ISSN 1094-2246

Contemporary Herpetology

Volume 2006, Number 1 www.contemporaryherpetology.org 27 March 2006

IMMACULATE CONCEPTION, INCUBATION PROTOCOLS, AND EGG CHARACTERISTICS OF THE GANGES SOFTSHELL TURTLE (*ASPIDERETES GANGETICUS*)

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ABSTRACT: Reproductive biology of *Aspideretes gangeticus* was studied between 1986 and 2001. Clutch size averaged 17.9 eggs and ranged between six to thirty-five eggs. Egg length averaged 30.6 mm, egg width averaged 30.22 mm, and egg weight averaged 16.85 g. Clutch volume averaged 253.75 ml. No significant difference was observed in clutch size between dry and wet seasons. Of the various incubation protocols tested, one that involved transitional temperatures of 28º – 31º C, to chilling at 15º – 18º C, and then 23º – 26º C resulted in the highest hatching success. *Aspideretes gangeticus* exhibit two forms of development arrest during incubation, embryonic diapause early in incubation and embryonic aestivation in the latter trimester of incubation. The two *Aspideretes gangeticus* females that produced clutches for the current study produced eggs with a high fertility percentage throughout the fifteen years for which they stored sperm.

Key Words: Aspideretes gangeticus; egg characteristics; embryonic development; incubation protocols; sperm storage

INTRODUCTION

The Ganges soft-shell turtle (*Aspideretes gangeticus*) is a large trionychid from India, Nepal, Pakistan, and Bangladesh. The species is thought to have radiated from this region along with its three sister species (Das 2001).

The breeding biology of *Aspideretes gangeticus* is poorly known. Apart from reports by Menon (1988), Whitaker & Andrews (1997), Das (1995), and Whitaker (2000), nothing is known of developmental diapause in this species. Schmidt (1943) studied development of the mineral layer of the eggshell of *Aspideretes gangeticus*, while Rathke (1848) studied the embryology of a closely related species, *A. hurum*. Rao (1987) studied spermatogenic cycles in male *A. gangeticus*, the intrinsic nerve supply of the female reproductive tract (Rao 1983), and reported on egg laying in captivity (Rao 1985). Vasudevan (1995) and Vyas and Patel (1992) studied reproductive biology of *A. gangeticus* in the wild and in captivity respectively. Mishra (1987) reported egg collection of this species for the Ganga river pollution control project and studied nesting of the species on the Chambal River (Mishra 1986).

The ability of this species to store sperm for up to 13 years has been previously reported (Whitaker 2000). Seminal receptacles are present in the infundibular region of *A. gangeticus* females (Rao 1986). These receptacles are morphologically unspecialized tubles of albuminous glands in the caudal part of the albuminous region of the oviduct (Diwan & Dhakad 1995). Within Class Reptilia, sperm storage is known to occur in several lizards and snakes (Smyth & Smith 1968, Girons 1962, Cuellar 1966, Bellairs 1971), and testudines (Barney 1922, Ewing 1943, Smith 1956, Galbraith 1993, Gist & Fischer 1993, Gist & Fine 1992, Jones 1986, Palmer et al. 1998, Pearse et al. 2001), all from temperate regions. To date, *A. gangeticus* the only tropic-zone reptile known to exhibit sperm storage (Das 1995; Whitaker 2000).

Here, I present data on clutches collected between 1986 – 2001 at the Madras Crocodile Bank/Centre for Herpetology (MCB/CFH), produced by two females. No males of the species or closely related species were present in the enclosure for the duration of this 15-year period. The two *A. gangeticus* females inhabited both a temple turtle tank and a *Gavialis gangeticus* breeding enclosure, which has been previously described (Whitaker 2000). Long term sperm storage, seasonal variation in clutch size, incubation protocol experiments, growth of breeders, and egg characteristics and development are presented and discussed.

