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Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise

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Abstract

Incubation of northern snake-necked turtle (*Chelodina rugosa*) eggs and subsequent sale of hatchlings for the pet industry has the potential to provide culturally suitable employment for indigenous communities in northern Australia. Developmental arrest in response to egg inundation is unique to *C. rugosa*. Eggs can be stored under water for up to 10 weeks without appreciable impact on egg or embryo survival, allowing the transport and sale of eggs into niche markets without high levels of mortality, and permitting eggs to accumulate in diapause until there are sufficient numbers to incubate as batches. Eggs that are not inundated or inundated for short periods experience similar survival rates to eggs inundated for lengthy periods. Incubation temperature influences embryo survival and development period in *C. rugosa*. Embryonic survival is greatest at 26 °C, steadily declining as temperature increases to 32 °C. A similar increase in incubation temperature decreases incubation period by approximately 40 days, however almost half of this variation is attributed to the increase in incubation temperature from 26 to 28 °C. Hatchling growth in *C. rugosa* is characterized by two phases. There is an initial phase of relatively slow growth under the partial influence of initial egg size and incubation duration, followed by a second phase of relatively rapid growth under the partial influence of water temperature and mass at hatching. Post-hatching survival is negatively correlated with duration of egg inundation and water temperature. Evidence suggests that inundation of *C. rugosa* eggs for 6 weeks, incubation of embryos at 28 °C and raising hatchlings in 28 °C water will yield the best overall outcomes.
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Keywords: Chelidae; Embryonic diapause; Growth; Incubation; Reptile; Survival

1. Introduction

Production of turtle meat and turtle products is an important component of some local economies in both the developed (Kafuku and Ikenoue, 1983; Hughes, 2000; Lutz, 2000) and developing world (Chen et al., 2000; Sharma and Tisen, 2000; Mai and Tan, 2002). Turtle products are in high demand in Asia (van Dijk and Palasuwan, 2000) driven by the combination of ancient tradition and newfound wealth (Behler, 1997). This demand is currently met in a large part by the harvest of natural populations at a rate that is unsustainable. Many species of freshwater turtle that were once widespread and abundant throughout Asia are now considered locally extinct or regionally endangered as a direct result of over-harvesting for international trade (van Dijk et al., 2000). However, freshwater turtles are also a significant component of aquaculture in many developing countries of the

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region. For example, China produced 92,000 tonnes of softshell turtle (*Pelodiscus sinensis*) in 2000, primarily for human consumption, equal to 0.6% of their total freshwater aquaculture production in that year (Mai and Tan, 2002). Aquaculture has the potential to mediate the impact of commercial demand for turtle products on wild populations.

In the developed world, demand for freshwater turtles is focused more on their value as pets than on meat or medicinals. A substantial aquaculture industry developed in Louisiana in the late 1950s and by 1969, there were 75 producers and 15 million hatchling red-eared slider turtles (*Trachemys scripta*) passing though the domestic trade (Wood, 1991; Hughes, 2000). In 1975, the US Food and Drug Administration banned domestic sales of turtles less than 10 cm in length in response to concerns over salmonella infections in children (Lutz, 2000). The industry shifted its focus to the international trade in pet turtles (Kuzenski, 1976). In 1996, the United States exported over 8.4 million slider turtles, primarily to Europe and Southeast Asia, representing 85–90% of global trade in pet turtles and establishing the potential for the industry to make a substantial contribution to local economies (Hughes, 2000).

Indigenous communities are showing increasing interest in aquaculture (Skladany et al., 2004), particularly where it has the potential to contribute to the local economy and self-determination. Indigenous enterprises often involve species that have been hunted traditionally, because it is seen to engage young people in activities that connect them with their traditional lands and culture (Webb, 1996). In Australia, the Bawinanga Aboriginal Corporation (BAC), representing the indigenous Maningrida community in northern tropical Australia, is keen to develop local industries that contribute to economic independence and self-determination through the sustainable development of their land and sea resources (Altman and Cochrane, 2005). A key objective of BAC is to provide employment opportunities for Aboriginal people that draw upon cultural knowledge and provide young people with the prospect to regularly move onto their lands and interact with people that live a more customary (subsistence) lifestyle (Fordham et al., 2004). As part of this initiative, they have established a local industry to produce hatchlings of the distinctive northern snake-necked turtle (*Chelodina rugosa*), initially for the domestic pet turtle market (Fordham et al., 2004), but with an eye to expansion in response to the growing demand for wild meats and natural medicinals, domestically and internationally.

