

The original protocol for testing the model was to evaluate predictions of proportional development based on the assumption that maximum development (HWR) occurred at stage 26. In other words, the model predicted embryo HWR, as a proportion of hatchling HWR, on the basis that the time to reach maximum HWR was the same as the time to reach stage 26 (77 days – see Section 3.1). However, results from the ageing work revealed that HWR reached its maximum at stage 24 (see Section 3.1.3). Consequently, a second set of predictions was made based on the assumption that

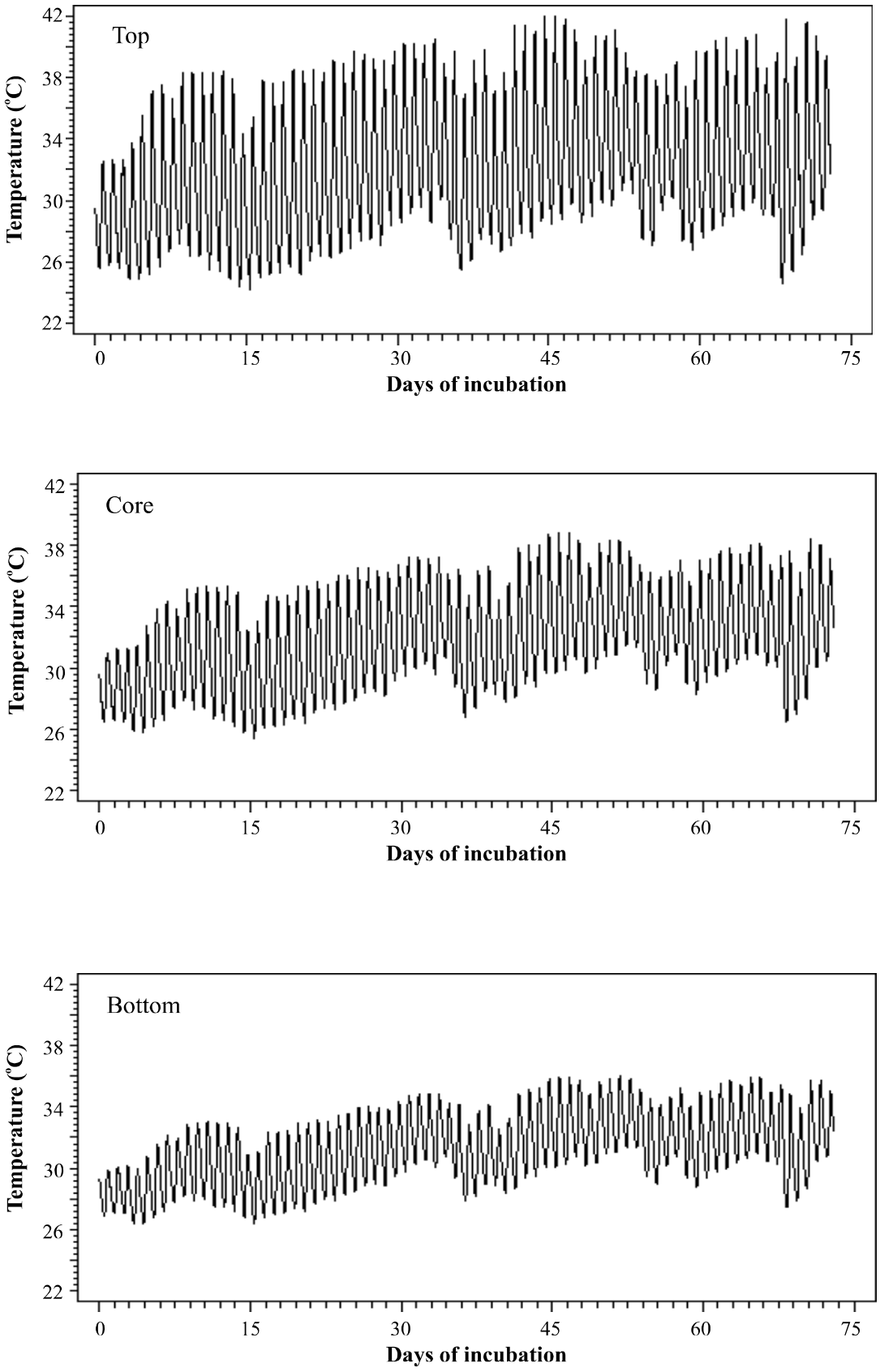


Figure 3.28. Temperatures recorded at the top, core and bottom of Nest 9, throughout incubation.

maximum development occurred at stage 24 (using 66 days, as the time to complete development, in the model).

In the first set of predictions, the model generally underestimated proportional embryonic development (Table 3.7 and Fig. 3.29). The predicted proportional development was within 5% of the observed development in Nests 7, 9, 12 and 13 (Table 3.7 and Fig. 3.29). Development was slightly underestimated (by between 5% and 10%) by the model in Nests 6, and 11 (one embryo) (Table 3.7 and Fig. 3.29). However, development was more substantially underestimated (by between 10% and 20%) by the model in Nests 1, 2, 3, 4, 5, 8 and 11(one embryo) (Table 3.7 and Fig. 3.29). Conversely, development was slightly overestimated by the model in Nests 10 and 14 (Table 3.7 and Fig. 3.29). Observed and expected development were related linearly ($F = 1551.5$, $df = 1, 13$, $P < 0.0001$) and overall (excluding Nests 10 and 14), the model significantly underestimated development by about 10% ($T = -6.0$, $df = 8.8$, $P < 0.001$). The total time to complete development, that is, the time to reach maximum HWR (stage 26) was consistently underestimated by the model, although only by two days, for Nest 11 (Table 3.7). However, estimates of the observed time to complete development were considered unreliable (possibly overestimated), because hatching was not tested regularly and embryos may have completed development but undergone a hatching delay.