MATERIALS & METHODS

Eggs from 30 clutches were collected from the temple turtle tank and *G. gangeticus* breeding enclosure between 1986 – 2001. Clutches were collected soon after laying, and oviposition dates are known for 1986. Incubation period for clutches with known oviposition dates are referred to as TIP (true incubation period) while eggs that were collected soon after laying are referred to as LIP (number of laboratory incubation days elapsed). Eggs were marked at the upper-most surface as collected in

the nest with a Sharpie ™ marker or lead pencil, after which they were candled for presence of sub-embryonic fluid (Andrews & Whitaker 1993). Next they were assigned a clutch number and egg number. Initially, determining viability by candling can be difficult (Whitaker & Andrews 1997), but viable eggs always chalk within 24 hours of oviposition. Egg mass (EWT) and hatchling turtles were weighed with an ACCULAB™ weighing scale (0 – 250 ± 0.2 g), and measured with a KWD type dial vernier caliper (± 0.1 mm). Previous authors, when measuring spheroid (i.e. trionychid) eggs refer to either egg diameter (Rashid 1991) or length (Vasudevan 1995). Here the measurements of egg length (EL, in mm) and egg width (EW, in mm) used are essentially measurements of egg diameter at the maximum and minimum diameter, respectively. Measurements of eggs are expressed as average of the total, indicating that figures are averages of separate clutch averages. Clutch volume (CV, in ml) was calculated from the formula $CV = \pi / 6.d^3$ x clutch size, where d=mean egg diameter (Vasudevan 1995). Adult turtles were weighed with a Pesola™ (0 – 50 kg, ± 100 g) weighing scale, and measured with a steel ruler. Abbreviations used in the text are SCL (straight carapace length in cm), SCW (Straight carapace width in cm), PL (Plastron length in cm), PW (Plastron width in cm), and BM (Total body mass in kg).

There are no published standard methods for incubation *A. gangeticus* eggs, apart from studies by Andrews & Whitaker (1993) and Whitaker (2000). Hence, a variety of incubation protocols were tested to determine those that produced maximum hatchability of viable eggs. Protocols involved one to three major shifts in incubation temperature regime, and these are described in Table 1. The exact number of days that eggs underwent incubation at each phase is noted in the results.

Chilling of eggs was accomplished by placing them in the lower shelf of a refrigerator, and this maintained eggs between 15º – 17º C. Constant temperature treatments were conducted using high (albeit unmeasured) humidity incubators. Accuracy was ± 0.3º C of designated temperature. Components and design of these incubators have been described by Lang et al. (1989). Eggs were maintained at 23º – 26º C by incubating them in an air-conditioned laboratory, and were maintained at 28º – 31º C in a ventilated room. Unless otherwise stated, eggs were incubated in plastic boxes with tight-fitting lids and numerous holes. The substrate was large grained sand which was periodically moistened when required. Ventilation holes in boxes were plugged with cotton to prevent ingress of fruit flies. Eggs were immersed in the sand to the point at which ~½ of the egg was buried. Eggs were candled through incubation at irregular intervals and development was gauged by expansion of the chorio-allanotoic network along with development of the opaque patch.

Statistical analysis follows Fowler et al. (1998). Alpha-levels were $p<0.05$ unless otherwise stated. Standard deviations are provided following the mean. All data met assumptions of parametric statistical tests.

RESULTS

Growth of breeding females

Both *A. gangeticus* females were collected from the Jamuna-Naraz River in Orissa in 1985 as adults by the late MCB research associate J. Vijaya and Edward O. Moll. These females were measured at five intervals. Growth in carapace and plastron size was exponential, however mass of both females exhibited large fluctuations (Table 2). Larger size favored higher growth rates in the larger female, and accordingly this female increased 10 cm CL between March 1993 and July 1998, while the smaller female increased 5.5 cm. Mass of the larger female increased 3.5 kg in these five years, while it remained relatively constant in the smaller female.

Clutch and egg characteristics

Clutch size in the current study (n=30) averaged 17.9 ± 7.75 (range 6 - 35 eggs). Egg length (479 eggs from 27 clutches) averaged 30.60 ± 1.41 mm (range 27.43 – 33.53), egg width averaged 30.22 ± 1.83 mm (range 27.7 – 32.5) and egg mass averaged 16.85 ± 1.83 g (range 12.73 – 20.08). Total clutch mass (n=27) averaged 294.39 ± 138.96 g (range 106.4 – 672.1). Clutch volume (n=27) averaged 253.75 ± 113.030 ml, and ranged between 89.57 – 578.18 ml.