The aim to develop an aquaculture enterprise focused on culturally identifiable employment in part influences the technical challenges of egg collection, incubation and hatchling husbandry. These challenges include (a) obtaining eggs from remote satellite communities (outstations) in sufficient numbers and at an appropriate time to enable them to be incubated in batches; (b) determining the conditions of incubation that optimize egg survival and hatchling viability, and minimize incubation duration; and (c) determining the conditions that are optimal for hatchling survival and growth in preparation for sale.

C. rugosa in many ways lends itself to aquaculture. It is widespread and abundant in the wet-dry tropics of northern Australia (Cogger, 2000), where it lays its eggs underwater during the late wet season at the edge of the waterholes it inhabits (Kennett et al., 1993a). Eggs survive inundation because they have a modified vitelline membrane (Seymour et al., 1997), allowing oviducal arrest to continue in the form of embryonic diapause until the waters recede and the inundation-induced hypoxia ends (Kennett et al., 1993b). Tolerance of immersion in water means that eggs can be readily transported and stored to await incubation. This species is highly fecund compared to other freshwater turtles of a similar size, laying between 6 and 21 eggs per clutch (Kennett, 1999), and 3–4 clutches of eggs per season (Fordham, unpubl. data). Offspring sex of all chelid turtles so far examined is determined genetically (Georges, 1988; Georges and McInnes, 1998). Young turtles grow rapidly, reaching sexual maturity in as little as 4.2 years for males and 7 years for females (Kennett, 1996).

Turtle aquaculture often lacks a solid foundation in science for the collection, incubation and post-hatching husbandry of young turtles, as approaches are often founded in knowledge accumulated in ad hoc fashion (Shi and Parham, 2000). In this study, we investigate the influence of duration of inundation, incubation temperature and water temperature on the survival and quality of hatchlings of *C. rugosa* to better inform the Maningrida industry in obtaining hatchling turtles for sale in the domestic Australian pet trade. We show that conditioning of the pre-incubation and incubation environment, not only influences embryological development in *C. rugosa*, but also hatchling fitness and growth. This paper is a continuation of an earlier paper exploring the evolutionary implications of these data on incubation period and egg survival (Fordham et al., 2006).

2. Materials and methods

2.1. Egg inundation and incubation

Inundation and incubation procedures are documented in detail elsewhere (Fordham et al., 2006). In summary, eggs were obtained by hormonal induction with Oxytocin® (Troy Laboratories, Smithfield, NSW, Australia) at a dosage rate of 1 unit/100 g body mass (Ewert and Legler, 1978), weighed (±0.1 g) and labelled with a unique number. Two eggs were selected at random from each of five clutches and systematically allocated to each of five constant temperature treatments (26, 28, 29, 30 and 32 °C±0.1 °C) and five inundation treatments (0, 2, 6, 10 and 25 weeks) in a Latin Square Design. This basic experiment was replicated four more times using eggs from the remaining 20 clutches. The response variables were duration of incubation (time to pipping) and survival rate. Hatchlings were removed from the incubator once their egg yolk sac had been fully internalized. The scutes of each hatchling were notched with a unique number system and they were then transferred to the husbandry experiment.

2.2. Husbandry

Hatchlings were grown in one of three Reln 1000 Litre (L) oval water tubs (Reln Plastics Pty Ltd; Ingleburn, NSW, Australia), each with a separate 80 L bio ball filtration system fed by a Pond Master PM-1500 (Kong's [Aust.] Pty. Ltd; Ingleburn, NSW, Australia) submersible pump (flow rate of 1450 L/h). Two 48″ NEC UV tubes (NEC Lighting, Ltd.), one black tube (emitting 290–320 NM of UVB and 320–400 NM of UVA) and one white tube (emitting 400–700 NM of UV, 280–320 NM of UVB and 320–400 NM of UVA) were suspended 28 cm above the water surface, providing a full spectrum light source. Lights were set on timers to provide a day night cycle. Ultraviolet B exposure during basking is important for endogenous production of vitamin D (Ferguson et al., 2003). Basking platforms constructed from 10 mm thick high-density foam, surrounded by plastic mesh, were available to hatchlings in each tank. Tanks were maintained at a constant water temperature of 26, 28 or 30±0.2 °C. Hatchlings were allotted to a water temperature according to egg history, allowing inundation and incubation treatments to be balanced across water temperature treatments. However, the experimental design did not allow for the effects of clutch to be balanced across the husbandry treatments. Water temperature was monitored daily via probes connected to a Datataker 500® series data logger (Esis Pty Ltd; Pennant Hills, NSW, Australia). Probes were calibrated against a reference (±0.1 °C) certified by NATA.

