

EFFECTS OF INCUBATION TEMPERATURES ON
CHARACTERISTICS OF HATCHLING
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ABSTRACT: We examined the effects of egg qualities and incubation temperatures on characteristics of hatchling American alligators (*Alligator mississippiensis*). Alligator eggs were collected from the Rockefeller Wildlife Refuge, Louisiana, and transported to the Savannah River Ecology Laboratory near Aiken, South Carolina. Eggs from each clutch were initially incubated at either 30 C to produce only females or 34 C to produce only males. After 40 days of incubation, the eggs were distributed among four final temperatures (28, 30, 32, and 34 C) for the remaining third of development. Egg and hatchling lipids were extracted with petroleum ether and hatchling nitrogen content was determined using the micro-Kjeldahl procedure.

Egg yolk averaged 40.7% non-polar lipids and parental investment ratios (hatchling yolk lipids/egg lipids) averaged 66% and did not vary significantly among incubation treatments. Individual hatchling characteristics (snout-vent length, carcass lean dry and lipid mass, yolk sac lean dry and lipid mass) were not significantly influenced by initial incubation temperature and, with the exception of hatchling yolk lean dry mass, were not significantly influenced by final incubation temperatures. Multivariate analysis of hatchling characteristics revealed that maternal effects and incubation temperatures during the final third of development appear to have major impact on hatchling characteristics. Based on these results, the "decision" to produce primarily male or female offspring would first be based on characteristics of the female parent and secondarily on the nest temperatures influenced by nest site selection. The effects of nest temperatures would first determine the sex through the first two-thirds of incubation and subsequently influence the quality of the offspring during the final third of development.

Key words: Alligator; *Alligator mississippiensis* Eggs; Embryo development; Hatchlings; Incubation temperatures; Lipids; Yolk reserves

FEMALES may influence the quality of their progeny through mate choice, the amount of resources that they allocate to each egg, and the amount of parental care that they provide. In some cases, females may also influence offspring quality through selection of the post-natal environment.

The first environment that oviparous reptiles experience is within the egg. An embryo's environment is influenced by its own development and externally by the egg's microenvironment within the female or within a nest. Interest in how the egg contents and the immediate nest environment affect reptile hatchlings has increased in recent years, both independent of and due to the discovery that the thermal environment determines the sex of

many species of turtles and crocodylians. Thermal and hydric conditions within a nest influence (1) duration of incubation, (2) hatchling size, (3) yolk sac size, and (4) sex of hatchlings in species with environmental sex determination (Congdon and Gibbons, 1990; Ferguson and Joanen, 1982, 1983; Gutzke and Packard, 1986; Packard and Packard, 1984a; Packard et al., 1985, 1987; Paukstis et al., 1984).

We examined characteristics of a sample of eggs (total wet mass, dry mass of yolk, non-polar lipids, lean dry mass, and shell dry mass) of American alligators (*Alligator mississippiensis*). Additional eggs were incubated at two temperatures for the first two-thirds of incubation and at four temperatures during the last one-third of incubation. At the end of incubation, we examined the following hatchling characteristics: (1) survival, (2) snout-vent length (SVL), (3) total body mass, (4) lean

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carcass mass, (5) lean yolk mass, and non-polar lipid mass (NPL) in (6) the carcass and (7) the yolk sac. We examined an additional hatchling characteristic of pre-ovulatory parental investment in care (PIC). We use the ratio of hatchling total NPL/egg total NPL as an indicator of PIC (Congdon, 1989; Congdon and Gibbons, 1989, 1990; Congdon et al., 1983; Fischer et al., 1991). We based the following experiment on the assumption that temperatures during incubation sequentially influence tissue development, and then organ development and sex determination, and finally embryo growth. The experimental protocol outlined below was established to test the following working hypothesis: Hw—effects of temperature on hatchling characteristics are expressed more during the last third of development (embryo growth phase) than during the first two-thirds of development (from the beginning of embryo formation through the sex determining period). In addition, data from this study were compared to data from a previous study of alligator eggs collected in 1986 from the Rockefeller Wildlife Refuge, Louisiana (Fischer et al., 1991).