Sperm storage as reflected by egg viability

Despite the absence of males in the enclosure in which the two *A. gangeticus* females were housed, they continued to produce viable eggs. Viability remained high throughout these 15 years, and averaged 95.85 % (range 83.3 – 100 %; n=27 clutches).

While embryonic mortality in this study was high in eggs incubated under experimental temperature and substrate regimes (see below), eggs that were incubated under favorable conditions did survive well into incubation, whilst yet others hatched. Further, the absence of a male in the reproductive cycle of these females did not appear to affect frequency of or the size of clutches.

Influence of season on clutch size

Clutch size in the wet season (October – February, when ca. 65% of annual rainfall occurs) averaged 18 eggs ± 8.39 and averaged 17.82 eggs in the dry season (March – September; n=17 clutches; Figure 1). Clutch size peaked in the wet season (35 eggs) and minimum recorded clutch size was in the dry season (7 eggs). There was no significant difference in clutch size between dry and wet seasons (ANOVA; F=0.00036; N=30).

Embryonic mortality and incubation protocols

Protocol A. 28º-31º C to chilling to 23º – 26º C

Table I. Temperature shift protocols used for incubation of *Aspideretes gangeticus* eggs (ºC), and the number of clutches/eggs utilized for each protocol.

Protocol	Phase I	Phase II	Phase III	#eggs	# clutches
A	28º – 31º	Chill	23º – 26º	41	2
B	Chill	28º – 31º	23º – 26º	5	1
C	Chill	23º – 26º	32.5º (constant)	10	1
D	32.5º (constant)	28º – 31º	Chill	9	1
E	23º – 26º	28º –31º	23º – 26º	5	1
F	31.0º (constant)	Chill	31.0º (constant)	4	1

For this treatment, two separate clutches were used. Incubation period is LIP. One was collected on 7th February 2001. Total clutch size was 17 viable eggs of which six were used in this treatment. Eggs from this treatment were incubated from day 1 to day 12 at 28º – 31º C, from day 12 to 64 they were chilled by the technique mentioned in the methods, and from day 64 until the remainder of the incubation period they were at 23º – 26º C. The eggs were candled on 24 April 2000 (day 76 of incubation), and one egg exhibited development when candled. The remaining five eggs appeared the same upon candling as when initially collected. No eggs hatched from this treatment.

The second clutch of eggs was collected on 19 October 2000, and all 35 eggs were chalked at collection. Eggs were incubated from day 1 to day 16 at 28º – 31º C, chilled from day 28 to 47, and from day 47 until the remainder of incubation they were at 23º – 26º C. These eggs were candled on 1 January 2001 (day 72 of incubation), and 18 eggs were judged to be inviable and were discarded. The remaining 17 eggs exhibited positive development upon candling. They were candled again on 24 April 2001 (day 186 of incubation), and 14 eggs continued to show development. Two eggs were discarded at this stage as chalking had regressed. Between 26 and 27 March 2001 (216 – 217 days incubation), 12 of these eggs hatched.

Protocol B. 28º – 31º C to 23º – 26º C

A portion of 5 of 11 viable eggs was used for this treatment from a clutch collected on 7 February 2001. Incubation period referred to is LIP. From day 1 to 12, eggs were kept chilled. From day 12 to 67 of incubation, eggs were maintained at 28º – 31º C. From day 67 until hatching, eggs were kept at 23 – 26. These eggs were candled on 24 April 2001 (day 76 of incubation). Three eggs appeared to have not developed, one egg had rotted, and one egg exhibited development. This particular egg hatched on 14 July 2001, after an incubation period of 157 days.