Upon absorption of egg yolk sac, hatchlings were immediately housed in one of the three 70 L interim tanks heated at 26, 28 and 30±0.2 °C for a standard period of seven days. These tanks were stocked to excess with brine shrimp. Hatchlings were then removed and weighed (±0.01 g) using a HF-6000 electronic balance (A & D Mercury Pty. Ltd. Seven Hills, NSW, Australia). Maximum straight-line carapace and midline plastron length (±0.01 mm) were measured using vernier callipers; marks were re-notched where necessary. The hatchlings were then released into grow out tanks. Turtles were fed premium beef mince and vitamin supplements were added to this at a dosage of one tablespoon of Herptivite Multivitamin and one tablespoon of calcium with vitamin D3 (Rep-Cal®; Herp Shop™, Ardeer, VIC, Australia) per kg of mince. A diet of mince was chosen primarily because of its ready availability at low cost in the Maningrida community. Turtles were fed daily to excess and had equal access to food; thus food competition was eliminated. Water fouling was prevented by daily removing uneaten food and by flushing each tank with 200 L of fresh, pre-heated water. Turtle weight and maximum straight-line carapace and midline plastron lengths were measured at weekly intervals. Turtles were removed from the experiment after 26 weeks.

2.3. Analysis

Data from the Latin Square experiment were analysed to determine the impact of inundation duration, temperature and clutch on incubation period as reported elsewhere (Fordham et al, 2006). The response surface for incubation period as a function of inundation duration and incubation temperature was obtained using a general linear model (PROC GLM) following appropriate transformation. The residuals were calculated for the presentation and interpretation in the present paper. Hatchling survival was related to inundation duration, incubation temperature, water temperature and clutch using generalized linear models (PROC GENMOD) in Program SAS Version 8 (SAS Institute, 2001) with a logistic link function and binomial error structure.

Split stick regression (Freund and Little, 1991) was used to estimate the parameters of two linear segments of the post-hatching growth curve and to identify the junction point K (knot). All other analyses are standard (Sokal and Rohlf, 1995). Means are presented with their standard errors, unless otherwise specified and main effects in the analyses of variance were not interpreted without qualification before first testing for interaction.

3. Results

3.1. Size at hatching

Hatchlings weighed 7.1±0.1 g on average (3.2–10.3 g; n=116) emerging from eggs that initially weighed 14.0±

0.2 g on average (7.4–21.1 g; $n=250$). The hatchlings had a mean maximum straight-line carapace length of 33.3±0.25 mm (22.9–38.6 mm; $n=112$) and a midline plastron length of 25.6±0.20 mm (17.7–29.2 mm; $n=112$).

Both incubation temperature ($F=4.94$; $df=4$, 70; $p<0.002$) and clutch of origin ($F=11.95$; $df=23$, 70; $p<0.0001$) had a significant effect on hatchling weight, but the magnitude of the effect in each case was marginal (Fig. 1). Duration of inundation did not have a significant effect on hatchling weight ($F=0.43$; $df=4$, 70; $p=0.79$). By far the most influential variable on hatchling weight was initial egg weight. The two were very strongly positively correlated ($R^2=0.72$, $F=286.3$; $df=1$, 114; $p<0.0001$). Of the variation explained by this correlation, 88.3% could be explained by clutch of origin. Variation in egg weight among clutches was much greater than variation within clutches. We therefore calculated the predictive relationship between mean hatchling weight (HW) and egg weight (EW) [grams] by clutch using the following equation:

$$\overline{\mathrm{HW}} = 0.468\overline{\mathrm{EW}} + 0.4 \text{ grams} \times \left(R^2 = 0.81; F = 95.2; df = 1, 23; p<0.0001\right)$$

3.2. Post-hatching survival and growth

Both period of inundation ($X^2=7.93$; $df=1$; $p<0.005$) and water temperature ($X^2=6.27$; $df=1$; $p<0.02$) significantly influenced post-hatching survival

