

# Temperature-Dependent Sex Determination in Crocodylians

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**ABSTRACT** Half of the 22 extant crocodylians show evidence of temperature-dependent sex determination (TSD). We examine evidence for TSD in 11 species by reviewing reports on five and presenting new data for six. The female-male pattern (FM; females at low temperature, males at high temperature) attributed to *Alligator mississippiensis* and *Caiman crocodilus* are here revised to be female-male-female (FMF; males at intermediate temperature, females at low and high temperatures). A similar pattern characterizes *Crocodylus palustris*, *C. moreletii*, *C. siamensis*, and *Gavialis gangeticus* based on new data; published accounts establish a FMF pattern in *Crocodylus porosus*, *C. johnstoni*, and *C. niloticus*. TSD apparently occurs in *Paleosuchus trigonatus* and *Alligator sinensis*, but patterns are not yet documented. In the well-studied species, the incubation temperatures for FM transitions are congruent, but MF transition temperatures differ among species. In *A. mississippiensis*, 100% males are produced over a range of constant incubation temperatures, whereas in *C. johnstoni*, only low proportions of males are produced at any constant temperature.

The thermosensitive period (TSP) for *A. mississippiensis* occurs during stages 21 to 24 (days 30-45 at intermediate temperatures) and coincides with gonadal differentiation. A similar scenario is suggested in other species. The TSP in *A. mississippiensis* (and possibly other crocodylians) encompasses the third quarter of development and occurs later than in turtles and a lizard. In *A. mississippiensis* as in turtles, the duration (cumulative effect) and/or the magnitude (potency effect) of incubation temperatures during the TSP predictably alter sex ratios. TSP chronologies and features which are shared among TSD reptiles suggest common, underlying mechanisms; *A. mississippiensis* is an appropriate model for further study. In crocodylians, clutch effects are a significant source of variation in TSD response. Hatchling sex ratios previously reported for *A. mississippiensis* are reconsidered in light of our new data. © 1994 Wiley-Liss, Inc.

Efforts to understand temperature-dependent sex determination (TSD) in reptiles have largely proceeded along two intersecting paths. The ecological and evolutionary consequences of TSD have invited attempts to provide adaptive explanations for its maintenance and evolution (e.g., Bull, '80; Ewert and Nelson, '91; Burke, '93). On the other hand, a search has been under way to elucidate the mechanistic basis of TSD, posited on the assumption that common pathways are operating at cellular and molecular levels, not only in TSD species, but among all vertebrates (e.g., Spotila et al., this issue; Wibbels et al., this issue).

TSD as well as chromosomal or genetic sex determination (GSD) both occur in representative turtles and lizards (Ewert et al., this issue; Viets et al., this issue). In these groups, TSD and GSD coexist within and among phylogenetic assemblages. TSD species typically exhibit diverse patterns of sex

ratios and associated incubation temperatures. Initial reports in alligators (Ferguson and Joanen, '82, '83) and crocodiles (Webb and Smith, '84; Webb et al., '87) suggested similarly diverse patterns. But the absence of sex chromosomes in the group (Cohen and Gans, '70) prompted speculation that, in contrast to turtles and lizards, TSD may be universal in crocodylians (Ferguson, '85).

Here we examine TSD in eleven species of crocodylians, reviewing previous studies and presenting new data on six species. Our primary focus is to compare species with respect to TSD patterns and parameters, thermosensitive periods, and clutch effects in an effort to highlight important similarities and differences. We then compare the chronology of TSD in *Alligator mississippiensis* with that in other TSD reptiles and discuss features common in crocodylians, turtles, and lizards. Finally, we comment briefly on the significance of

clutch effects in these studies and reinterpret previously reported data on hatchling sex ratios in *A. mississippiensis* in light of our results.

## METHODS

Our methodology follows that described previously (Lang et al., '89). The eggs of *A. mississippiensis* were collected within 1 week of oviposition at the Rockefeller Refuge, Grand Chenier, Louisiana; this is the same source of eggs used for previous studies (summarized in Deeming and Ferguson, '91). Eggs of other species were collected within 0–3 days of oviposition in breeding enclosures at the Madras Crocodile Bank, Madras, Tamil Nadu, India. All eggs were incubated in custom-designed, foam box incubators at constant temperatures maintained within  $\pm 0.1^\circ\text{C}$  of the set temperature, calibrated to a NBS traceable certified thermometer. Eggs were candled to monitor development; representative embryos were collected at fixed intervals throughout incubation and staged according to Ferguson ('85). Staging tables were constructed for reference, relating morphological stage to known age over the range of incubation temperature (Table 1). Caution is required in interpreting differences in stage. As defined, stages emphasize differentiation during initial development; during the final phase of development, the embryo grows in size. Consequently, the intervals between advanced stages occur over longer time periods and are associated with appreciable differences in embryo mass not evident in intervals between earlier stages. Incubation time was calculated from oviposition to pipping.

Hatchlings were marked individually and sexed at various ages by examination of gonads and/or

genitalia; representative gonads were examined in histological section for diagnostic sex-specific features. In alligators (Allsteadt, '93) and in other crocodilians (Webb et al., '84; Lang and Andrews, unpublished data), genitalia are dimorphic at hatching; these differences are also evident at subsequent ages. Sample sizes of sexed animals for each species are listed in Table 2 or in the text. The TSD pattern becomes evident when sex ratio (% male) is plotted on the y-axis against constant incubation temperature on the x-axis. These patterns fall into three categories, moving from left to right along the x-axis: female-to-male (FM), female-to-male-to-female (FMF), male-to-female (MF). These patterns are equivalent to those described with varied terminology (see Ewert et al., this issue).

In the following section, new and reviewed information is presented for each species organized within the major taxonomic groups (i.e., alligators and caimans, crocodiles, and gharials). However, the level of detail varies with each species.

## RESULTS

### Alligator mississippiensis

#### Embryonic survival and development

Our new data substantially revise previous reports (summarized in Deeming and Ferguson, '91). We incubated fertile eggs at constant incubation temperatures of 28–36°C at intervals of 0.5–1.0°C (Table 2). At 28°C, most eggs complete development but fail to hatch; embryo mortality exceeded 90%. At 36°C, embryos fail to develop beyond stage 18. At 35 and 34.5°C, embryonic survival was 11% and 29%, respectively; but viability increased markedly to 87% at 34°C. Additional observations indicate that embryos tolerate temperatures for 1–2 days that range from 1–3°C above and below the viable range. Embryonic survival is seemingly unaffected by constant incubation temperatures between 29.0 and 33.5°C; mortality was less than 10%. However, when it occurred, mortality was clearly clutch-related.

The rate of embryonic development is strongly temperature dependent. Representative stages at different temperatures during development are tabulated in the Table 1. At 31°C, total incubation time averages 71 days, which is 1.18 times shorter than at 29°C and 1.12 times longer than at 33°C.

#### Sex determination

Previously, Deeming and Ferguson ('91) and earlier reports had indicated that the TSD pattern

TABLE 1. Relationship for Alligator mississippiensis between age (in days) and developmental stages at constant incubation temperatures of 29–33°C<sup>1</sup>

Age (days)	29°C	30°C	31°C	32°C	33°C
10	9	9	10	10.5	11
15	11.5	13	13.5	14.5	14.5
20	14	45.5	16	16.5	18
25	15	18	18.5	19.5	20
30	17	20	20.5	21	21.5
35	19.5	21	21.5	22	22.5
40	20.5	22	22.5	23	23.5
45	21.5	23	23.5	24	24.5
50	22.5	24	25	25	25.5
Hatch (days)	84	77	71	66	63

<sup>1</sup>Incubation time in mean number of days from oviposition to pipping (eggshell membranes broken by hatchling) shown at bottom. For sample sizes for total incubation time, see Table 2.