Protocol C. Chilling to 23º – 26º C to constant 32.5º C

Eggs used in this treatment were from a clutch of 10 eggs all that were viable, collected on 2 December 2000. Incubation period is LIP. From day 1 to 40, eggs were chilled. From day 41 to 88, eggs were maintained at 23º – 26º C, and from day 88 until hatching eggs were maintained at a constant 32.5º C. These eggs were candled on 1 February 2001 at 61 days of incubation, and upon candling only one egg showed development. An etched disk with a prominent nucleus was visible in this egg. Candling on 24 April 2001, day 143 of incubation, revealed that four eggs had visibly developed, four appeared to have not developed, and two eggs had rotted. Candling on 31 May 2001, day 178 of incubation, revealed that the remaining eight eggs had rotted.

Protocol D. Constant 32.5º C to 28º – 31º C to chilling

Eggs for this protocol came from a clutch collected on 2 December 2000. Clutch size was 9, all of which were deemed viable by candling. Incubation period is LIP. From day 1 to day 20 of incubation, eggs were maintained at 32.5º C. From day 20 to 39, they were kept at 28 – 31º C, and from day 39 onwards they were chilled. Candling on 1 January 2001, day 29 of incubation, revealed no positive development in all eggs, and some eggs showed signs of regressing bands. On 24 April, day 143 of incubation, candling revealed no development since the last candling, and the incubation media was moistened. On 31 May 2001, day 179 of incubation, all eggs were discarded as they had rotted.

Protocol E. 23º – 26º C to 28º – 31º C to 23º – 26º C

Eggs for this treatment resulted from a clutch collected on 11 November 2000. Total clutch size was 17 eggs, but only 9 eggs which were viable appeared stable, and the others had rotted. Of the 9 viable eggs, 5 were used for this treatment. Incubation period refers to LIP. From day 1 to 30, eggs were maintained at 23º – 26º C, from day 30 to 52 they were maintained at 28º – 31º C. From day 52 until the remainder of incubation, eggs were switched back to 23º – 26º C. Candling on 2 December 2000, day 21, revealed no visible development when candled, and 3 eggs had developed air spaces. Candling on 1 February 2001 revealed that 5 of the eggs exhibited no development since the last candling, and bands were regressing. Upon inspection on 24 April 2001, day 164, all eggs were found to have rotted.

Protocol F. Constant 31.0º C to chilling to constant 31.0º C.

The remaining four of the nine eggs from the clutch collected on 11 November 2000 as described in Protocol E, were used for this treatment. Incubation period refers to LIP. Eggs were incubated at a constant temperature of 31.0º C, on plastic trays (no media) from day 1 to 22. Following this treatment, eggs were placed in a plastic tray with vermiculite as the medium, with water relations as described by Whitaker (2000). From day 22 to day 44, they were chilled. After this, from day 44 until the remainder of the incubation period they were removed form the vermiculite media and returned to a constant 31.0º C on plastic trays without media. Candling on 2 December 2000 revealed that all eggs had developed air spaces that weren't evident at the initial candling. On 1 February 2001, day 82, all eggs exhibited positive development. One egg had a full term embryo visible upon candling, while the other three had extensive vasculature visible. Candling on 24 April 2001, day 165, revealed that two eggs showed development in vasculature, and one egg had rotted and was discarded. Candling on 27 May, day 198, revealed that all eggs had rotted.

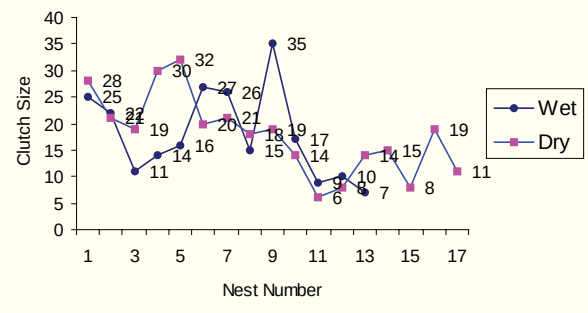

Figure 1. Variation in clutch size in *Aspideretes gangeticus* in dry and wet seasons.

Table 2. Measurements of *Aspideretes gangeticus* females (CL, CW, & PL in cm; mass in kg).