MATERIALS AND METHODS

Freshly laid eggs ($n = 177$) were obtained from six nests located on the Rockefeller Wildlife Refuge, Louisiana during the late spring of 1987. Eggs were packaged in vermiculite and shipped by air from Louisiana to Augusta, Georgia, and were then transported by automobile to the Savannah River Ecology Laboratory. From 3–5 eggs from each of five clutches and all four eggs from the sixth clutch ($n = 25$) were frozen for analysis of egg components. We examined a sample of eggs from each clutch to assure that there had been little or no embryonic development past the gastrula stage. Each egg was then divided into shell and egg contents (hereafter referred to as egg yolk).

We used the remaining 152 eggs in an experiment that manipulated incubation temperatures during the early (first 40 days, hereafter referred to as initial incubation temperatures) and late stages (from 40 days

to hatching, hereafter referred to as final incubation temperatures) of embryonic development. The first 40 days of development correspond to the sex determining period (Ferguson and Joanen, 1982, 1983) and formation of a miniature version of the hatchling; the remaining period of incubation (approximately 20 days) is characterized mainly by embryo growth (Ferguson, 1985).

The experiment was a nested design with two treatments, treatment 1 having two initial incubation temperatures (30 and 34 C) for the first 40 days of incubation. Treatment 2 consisted of four final incubation temperatures (28, 30, 32, and 34 C) nested within treatment 1 for the final 20 days of incubation. Eggs from each clutch were initially incubated at 30 C to produce only females or 34 C to produce only males (Ferguson and Joanen, 1982, 1983). At the beginning of the initial incubation temperatures (30 and 34 C), and at the beginning of the final incubation period (28, 30, 32, and 34 C), we distributed viable eggs from each clutch as uniformly as possible to each temperature. Incubation of each embryo was terminated within 10 h of the time that the hatchlings pipped the eggs.

The 152 eggs were incubated in covered plastic containers that contained a minimum of 1.5 kg of vermiculite and water at a ratio of one part vermiculite to 1.5 parts distilled water by mass (approximately -250 kPa). Although we did not measure the water potential of the incubation substrate every other day, vermiculite moisture content was maintained by weighing all containers and eggs and restoring each container to its initial mass by adding <2 ml (0.2% of the total water in the incubation substrate) of water by misting the eggs and substrate every other day. We moved containers among shelves in the incubators every other day to decrease any effects of thermal stratification within the incubator. After pipping, hatchlings were cooled in a refrigerator and then frozen and stored for later analysis.

Wet masses of eggs and hatchlings were obtained to the nearest 0.01 g. Hatchlings were thawed, SVL and tail length measured to 1.0 mm, and each individual was

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TABLE 1.—Characteristics of eggs of American alligators from Louisiana. Data are mean of clutch means of 25 eggs from six clutches (* = component of yolk dry mass).

	Length (mm)	Width (mm)	Wet mass (g)	Water (ml)	Shell dry mass (g)	Yolk dry mass (g)	Yolk lipid mass (g)*	Yolk % lipids
Mean	70.3	41.0	71.4	48.6	7.99	14.82	6.08	40.7
Standard error	1.9	0.5	1.3	0.9	0.19	0.26	0.11	0.30
Minimum	67.1	39.6	60.2	40.1	6.49	11.87	4.80	37.4
Maximum	74.7	42.6	82.6	56.9	9.68	17.06	7.06	44.8

then separated into carcass and yolk sac. Egg yolks, and hatchling bodies and yolk sacs were oven dried at 60 C to a constant mass (nearest 0.001 g) and ground with mortar and pestle. We extracted non-polar lipids (NPL) from all samples for a minimum of 5 h using petroleum ether in a Soxhlet apparatus. The amount of NPL in a sample was determined by subtracting mass of the sample after extraction from the sample mass before extraction. We determined total nitrogen content of lean dry samples of hatchling body and yolk sac using the micro-Kjeldahl procedure and calculated total protein by multiplying total nitrogen by 6.25 (Card and Nesheim, 1966). For eggs not incubated, the dry masses of shells were determined (to 0.001 g) after oven drying at 60 C. We determined inorganic content of hatchling bodies and yolk sacs by burning aliquant samples in a muffle furnace at 550 C for a minimum of 12 h and weighing the remaining ash.

We analyzed the data using the SAS statistical package for micro-computers (SAS, 1985). Individual traits were analyzed using ANOVA and ANCOVA with mean egg yolk dry mass of each clutch as a covariate. We think that the mean egg yolk dry mass of the sample of eggs taken from each clutch is a better covariate than the initial wet mass of individual eggs that were incubated. Use of mean egg yolk dry mass as a covariate prevents the analyses from being confounded by variation in water content and egg shell mass of eggs. We analyzed parental investment ratios using the nonparametric Kruskal-Wallis test. Because many hatchling characteristics are highly correlated, we also analyzed the effects of incubation temperatures on

hatchling characteristics using a multivariate approach (MANOVA).