TABLE 2. Summary of TSD patterns (top) expressed as proportions of males (% males; top) resulting at constant incubation temperatures (degrees C) and sample sizes (total number of individuals sexed; bottom) for five species of crocodylians<sup>1</sup>

Species	28	28.5	29	29.5	30	30.5	31	31.5	32	32.5	33	33.5	34	34.5	35	36	Total	Clutch	Reference
% male																			
AM	0		0		0	0	0	0	66	100	100	84	35	7	0				
CPA	0	0	0	0	0	0	0	22	59	93	31								
CC		0		0	0		0	52	95	90	85	75	50						
CPO	0		0		0		16		86		17								
CJO	0		0		0		0		39		7								
# sexed																			
AM	(10)		113		27	27	197	125	342	122	97	19	54	(15)	(6)	eed	1,154	46	1
CPA	27	35	32	22	46	17	51	9	32	297	29	eed	eed				597	64	1,2
CC		9		9	19		18	23	40	10	34	12	(2)		eed		176	15	1,2
CPO	4		27		70		19		72		30		eed				222	?	4
CJO	4		31		48		148		148		151				eed		530	?	3,5,6

<sup>1</sup>AM, *Alligator mississippiensis*; CC, *Caiman crocodilus*; CJO, *C. johnstoni*; CPA, *Crocodylus palustris*; CPO, *Crocodylus porosus*. Total numbers and number of clutches for each species are listed (right columns) Numbers in parentheses indicate late embryonic death or dead at hatching; eed, early embryonic death (described in Results). References: 1, this study; 2, Lang et al., '89; 3, Webb et al., '87; 4, Webb and Cooper-Preston, '89; 5, Webb et al., '90; 6, Webb et al., '92.

is FM; females are produced at low incubation temperatures (<32°C) and only males at high temperatures (>32°C). In contrast, our results indicate that females are produced at both high and low incubation temperatures, with males predominating at intermediate temperatures, a FMF pattern (Fig. 1). The initial transition from low-temperature females to males is relatively steep; interpolation of the TSD curve (Fig. 1) indicates an increase from 0% to 50% males (FM pivotal temperature) within a span of 0.3°C, from 31.5–31.8°C. Exclusively males are produced at 32.5 and 33.0°C. The transition from males to high-temperature females is more gradual; a decrease from 100% to 50% males (MF pivotal temperature) occurs within a span of 0.8°C, from 33.0–33.8°C. Various TSD parameters are summarized in Table 3.

The high-temperature females produced at 33.5 and 34°C are normal in size, appearance, and behavior. These hatchlings grew at least as well as their low-temperature female siblings when maintained under identical feeding and thermal regimes for 6 months (Lang, unpublished data). At 34.5 and 35°C, the frequency of "runts" increases; but surviving animals, though smaller, were healthy despite slower growth rates. The ovaries and oviducts of representative high-temperature females appeared similar in size and appearance to those of low-temperature females. Histological examination revealed the distinct ovarian sacs or lacunae and similar cortical layers of germ cells characteristic of hatchling ovaries produced at 30–31°C (Lang and V. Lance, unpublished data). In addition to the results presented here, three

independent laboratories have recently produced females with incubation at 34–35°C (Schulte, '89; H. Austin, unpublished data; V. Lance, unpublished data).

### Thermosensitive periods

Switch experiments, in which incubating eggs are transferred at specified times from one constant incubation temperature to another, have been conducted previously (see review by Deeming and Ferguson, '91). These studies postulate putative thermosensitive periods for single shifts between 30 and 34°C and vice versa. In addition, "double" switch experiments involving 7 day "pulses" of either 30 or 33°C failed, in most instances, to "reverse" the sex as determined by the initial or background incubation temperatures (i.e., either 30 or 33°C). These results require re-interpretation in light of the revised FMF pattern reported here. For example, 34°C is not an exclusively male-producing temperature. Consequently, the expected outcome for single shifts between 30 and 34°C is a mix of males and females. With such results, it is difficult to define thermosensitive periods (TSPs) (defined by Mrosovsky and Pieau, '91).

We conducted new shift experiments that involved (1) single shifts between an initial and a final temperature, (2) double shifts resulting in a pulse of either low or high temperatures relative to the background temperature, (3) shifts in which the magnitude and/or duration of low or high temperature exposure (from equivalent background temperatures) differed during the TSPs, and (3) shifts consisting of multiple, progressive increases in temperature during the TSPs. Individual

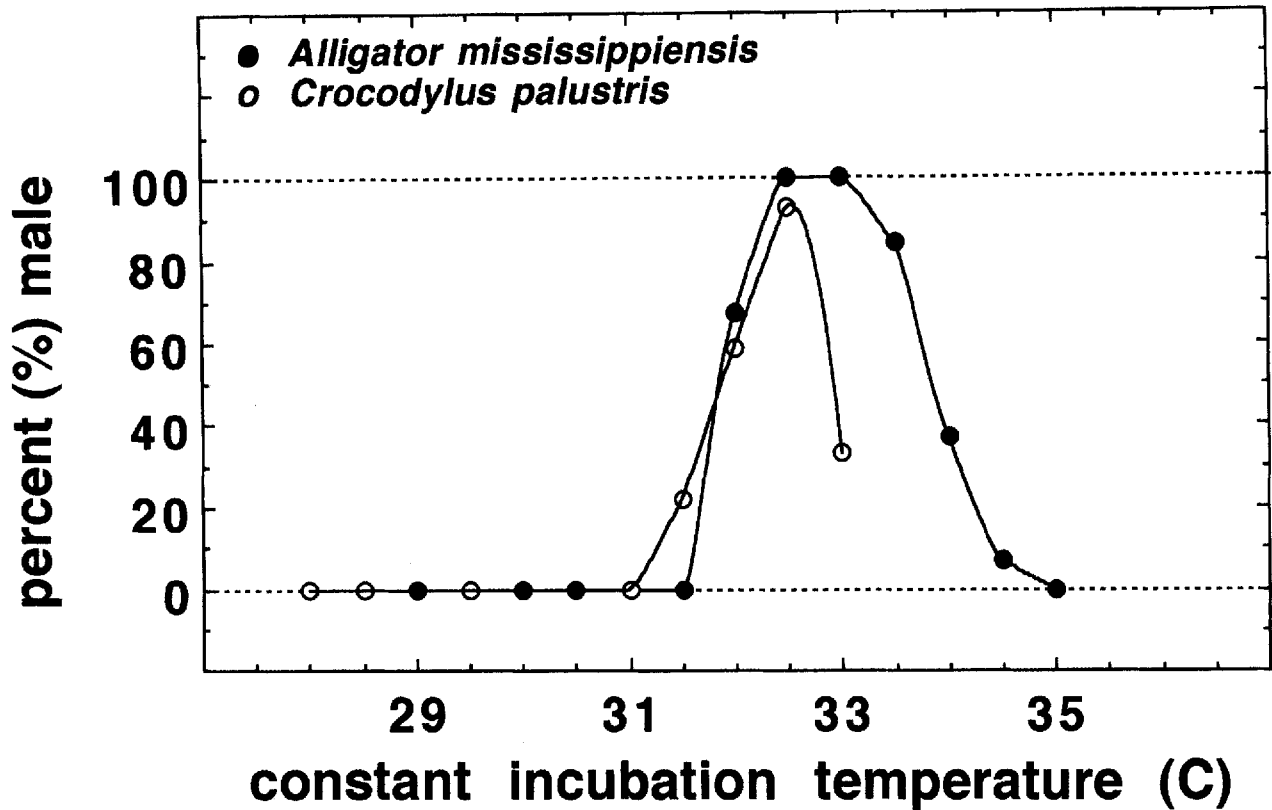


Fig. 1. Sex ratio expressed as % males vs. constant incubation temperatures (degrees C) for the American alligator, *Alligator mississippiensis* (closed circles) and the Indian mugar crocodile, *Crocodylus palustris* (open circles). Both species exhibit female-male-female (FMF) patterns of temperature-dependent sex determination (TSD), with similar FM transitions, but differ in the % males produced and at MF transition temperatures. TSD parameters for these curves are listed in Table 3. Sample sizes and tabulated values are shown in Table 2.

clutches of known age were utilized for all treatments in order to control for clutch effects (see below). A total of 639 eggs from 24 clutches produced hatchlings and/or full term embryos which could be sexed. These results will be elaborated elsewhere (Lang, unpublished data), but some trends are clearly apparent.