Date	Large female SCL	SCW	PL	mass	Small female SCL	SCW	PL	mass
03/18/93	64.0	49.5	51.0	30.0	47.0	34.5	36.5	14.5
08/09/94	67.5	48.5	51.3	30.0	47.0	-	37.0	13.5
04/05/95	69.0	49.5		33.4	48.2	33.5	37.0	15.4
11/21/95	70.0	55.0	52.0	31.0	51.0	37.0	37.0	14.5
12/13/96	74.0	-	52.0	31.5	52.5	-	37.0	13.5
07/26/98	-	-	-	33.5	-	-	-	14.5

DISCUSSION

Embryonic mortality and incubation protocols.

Of the four types of developmental arrest described by Ewert (1985), *A. gangeticus* exhibit embryonic diapause earlier in development, and embryonic aestivation at the trimester of incubation. This appears to be unique; no other testudine is known to exhibit this feature of "bi-arrest" during incubation.

Although frequently associated with temperate turtles, embryonic diapause may be an adaptation by *A. gangeticus* to survive the major temperature fluctuations experienced in the northern Indian habitats in which they occur. Annual fluctuation was 4º – 47º C at one locality, Etawah District, in Uttar Pradesh (Vasudevan 1995). Species that exhibit this form "advance through development extremely slowly though the incubation temperature is conducive to rapid development of their later states" (Ewert 1985). In temperate zone testudines known to exhibit embryonic diapause, chilling may on one hand shorten the period of diapause (*Kinosternon scorpioides*), or be essential for development to resume (*Rhinoclemmys pulcherrima*) (Ewert 1985). The later scenario is the situation in *A. gangeticus*.

The duration of embryonic diapause in *A. gangeticus* remains unclear. However, based on results from the current (Protocol A), and previous (Whitaker & Andrews 1997, Whitaker 2000) studies, the duration that eggs tolerate induced chilling ranges between 19 and 60 days. The stage of incubation at which chilling has been induced is highly variable. Previously, Whitaker (2000) chilled eggs between 14 – 37 TIP at 13º – 15º C, whilst Whitaker & Andrews (1997) chilled eggs at 30, 45, and 60 days (TIP) at 17º C. In the latter study, eggs that were chilled for periods shorter than 60 days did not develop past diapause, whilst eggs that were chilled for 60 days exhibited development within 20 – 30 days. In the current study, chilling was induced between 28 – 47 LIP days. The relationship between the number of days eggs were chilled and incubation period, if any, is unclear in *A. gangeticus*. Eggs chilled for 19 days (LIP) in the current study hatched after 216 – 217 days, whilst in another study (Whitaker 2000) eggs chilled for 23 days (TIP) hatched at 174 days.

Of the six incubation protocols tested here, protocols A & B were the sole treatments that resulted in production of any hatchlings. Of these, protocol A produced the highest hatchability of fertile eggs (34.29%). Hatching success of *A. gangeticus* may be naturally low; Whitaker & Andrews (1997), found from a total of 10,372 eggs collected for the Ganga Cleanup Project, that 3,928 hatched (37.87%). In an earlier protocol, I hatched three *A. gangeticus* from seven chalked eggs incubated in a media of vermiculite after a laboratory incubation period of 174 days (Whitaker 2000), and hence egg survival was 42.86 %. In this treatment, eggs were maintained at 29º – 30º C from days 2-14 (LIP), chilled at 13º – 15º C from days 14 – 37, and then kept in an air-conditioned room at 24º – 25º C until they hatched.

Vasudevan (1995) recorded hatching percentage of 12 nests at the Chambal River, Uttar Pradesh, and this was highly variable. Hatching percentage here is expressed as the number of hatchlings resulted from a clutch of un-candled eggs. Hatchling percentage (calculated from Vasudevan 1995 ; Table 2) averaged 62.86, and ranged from 0 - 100 %. Of interest in this study is that three clutches produced a 100 % hatch, despite nest temperature maxima in the range of 35.3º – 36.3º C. In addition, nest temperature maxima were negatively (albeit significantly) related to hatching percentages, the latter decreasing as the former increased (r=-0.78, N=12; calculated from Vasudevan 1995; Table 2). Incubation temperatures of 38º C are known to be lethal to Australian *Chelonia mydas* embryos (Bustard & Greenham 1968 In Ewert 1985). Eggs of *Graptemys ouachitensis* and *G. pseudogeographica* from the United States are marginally tolerant to incubation temperatures of 35º C (Vogt 1980 In Ewert 1985).