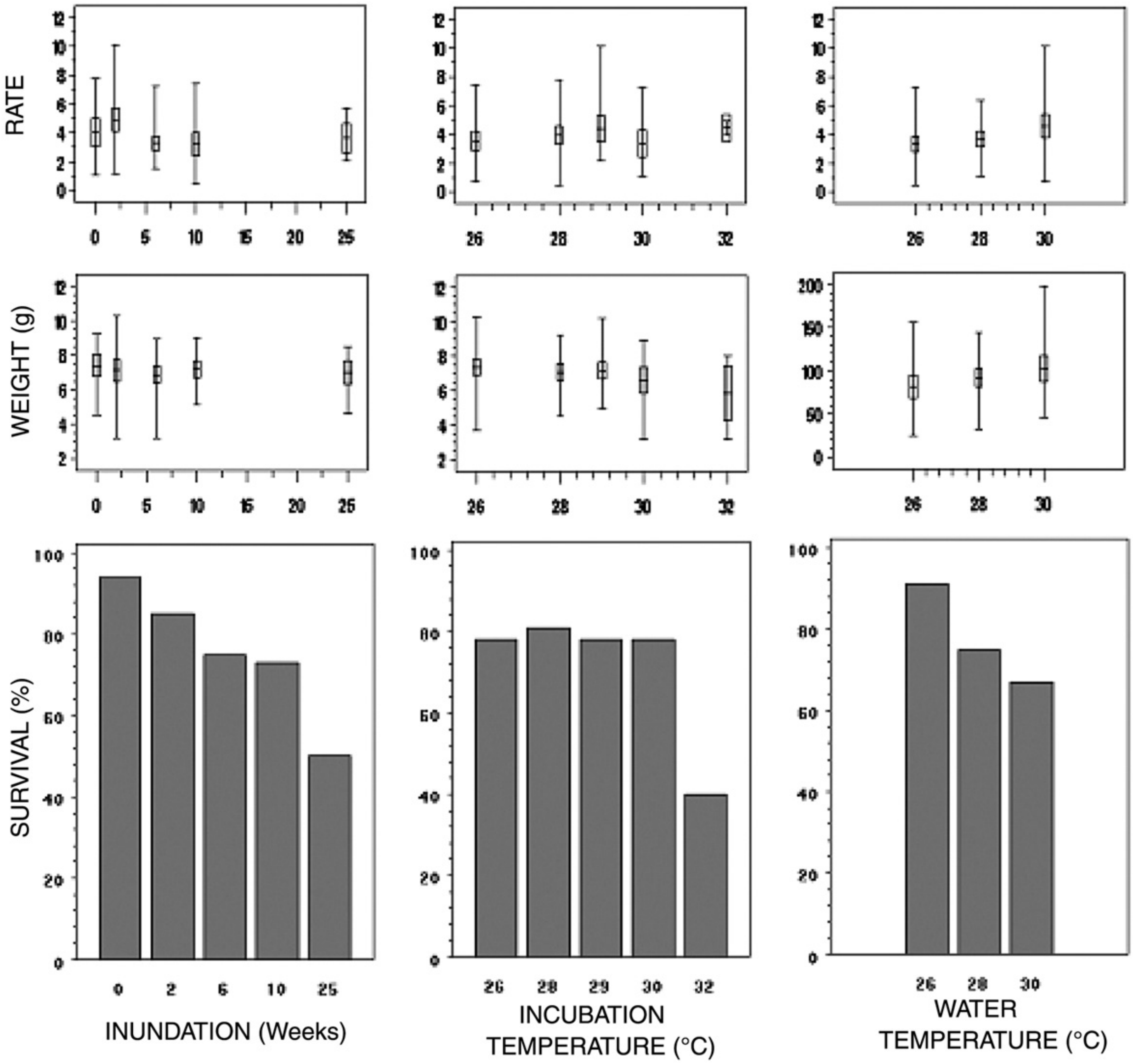


Fig. 1. Effect of inundation (0, 2, 6, 10, 25 weeks), incubation temperature (26, 28, 29, 30 and 32 °C) and water temperature (26, 28, 30 °C) on post-hatching survival, initial hatchling weight (final hatchling weight in the case of water temperature) and growth rate (g/week) post-yolk exhaustion.

(Fig. 1). Highest post-hatching survival occurred in hatchlings that were from eggs that were not inundated or inundated for a relatively short period and housed, following hatching, in the coolest water temperature of 26 °C. There was a significant positive relationship between survival and hatchling weight ($X^2=14.72$; $df=1$; $p<0.0001$) and this remained significant after correcting for the effect of clutch ($X^2=4.04$; $df=1$; $p<0.05$). Incubation temperature did not influence post-hatching survival ($X^2=3.55$; $df=4$; $p=0.47$) though survival at 32 °C was extremely low (Fig. 1).