The second set of predictions were generally closer to the observed figures, although, in contrast to the first predictions, the model generally overestimated development. Expected proportional embryonic development was within 5% of the observed development in Nests 1, 2, 3, 5, 8, and 11 (one embryo) (Table 3.7 and Fig. 3.29). Development was slightly overestimated (5% to 10%) by the model in Nests 4, 6, 7, 11 (one embryo) and 13 (one embryo) (Table 3.7 and Fig. 3.29). Furthermore, development was more substantially overestimated by the model in Nests 9, 12 and 13 (one embryo) (12% to 14%), and greatly so in Nests 10 and 14 (19% to 23%) (Table 3.7 and Fig. 3.29). Observed and expected development were again related linearly ($F = 1648.6$, $df = 1, 13$, $P < 0.0001$) and overall (excluding Nests 10 and 14), the model significantly overestimated development by about 5% ($T = 2.8$, $df = 8.8$, $P < 0.001$). As this set of predictions was based on maximum development (HWR) occurring at stage 24, the expected time to complete development could not be compared with the

incubation period recorded for each nest (as this was for stage 26). Instead, these predictions were

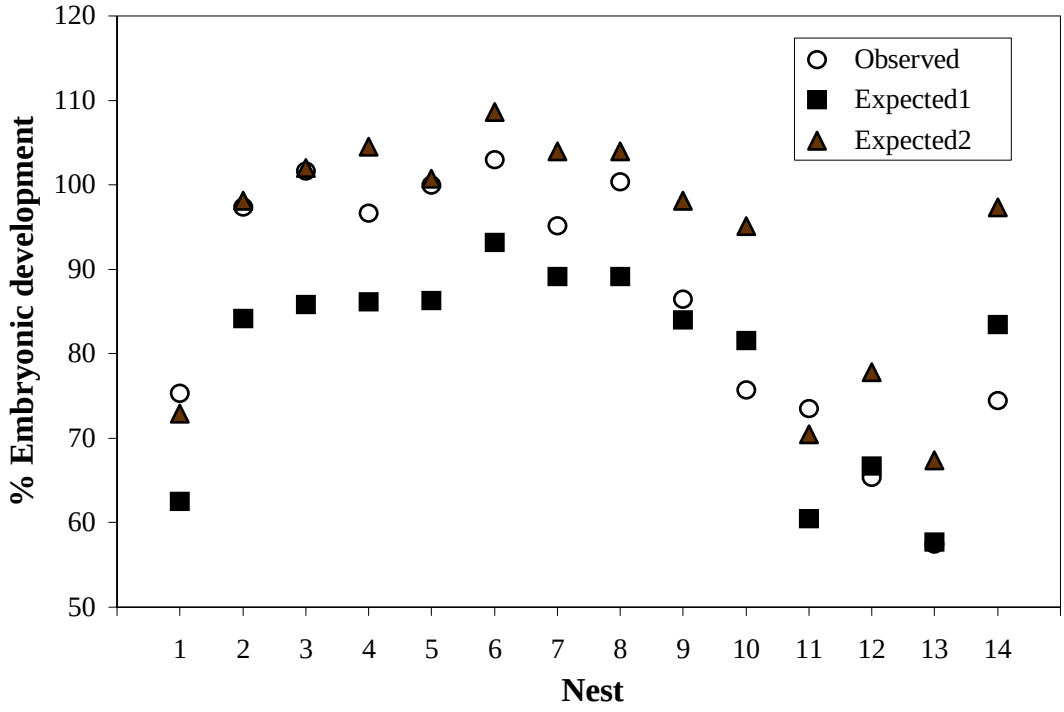


Figure 3.29. Observed and expected proportional (%) development for fourteen natural *Carettochelys insculpta* nests. Observed values are means for each nest; expected values were predicted using the Linear Development Model based on maximum development (HWR) occurring at 1. stage 26 ($expected_1$) and 2. stage 24 ($expected_2$).

assessed in relation to the timing of stage 24. In four nests (Nests 2, 4, 5 and 9), the observed and expected timing of stage 24 was very close or the same (Table 3.7). Furthermore, the predicted timing of stage 24 was considered probable in relation to the observed timing of this or other stages, in all other cases except Nests 10 and 13 (Table 3.7). In these nests (for which embryos were between stage 21 and 22 when eggs were opened), the model predicted that embryos would be at stage 24 sooner than was likely (Table 3.7).

3.3.2 Artificial incubation of eggs in cyclic thermal regimes

Information regarding the cyclic incubation thermal conditions and embryonic development is summarised in Table 3.8. The actual thermal conditions experienced by eggs were very close to the nominal temperature cycles with temperatures deviating no more than 0.6°C from their programmed settings (Table 3.8).

As for the natural nests, two sets of predictions were made using the Linear Development Model. In the first case, developmental rate (% HWR day^{-1}) was underestimated by model by between 0.26 and 0.47 units (in relation to mean observed values), for most of the regimes (Table 3.8 and Fig. 3.30). However, the observed and expected developmental rate was very similar (0.04 units difference) in Regime 2 (25 ± 7°C), and for Regime 7 (30.5 ± 6.5°C) the opposite trend was observed with the model overestimating developmental rate by 0.44 units (in relation to the mean observed rate) (Table 3.8 and Fig. 3.30). Observed and expected development were related linearly ($F = 42.3$, $df = 1, 6$, $P < 0.001$) and overall (excluding Regime 7), the model significantly underestimated development by about 26% ($T = -10.1$, $df = 5$, $P < 0.001$).

The second set of predictions were generally closer to the observed developmental rates, as was found in the natural nests. Developmental rate was underestimated by the model only by between 0.11 and 0.26 units (in relation to mean observed values), for most regimes and the observed and expected rates for Regime 2 (25 ± 7°C) were again similar (0.06 units difference) (Table 3.8 and Fig. 3.30). As for the first set of predictions, the model substantially overestimated developmental rate (by 1.00 units) for Regime 7 (30.5 ± 6.5°C) (Table 3.8 and Fig. 3.30). Observed and expected development were related linearly ($F = 43.8$, $df = 1, 6$, $P < 0.001$) and overall

(excluding Regime 7), the model significantly underestimated development by about 13% ($T = -4.6$, $df = 5$, $P < 0.01$).