RESULTS

Egg Characteristics and among Year Variation

Water comprised 68% of the total wet mass of freshly laid eggs. Egg total dry mass consisted of 35.0% shell, 38.3% lean material, and 26.7% NPL. Yolk consisted of 40.7% NPL (Table 1). Analyses of variance indicated that there were significant differences in both total egg lean mass ($F_{5,18} = 20.16$, $P = 0.0001$) and egg lipid mass ($F_{5,18} = 14.76$, $P = 0.0001$) among clutches.

We compared characteristics of alligator eggs obtained from the same area in Louisiana in 1986 (Fischer et al., 1991) to those collected for this study in 1987. Clutch means of dry mass of egg yolks (17.86 g) from 1986 were significantly heavier (3 g) than those from 1987 (14.82 g; ANOVA; $F_{1,9} = 7.82$, $P = 0.021$). The difference was primarily due to an increased NPL mass ($F_{1,9} = 64.82$, $P = 0.0001$) and only secondarily due to increased lean mass of eggs ($F_{1,9} = 4.69$, $P = 0.058$). Linear regressions of natural log-transformed clutch means of the dry masses of egg NPL and yolk were used to examine whether NPL were added in proportion to lean dry yolk material of the egg. Slopes of the relationships between egg NPL mass and total yolk dry mass were not significantly different from 1.0 (based on overlap of standard errors of the slopes with 1.0). Overlap of standard errors with one indicated that NPL were added to eggs in proportion to each unit of lean dry mass in 1986 ($\ln \text{Egg NPL mass} = -0.884 + 0.999 \cdot \ln \text{Yolk Dry Mass}$, $R^2 = 0.996$, $P =$

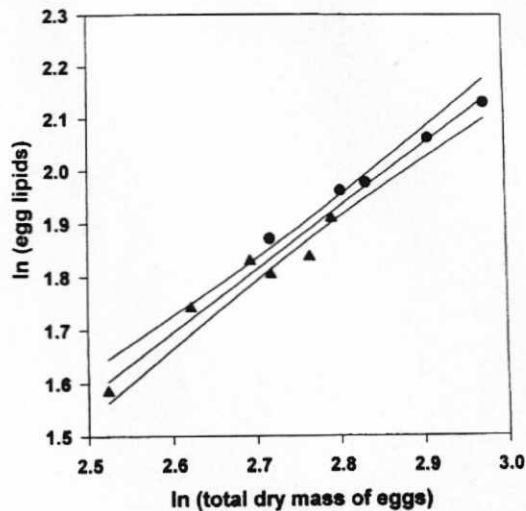


FIG. 1.—A regression of log-normal transformed clutch means of egg NPL mass and yolk total mass for eggs collected in 1986 (circles) and 1987 (triangles) resulted in the following statistics: \ln Egg NPL Mass = $-1.38 + 1.18 \cdot \ln$ Yolk Dry Mass, $R^2 = 0.967$, $P = 0.0001$, $n = 11$, $2 \text{ SE} = 0.14$.

0.0001, $n = 5$, $2 \text{ SE} = 0.070$), and 1987 (\ln Egg NPL mass = $-1.15 + 1.09 \cdot \ln$ Yolk Dry Mass, $R^2 = 0.936$, $P = 0.0016$, $n = 6$, $2 \text{ SE} = 0.28$). However, when data from both years were combined, the slope of the regression was significantly >1 (i.e., proportionally more lipids were added to the larger eggs than was lean material: Fig. 1).

Embryo Mortality

A total of 58 (38%) of the eggs incubated produced hatchlings. Percent mortality among clutches ranged from 38–83% and was significantly different ($\chi^2 = 24.58$, $P = 0.0001$). However, because clutches were distributed uniformly between initial treatments and among final temperature treatments, percent mortality was unaffected by treatment ($\chi^2 = 6.337$, $P = 0.501$). Mortality was not associated with initial incubation temperatures ($\chi^2 = 0.45$, $P = 0.504$) but was significantly higher in the 34 C final incubation temperature treatment ($\chi^2 = 8.20$, $P = 0.042$). In addition, we found no effects on hatchling wet mass that could be attributed to the interaction between mortality and treatments ($F_{7,27} = 0.97$, $P = 0.519$).