Post-hatching growth (change in mass) followed a pattern that could be characterized by two distinct segments (Fig. 2). Growth was at first slow (Phase I growth), then suddenly accelerated to a new higher rate (Phase II growth), which persisted until the end of the experiment. We interpret this as two periods of growth, the first dominated by drawing upon its residual yolk (Phase I), and the second dominated by a period of growth under the influence of food intake (Phase II). The junction point between Phase I to Phase II growth (K, in days) provided an index of the relative duration of each of the two linear segments of the post-hatching growth curve in the 6 months of examination.

Incubation period ($F=12.87$; $df=1$, 97; $p<0.001$; $R^2=0.12$) and egg mass ($F=8.77$; $df=1$, 96; $p<0.005$; partial $R^2=0.07$) were identified as influential on rate of growth in Phase I by a stepwise regression on incubation period, egg mass, duration of inundation, incubation temperature, water temperature and hatchling mass. An increase in either incubation period (decrease in development rate in the egg) or an increase in egg mass results in a marginal increase in the rate of early post-hatching growth. Incubation period was also weakly influential on parameter K ($F=4.93$; $df=1$, 98; $p<0.05$; $R^2=0.04$), which determines the duration of the early phase of growth. An increase in incubation period (decrease in embryonic development rate) would be expected to prolong early post-hatching Phase I growth.

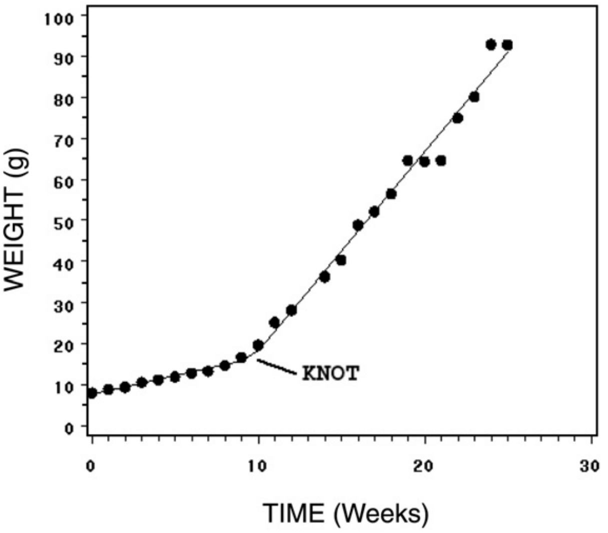


Fig. 2. Hatchling growth is characterized by two distinguishable segments. Growth during Phase I (left of the knot) is thought to be dominated by utilization of residual yolk. Growth during Phase II (right of the knot) is thought to be dominated by energy derived from feeding (example shows growth for a hatchling reared at 28 °C).

Post-hatching growth after the initial phase of relatively slow growth was positively influenced by water temperature ($F=7.94$; $df=1$, 98; $p<0.01$; $R^2=0.08$) and mass at hatching ($F=6.57$; $df=1$, 97; $p<0.02$; partial $R^2=0.05$). Final size of hatchlings raised in 30 °C water (102.4±7.46 g; 46–198 g) was 25.2% greater on average than those raised in 26 °C water (81.8±6.42 g; 25–156 g).

4. Discussion

This research emerged from the desire of the Bawinanga Aboriginal Corporation to develop an indigenous enterprise initially focused on egg harvesting for the production and sale of hatchling turtles, with a view to expanding to rearing turtles for meat and medicinal purposes. Egg harvesting focuses on the least sensitive stage class for population growth (Cunnington and Brooks, 1996; Heppell, 1998), and as such, egg harvesting provides little threat to population persistence. The optimal conditions for the storage of eggs, their incubation and the subsequent growth of hatchlings require integration of previously studied data with those presented in this paper.

Eggs of *C. rugosa* can withstand extended periods of hypoxia through inundation, up to 10 weeks without suffering appreciable mortality, and an overall tolerance of 25 weeks (Fordham et al., 2006). Developmental arrest in response to hypoxia in *C. rugosa* carries with it a number of unique advantages for the industry. The ability to store eggs immediately after laying for up to 10 weeks without appreciable impact on egg or embryo survival permits egg transportation (under water) without incurring high levels of mortality similar to those reported during the transport of eggs of other turtle species (Harry and Limpus, 1989; Parmenter, 1980). Six weeks inundation actually improves embryonic survival compared to no inundation (Fordham et al., 2006). This unique feature of the biology of *C. rugosa* opens up potential niche markets such as that provided by research laboratories nationally or internationally for eggs of this species, but perhaps more importantly, it allows the accumulation of eggs in diapause until there are sufficient numbers to incubate as a batch. A window of 2–10 weeks inundation is sufficiently broad to allow the accumulation of eggs without a marked cost in egg survival.