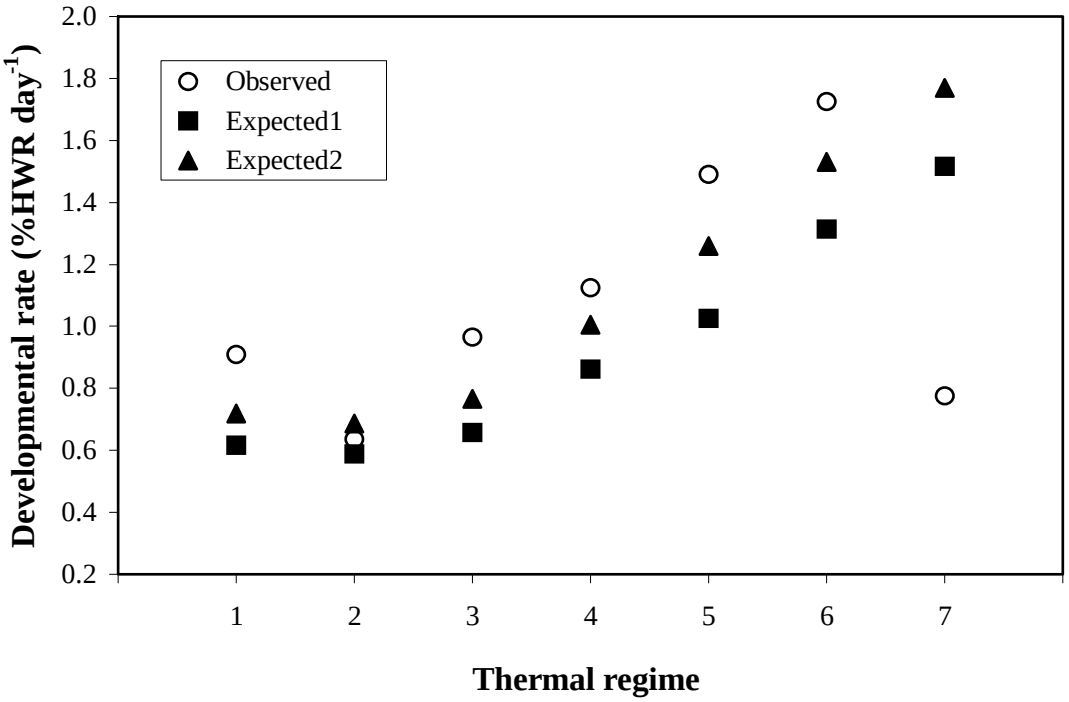


Figure 3.30. Observed and expected developmental rates for *Carettochelys insculpta* embryos from seven cyclic incubation regimes. Observed values are means for each regime; expected values were predicted using the Linear Development Model based on maximum development (HWR) occurring at 1. stage 26 ($expected_1$) and 2. stage 24 ($expected_2$). See Table 3.8 for explanation of thermal regimes.

CHAPTER FOUR

4. Discussion

4.1 Techniques for ageing embryos

4.1.1 Candling: qualitative observations and measurements

The value of candling for ageing turtle embryos was first demonstrated by Ewert (1985) who established a chronology of developmental stages based on qualitative candling observations for 37 turtle species from six families. This work was limited to descriptions of the timing of appearance of characters defining four stages only, though it did include a range of temperatures (see Section 1.3.1).

Both qualitative observations and measurements of candling attributes were shown to be useful for ageing *C. insculpta* embryos (Tables 3.1 and 3.2; Figs 3.1 to 3.6). There were three main periods during development (early, mid and late) for which different candling characteristics were applicable. In what I refer to as a "pre-embryo" period, early in development (from zero to 2.5 days, < stage 5), ageing was possible using measurements of the opaque patch. Following this period, there was an "embryo" period (between approximately seven and 52 days, stage 5 to 22) during which ageing could be achieved using qualitative embryo observations and embryo measurements. During the final period of development, the "post-embryo measurement" period (from about 52 to 77 days, stage 23 to 26), ageing was based on the proportion of the volume of the egg occupied by the embryo and degree of yolk internalisation, rather than specific embryonic features or measurements.

Candling features were more useful at certain times than at others. Between 2.5 and 7 days, the opaque patch index was constant yet blood islands had not formed. Hence, it was not possible to age or stage embryos within this period. Similarly, accurate ageing in the "post-embryo measurement" period was difficult because the qualitative estimates of embryonic size and degree of yolk internalisation were subject to considerable error. Despite these limitations, accurate ageing is possible at all other times, including the thermosensitive period for TSD (stage 15 to 22 in *C. insculpta*; J. Young, pers. comm.), important in studies of temperature dependent sex determination.

This is a major advance in the estimation of age using candling observations, substantially extending the published work of Ewert (1985).

Few studies have utilised candling measurements, as opposed to qualitative observations, for ageing embryos. Ewert (1985) presented none and Miller (1985a) presented no candling observations at all. Spread of the opaque patch with time has been reported for crocodilians (*Crocodylus johnstoni*: Webb *et al.* 1983a; *Crocodylus porosus*: Webb 1983b) and turtles (*Emydura macquarii*: Thompson 1985; *Carettochelys insculpta*: Webb *et al.* 1986). In these studies, the opaque patch spread rapidly (and approximately linearly) within the first few days of incubation, then slowed to remain roughly of constant size, so that eggs appeared about 40% to 60% opaque (Webb *et al.* 1983a; 1983b; Thompson 1985; Webb *et al.* 1986). The pattern of spread of the opaque patch index found during the present study was similar (Fig. 3.1), though no data was collected beyond the time the patch equalled egg diameter. In those studies where spread of the patch was recorded throughout development, it was found that patching increased to 100% by the time development was complete (Webb *et al.* 1983a; 1983b; Thompson 1985; Webb *et al.* 1986). Apart from measurements of the opaque patch, no studies have used quantitative measurements from intact eggs to assist in staging embryos. The present study clearly demonstrates the potential utility of quantitative measurements obtained during candling of intact eggs, as indicated by high coefficients of determination. When the quantitative measurements are coupled with the qualitative observations, many more stages than the four identified by Ewert (1985), spanning most of development, were shown to be diagnosable.