Shifting eggs from 31°C (female producing) to 33°C (male producing) resulted in 100% males at stage 11.5 at day 15, stage 16 at day 20, stage 18.5 at day 25, and stage 20.5 at day 30, in 92% males at stage 21.5 at day 35, in 33% males at stage 22.5 at day 40, and in 0% males at stage 23.5 at day 45. These data bracket the correspond-

TABLE 3. Temperature-dependent sex determination (TSD) pattern type and thermal parameters for five species of crocodylians<sup>1</sup>

Sp	Pattern	F-M trans	50%	Max male	50%	M-F trans	viable	TSP
AM	F-M-F	>31.5<32.5	31.8	32.5–33.0 [100%]	33.8	>33<34	>28–34	s21–24
CC	F-M-F	>31<32	31.5	32.0–32.5 [95%]	(34)	>32.5<33.5	>28–33.5	?
CPA	F-M-F	>31<32.5	31.8	32.5 [93%]	32.8	>32.5<33	28–33	(s21–24)
CPO	F-M-F	>30<32	31.5	32.0 [86%]	32.7	>32<33	28–33	(s18–23)
CJO	F-M-F	>31<32	(31.5)	32.0 [39%]	(32.5)	>32<33	28–33	(s19–24)

<sup>1</sup>AM, *Alligators mississippiensis*; CC, *Caiman crocodilus*; CJO, *Crocodylus johnstoni*; CPA, *Crocodylus palustris*; CPO, *Crocodylus porosus*. Parameters listed in order (left to right) in degrees C: female-male (F-M) transition range, F-M pivotal temperature (50% male), maximum male-producing temperatures [maximum % males], male-female (M-F) pivotal temperature (50% male), M-F transition range, viable range for constant incubation, thermosensitive period (TSP) with morphological stages listed. Parentheses enclose approximate values. References cited in text and Table 2.

ing TSP for this series of shifts by stages 20.5 to 23.5, or an interval of 15 days, from day 30 to day 45. Sex ratios are labile from stage 21 to stage 23, an interval of 10 days between day 32 to day 42 in real development time. Additional single shifts from 30 to 33°C, from 31 to 34°C, and from 31.5 to 32.5°C identify corresponding TSPs at stages 20–22, 21–23, and 20–23, respectively.

Shifting eggs from 33°C (male producing) to 31°C (female producing) resulted in 0% males at stage 21.5 at day 30, 13% males at stage 22.5 at day 35, 75% males at stage 23.5 at day 40, and 100% males at stage 24.5 at day 45. These data bracket the corresponding TSP for this series of shifts by stages 21.5 to 24.5, or an interval of 15 days, from day 30 to day 45. Sex ratios are labile from stage 22 to stage 24, an interval of 10 days between day 32 and day 42. An additional single shift from 32.5 to 31.5°C identifies the corresponding TSP between stage 21.5 and stage 24.5.

Shifting eggs twice produces an effective pulse of temperature exposure that may reverse the sex as determined by the background or initial incubation temperature. For example, shifts from 31 to 33 and back to 31°C between stages 21.5 and 25.5 for a period of 20 days at days 30–50 resulted in 100% males against an otherwise female-producing background temperature (i.e., 31°C). Reciprocal shifts from 33 to 31 to 33°C between stages 20.5 and 24.5 at days 30–50 resulted in

100% females against an otherwise male-producing background temperature (i.e., 33°C). In both cases, the pulse of opposite temperature exposure encompasses the corresponding TSPs identified in single shift experiments, stages 21.5–24.5 and 20.5–23.5, respectively.

The results of the shift twice or double shift experiments depend on the duration and on the magnitude of the shifts, as well as on the stages/ages when the shifts were carried out. These features are illustrated in Figure 2. Shifts from 31 to 33 to 31°C for intervals of 10, 15, or 20 days produced 0%, 55%, or 100% males, respectively. Shifts from 31 to 34 to 31°C for 10, 15, or 20 days produced 0%, 100%, and 73% males, respectively (Fig. 2a). Presumably, these percentages represent low-temperature females at 10 days, males at 15 days, and males and high-temperature females at 20 days. By comparison, a 15 day pulse of 33°C exposure resulted in 55% vs. 100% males for the equivalent period at 34°C. Thus, the magnitude of the temperature pulse is related to the percentage of males produced.

The reciprocal double shifts from 33 to 31 to 33°C (from male producing to female producing and back) at varying intervals produce increasing proportions of females as the duration of the pulse increases between stage 21.5 and stage 25.5. A duration of 20 days produced 0% males. In contrast, a 15 day pulse resulted in 33% males, and several 10 day pulses (final 10 days between stage

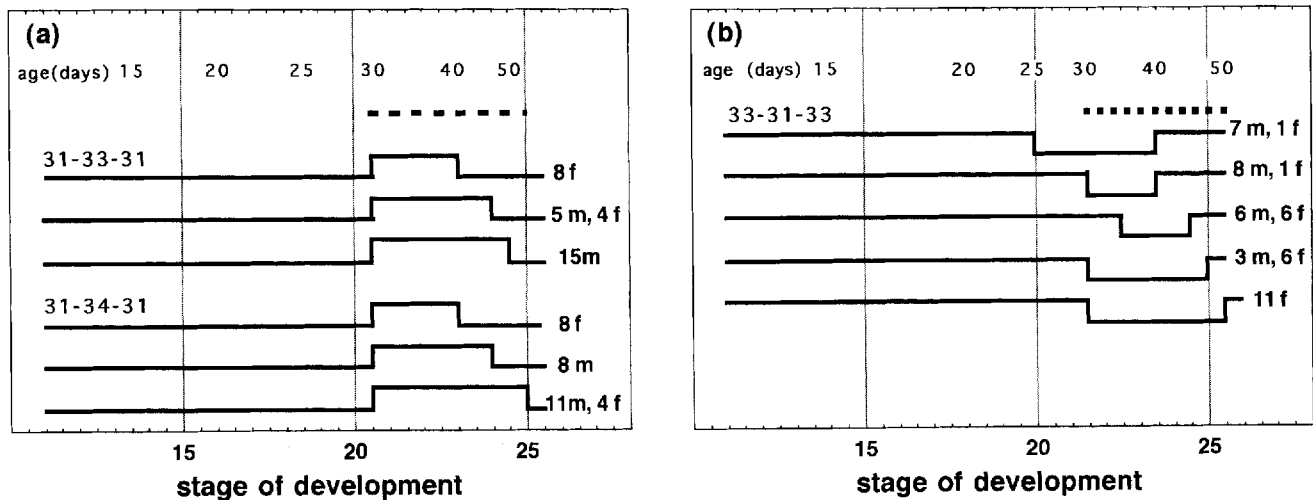


Fig. 2. Double shifts (temperature pulsed against background incubation temperatures) of eggs of *Alligator mississippiensis* from female-producing temperatures to male (and/or high-temperature female) and back to female-producing temperatures for differing intervals of time (shown in days at top) at specific morphological stages (horizontal axis; **panel a**) and vice versa (**panel b**). Detailed explanation in Results.

21.5 and 25.5 vs. initial 10 days) resulted in 50% and 89% males, respectively (Fig. 2b).

Multiple, progressive shifts in incubation temperature above 32°C during days 30–50 produce increasing proportions of males at mean temperatures of 33–34°C (average for 20 day period) and increasing proportions of females at higher temperatures. For example, eggs shifted from 30 to 32°C at day 20, from 32 to 33°C at day 30, and from 33 to 34°C at day 40 until hatch produced 100% males. Eggs shifted from 32 to 34°C at day 30, from 34 to 35°C at day 40, and from 35 to 36°C at day 50 until hatch produced males (55%) and high-temperature females. In these experiments, the resultant sex ratios produced by exposure to increasing temperature during stages 21–24 closely paralleled the results of the double switch experiments.