Embryonic survival, developmental period and size at hatching are often influenced by incubation temperature (Booth, 2000; Brooks et al., 1991; Georges et al., 2005; Hewavisenthi and Parmenter, 2001; Reece et al., 2002). An increase in incubation temperature, within bounds, accelerates development without adversely affecting morphogenesis (Ewert, 1985), so the optimal temperature for incubation is a trade-off between maximizing hatching success, minimizing incubation period and possibly maximizing hatchling size. Fordham et al. (2006) found that embryonic survival of *C. rugosa* was greatest at 26 °C, steadily declining as temperature increased to 32 °C (Fig. 3). Similarly, an increase in incubation temperature decreased incubation period by approximately 40 days; however almost half of this variation in incubation period could be attributed to the increase in incubation temperature from 26 to 28 °C. These results suggest that 28 °C would be a good compromise to ensure both high survival of the embryos and a relatively short incubation period (Fig. 3). The effect of incubation temperature on size at hatching was marginal, it being dominated by initial egg size.

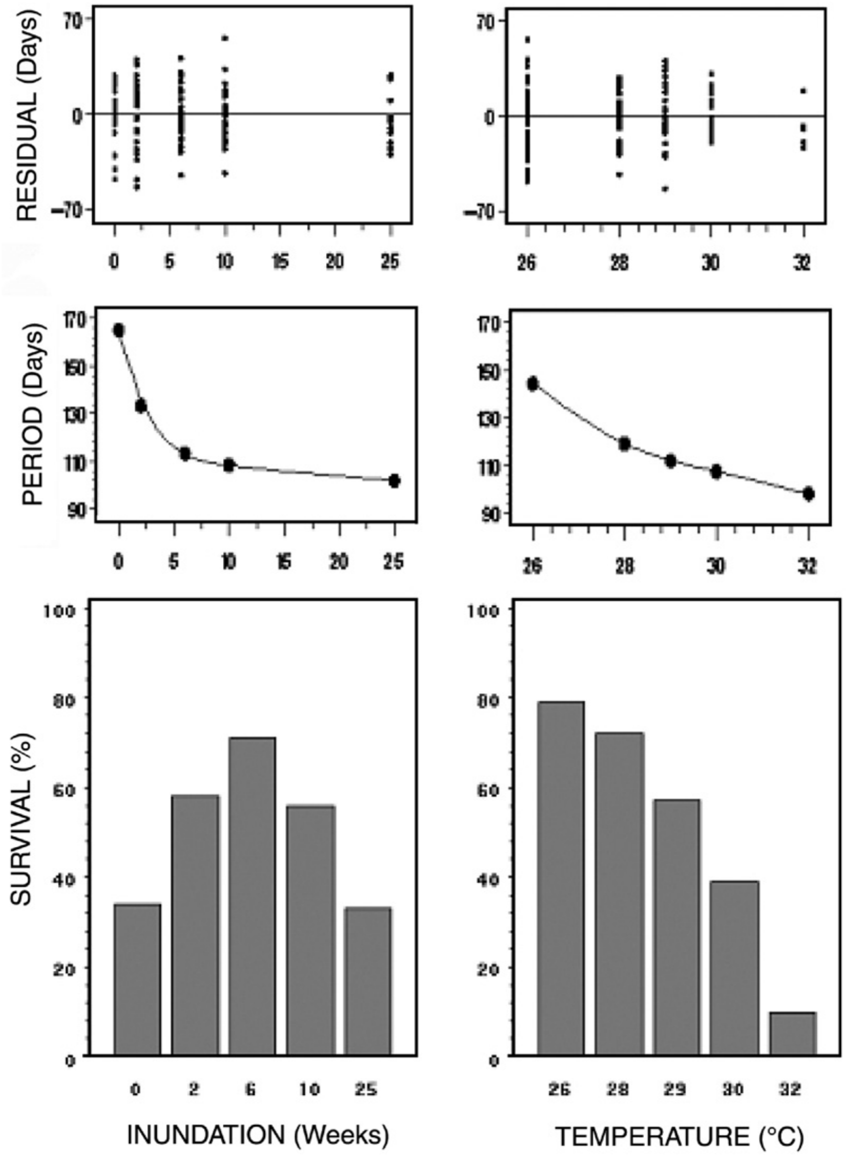


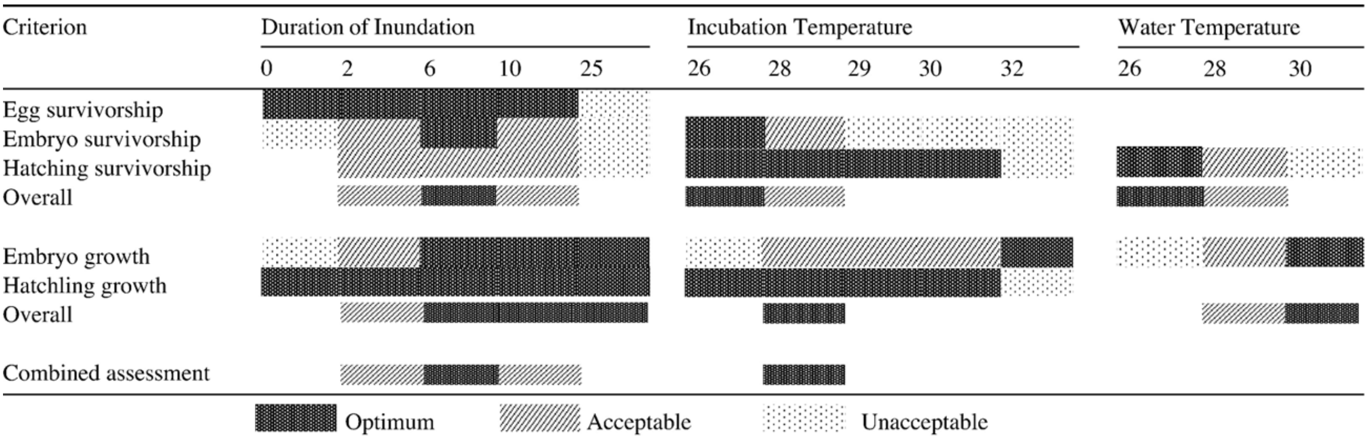
Fig. 3. Effect of inundation (0, 2, 6, 10, 25 weeks) and incubation temperature (26, 28, 29, 30 and 32 °C) on egg survival, incubation period and residual variation among eggs in incubation period (after Fordham et al., 2006). Incubation period is plotted as the marginal means from the Latin Square ANOVA, noting that the inundation and temperature effects were additive (Fordham et al., 2006). The residual variation in incubation period as shown is after the effects of temperature, inundation and clutch have been removed.