The reason both qualitative and quantitative observations are needed for adequate staging of intact eggs is evident from the range of ages and stages that each is most effective (Tables 3.1 and 3.2). If only candling observations are used to age embryos, stage or age could often only be determined as a range (e.g. between stages 14 to 16, or stages 17 to 18 – see Table 3.1). Ageing based solely on candling measurements will be similarly limited. The embryo crown-rump (ECR) length index decreased between stages 12 and 14 (because of torsion), was constant until stage 16, and then increased (Fig. 3.5). Consequently, similar ECR values exist for stages 12 and 17. Qualitative candling observations clearly distinguish these stages (Table 3.1), so ageing is best achieved using the complete set of candling information.

Most of the candling work in the present study was done with eggs incubated at one temperature only (30°C). Some eggs were incubated at other constant temperatures, and the influence of temperature on the timing of appearance of certain candling features (opaque patch and haemodisc) was also assessed (Figs 3.1 and 3.3). The effect of temperature on these early developmental indices was varied. The rate of spread of the opaque patch was not affected by incubation temperature (30°C versus 34°C) over the period monitored (Fig. 3.1). As discussed earlier, there is little published work on the development of the opaque patch, and only one study (Webb *et al.* 1983a) incubated eggs at more than one temperature (26°C, 28°C, 30°C, 34°C). Webb *et al.* found that the spread of the opaque patch was generally faster at warmer temperatures than at cooler temperatures. Eggs from warmer conditions were completely opaque sooner than eggs from cooler incubation temperatures, but there was little difference in the rate of patching during the initial period of patching (Webb *et al.* 1983a). The findings of the present study are therefore consistent with those of Webb *et al.* (1983a). They suggest that very early in development, the rate of embryonic growth may be little affected by incubation temperature, within limits. Alternatively, the spread of the opaque patch may not be closely related to embryonic growth. Temperature may affect embryonic growth, but this may not be reflected in the rate of patching.

Temperature clearly had an effect on the rate of growth of the haemodisc. The haemodisc index reached its maximum in about 15 days, for eggs incubated at 32°C and 34°C, compared to 32 days, for 28°C (Fig. 3.3). Estimates of development time, based on rate of haemodisc growth, were in line with those reported for the total incubation period for *C. insculpta* – 53 days, at 32°C, and 101 days, at 28°C (Webb *et al.* 1986). In either case, development rate at 32°C was about double that at 28°C. This is consistent with expectation based on rates of embryo growth, but contrasts with the observed temperature independence in the rate of spread of the opaque patch. Hence, lack of dependence between spread of the opaque patch and temperature probably indicates that spread of the opaque patch and embryo growth are uncoupled early in development.

Rates of embryonic development are temperature dependent. If the standard 30°C series of candling observations and embryo measurements are to be useful across a wide range of conditions, correction for incubation temperature will be necessary, as outlined in Section 3.1.3. Embryonic stages are primarily defined by morphological features (Ewert 1985) and as many candling features are morphological, the association between

candling attributes and particular stages would be the same regardless of incubation temperature. However, the timing of candling observations and rate of growth of candling indices will vary with incubation temperature. Adjusting for temperature may not be simply a case of adjusting for development time. It is well established that warm incubation temperatures produce relatively small reptile hatchlings compared with cooler temperatures (crocodilians: Joanen *et al.* 1987; Allsteadt and Lang 1995; monitor lizards: Phillips and Packard 1994; turtles: Janzen 1993a; Bobyn and Brooks 1994). Therefore, the ranges of the ECR and ECL established for each stage at 30°C might not be applicable for ageing embryos incubated at 34°C. Even after adjusting for the differences in development time, an embryo at any particular stage incubated at 34°C would most likely be smaller than an embryo at the same stage, incubated at 30°C. These considerations will inject an error into corrections for incubation temperature, but the success of temperature similar corrections applied by Webb *et al.* (1986) suggest that the effect is small. The method for correcting for temperature presented in this paper is therefore considered to be adequate, and greatly increase the utility of the age-stage and age-morphometric relationships.

4.1.2 Embryo characteristics and measurements

Describing an embryological series to the level of detail presented by Yntema (1968) for *Chelydra serpentina*, and presented by Miller (1985a) for several marine turtles, is a major undertaking. Many embryos need to be sacrificed and examined. Such detailed work was not the objective of the present study. Rather, the objective was to use Yntema's series as a basis, refining it as necessary, to incorporate features peculiar to the embryonic development of *Carettochelys insculpta*. For the large part, the results show this to have been a successful strategy, with a good general correspondence between the developmental series of the two species.

Primary differences lay in the development of eyelids, forelimbs and claws and nose, and in the timing of appearance and degree of pigmentation (Table 3.3; Figs 3.7 to 3.13). The refined embryological series presented here will provide a useful tool for ageing nests of *Carettochelys insculpta* where it is possible to open a representative egg.

Embryo measurements, as opposed to qualitative descriptions, also provided information useful for ageing. In this study, HWR was used as the index to size, and strong predictive relationships with stage and age were presented (Figs 3.14 and 3.15). HWR increased linearly with time until it reached a maximum, at around stage 24. Thereafter, HWR remained constant until development was complete, at stage 26. This confirms the conclusion of Webb *et al.* (1986), also working with HWR of *Carettochelys insculpta*, that embryonic size reaches a maximum before qualitative changes are complete at stage 26. During these last stages of development (stage 24 to 26), embryos underwent minor morphological changes (e.g. in eyelid development and pigmentation) and internalised their yolk, rather than growing in size. However, the only index of embryonic size used in this study and that of Webb *et al.* (1986) was HWR. Alternative measures may have revealed different trends. For example, length of the embryo has been found to increase until development is complete in turtles (Yntema 1968) and crocodilians (Deeming and Ferguson 1989). Embryo weight increases throughout development in turtles (Pieau and Dorizzi 1981; Ewert 1985) and crocodilians (Deeming and Ferguson 1989), although this is not always a reliable an index of size, as weight can be confounded by embryonic water content and the amount of residual yolk (Piersma and Davidson 1991; Cagle *et al.* 1993). In light of these established trends, it seems likely that *C. insculpta* embryos continue to grow between stages 24 and 26, but that this growth was not reflected in HWR measurements. Consequently, the application of relationships among HWR, age and stage to ageing was limited to the period of development up to stage 24. The exception was the relationship between stage and age, which was strong throughout development and hence useful as a staging tool beyond stage 24 (Fig. 3.16). Further work involving alternative growth indices is needed to identify the best measure of embryo size – preferably one that is linearly related to time, that is not subject to great errors in its determination, and one that continues to increase throughout development.