Taken together, the results of these experiments support the stages identified as TSPs by the single switch results (Table 3). They also demonstrate a quantitative effect of incubation temperatures in terms of the duration (cumulative effect) and/or magnitude (potency effect) of incubation temperatures experienced by embryos during the TSP.

#### Clutch effects

In our studies, the sex ratios of individual clutches differed markedly at transition temperatures. These differences were evident despite incubation at uniform and constant temperatures ( $\pm 0.1^\circ\text{C}$ ). Furthermore, eggs were randomized in position within several incubators and rearranged on trays within incubators. Thus, the observed differences among clutches are not attributable to local gradients, if any, within incubators.

Within the FM transition range at a temperature of 32.0°C, a total of 28 clutches (mean egg number =  $11.4 \pm .6$  SEM; range = 6–15) yielded sex ratios ranging from 0–100% males. The frequency distribution was as follows: 0–25% males = 4 clutches, 26–50% males = 4 clutches, 51–75% males = 4 clutches, and 76–100% males = 16 clutches. For all of these clutches, representative eggs ( $n = 5$ –10) incubated at 31.5°C produced only females and at 32.5°C produced only males.

Within the MF transition range at a temperature of 34.0°C, a total of 6 clutches (mean egg number =  $7.4 \pm .4$  SEM; range = 7–9) yielded sex ratios ranging from 0–71% males. The frequency distribution was as follows: 0–25% males = 3 clutches and 51–75% males = 3 clutches. Eggs from these same clutches incubated at 33.0°C produced 100% males.

Clutch effects were also evident in some instances when multiple clutches (equivalent in age and stage) were subjected to identical switch protocols. For example, when eggs incubated at 31°C were subjected to a 20 day pulse of high temperature between day 30 and day 50, the pattern of resultant sex ratios was clearly different in the two clutches tested. One clutch produced 78%, 100%, and 73% males at 33, 34, and 35°C, respectively, whereas the other produced 87%, 92%, and 100% males. Presumably, the former clutch produced low-temperature females when pulsed at 33°C and high-temperature females when pulsed at 35°C. In the latter clutch, low-temperature females were produced by pulses at 33 and 34°C.

#### Exogenous steroid hormones

In previous studies on *A. mississippiensis*, exogenous estradiol injected into eggs prior to gonadal differentiation resulted in the production of females when eggs were incubated at male-producing temperatures (Bull et al., '88; Lance and Bogart, '92). High egg mortality was attributed to infection associated with the injection treatment. In pilot studies, we applied estradiol topically (following the methodology of Crews et al. ('91); estradiol 17 $\beta$  in ethanol, dose = 100  $\mu\text{g}/5\mu\text{l}$ ) just prior to the thermosensitive period (i.e., days 25–30). Mortality in these trials was less than 5%. When eggs were incubated at 32.5°C (male-producing temperature), estradiol treatment resulted in 100% female hatchlings ( $n = 26$ ); controls produced 100% males ( $n = 24$ ). Treatment females were similar in appearance, size, and behavior to hatchlings from female-producing temperatures. Ovaries of treated animals appeared to be normal macroscopically, but oviducts were hypertrophied in some animals relative to normal females. In addition, there was a marked reduction in the size of the genitalia of the treatment females relative to the genital dimensions of normal females.

#### Alligator sinensis

Preliminary data establishes the presence of TSD in this species but are insufficient to identify a TSD pattern. A sample of 40 eggs was incubated at 33–35°C; of the resultant 36 hatchlings, 91% were males, 6% females, and 3% undetermined. Females were produced at low incubation temperatures, ~28°C. Survivorship was poor at temperatures above 36°C and below 27°C (Chen, '90; cited and summarized in Webb and Vernon, '92).

### **Caiman crocodilus**

#### **Embryonic survival and development**

Preliminary data on *Caiman crocodilus* was reported in Lang et al. ('89). Here we summarize results based on our continuing studies at the Madras Crocodile Bank. The origin of the breeding adults was Central America, but specific locality is unknown. We incubated fertile eggs at constant temperatures of 28.5–35 at intervals of 0.5–1.0°C. At 34.5 and 35°C, embryos died prior to stage 20. At 34°C, most embryos fail to develop to stage 25; the few that developed beyond stage 25 failed to hatch. Between 28.5 and 33.5°C, viability is >85% and is dependent on clutch. At 31°C, total incubation time averages 79 days, which is 1.14 times shorter than at 29°C and 1.10 times longer than at 33°C.

#### **Sex determination**

Initially, we reported a FM pattern for this species. Recent data indicate that the pattern is FMF. At 31°C and below, exclusively females were produced. At 31.5°C, 52% were males. At 32 and 32.5°C, 95% males and 90% males resulted, respectively. At 33, 33.5, and 34°C, 85%, 75%, and 50% males resulted, respectively (Table 2). Further information is needed to delineate the MF transition. However, it is clear that some females are produced at high temperatures. These appear to be similar in size, appearance, and behavior to females produced at lower temperatures. We have no data on thermosensitive periods.

#### **Clutch effects**

Sex ratios of individual clutches were variable at male-producing temperatures. At 31.5°C, for clutches produced sex ratios of 20%, 33%, 50%, and 100% males. At 32°C, seven clutches were 100% males; two others were 89% and 75% males. At 32.5°C, two clutches were 100% males, and another was 50% male. At 33°C, four clutches were 100% males, and another was 67%. At 33.5°C, 2 clutches were 100% males, and two were 50% males. In two clutches, incubated at 32, 33, and 33.5°C, only males were produced across all temperatures. In two others incubated at 32 and 33°C, mixed sex ratios resulted at both temperatures.

### **Paleosuchus trigonatus**

Preliminary data in this species establish the presence of TSD but are insufficient to define the pattern of TSD. Females were produced at temperatures of 31°C and below, and males resulted

at 32°C. Survivorship was reduced below 27°C (Yamakoshi et al., '87; Magnusson et al., '90). In the field, high nest temperatures (>31.5°C) produced mostly males, and low nest temperatures (<30.5°C) produced only females (Campos, '93).

### **Crocodylus palustris**

#### **Embryonic survival and development**

TSD in *Crocodylus palustris* has been reported previously (Lang et al., '89) and is summarized here with new data. We incubated fertile eggs at constant incubation temperatures of 28–34°C at intervals of 0.5–1.0°C. Eggs incubated at 28°C produce viable hatchlings. At 33.5 and 34°C, embryos died at early stages (< stage 20; prior to day 30). At 31°C, total incubation time averages 73 days, which is 1.22 times shorter than at 29°C and 1.15 times longer than at 33°C.

#### **Sex determination**

At 31.0°C and below, exclusively females were produced. At 31.5°C and above, varying proportions of males and females resulted (Lang et al., '89). Previously, we reported that 100% males were produced at 32.5°C, based on a sample of eight eggs from a single clutch. Additional constant temperature incubations of 130 eggs from 15 clutches in 1989 and of 159 eggs from 14 clutches in 1990 resulted in overall values of 92% and 89% males, respectively, at 32.5°C. The combined value for all years (297 eggs from 30 clutches; 8–12 eggs/clutch) was 93% males at 32.5°C.

The pattern of TSD is FMF but differs in several important features from the pattern in *A. mississippiensis* (Fig. 1). First, some females are produced at all viable temperatures; no single incubation temperature results in 100% males in all clutches. Second, the range of male-producing temperatures is narrow. The MF pivotal temperature (50% male) occurs at 32.8°C, about a degree lower than in *A. mississippiensis*. Third, the range of viable temperatures is low; hatchlings are produced at 28°C but failed to develop at 33.5 and 34.0°C (Table 3).