Aspideretes gangeticus eggs appear to exhibit embryonic aestivation in the latter trimester of incubation. Ewert (1985) describes embryonic aestivation as "a form of late embryonic dormancy that occurs under warm weather conditions". Some evidence for this mechanism operating in the species comes from Vasudevan's (1995) study. There, residual yolk in dead full term embryos was found to be more than that of live hatchlings. It was inferred that the embryos died well before hatching, and that the fully developed embryo aestivates, using yolk reserves until environmental conditions outside of the nest improve. External stimuli play a role in inducing hatching.

In a previous report (Whitaker 2000) I might have induced *A. gangeticus* hatching earlier than it would have occurred if the eggs were left undisturbed. In another instance, Whitaker & Andrews (1997) found that *A. gangeticus* eggs hatched soon after being soaked in water and observed this in a field situation also. Vijaya (1983 in Ewert 1985) reported that *Lissemys punctata* were fully developed by around 180 days of incubation, but may not emerge from eggs until rains occur.

Seasonal variation in clutch size.

Clutch size in the current study was found to not differ significantly between seasons, (ANOVA). Gibbons & Greene (1990) reported that a comparison of early and late nesting *Trachemys scripta* indicated a difference in average clutch size seasonally, being smaller at the end of the egg-laying season. Gibbons et al. (1979) suggested that variables such as clutch sequence, length of the previous growing period, season, and reproductive output in previous years were among factors to examine for understanding patterns of clutch size variation in aquatic turtles.

Sperm storage.

Spermatozoa are fragile cells that do not survive for long outside of the male reproductive tract (Restall 1967 in Gist et al. 2000). However, fertility is preserved in those species that store spermatozoa within the female reproductive tract (Gist et al. 2000). Spermatozoa are stored within the oviducts of many reptiles and fertile eggs may be deposited for up to several years after isolation from males (Gist & Jones 1987).

In the current study, *A. gangeticus* females produced clutches of eggs with near 100 % viability throughout the 15 years that they stored sperm. This is in marked contrast to trends observed in *Terrapene carolina carolina*, where the proportion of infertile eggs increased as access to males declined (Halgren-Scaffidi 1986, Dodd 2001 in Bezler 2002). Results from the current study also refute the hypothesis that the ability of turtle sperm to fertilize eggs declines in the long term during retention in the female reproductive tract (Hildebrand 1929, Gist & Congdon 1988).

Sever and Hamlett (2002) reported that sperm are found in glands located at the periphery and not the center of the major glandular regions, irrespective of the location of sperm storage tubules. They further note that testudines lack glands specialized for sperm storage, and that turtles have perhaps the longest effective sperm storage (up to four years) among vertebrates. Published records of sperm storage extremes among reptiles are up to seven years in the ophidian *Acrochordus javanicus* (Magnusson 1979), and possibly nine years in the marine turtle *Chelonia mydas* (Fitzsimmons 1998). In the current study, *A. gangeticus* females stored sperm for 15 years. This appears to be a new record for sperm storage in any vertebrate.

ACKNOWLEDGEMENTS

Thanks must be given to Harry V. Andrews, Indraneil Das, Chandani Menon, Manjula Tiwari, and J. Vijaya for maintaining records and carrying out several incubation trials. It is because of their systematic collection and diligence that this work is possible. Thanks to Arthur Georges of the University of Melbourne, Daniel Gist of the University of Cincinnati, Bill Bezler at Clarion University, and Brian A. Stacy for gifts of reprints. Indraneil Das, Karthik Vasudevan, Grahame Webb, John Applegarth, and Allen Salzberg provided literature citations. Thanks to my Irula friend C. Kali for his enviable skills in turtle nest location, and for his patience in trying to teach me the same. Thanks to Janaki Lenin for help with the title. I am grateful to the Trustees of the Madras Crocodile Bank/Centre for Herpetology.

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