Relationships between embryonic age, size and stage, such as those derived in the present study, are potentially very useful for embryological work. The relationships could be used to predict size of *C. insculpta* embryos or their stage at any particular time during incubation. Alternatively, inverse predictions could be made using HWR to estimate age (at 30°C). Relationships between morphometrics and time have been used successfully to age crocodile embryos (*Crocodylus johnstoni*, Webb *et al.* 1983a; *Crocodylus porosus*, Webb *et al.* 1983b). Morphometric formulae are particularly

useful for ageing incomplete embryos (e.g. damaged or partly decomposed embryos), which may be missing features crucial for staging using morphological descriptions (Webb *et al.* 1983b).

Embryos in this section of the study were incubated at one temperature only (30°C constant), therefore it was difficult directly assess the effect of temperature on embryo growth. As with candling attributes, other studies have shown that incubation temperature has a profound effect on the relationships between developmental rate and time (Miller and Limpus 1981; Webb *et al.* 1987a; Deeming and Ferguson 1989; Viets *et al.* 1993), including *C. insculpta* (Georges, unpublished data). To increase the generality of the results of incubation experiments at 30°C, corrections are necessary as outlined in the results (Section 3.2). The results together with these corrections provide a tool for ageing eggs and embryos of *C. insculpta* under a wide range of conditions, where it is possible to sacrifice the egg.

4.1.3 Summary

Two highly effective methods of ageing *Carettochelys insculpta* embryos are presented in this study. The ability to accurately age embryos from qualitative candling attributes and measurements represents a significant advance not only for embryological work involving *C. insculpta*, but potentially for embryological studies of oviparous reptiles in general. The standard series of development for *Chelydra serpentina* (Yntema 1968) is applicable to *Carettochelys insculpta* embryos, provided it is refined, as outlined in this study, to incorporate developmental features peculiar to *Carettochelys insculpta*. Relationships between embryonic size, age and stage established during the present study and are also potentially valuable ageing tools. A method for correcting the ageing systems established at 30°C was provided, thus rendering the ageing techniques applicable to a range of incubation temperatures.

4.2 Thermal gradients and embryonic development

4.2.1 Thermal gradients in the soil profile

The term “thermal gradient” usually applies to a gradient in temperature through a nest at a particular time. Such gradients are common because the cycle in ambient temperatures and solar irradiation passes as a wave through the soil. The thermal maximum experienced at the surface is not experienced at depth until some time later. This coupled with dampening of the daily cycle results in differences in temperature with depth at a particular time. However, the effect of such gradients on embryonic development is far from clear, as eggs at different depths experience much the same average temperatures, only at different times.

The main differences in thermal regime associated with depth were in the diurnal variability and timing of maximum (and minimum) temperatures (Figs 3.18 to 3.20). The temperature cycle was damped at greater depths, with the diurnal range at 50cm being two orders of magnitude less than what was experienced at the surface. Also, temperatures reached a maximum at about 1600 hours at the surface, but maximum temperature occurred progressively later in the day with increasing depth. These trends were in line with those well established in and can also be explained by the principles of soil physics (Carson and Moses 1963; Marshall and Holmes 1979; Hanks 1992). In the upper 50cm of the soil profile, the primary determinant of temperature is solar radiation (Marshall and Holmes 1979; Hanks 1992). Short-wave radiation reaching the ground during daylight hours warms the surface layer and leads to a subsequent flow of heat downward (Carson and Moses 1963). Heat begins to flow to lower depths only after a temperature gradient develops, and thermal capacity and conductivity of the soil ensures that there is a time lag before the maximum temperature occurs at lower depths (Hanks 1992). Conversely, emission of long-wave radiation at night cools the surface and cause heat to flow upward through the profile (Carson and Moses 1963). Again, upward heat flow only occurs after a temperature gradient develops and a time lag also occurs in minimum temperature (Hanks 1992). Soil temperature is increasingly “buffered” from the highly variable temperatures at the surface, at greater depths (Carson and Moses 1963; Hanks 1992).

Another notable thermal characteristic of the soil profile was the similarity of mean temperatures within the depth profile. Mean temperature varied less than 2°C within the soil profile. This was an interesting finding as more substantial differences in mean temperature between depths are often found (Hanks 1992). However, a number of factors associated with soil type (e.g. organic content and colour), as well as moisture

content, influence the flow of heat through the soil profile (Hanks 1992). Consequently, the thermal characteristics of soil profiles are likely to vary widely across soil types. The nesting substratum used by *Carettochelys insculpta* is particularly homogeneous and therefore simple compared to other soil types.

Relationships between mean and variability in temperature with depth were particularly strong ($r^2 > 0.95$, Fig. 3.20) and are useful for predictive purposes. For example, as mean temperatures were generally similar across all depths, and the temperature at 50cm was essentially constant, the thermal regime at any depth can be estimated using a 50cm spot temperature and the derived predictive relationships. This could be particularly valuable in broadscale surveys of sex ratios across nesting beaches.