#### **Thermosensitive periods**

The utility of single switch experiments to delineate TSPs is limited because no single incubation temperature produces 100% males. Nevertheless, we report the results of several single switch protocols. Shifting eggs from 32 to 30°C produced 0% males at stage 21.5 at day 30, 20% males at stage 22.5 at day 35, 50% males at stage 23.5 at day

40, and 100% males at stage 24.5 at day 45. These data indicate that the corresponding TSP is bracketed by stages 21.5–24.5, or an interval of 15 days between days 30 and 45. Shifting eggs from 30 to 33°C at stage 18 at day 30 or stage 20 at day 35 produced 100% males, whereas a later shift at stage 23 at day 45 resulted in 0% males. These data suggest that 33°C is sufficient to produce males prior to stage 21 but not after stage 23. Taken together, these shifts delineate an approximate TSP during stages 21–24, stages corresponding to 28–42 days at 32°C.

### Clutch effects

Sex ratios of individual clutches varied at male-producing temperatures. Within the FM transition range at a temperature of 32°C, eggs from three clutches were 25%, 69%, and 70% males. Within the MF transition range at a temperature of 33°C, eggs from three clutches were 21%, 27%, and 75% males (Lang et al., '89). Inter clutch variation was also evident at 32.5°C. At this intermediate male-producing temperature, the overall sex ratio for all years was 93% (see above); but individual clutches yielded 100% males (n = 17), 91–99% males (n = 2), 81–90% males (n = 7), 71–80% males (n = 1), 61–70% males (n = 2), and 51–60% males (n = 1).

### Exogenous steroid hormones

In pilot studies with *C. palustris*, we have used topical application of estradiol to eggs (following the methodology of Crews et al. ('91); estradiol 17 $\beta$  in ethanol, dose = 100  $\mu$ g/5 $\mu$ l) just prior to the TSP (i.e., days 25–30). Mortality in these trials was less than 5%. Eggs were incubated at male-producing temperatures in six natural nests within outdoor breeding enclosures. In three nests in which the controls produced 100% males (n = 52), treated eggs produced 100% females (n = 58). This result indicates that exogenous estradiol exerts a feminizing effect on embryonic crocodilian gonads when eggs are incubated at male-producing temperatures in simulated natural nests; in these nests, eggs experienced daily and seasonal changes in temperature throughout incubation.

### *Crocodylus porosus*

In *Crocodylus porosus* and *Crocodylus johnstoni*, TSD has been studied by G. Webb and colleagues (summarized in Webb et al., '87; Webb and Cooper-Preston, '89). Detailed analyses of embryonic survival and development as well as comprehensive data on sex determination and thermosensi-

tive periods in both species were presented by Webb et al. ('87). Information on clutch effects on TSD was not included in any of these studies. Here we summarize relevant TSD parameters of these species for interspecific comparisons and attempt to reinterpret the data on TSPs in both species in light of our recent studies of TSPs in *A. mississippiensis*.

### Sex determination

In *C. porosus*, the pattern of TSD is FMF. At 28, 29, and 30°C, all females were produced. At 31, 32, and 33°C, a total of 16%, 86%, and 17% males resulted, respectively (Table 2). Note that incubation was not carried out at 0.5°C intervals, particularly in the male-producing region. Consequently, the resultant TSD pattern lacks the resolution of the patterns for *A. mississippiensis*, *Caiman crocodilus*, and *Crocodylus palustris* presented above. Nevertheless, many features of the FMF pattern in *C. porosus* closely correspond to those of *C. palustris* (Table 3).

### Thermosensitive periods

With regard to TSPs in *C. porosus*, interpretation of switch experiments is confounded because, unlike *A. mississippiensis*, no single incubation temperature consistently produces 100% males. In general, females (presumably low-temperature females) are produced when eggs are shifted early from high to low temperatures and when eggs are shifted late from low to high temperatures (Fig. 11 in Webb et al., '87). These authors conclude that "early switches adopted the sex of the final temperature whereas later switches the sex of the initial temperature." The transition point in a single shift upward from a constant temperature of 30 to 32°C (86% males) was at about stage 18 at day 30. Other transitions occurred at earlier stages/ages as the magnitude (difference between initial and final temperatures) of the switch increases. An upward shift from 31 to 32°C did not extend beyond day 40 and was inconclusive. The transition points in single shifts downward from 32°C and from 33°C to 30°C occur at stages 22 at day 40 and 23 at day 45, respectively. Thus, in *C. porosus*, we interpret these single shift experiments to indicate a transition at stages 18–20 at days 30–34 and another transition at stage 22–23 at days 40–45. We suggest that these transitions bracket, in an approximate manner, the region of TSPs in *C. porosus* (Table 3).



## *Crocodylus johnstoni*

### Sex determination

In *Crocodylus johnstoni*, the TSD pattern is FMF. However, available data suggest that no single constant temperature produces the high proportion of males (~80–100% males) characteristic of the other species studied to date. A composite of original values (Webb et al., '87) and revised values (Webb et al., '91; Webb et al., '92) for % male at constant temperature incubation is presented here (Table 2). As in *C. porosus*, not all 0.5°C intervals have been tested, particularly in the male-producing region. At 29, 30, and 31°C, all females are produced. At 32 and 33°C, 39% and 7% males result, respectively. A similar TSD pattern was produced for *C. johnstoni* using another incubation methodology (method A; Webb et al., '87). Important TSD parameters for *C. johnstoni* are summarized in Table 3.

### Thermosensitive periods

With respect to TSPs, interpretation of switch experiments for *C. johnstoni* was confounded because male production at any single constant temperature is low (maximum % male = 39% at 32°C). Using male production as the primary criterion to identify transition points, we identify stages 19–24 at days 35–42 as the end points for male production in various single shift experiments upward, from low to high temperatures (Fig. 9 in Webb et al., '87). In these upward shifts, females prior to day 35 are interpreted to be high-temperature females, whereas females subsequent to days 35–40 are considered to be females produced at low temperature. Only one downward single shift from 32 to 30°C resulted in male production and a transition at stage 24 at day 42; no males were produced in shifts from 33 to either 30 or 32°C (Fig. 9 in Webb et al., '87). We interpret these data to indicate transitions in *C. johnstoni* occurred at stages 19–24 at days 35–42, depending on the shift protocol. We suggest that these transitions bracket approximate TSPs in *C. johnstoni* (Table 3).

A paradoxical feature of the TSD pattern in *C. johnstoni* is the discrepancy between consistently low proportions of males incubated at constant incubation temperatures vs. the high proportions of males (~100% males) found in some wild nests (Smith, '87). Simulations of possible male-producing incubation regimes in the lab have demonstrated that daily fluctuating temperatures between 31 and 33°C (mean = 32°C) do not increase male

production when compared to constant temperature incubation at 32°C (Webb et al., '90). On the other hand, protocols utilizing increases in incubation temperature (either in step increases or in steadily rising, daily increments) result in 70–100% males (Webb et al., '92).

Of interest here is that step increases resulting in mean temperatures of 31–32°C during stages 19–24 (approximately days 25–45) produced 72–80% males. In contrast, lower or higher mean temperatures (from ~29–31°C; ~32.5–33°C) during this developmental period resulted in proportionately fewer males (0–53% and 56 to 36% males, respectively, at lower and higher temperatures). The steadily escalating protocol (from 30 to 35°C in daily incremental increases throughout incubation) produced an approximate mean temperature of 32°C during the equivalent developmental period. This protocol produced even higher proportions of males, 88–100%. Thus, incubation temperatures of 31–32°C during the putative TSPs in *C. johnstoni* (stages 19–24 as suggested above) are associated with maximum male production in both types of protocols involving increasing temperatures.

## *Crocodylus niloticus*

In *Crocodylus niloticus*, the pattern of TSD is FMF; the following summary is based on Hutton ('87). For eggs incubated at constant temperatures of 28 (n = 53), 31 (n = 57), and 34°C (n = 54) at 15 days of age or younger, 0% males resulted at 28 and 31°C and 81% males at 34°C. In a separate experiment, eggs incubated at 31, 32.5, and 34°C (n = 11/treatment) within 14 days of laying produced 0%, 91%, and 82% males, respectively.