The high variability in incubation period observed in *C. rugosa* at a given inundation and incubation regime is rare among turtles, hatching tending to be synchronous (Booth, 1998; Hewavisenthi and Parmenter, 2001; Spencer et al., 2001; Ashmore and Janzen, 2003). Our analysis of residual variance (Fig. 3) showed variation in hatching time of ± 50 days for eggs treated in the same way. When incubated as a batch, this variation greatly increases the duration of attention the eggs need, with consequential inefficiency. It is not known whether this high variability is a feature of the life history of *C. rugosa* to cope with unpredictability in the onset of conditions suitable for hatching in the wet–dry tropics (Fordham et al., 2006), or whether, in addition to inundation-induced diapause, there is some form of mid-term diapause or late-term embryonic aestivation that has not been taken into account. A mid-term diapause terminated by some environmental cue such as a low temperature pulse (as occurs in *Chelodina expansa*; Booth, 2002), or late-term embryonic aestivation terminated by increased moisture (as occurs in *Carettochelys insculpta*; Webb et al., 1986), would serve to entrain embryonic development and reduce variability in hatching times for eggs inundated and incubated under identical conditions. The existence, or otherwise, of additional periods of embryonic diapause or aestivation during the embryonic development of *C. rugosa* awaits further research.

Our research confirms the findings of other studies of turtles (Finkler, 1999) and other reptiles (Hare et al., 2004) in demonstrating an effect of incubation environment on post-hatching survival. Post-hatching survival of *C. rugosa* was negatively correlated with duration of inundation (Fig. 1). Calcium is leached from the shell during egg immersion in *C. rugosa* (Seymour et al., 1997). Since the eggshell provides a substantial source of calcium for hatchling reptiles (Packard et al., 1992; Shadrix et al., 1994), the negative relationship between hatchling survival and egg immersion may reflect a cost associated with calcium leaching during inundation. Incubation temperature did not appear to influence post-hatching survival, though the survival of hatchlings that emerged from eggs incubated above 32 °C was very low (Fig. 1).