Pre-ovulatory Parental Investment in Care

Over all final temperature treatments, PIC averaged 66% ($\text{SE} = 0.021$), mean values for 28 C, 30 C, 32 C, and 34 C were 64.2 ($\text{SE} = 2.6$)%, 68.4 ($\text{SE} = 1.6$)%, 65.2 ($\text{SE} = 1.9$)%, and 63.4 ($\text{SE} = 2.2$)%, respectively. PIC did not vary significantly (Kruskal-Wallis test) among final incubation temperatures ($\chi^2 = 0.209$, $P = 0.273$) nor among clutches ($\chi^2 = 0.168$, $P = 0.551$). Furthermore, PIC did not vary between hatchlings incubated at the two sex determining temperatures during both the initial and final incubation periods (30 C, \bar{x} PIC = 68.4%; 34 C, \bar{x} PIC = 64.6%: $\chi^2 = 2.380$, $P = 0.123$).

Hatchling Characteristics

Hatchlings averaged 110 ($\text{SE} \pm 0.70$) mm in SVL and 230 ($\text{SE} \pm 1.0$) mm in total length. Water comprised 75.4% of hatchling total mass, and hatchling total dry mass (11.4 g) averaged 85.9% carcass and 14.1% yolk sac (Table 2). Non-polar lipids averaged 30.2% of the carcass dry mass and 66.2% of yolk sac dry mass. Hatchling total dry mass consisted of 35.2% NPL, and 67.0% of the original egg NPL (6.0 g) remained as total hatchling NPL (4.0 g) in the carcass and yolk sac (Tables 1 and 2). Hatchlings averaged 47.3% protein and 9.7% ash (Table 2). An average of 50.2% of the total dry mass of the egg was converted to hatchling, and 76.9% of the total dry mass of egg yolk was converted to total hatchling dry mass.

Analyses of variance indicated that (1) SVL, (2) tail length, (3) carcass lean dry mass, (4) carcass NPL mass, (5) yolk sac lean dry mass, and (6) yolk sac NPL mass did not vary significantly with initial incubation temperature (the sex determining temperatures; P values ranged from 0.23–0.84) or with the interaction between initial incubation temperatures and final incubation temperatures (P values ranged from 0.43–0.89). Similarly, neither SVL, tail, hatchling carcass lean dry mass, NPL dry mass, nor yolk NPL mass varied with final incubation temperature (P values ranged from 0.24–0.68). However, hatch-

TABLE 2.—Characteristics of hatchlings of American alligators from Louisiana. Data are means of clutch means of hatchlings from five clutches (* = components of yolk dry mass).

	SVL (mm)	Tail (mm)	Total wet mass (g)	Body dry mass (g)	Body lipid mass (g)	Yolk dry mass (g)	Yolk lipid mass (g)*	Body protein mass (g)*	Yolk protein mass (g)*	Total ash mass (g)
Mean	109.6	118.7	46.0	9.81	2.96	1.60	1.03	5.01	0.38	1.11
Standard error	0.7	0.11	0.41	0.37	0.14	0.14	0.09	0.08	0.03	0.03
Minimum	96.0	107.0	39.2	8.64	2.47	1.14	0.72	3.90	0.07	0.56
Maximum	126.0	132.0	52.5	10.64	3.29	1.93	1.23	6.49	1.10	2.21

ling yolk lean dry mass varied significantly with final temperature ($F_{3,46} = 6.50, P = 0.0009$). Because egg variables differed among clutches, we re-analyzed the same hatchling variables used in the above ANOVA using analysis of covariance with the clutch mean of egg yolk dry mass as a covariate. These analyses revealed no significant differences associated with either initial incubation temperature or the interaction between initial and final incubation temperatures. Only hatchling tail length (ANCOVA: $F_{3,24} = 4.13, P = 0.02$) and hatchling yolk dry mass (ANCOVA: $F_{3,24} = 3.15, P = 0.043$) exhibited significant variation associated with final incubation temperatures. Hatchling characteristics associated with experimental treatments are summarized in Table 3.