With respect to TSPs, shift experiments were not conducted, but some insight about thermosensitivity may be deduced from the constant temperature incubations because these were initiated at different times during development. Three clutches of eggs were incubated at 28, 31, and 34°C beginning at 30 days of age. These produced females at 28 and 31°C (as above) but only males at 34°C. In contrast, some females were produced at 34°C when incubation was initiated early (summarized above). One interpretation of these data is that incubations initiated early (at 15 days of age or younger) produced some high temperature females at 34°C (~20%), whereas incubation initiated later failed to do so. This scenario, in turn, suggests some degree of thermosensitivity at 30 days but not at 15 days of age or younger.

Although experiments are limited, sex ratios

varied among clutches. For six clutches incubated at 34°C (at 15 days of age or younger), sex ratios ranged from 33–100% males for individual clutches. Embryonic survival and development and incubation times are incompletely documented (Hutton, '87).

### ***Crocodylus moreletii***

In *Crocodylus moreletii*, the pattern of TSD is FMF, based on constant temperature incubation of five clutches at the Madras Crocodile Bank in 1991–1992. Eggs were produced by animals originating from Zoo Atlanta, Georgia. Eggs incubated at 31 (n = 6), 31.5 (n = 19), and 32°C (n = 12) produced 0%, 5%, and 33% males, respectively. At 33 (n = 11) and 33.5°C (n = 19), 55% and 5% males resulted, respectively. Although samples are small, the results establish the presence of low-temperature and high-temperature females in *C. moreletii*. Further study is required to delineate the male-producing incubation temperatures in this species.

Fertile eggs develop at constant incubation temperatures of 30–33.5°C. At 31°C, total incubation time averages 78 days, which is 1.21 times shorter than at 29°C and 1.11 times longer than at 33°C.

### ***Crocodylus siamensis***

In *Crocodylus siamensis*, the pattern of TSD is FMF. The following summary is based on Lang ('87) and recent results from eggs incubated at the Madras Crocodile Bank. The eggs (five clutches from three females) used in both studies were produced by animals originating from the New York Zoological Society. Eggs incubated at 28 (n = 4) and 29°C (n = 8) produced exclusively females. At 31 (n = 6) and 32°C (n = 13), all females resulted. At 32.5 (n = 15) and 33°C (n = 15), 100% and 60% males were produced, respectively.

Fertile eggs are viable over constant incubation temperatures of 28–33°C. At 31°C, total incubation time averages 76 days, which is 1.20 times shorter than at 29°C and 1.12 times longer than at 33°C.

### ***Gavialis gangeticus***

In *Gavialis gangeticus*, the pattern of TSD is FMF, based on incubation of three clutches of eggs at the Madras Crocodile Bank in 1990–1991. The eggs were produced by animals acquired as juveniles in the late 1970s from the Kukrail Gharial Centre, Lucknow, Uttar Pradesh, India. At 31.5 (n = 3) and 32°C (n = 9), 0% and 89% males resulted, respectively. At 33 (n = 5) and 33.5°C (n = 13), 20% and 15% males were produced, respec-

tively. Although samples were small, these data establish the presence of low-temperature and high-temperature females; further study is required to delineate the male-producing region in this species. It should be noted that sexing gharials by examining differences in the size and shape of the cliteropenis is very difficult during the first 1–2 years of age (<1 meter in total length). Young gharials do not have sexually dimorphic genitalia as do other crocodylians examined (Andrews and Lang, unpublished observations); in larger, older individuals, dimorphic genitalia are diagnostic of sex (Lal and Basu, '81).

Fertile eggs develop at incubation temperatures from 29–33.5°C. At 31°C, total incubation time averages 70 days, which is 1.20 times shorter than at 29°C and 1.17 times longer than at 33°C. Although the eggs of this species are large (mass = ~150–200 grams), development is rapid, particularly at high temperature.

## **DISCUSSION**

### ***TSD patterns***

To date, TSD has been documented in nine species and is supported by preliminary data in two additional species. These eleven species comprise half of the 22 extant species of Crocodylia and include one or more representatives in each of the three major lineages (i.e., alligators/caimans, crocodiles, and gharials) (King and Burke, '89). Within the order, the widespread distribution of TSD and the universal absence of heteromorphic sex chromosomes (Cohen and Gans, '70) point to the likelihood that all living crocodylians exhibit TSD. If so, the universality of TSD in this group contrasts sharply with the diversity of sex-determining modes in the turtles and lizards so far examined (Ewert et al., this issue; Viets et al., this issue).

With respect to TSD patterns, five species, including representatives of the two major lineages, have been examined in detail. All exhibit uniformity in TSD pattern, namely a FMF pattern within a relatively restricted range of viable incubation temperatures. The relationship between temperature and sex ratios in individual species are remarkably similar. Exclusively females are produced at 31°C and below, with the FM pivotal temperatures between 31 and 32°C. Maximum male-producing temperatures range from 32–33°C (Table 3). The TSD patterns in four additional species, although incompletely described, are also clearly FMF. Certain features, such as exclusive female production at low temperatures, appear

constant in all species (Table 3). The consistent pattern and similar pivotal temperatures are unprecedented in other reptiles with TSD, particularly in comparison with turtles (Ewert et al., this issue). Sea turtles are the only chelonians which do not show substantial interspecific variation in pivotal temperatures (Mrosovsky, this issue).

The ubiquitous FMF pattern in crocodylians, with dual transitions (males predominating at intermediate temperatures), contrasts with the MF pattern evident in many turtle species. It is particularly noteworthy given the antiquity of phylogenetically distinct lineages (alligators vs. crocodiles), diversity of nesting habits (mound vs. hole nesting), and the widespread distribution of representatives in wet and dry tropical and warm temperate habitats. Furthermore, the FMF pattern of TSD in crocodylians supports the suggestion that this pattern may be the basal pattern in reptiles, from which single transition patterns (e.g., MF) have been derived (Webb and Smith, '84; Deeming and Ferguson, '88).

Similarities among crocodylians notwithstanding, comparisons of TSD patterns and parameters reveal important interspecific differences. Most notable is the exclusive male-producing region in *A. mississippiensis*, which is absent in other species and rare among other TSD reptiles with the FMF pattern (Ewert et al., this issue; Viets et al., this issue). In addition, in *A. mississippiensis*, this region spans a wider range of temperatures, and the upper limit of viable incubation temperatures is higher, relative to other species (Table 3). In *A. mississippiensis* and, to a lesser extent, in *Caiman crocodylus*, the MF transition and pivotal temperatures are high, relative to those in the other well-studied species (Table 3). The other major difference among these species is the atypical FMF pattern in *C. johnstoni*, in which the constant temperature that produces maximum % males produces only low percentage of males (Webb et al., '87).

#### ***Thermosensitive periods***

Thermosensitive periods vary with experimental procedures and with the criteria used to define the window of sensitivity to incubation temperatures (Mrosovsky and Pieau, '91). Previously, the TSPs for *A. mississippiensis* were reported to be stages 14–16 (female to male; 30–34°C) and stages 21–23 (male to female; 34 to 30°C) (Ferguson and Joanen, '83; Deeming and Ferguson, '89). In our study, the primary period of thermosensitivity extended from stage 21 to stage 24, correspond-

ing to an approximate developmental time between days 30 and 45. This disparity in timing is likely attributable to the magnitude of the shift used in the earlier experiments (a difference of 4°C) and to the fact that 34°C produces both males and females (Fig. 1).

Concise definition of TSPs in other crocodylians so far studied is confounded because male production does not appear to reach 100% at any constant incubation temperature. Nevertheless, the window of thermosensitivity has been identified tentatively for three species of *Crocodylus* by considering the available data from switch protocols in light of the recently defined TSPs in *A. mississippiensis*. Despite an equivalent lack of precision in specifying end points, the revised TSPs in *Crocodylus* species are in general agreement with the TSPs in *A. mississippiensis*. Even the atypical pattern noted in *Crocodylus johnstoni*, namely high proportions of males associated with increasing incubation temperatures (vs. constant incubation temperatures), is consistent with the embryos being subjected to a narrow range of male-producing temperatures during the TSP (Table 3).