The existence of thermal gradients in the soil profile has implications for the design of field studies involving the monitoring of soil and nest temperatures. A number of studies involving natural turtle nests (e.g. *Chelydra serpentina*: Packard *et al.* 1985; *Emydura macquarii*: Thompson 1988; *Dermochelys coriacea*: Chan and Liew 1995) have used "spot" temperatures, which were typically taken at multiple depths, a couple of times each day. The time chosen for temperature readings was often noon (Packard *et al.* 1985; Thompson 1988) or at the times of expected minimum and maximum temperature at "nest depth" (Chan and Liew 1995). However, the complex thermal regime within nests, involving lags and damping, may result in the temperatures taken at different depths appearing more similar to each other or less similar to each other depending on when they are taken. For example, in the present study, spot temperatures taken at noon, at different depths, varied little (Fig. 3.18); those taken at 1800 hours varied a great deal. Unless time lags in the temperatures experienced at depth are taken into account, very misleading data on thermal gradients will result. The temporal dimension needs to be considered when designing protocols for monitoring nest or soil temperatures at a range of depths.

4.2.2 Thermal gradients and embryonic development

The relationship between soil depth and temperature in the depth experiments was similar to that reported for the soil profile (Figs 3.21, 3.23, 3.25 and 3.27) in that mean temperatures across depths were generally similar; daily range varied greatly across

depths. Temperatures were above the developmental zero for *C. insculpta* ($T_0 \cong 24.5°C$, Georges, unpublished data) at all times.

No embryonic developmental asynchrony was observed, despite clear differences in thermal regime with depth. Shine and Harlow (1996) obtained similar results for eggs of the skink *Bassiana duperreyi*, in both natural nests and cyclic incubation experiments. Lack of developmental synchrony also supported the hypothesis of Georges (in prep), which states that fluctuating temperatures during incubation will not cause developmental asynchrony provided they cycle about similar means and remain above T_0.

Shallower eggs experienced a wider range of temperatures than did deeper eggs, although the mean temperatures at various depths were not substantially different. The shallow eggs were subjected to warmer temperatures at times than deeper eggs, however, they were also subjected at times to colder temperatures. Developmental rate would be accelerated during warmer conditions, cancelling the decelerated development during colder conditions. Thus, the effects of warmer and cooler temperatures on developmental rate were balanced, and the overall outcome was developmental synchrony. Shine and Harlow (1996) interpreted their results in a similar way. However, if temperatures fluctuated to the extent that they dropped below T_0 , developmental asynchrony would occur according to Georges (in prep.), as was observed by Shine and Harlow (1996). During cyclic temperature experiments, the incubation period of *B. duperreyi* embryos which experienced high fluctuations in incubation temperature, including periods below T_0 , was dramatically reduced compared to what was expected on consideration of the means (Shine and Harlow 1996). This occurred because the higher temperatures accelerated embryogenesis, but excessively low temperatures did not retard development to the same extent (as developmental rate could not drop below zero). The net effect of increasing fluctuations was hastened development (Shine and Harlow 1996) compared to what would have occurred if temperatures were held constant at the mean. Under such conditions, developmental asynchrony – with the shallower embryos developing more quickly than deeper embryos, is likely to occur.

Nests of *C. insculpta* did not drop below the developmental zero, so the findings of Shine and Harlow (1996) and the predictions of Georges (in prep) are academic. They

do, however, experience high temperatures. Just as there is a developmental zero, a high temperature developmental threshold, T_{max}, is also likely. Above this threshold, increase in developmental rate with increasing temperature is no longer linear, and development rate may even decrease. There is some evidence for this in the reptilian literature. Muth (1980) found that incubation period was greater for iguana embryos incubated at 40°C, relative to 36°C. High embryo mortality has been observed in hot turtle nests (e.g. *Emydura macquarii*: Thompson 1988; present study – see Section 3.3.1), an extreme of developmental retardation. Therefore, in cases when temperature fluctuations are such that some time is spent above T_{max} each day, but not below T_0 , the net effect would most likely be retarded development. This idea is, as yet, untested and therefore speculative. However, there was evidence for this in the present study, to be discussed further in Section 4.3.

The results of this study did not support Thompson's (1989) ideas, as outlined in the introduction. In developing his hypothesis on developmental asynchrony, Thompson (1989) made no reference to T_0 . He also assumed that highly fluctuating temperatures represented overall warmer incubation regimes (Thompson, 1989: 251). In the present study, high variability in temperature at shallow depths resulted in both warmer and cooler temperatures at different times of the day, relative to that at greater depths, such that mean temperatures varied little with depth. If mean temperatures had varied substantially with depth (cooler at greater depths), the overall thermal regimes would have been significantly different. Developmental asynchrony would have been likely, in line with Thompson's predictions. This was not observed in the present study.

Furthermore, Thompson's (1989) "catch-up" hypothesis proposed that the peaked pattern of rate of oxygen consumption (VO_2) and late term metabolic depression (see Section 1.3.2, in the introduction) allowed simultaneous hatching, in species likely to experience developmental asynchrony within single nests. This may be a reasonable explanation for late term metabolic depression in nests that experience temperatures below T_0 for part of each day. In such nests, developmental asynchrony is likely. Such developmental asynchrony did not occur with *C. insculpta* embryos, a species with an attenuated peaked pattern of oxygen consumption and greatly extended period of late term metabolic depression (Webb *et al.* 1986). Alternative explanations need to be explored. It may well be that the delayed hatching that results from the late term metabolic depression is not related to preceding incubation conditions, but rather to

hatchling survival strategies. The delay may ensure that hatching occurs when conditions are conducive to hatchling survival. *C. insculpta* have a discrete and unambiguous cue for hatching – hatching can be stimulated by innundation or saturating rains (Webb *et al.* 1986; present study). If these conditions coincide with conditions conducive to hatchling survival, then the embryonic aestivation may bring the two into synchrony. Hatchling survival may be enhanced at this time because there is a greater volume of water available for dispersal and the increased turbidity associated with flooding may decrease susceptibility to predation (Webb *et al.* 1986).

Embryonic aestivation and hatching in response to a discrete and unambiguous external cue will also result in synchrony of hatching across nests, potentially saturating predators. This benefit may also explain the evolution of the late term metabolic depression and delayed hatching, rather than Thompson's hypothesis.