Multivariate analysis of the effects of clutch, and initial and final incubation temperatures on dry masses of yolk NPL and lean, and body NPL and lean material,

and SVL of hatchlings indicated that (1) maternal effects (clutch) were significant (Hotelling's $T: F_{20,162} = 9.262, P = 0.0001$), (2) effects of initial incubation temperatures were not significant (Hotelling's $T: F_{5,46} = 0.32, P = 0.312$), (3) effects of final incubation temperatures were significant (Hotelling's $T: F_{15,134} = 3.38, P = 0.0001$), and (4) the effect of interaction between initial and final incubation temperatures was not significant (Hotelling's $T: F_{15,134} = 0.88, P = 0.590$).

We examined the same set of characteristics for just those hatchlings incubated at sex determining temperatures (30 C and 34 C) during both the initial and final incubation periods and found a significant difference in hatchling characteristics (Hotelling's $T: F_{5,10} = 4.08, P = 0.028$). In addition, we examined SVL and tail length of the same group of hatchlings and found no significant difference in size between males and females incubated at sex deter-

TABLE 3.—Least square means (with clutch means of egg dry yolk mass as a covariate) and standard errors (SE) of hatchling components grouped by (A) initial incubation temperatures and (B) final incubation temperatures. (* = variables with significant covariate effects during final incubation.)

		SVL (mm)	Tail (mm)*	Total wet mass (g)	Body dry mass (g)	Body % lipid	Yolk dry mass (g)*	Yolk % lipid
A. Initial incubation								
30 C	Mean	107.1	117.6	45.97	9.69	28.79	1.58	64.90
	SE	1.31	1.48	0.64	0.64	0.97	0.14	2.06
34 C	Mean	109.7	120.3	45.83	9.83	31.49	1.55	67.46
	SE	1.14	1.66	0.71	0.71	1.07	0.16	2.28
B. Final incubation								
28 C	Mean	110.3	116.4	44.18	9.88	29.99	1.22	75.64
	SE	2.12	1.91	0.97	0.88	1.63	0.19	1.85
30 C	Mean	107.1	124.2	46.38	9.92	30.51	1.44	67.86
	SE	1.28	1.74	0.87	0.16	1.47	0.17	1.66
32 C	Mean	108.5	118.3	46.64	9.69	30.09	1.69	61.12
	SE	1.93	1.85	0.92	0.17	1.54	0.18	1.74
34 C	Mean	108.3	116.8	45.42	9.37	28.95	2.05	57.24
	SE	2.21	2.23	1.14	0.21	1.92	0.22	2.17

mining temperatures for the initial and final periods of development (Hotelling's $T: F_{2,13} = 4.71, P = 0.635$).

DISCUSSION

Among Clutch Variation in Eggs

In the sample from 1987 (this study), variation in yolk dry mass among clutches resulted equally from differences in lean and NPL portions of the eggs. A comparison of eggs collected in Louisiana in 1987 and 1986 provided contrasting results. The yolk dry mass of eggs in 1987 averaged 3 g less than that of the sample of eggs taken from the same general area in 1986. The log-log regression of egg NPL on total mass of egg yolk for each year indicated that NPL were added to eggs in direct proportion to lean material. Contrasting results were obtained from an ANOVA and a linear regression analysis of combined data on egg yolk components between years. Both analyses indicated that the difference in egg yolk mass was primarily due to higher proportional allocation of NPL in the larger eggs in the sample from 1986. Two explanations for this disproportional allocation appear possible: (1) in larger eggs, NPL are added at a higher rate than lean material or (2) differences in relative proportions of egg components occur among clutches, and the differences between years are the result of sampling bias.

When combined, the variation in egg mass among clutches and the significant difference in egg masses between sampling years indicates a strong potential for maternal effects to be apparent in alligator offspring. Additional evidence for strong maternal effects is indicated in that, even with the rather extreme experimental manipulations, clutch effects associated with hatchlings were significant.

Hatchling Characteristics among Samples

Hatchlings from eggs in 1987 averaged 2.81 g less in total dry mass than did those from eggs in 1986 (11.41 versus 14.22 g; Fischer et al., 1991). The differences between hatchling masses (2.8 g) and egg masses (3.1 g) were similar because the

total dry mass conversions from egg yolk to hatchlings were similar for samples in both 1987 (77.5%) and 1986 (79.6%). Ranch-raised eggs from Florida (yolk mass = 13.0 g), which were substantially smaller than the eggs from Louisiana (average yolk mass 17.86 g in 1986 and 14.72 g in 1987), exhibited a dry mass conversion efficiency of 76.9% (Congdon and Gibbons, 1989), which is similar to eggs from Louisiana.