It is generally stated that thermosensitivity in TSD reptiles extends for the middle third or half of development (Bull, '87; Wibbels et al., '91). With new data for *A. mississippiensis*, it is possible to refine comparisons among TSD reptiles in which TSPs have been specified. The period of temperature sensitivity, as outlined here, begins at 45% of the incubation period at an intermediate temperature (i.e., 32°C, and it encompasses 23%, or approximately the third quarter, of development. Comparable values for turtles, calculated at intermediate incubation temperatures for each species, range from 28–36% of development when the TSPs are initiated, and the various TSPs extend for periods ranging from 18–25% of development (data from Yntema, '79; Pieau and Dorizzi, '81; Bull and Vogt, '81; Wibbels et al., '91). Comparable data for the leopard gecko indicate that the TSP is initiated at about 20% of development and extends for about 30% of the incubation period (Bull, '87). The TSP for *A. mississippiensis* extends for proportionately the same amount of developmental time, 25% of the total; but it occurs later, during the third quarter, relative to the chronology of the TSPs of representative turtles and a lizard, occurring during the second quarter. Thus, the TSP occurs early in postovipositional development in geckos, somewhat later in turtles, and latest in *A. mississippiensis*. Comparisons of mor-

phological stages, based on equivalent development of cranial features and of the limbs, among these TSD reptiles indicate that, in *A. mississippiensis*, the TSP is associated with more advanced stages relative to the earlier and nearly equivalent thermosensitive stages reported in various turtles and a lizard.

In *A. mississippiensis*, sex is determined at female-producing temperatures at an earlier stage than at male-producing temperatures. Similar chronologies have been reported in some turtles (Yntema and Mrosovsky, '82; Wibbels et al., '91) and a lizard (Bull, '87), whereas chronologies differ in other turtles (Yntema, '79; Bull and Vogt, '81; Pieau and Dorizzi, '81). In single shift experiments with *A. mississippiensis*, male production requires initiation of the appropriate temperature regime 1–2 stages earlier than those required for female production. In double shift experiments, temperature pulses spanning four developmental stages effectively induce males or females against background temperatures that otherwise produce the opposite sex. Embryos at these male-inducing temperatures are one stage younger than those at female-inducing temperatures (i.e., stages 20.5–24.5, and stages 21.5–25.5, respectively) (Fig. 2). Of particular interest, both male-inducing high temperatures and female-inducing low temperatures are equally effective over the same length of time (i.e., 20 days). A similar effect of temperature pulses in both directions has been reported for the turtle *Emys orbicularis* (Pieau and Dorizzi, '81); pulses of equivalent duration are equally effective in determining either sex. But in other turtles, differences in the time of the temperature pulse, its duration, and its direction affect male vs. female production asymmetrically. In some species, short pulses of warm temperature induce females more effectively than reciprocal shifts (Yntema, '79; Yntema and Mrosovsky, '82; Wibbels et al., '91), whereas, in other emydids, cool temperature pulses more readily induce males than the reciprocal shifts (Bull and Vogt, '81).

In *A. mississippiensis*, the temperature experienced over a range of stages spanning a discrete period of development clearly has a quantitative effect on sex determination. In double shift experiments, the duration (cumulative effect) and the magnitude (potency effect) of the temperature pulse influence the resultant sex. Longer duration pulses and/or shifts of greater magnitude are relatively more effective at inducing males and high-temperature females against background

temperatures which produce only low-temperature females. A similar result is obtained with multiple, progressive increases in incubation temperatures above 32°C during the TSP. These results in *A. mississippiensis* are consistent with studies in turtles that have demonstrated that sex determination is sensitive to the cumulative and/or potency effects of incubation temperatures experienced during the TSPs (Yntema, '79; Bull and Vogt, '81; Pieau and Dorizzi, '81; Bull et al., '90; Wibbels et al., '91).

### *TSPs and gonadal differentiation*

Gonadal differentiation in *A. mississippiensis* conforms to the general pattern described in other reptiles (reviewed in Raynaud and Pieau, '85) and has recently been described at the histological and ultrastructural levels by Smith and Joss ('93). At 33°C, the onset of testis formation is signaled by the formation of pre-Sertoli cells, occurring during stages 21–22. These cells differentiate into Sertoli cells during stage 23. In our shift experiments, 100% male production required that eggs be transferred to male-producing temperature (33°C) by stages 20.5–21; sex is irreversible after stages 23 via single shifts. At 30°C, ovarian differentiation is characterized by the proliferation of cortical germ cells during stages 22–23, followed by an increase in somatic cells in the cortex. In our shift experiments, 100% female production occurred when eggs were transferred to female-producing temperatures by stages 21.5–22; sex is irreversible after stage 24 via single shifts. In summary, testis formation began a stage earlier than ovarian formation, and the critical period for gonadal differentiation spanned stages 21–24 (Smith and Joss, this issue).

The TSPs identified in the present study for *A. mississippiensis* encompass stages 21–24 and are aligned with the detailed observations of Smith and Joss (this issue) as summarized above. This scenario substantially revises previously reported chronologies for this species and is consistent with the relationship between gonadal differentiation and the TSPs detailed for several turtles (Pieau, '74; Pieau and Dorizzi, '81; Wibbels et al., '91). Of interest in these comparisons is the observation that, while the TSPs in turtles are associated with earlier morphological stages relative to those of *A. mississippiensis* (see above), the TSPs of both turtles and *A. mississippiensis* occur during similar stages of gonadal differentiation.

### ***Mechanisms and models***

Despite an evident diversity among TSD reptiles in pattern type and associated pivotal/transition temperatures, general features shared by all species suggest a commonality in the underlying mechanisms (Wibbels et al., this issue). Shared features include a thermosensitive period (TSP), quantitative effects of incubation temperature in terms of duration and magnitude, correspondence between the TSP and gonadal differentiation, and alteration by exogenous steroid hormones during the TSP. The findings presented and reviewed here provide strong evidence that these features characterize the TSD system in *A. mississippiensis* and may apply to other extant Crocodylia exhibiting TSD. However, details of the proximal mechanism(s) inducing sexual differentiation in crocodylians, in TSD chelonians, and in TSD lizards may differ, based on the varied responses of different species to hormone manipulations (Rhen, '93).

Certain features of the TSD pattern in *A. mississippiensis* make it an attractive model for further studies of TSD mechanism(s). In particular, both low-temperature and high-temperature females are produced at viable temperatures. Male production is 100% within a range bounded by transition temperatures characterized by clutch-related male and female production (Fig. 1). The shift experiments presented here clearly demonstrate, for the first time in a TSD species with a FMF pattern, that unidirectional increases in the magnitude and/or duration of the temperature pulse (e.g., higher temperatures and/or longer periods) produce one or both sexes (e.g., males and/or high-temperature females) in accordance with predicted sex ratios based on various outcomes at constant temperatures. Thus, in *A. mississippiensis*, high temperatures during the TSP facilitate male production, resulting in increasing proportions of males as incubation temperatures increase. Temperatures above those that produce 100% males inhibit male production and/or stimulate the production of females, as evidenced by decreasing proportions of males as incubation temperatures increase further to the upper viable limit.

Various hypotheses have been postulated in which threshold levels of sex factor(s) within a specified time period ultimately result in gonadal differentiation (Deeming and Ferguson, '89; Haig, '91). The MF transition to high-temperature females in *A. mississippiensis* and other TSD reptiles with similar patterns presumably is the

result of a temperature-mediated inhibition of male-determining factor(s) and/or an activation of female-determining factor(s). Thus, in FMF species, it may be profitable to test hypotheses about events leading to gonadal differentiation at the MF transition as well as at the FM transition.

Smith and Joss (this issue) examined gonadogenesis in low-temperature females and males of *A. mississippiensis* and postulate a mechanism of developmental asynchrony resulting in a temperature-dependent mismatch between testis vs. ovary determination (a refinement of Joss ['89] and Haig ['91]). At male-determining temperatures, a threshold number of Sertoli cells is reached, preempting ovarian development and resulting in testis differentiation. In some embryos incubated at the nominal, male-producing temperature (33°C), they noted few Sertoli cells and suggest that variation among embryos may be responsible for production of both sexes at intermediate temperatures (Smith and Joss, '93). In light of the high incubation temperature, these embryos may represent high-temperature females occurring at the MF transition in *A. mississippiensis*. Examination of gonadal differentiation in high-temperature females and in embryos of different clutches incubated within the transition range of temperatures would be instructive in establishing whether similar developmental mismatches occur at other female-producing temperatures.