Finally, the evolution of late term metabolic depression may be explained in terms of phenotypic plasticity. There may have been times in the evolutionary history of the species when temperatures dropped below the developmental zero, and developmental asynchrony may have been a common feature of the species life history. Late term metabolic depression may have evolved initially in response to this, ensuring hatching synchrony, and is retained in present day forms that, under current weather patterns, do not experience developmental asynchrony. The retention of the trait may have been facilitated by the added advantages of synchronous hatching across nests and timing of hatching to match favourable conditions.

This study has demonstrated that fluctuating temperatures in shallow nests of reptile species do not result in developmental asynchrony, when temperatures remained above developmental zero. Thus, explanations as alternatives to that presented by Thompson (1989) need to be considered to explain late term metabolic depression in these species. Of course, other developmental attributes may be influenced by variation in thermal regimes with depth, as is the case for embryo sex, where it is the proportion of development occurring above and below the threshold for sex determination in species with TSD (Georges *et al.* 1994). Indeed, Georges (1992) found that in nests of *C. insculpta* with mixed hatchlings sex ratios, the eggs at the top of the nest generally produced female hatchlings, whereas those at the bottom produced male hatchlings, even in the absence of gradients in daily mean temperature.

4.3 Testing the Linear Development Model

This study demonstrated that embryonic development in field nests could be satisfactorily predicted from incubation temperatures using the Linear Development Model of Georges (1989; 1994, in prep.), provided nest temperatures did not get too hot (Tables 3.5 and 3.6; Figs 3.30 and 3.31). The model required some adjustment to account for the observation that development was linearly related to time up to stage 24, not stage 26 as assumed by Georges (in prep). In the natural nests, proportional development, and especially incubation period, was often underestimated by the model using stage 26 as the terminal stage for growth in HWR. This most likely reflected difficulty in determining incubation period of natural nests, when *C. insculpta* embryos enter a period of aestivation at the end of development but before hatching. (Webb *et al.* 1986). Predictions noticeably improved when the model was adjusted to use stage 24 as the time of maximum development (see Section 3.1.3). Development in a few nests was consistently overestimated by the model and interestingly, these were the nests that experienced the hottest incubation regimes and also recorded the highest mortalities.

There were similar trends in the predictive ability of the model for embryos incubated under artificial, cyclic thermal regimes, except development was only substantially overestimated in one regime. As for the natural nests, the model predictions were better when stage 24 was used as the time of maximum development. Again, the regime for which the model overestimated development was the hottest regime.

These observations suggest that high temperature inhibition of development occurs in some nests, and that the associated lack of linearity introduces a bias to the predictions of the Linear Development Model. The relationship between temperature and development rate may only be linear up to a point. At some high temperature threshold, T_{max} , development rates may initially be retarded below what would be expected from the linear model, as was reported by Muth (1980). At higher temperatures, development rates may actually be less than at lower temperatures, and at higher temperatures still, embryos may die. In the present study, the highest temperatures experienced may have retarded development of some embryos and killed others. The Linear Development Model assumes that developmental rate simply

increases linearly with temperature (Georges 1989; 1992b) and therefore does not incorporate any high temperature inhibition. Consequently, in these high temperature eggs, predictions overestimated observed accrued development.

There are other sources of error that could explain some of the differential between predicted and observed. One is the error associated with using average hatchling HWR to obtain proportional development. A dilemma common to all work requiring direct embryonic measurements is that the development of an individual embryo can not be followed to completion. The embryo is killed in the process of measurement. In this study, average hatchling HWR (from each nest, or from the complete set of hatchlings obtained during the study) was used as the estimate for maximum HWR with which embryo HWR was compared. The error would arise in cases where particular embryos were destined to become significantly larger or smaller hatchlings (HWR), in relation to the rest of their clutch, or overall average. In these cases, the estimated proportional development may have been inaccurate. For the cyclic incubations, this problem was somewhat overcome by calculating observed developmental rates from the range in hatchling HWR and the average hatchling HWR. However, there remained a small but significant bias in the predictions of development rate compared to the observed development rates.

In conclusion, the Linear Development Model of Georges (1989; 1994) was best able to predict proportional embryonic development when the time of maximum development was considered to occur at stage 24. Also, the model did not incorporate the curvelinearity in the relationship between developmental rate and temperature likely to occur at high temperatures. Therefore, proportional embryonic development could only be predicted for embryos incubated in the cool to warm range ($< 36^{o}C$) of natural and artificial thermal regimes. The effect of high temperatures should be incorporated into the model. Predictive ability of the refined model should be evaluated using a developmental index which increases throughout development. One choice might be embryonic weight, which generally does increase throughout development (Pieau and Dorizzi 1981; Ewert 1985), and which can be accurately measured as dry weight minus yolk.

4.4 Synopsis

The present study investigated certain aspects of the embryonic development of *Carettochelys insculpta,* under constant and fluctuating temperature regimes. A number of significant findings have resulted from this research, which may be applicable not only to further embryological work on *C. insculpta,* but oviparous reptiles in general.

This research has clearly demonstrated the potential utility of qualitative observations and quantitative measurements obtained during candling of intact eggs as methods of ageing embryos. These techniques may be particularly useful for species with temperature dependent sex determination (TSD), as the thermosensitive period of development was within the time during which eggs can be most accurately aged from candling attributes. Importantly, the candling ageing system developed during this study is, to date, the only one of its kind and is hence the only non-lethal method available for accurately ageing reptile embryos. Taking into account the morphological differences between *Carettochelys insculpta* and *Chelydra serpentina,* embryos could be successfully staged using a developmental series based on that of Yntema (1968). Relationships between embryonic size, age and stage established during the present study and are also potentially valuable ageing tools. Furthermore, a method for adjusting the 30^{o}C ageing work for the effect of incubation temperature on development is presented. Therefore the relationships between candling attributes, embryo morphometrics, embryonic stage and age established during the study will be applicable to eggs and embryos from a range of incubation environments.