We used ash content data from alligator eggs collected in 1986 (Fischer et al., 1991) to calculate the total inorganic content of eggs from the sample in 1987. Both the study in 1986 and the present study indicate that the mass of inorganic material in the egg yolk was, respectively, 80% and 77% of the total ash in hatchlings. These data suggest that approximately 20% of the inorganic material in hatchlings may come from the egg shell. The use of the egg shell as a major source of inorganic nutrients for hatchling development has been observed in previous studies of alligators (Packard and Packard, 1989), turtles (Packard and Packard, 1984b, 1986; Packard et al., 1984a, 1985), lizards (Packard et al., 1992), and snakes (Packard and Packard, 1988; Packard et al., 1984b).

Pre-ovulatory Parental Investment in Care

Levels of PIC and hatchling percent NPL were similar among years and between locations [Louisiana in 1986 (74% and 43%) from Fischer et al. (1991); 66% and 40.0% from the present study; and Florida (74% and 38.4%) from Congdon and Gibbons (1989)]. The contention that the amount of PIC is an important reproductive trait that is resistant to environmentally induced variation (Congdon, 1989) is supported in that differences in collection years, locations, clutches (parental effects), egg sizes, or relatively extreme differences in incubation conditions did not result in substantial variation in pre-ovulatory PIC and hatchling reserves.

Embryo Mortality

In the sample from 1987, mortality varied significantly among clutches and was substantially higher than it was in the sam-

ple from 1986 (7.0%). Higher mortality in 1987 compared to 1986 may have been due to some intrinsic feature of the eggs or, more likely, due to treatment during transport from Louisiana to the Savannah River Ecology Laboratory. Eggs from both years were incubated under similar hydric conditions, although the thermal manipulations in 1987 were more extreme. Because eggs from each clutch were distributed uniformly among initial and final temperature treatments in 1987, mortality did not interfere with our ability to detect the effects of incubation temperature treatments on hatchling characteristics except by the reduction in sample size.

Experimental Effects on Hatchling Characteristics

Whereas initial incubation temperatures play an important role in the early stages of tissue and organ development and sex determination, it is the final incubation temperatures and their differential effects on embryo growth that are the primary cause of variation in hatchling characteristics and size. Although variation in hatchling characteristics occur during the last third of development, with the exception of the amount of tail length and yolk lean material used prior to hatching, differences were not detectable at the univariate level.

Our results support the working hypothesis that hatchling characteristics are influenced more during the last third of development (embryo growth phase) than during the first two-thirds of development (from the beginning of embryo formation through the sex determining period). Furthermore, when incubation temperatures vary during embryo development, hatchling characteristics exhibit less variation than found in some laboratory studies based on single incubation temperatures (Packard and Packard, 1984a; Packard et al., 1985, 1987).

An adaptive scenario for ESD in alligators is based on the presumed association of sex determining incubation temperatures with variation in both body size and mass of hatchlings (e.g., hatchling females are larger than males, and body size dif-

ferences persist up to 1 yr after hatching and may allow females to mature earlier: Ferguson and Joanen, 1982, 1983). However, our results indicate that differences in hatchling size, or suites of characteristics that may occur due to incubation temperatures experienced by the embryo through the sex determining period, were not detectable at either the univariate or multivariate levels. The scenario of Ferguson and Joanen requires a correlation between nest temperatures during the initial incubation period when embryo development and sex determination take place and the last third of development, which corresponds to embryo growth. It seems reasonable to assume that the correlation exists in some years, and possibly in most years; however, no data are available from natural nests to support the assumption. A striking result from our study was that maternal effects on hatchling characteristics were surprisingly detectable regardless of experimental treatments. That maternal effects are so strongly detectable over a broad range of incubation temperature treatments suggests that the characteristics of hatchlings are influenced as much or more by characteristics of reproductive investment of the female parent than by variation in nest environments resulting from nest site selection.

Two factors that appear to have major impact on hatchling characteristics are maternal effects and incubation temperature during the final third of development. Proposed adaptive scenarios are based on the "decision" to produce offspring of a particular sex based on an association of hatchling body size associated with future sexual roles as an adult. In contrast to the conclusions of Ferguson and Joanen (1982, 1983), the results from our study indicate that, with the exception of sex determination, hatchling traits are primarily related to characteristics of the female parent and secondarily on the influence of variation in both initial and final incubation temperatures related to nest site selection.

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