In *A. mississippiensis*, specification of pivotal (50% male) temperatures and thermosensitive periods will facilitate further investigation of the role of specific steroid hormones in TSD. For example, administration of exogenous androgens to eggs during appropriate developmental stages incubating at pivotal temperatures may augment male production, as has been demonstrated recently in a turtle (Wibbels et al., '92). Finally, the exclusive production of males at specified temperatures in *A. mississippiensis*, an unusual feature in species with FMF patterns, is a requisite for molecular studies in which male vs. female comparisons are critical (Coriat et al., this issue).

### ***Clutch effects***

In crocodylians, clutch of origin is potentially an important source of variation in TSD patterns and responses. Available data presented and reviewed here for various species indicate that the sex ratios of individual clutches vary substantially within the range of transition temperatures and at maximum male-producing temperatures in species not reaching fixation at 100% (e.g., C.

*palustris*). In *A. mississippiensis*, the sex ratios of individual clutches at two temperatures in the transition range (i.e., FM = 32°C, MF = 34°C) varied from 0 to 100% males. Clutch effects were also noted in switch experiments.

In various TSD species of turtles, clutch effects have been documented when eggs were incubated at constant temperatures and/or when eggs were subjected to shift protocols (Yntema, '79; Mrosovsky, '88; Mrosovsky and Pieau, '91; Etchberger et al., '91; Mrosovsky, this issue; Ewert et al., this issue). Possible factors contributing to clutch related variation include genetic and/or maternal differences which may be translated into variable TSD responses among offspring families. While the significance of maternal differences (e.g., egg size, yolk composition [see Ewert et al., this issue]) are largely unknown, sex ratio heritabilities have been calculated for several TSD turtles (Bull et al., '82; Janzen, '92).

From a practical standpoint, clutch effects in crocodilians are likely to be a significant source of variation in experimental procedures examining time-dependent and/or temperature-sensitive events in TSD. Definition of representative species and/or population-specific TSD patterns is dependent on multiple clutches of a number of females distributed across incubation temperatures. Interpretation of local or regional patterns will be facilitated by a better understanding of the range of individual patterns that combine to produce them. In *A. mississippiensis*, the magnitude of intrapopulation variation is small (e.g., a narrow range of transition temperatures) relative to those characteristic of several populations of the turtle *Chelydra serpentina* (Janzen, '92; Lang and Ewert, unpublished observations). To date, studies detailing interpopulation comparisons are lacking for any crocodilian species. In various TSD turtles, multiple examples of extensive geographic variation within species are apparent, as well as evidence of divergent individual TSD responses (e.g., strong bias for female production), within a population (Ewert et al., this issue).

### Crocodilian sex ratios

Sex ratios skewed toward females are apparently characteristic of some TSD reptiles (Bull and Charnov, '88, '89; Ewert and Nelson, '91). Published values for crocodilians include hatchling sex ratios estimated for two species, *Alligator mississippiensis* (Ferguson and Joanen, '83) and *Crocodylus johnstoni* (Webb and Smith, '84; Smith, '87). The many difficulties inherent in valid esti-

mations of natural sex ratios of reptiles with TSD are outlined by Mrosovsky (this issue). In a recent review, Mrosovsky and Provanča ('92) critically examined potential sampling biases in a number of TSD studies and suggested that the hatchling sex ratio of 17% reported for *A. mississippiensis*, based on 4 years of nesting and over 8,000 animals sexed, may have been biased by the methodology employed.

The results presented here for thermosensitive periods in *A. mississippiensis* confirm their suggestion. Egg collection from wild nests "at the end of the fifth week of incubation" (i.e., about day 35) (Ferguson and Joanen, '83), would have interrupted incubation within the TSP, dependent in part on prior nest temperatures. In our shift experiments, male production required 15–20 days continuously at male-producing temperatures (~33°C) during days 30–50 of age. Transferring eggs to 32°C (i.e., a FM transition temperature) would disrupt male production in eggs incubating at higher temperatures; in eggs incubating at lower temperatures in the nests prior to egg collection, 32°C is not sufficiently male-producing to alter an all female outcome. Thus, the sampling methodology probably biased the reported values by interrupting the TSP; our data suggest such an interruption with subsequent incubation at 32°C would have favored female production.

Clearly, additional data on sex ratios of *A. mississippiensis*, from various populations at various ages, are a necessary prerequisite to discussions of the ecological and/or evolutionary significance of TSD. Sex ratios of populations in Florida (Hines et al., '68), Louisiana (summarized in Nichols and Chabreck, '80; Carbonneau, '87; Rootes, '89; R. Elsey, unpublished data), and South Carolina (Bara, '72; Wilkinson, '84) have been variable, at times deviating significantly from a 1:1 sex ratio. These have frequently been biased toward males, even among juveniles in repeated samples, in various habitats and in numerous localities over decades. Recent data for other crocodilians suggest that sex ratios may deviate from 1:1, but not necessarily in the direction of a female bias (Ouboter and Nanhoë, '89; Thorbjarnarson, '90; reviewed in Cooper-Preston, '91).

Finally, we note that the female-biased, hatchling sex ratios reported in *C. johnstoni* may reflect the atypical pattern of TSD in this species (Table 3). Male production at any constant temperature is low relative to other species, although some wild nests produced 100% males. Sex ratios of juve-

niles and adults from various populations were female biased (Cooper-Preston, '91). In any event, the reported sex ratios in *C. johnstoni* may not be representative of other crocodylians.

In summary, a FMF pattern of temperature-dependent sex determination characterizes all species studied so far, including representatives of the three major crocodylian lineages. In *A. mississippiensis*, the thermosensitive period encompasses the third quarter of development and coincides with gonadal differentiation, as in turtles with TSD. Other shared features include a quantitative response to incubation temperature and alteration of gonadal sex via hormones. The FMF pattern in *A. mississippiensis* is an attractive model for testing whether incubation temperature inhibits male production, for testing whether it stimulates the production of females, or for testing alternative hypotheses. Our studies indicate that clutch effects are important and that hatchling sex ratios should be reexamined.

#### ACKNOWLEDGMENTS

This research was funded in India by grants from the Smithsonian Institution and the NSF-International, and in the USA by the NSF-EPSCoR-ASEND and faculty research grants at the University of North Dakota. Work in India was approved by the Departments of Education and of Environments and Forests, Government of India. Specimens were obtained with requisite permissions of state and central authorities within India and the USA and with the assistance of the staff, Division of Amphibians and Reptiles, USNM, Smithsonian Institution. Dr. P. Mehendiratta and the staff at the American Institute of Indian Studies provided numerous courtesies. We thank the staff at the Madras Crocodile Bank for their enthusiastic help, particularly Jeevanandam K. Encouragement and support were provided throughout the study by Romaine Andrews, Zai Whitaker, and Rom Whitaker. Work in the USA was made possible through the courtesies of the Louisiana Department of Fisheries and Wildlife, and we are especially grateful to Ted Joanen, Larry McNease, Ruth Elsey, and the staff at Rockefeller Refuge for collecting alligator eggs, for accepting hatchlings in return, and most of all for sharing their collective expertise about alligator reproduction. Many individuals at the University of North Dakota assisted in these studies, particularly J. Allsteadt, D. Casey, Xian Liu, M. Reiner, T. Rhen, and J. Sandeen. Val Lance at CRES, Zoo-

logical Society of San Diego, kindly provided data on alligator sex ratios as well as histological evaluations. For comments on the manuscript, we thank J. Allsteadt, C. Etchberger, M. Ewert, J. Joss, V. Lance, N. Mrosovsky, C. Smith, T. Rhen, B. Viets, G. Webb, T. Wibbels, and an anonymous reviewer.

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