Another significant finding of the present study was that differences in thermal regimes associated with soil depth did not cause developmental asynchrony in *C. insculpta* embryos. Variation in the magnitude of daily fluctuations in incubation temperature, about similar means, did not cause differential developmental rates, provided temperatures were conducive to development at all times. Therefore alternative explanations for the metabolic patterns observed in reptiles nests which experience variations in thermal regime may be more appropriate. The late term metabolic depression observed in such species may be related to hatchling survival strategies. Hatching may be delayed in order to take advantage of favourable hatching conditions. Furthermore, the evolution of late term metabolic depression may be explained in terms of phenotypic plasticity. Late term metabolic depression may have

evolved initially in response to conditions which produce developmental asynchrony, to ensure simultaneous hatching.

This study demonstrated that embryonic development in field nests could be satisfactorily predicted from incubation temperatures using the Linear Development Model of Georges (1989; 1994, in prep.). The model was best able to predict proportional embryonic development when the time of maximum development was considered to occur at stage 24. Also, the model did not incorporate the curvelinearity in the relationship between developmental rate and temperature likely to occur at high temperatures. Therefore, proportional embryonic development could only be predicted for embryos incubated in the cool to warm range (< 36^{o}C) of thermal regimes.

Collectively, the findings of the present study have implications for further reptilian embryological work and particularly that involving species with TSD. The ability to age embryos without killing them is an important advance, especially since some TSD are threatened (e.g. marine turtles). That fact that developmental asynchrony was not found to occur in reptile nests is important because it means that the developmental stage of a natural nest can be estimated from just one egg regardless of its position in the nest. The proportional development and sex ratios of nests can be determined from incubation temperatures using the Linear Development Model. Also, the ability to model developmental outcomes represents an important tool for making predictions about the potential impact changing climatic conditions may have on reptile populations.

In conclusion, the findings of this study have made an important contribution to the descriptive developmental embryology of the pig-nosed turtle, *Carettochelys insculpta*. It has also contributed to reptilian embryology, in general, through the development of a non-destructive ageing technique. Furthermore, this study has improved our understanding of and ability to model the thermal influences on reptilian embryonic development, under constant and fluctuating temperatures.

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Appendix 1: The time series analysis program

A time series analysis (TSA) program was developed by Arthur Georges (Unpublished work) to be used with detailed temperature traces, such as those recorded during this study. The TSA program is comprised of a series of stages and the following notes and figures explain how the program operates, in the context of its application with temperature traces from turtle nests.

1. Two traces are selected: firstly, a trace with one or more periods of erroneous or missing data (Fig. I); and secondly, a trace with complete data for the time period covered in the first trace. The latter trace is used as the predictor trace (x) with the former trace, the response trace (y). The most suitable predictor trace to use is one from a similar thermal regime (e.g. from the same nest or from a similar depth in a nearby nest). In the absence of more compatible data a less similar trace (e.g. from a different beach) may be used as the predictor trace.

2. As a time lag may exist between the x and y traces (Fig. II), the traces are first aligned. This is done by calculating the times of maximum and minimum temperature for each trace and subtracting one set of values from the other to obtain the time lag. The x trace is then adjusted by the calculated time lag to be in alignment with the y trace (Fig. III).

3. The next step is to separate each time series into two trends: a seasonal trend, and a daily cycle. The seasonal trend is extracted by calculating daily mean temperatures and therefore appears as a 24 hour moving average (Fig. IV). The daily cycle is then calculated with reference to the daily mean and therefore generally appears as a stationary time series (Fig. V).

4. Predictive relationships between the two traces, for each trend, are then developed by autoregressive modelling. The fit of each regression models to the y data can be assessed by viewing graphs of the trends (Figs VI and VII).

5. The final step is to fill the gaps in the incomplete y trace using the predictive relationships developed in the previous step. The missing data are predicted for the seasonal trend and daily cyclic data separately then the two are recombined to restore the y trace to its original format (Fig. VIII).

The effectiveness of the TSA program has been evaluated by testing on complete time series with sections of data removed. The gaps created in the time are series were filled using the program and then compared with the initial, complete data. The temperatures predicted using the TSA program were found to be highly accurate (A. Georges, pers. comm.).

Appendix 2: Estimating *Carettochelys insculpta* embryonic stage using the data of Webb *et al.* (1986)

Preliminary information on the embryonic development (embryo head width ratio HWR, stage and age) of *Carettochelys insculpta* was reported by Webb *et al.* (1986) (Table I). Webb *et al.* (1986) also reported developmental rate coefficients (DRC) for different incubation temperatures (28°C: DRC = 0.68, 30°C: DRC = 1.00, 32°C: DRC = 1.30). DRC represent the time for development to be completed, as a function of the time at 30°C (i.e. development at 28°C occurs at 0.68 times the rate of development at 30°C) (Webb *et al.* 1986). Webb *et al.* (1986) also investigated embryonic development in relation to time and reported the following predictive relationship between embryo head width ratio (HWR) and age, at 30^{o}C:

$$\text{Age (days)} = 182.8*\text{HWR} - 0.5 \quad (r^2 = 0.99) \quad \text{(for embryos up to stage 24)}$$

In order to estimate the timing of particular embryonic stages for a range of incubation temperatures, as was required during the present study, the HWR-age equation, stage and age data, and DRC presented by Webb *et al.* (1986) were used to develop stage-age models (for 28°C, 30°C and 32°C – T), through regression analyses. The relationships obtained were:

$$\text{HWR} = \frac{\text{Age} + 0.5}{182.8} \quad \text{(at 30°C)}$$

$$\text{Stage} = 85.75*\text{HWR} - 89.02*\text{HWR} + 5.33 \quad (r^2 = 0.99) \quad \text{(at 30°C)}$$

$$\text{Age (T °C)} = \text{Age (30°C)} \div \text{DRC (T °C)}$$

The above relationships were used to produce a table showing stage (up to stage 24), HWR and age for embryos incubated at 28°C, 30°C and 32°C (Table II). These tables were used during the present study to estimated dates for the retrieval and opening of *C. insculpta* eggs, from both the laboratory and field incubations. For eggs incubated in the field (in natural nests and the depth experiments), the thermal conditions were assessed in order to determine which constant temperature was most appropriate to use for estimating opening dates.