Hatchling growth in *C. rugosa* is characterized by two phases (Fig. 2). There is an initial phase of relatively slow growth under the partial influence of initial egg size and incubation duration (or correlates), followed by a second phase of relatively rapid growth under the partial influence of water temperature and mass at hatching (or correlates). This pattern was adequately modelled for the six-month period of this study by a regression comprising two linear segments. The timing of the switch from Phase I to Phase II growth varied from hatchling to hatchling and was weakly correlated with incubation duration. Residual egg yolk is important in meeting the initial metabolic demands of hatchling reptiles (Kraemar and Bennett, 1981; Webb et al., 1986; Whitehead, 1990). We suggest that Phase I growth is dominated by the period in which residual yolk, absorbed as a yolk parcel into the body cavity shortly before or after hatching, is used to sustain growth. We showed that an increase in either incubation period (decrease in development rate in the egg) or an increase in egg mass resulted in a marginal increase in the rate of early post-hatching growth. Incubation period reflects

Table 1
Summary of the responses to duration of inundation, incubation temperature and water temperature



Criterion	Duration of Inundation					Incubation Temperature					Water Temperature		
	0	2	6	10	25	26	28	29	30	32	26	28	30
Egg survivorship	▓	▓	▓	▓	░								
Embryo survivorship	░	▨	▓	▨	░	▓	▨	░	░	░			
Hatching survivorship		▨	▨	▨	░	▓	▓	▓	▓	░	▓	▨	░
Overall		▨	▓	▨		▓	▨				▓	▨	
Embryo growth	░	▨	▓	▓	▓	░	▨	▨	▨	▓	░	▨	▓
Hatchling growth	▓	▓	▓	▓	▓	▓	▓	▓	▓	░			
Overall		▨	▓	▓	▓		▓					▨	▓
Combined assessment		▨	▓	▨			▓						

▓ Optimum ▨ Acceptable ░ Unacceptable

Inundation of eggs for 6 weeks, incubation of embryos at 28 °C and raising hatchlings at a water temperature of 28 °C will yield the best overall outcomes, though trade-offs are required between incubation period versus embryo survival, and hatchling growth and survival.

overall rate of development within the egg, which is likely to affect anabolic efficiency (Booth, 2000). Egg mass is likely to be related to initial yolk mass (Packard et al., 1987). Hence, the two variables that have been identified in our analysis as being related to early hatchling growth also potentially influence the amount of residual yolk available to the hatchling. This is consistent with the above suggestion that the dominant influence on rate of growth soon after hatching is that of residual yolk.

We interpret Phase II growth as representing the period after the internalized yolk is exhausted, when the hatchling is actively feeding and where growth rate is primarily under the influence of food uptake. Other studies have shown that hatchling size (Valenzuela, 2001), incubation environment (Rhen and Lang, 1995; Roosenburg and Kelley, 1996; Du and Ji, 2003; Ji et al., 2003) and maternal identity (Brooks et al., 1991; Bobyn and Brooks, 1994; Steyermark and Spotila, 2001) can influence hatchling turtle growth. In our study, water temperature had the greatest influence on hatchling mass at 6 months. Hatchlings raised in 30 °C water tend to be 25.2% heavier on average than those raised at 26 °C, indicating that the higher temperature is more suitable for maintaining hatchling *C. rugosa*. This conclusion needs to be qualified by the reduced survival of hatchlings with increasing water temperature (Fig. 1), suggesting that rapid growth in *C. rugosa* may have associated costs (Forsman, 1993; Sorci et al., 1996; Warner and Andrews, 2002).

4.1. Conclusion

Integrating results of our experiments (Table 1) suggests that inundation of eggs for 6 weeks (2–10 weeks is acceptable), incubation of embryos at 28 °C and raising hatchlings in 28 °C water will yield the best overall outcomes. A trade-off exists between hatchling survival (highest at water temperature of 26 °C) and hatchling growth (greatest at 30 °C), somewhat limiting the possibility of industry expansion to the rearing of turtles for food. Further attention should be paid to increasing hatchling survival at 30 °C to achieve both high survival and the highest rates of growth. This, together with the issue of entraining embryonic development to achieve a short and predictable incubation period, awaits further research.

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