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Incubation temperature has long-term effects on behaviour of young Pine snakes (*Pituophis melanoleucus*)

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Summary. Eggs of pine snakes (*Pituophis melanoleucus*) were incubated at constant temperatures of 21°, 23°, 26°, 28°, 30° and 32° C to determine behavioural differences as a function of incubation temperature. For all behavioural and physiological measures hatchlings from medium incubation temperatures (26°, 28°) performed tests better than those hatchlings from eggs incubated at low temperatures (21°, 23°). For some behavioural tests hatchlings from eggs incubated at high temperature (30°, 32°) performed less well than medium temperature hatchlings. These differences were not due to ambient temperatures or age of testing since these were held constant. Some of the behavioural differences persisted for 24 weeks.

Introduction

Sex determination and sex differentiation have been studied extensively in most vertebrate classes. In some reptiles and fishes, sex is determined during incubation by environmental variables such as pH (Heiligenberg 1965), water potential (Gutske and Paukstis 1983), and temperature (Harrington 1971; Pieau 1971; Conover and Kynard 1981; Wagner 1980; Conover 1984). In laboratory studies, temperature dependent sex determination has been found for several species of turtles (Yntema 1976, 1979; Yntema and Mrosovsky, 1980; Bull 1985a), a lizard (Charnier 1966; Bull 1987), and an alligator (Ferguson and Joanen 1982). Field studies have shown similar results despite the fluctuating temperature of natural nests compared to the constant temperature of the laboratory (Bull 1985a).

Incubation temperature in reptiles also affects mortality and structure (Vinegar 1974; Osgood 1978; Gutzke et al. 1985; Gutzke and Packard 1987), but not behavior of offspring except for thermal selection behaviour of hatchling crocodiles (*Crocodylus*, Lang 1985). However, in most studies of sex-determination, rather than keeping the animals under observation, the experimenters sexed the embryos or hatchlings by dissection since there was no dimorphism of external morphology. In this paper I present data from the laboratory on behavioural differences as a result of incubation temperatures in pine snakes (*Pituophis melanoleucus*). In this species sex can be determined by eversion of the hemispenes, allowing me to keep animals alive for behavioural studies (Schaefer 1934; Fitch 1960; Gregory 1983; Gutzke et al. 1985). I was particularly interested in examining behaviour patterns that have relevance to survival of hatchlings in nature, and in determining if these differences persist for more than a few days. If incubation temperature effects subsequent behavior and physiology, it has evolutionary consequences for the temporal and physical features of nest site selection, range and distributions, conspecific and interspecific interactions, and ultimately for the fitness of individuals. There are few data on hatchling pine snakes, but their subterranean nests require them to dig out of the nests within a few days of hatching (Burger and Gochfeld 1985).

Methods

Pine snake eggs were collected under appropriate state permits in Ocean, Monmouth and Burlington counties, New Jersey. They were collected from the field within 4 days of laying or from females that laid in captivity. Pine snakes nests can be readily located by regularly checking nesting sites: females leave a characteristic nest opening and dirt pile (Burger and Zappalorti 1986). Further, we only used eggs that were not stuck together, ensuring that they were freshly-laid. Although development may take place before they were laid (Packard and

Packard 1988), I am confident the clutches used were freshly-laid.

In 1984 and 1985, I incubated eggs of pine snakes at six different temperatures to determine if temperature affected sex ratios or subsequent behaviour of hatchlings. In 1984, eggs were incubated at 21, 26 and 30° C and in 1985 eggs were incubated at 23, 28 and 32° C (±1° C). Each year clutches were collected from natural nest sites. Each clutch was from a single female. Females characteristically lay a clutch of 3–14 eggs (mean = 8.8; Zappalorti et al. 1983; Burger et al. 1987). Eggs from each clutch were randomly divided into 3 groups, and each group was placed in incubators at a different temperature under constant hydric conditions since substrate wetness can affect hatchling characteristics (Tracy et al. 1978; G.C. Packard et al. 1983). I maintained constant hydric conditions because the hydric environment of incubating reptile eggs affects hatchling mass in some studies (FG.C. Packard et al. 1981; Packard and Packard 1986, 1988; Gutzke et al. 1987), but not others (Tracy 1980; G.C. Packard et al. 1979, 1983), mortality and hatching success (Tracy 1980; M.J. Packard et al. 1982; Gutzke and Packard 1987), incubation time (G.C. Packard et al. 1983), and locomotary performance (Miller et al. 1987). Similarly, moisture during incubation affects hatchling snapping turtles (*Chelydra serpentina*): eggs incubated on a relatively wet substrate ran and swam faster than those from eggs incubated on a drier medium (Miller et al. 1987); however this was complicated by a size difference.

Each group of eggs was placed in a plastic shoe box on damp sand (equal amounts of sand and water added initially) and covered with damp sphagnum moss. Equal amounts of water were added on a regular basis to the sand. Eggs were checked every other day for moisture and egg condition. In nature, average temperatures in nests range from 21–35° C ($X = 27 \pm 2.6°$ C in 1986), and vary daily (2–10° C, Burger and Zappalorti 1988, unpublished data). Nest temperatures were recorded at 7 nests with continuously recording thermometers. Females normally dig nests in the open, exposing nests to continuous sunlight during the day (Burger and Zappalorti 1986).

Once the young hatched they were placed in individual cages, and maintained and tested at 26° C. All snakes were tested at the same number of days post-hatching, but on different dates since incubation period differed as a function of temperature. I performed two types of experiments: physiological (activity, drinking speed, and shedding time), and behavioural (3 m timed locomotion, righting response, incline, tube test, gap test, and anti-predaor test). This combination of tests was performed to evaluate the hatchlings' ability to move, climb, and avoid predators in the wild. In nature young pine snakes climb logs and trees, move up and down uneven ground, and go into and out of their nest tunnels and hibernacula burrows. Thus the battery of tests was designed to examine their ability to survive in the wild. All tests were performed by three technicians blind to the temperature identity of hatchlings. Each behavioral tests was conducted by only one technician to avoid variations among observers. Technicians were trained and tested on hatchlings that were not part of the experiments. Written criteria were provided for each test. Drinking speed, shedding times and activity were recorded by all three technicians. After training, all three technicians agreed on measures (see below).

Activity was ranked 1–5 and recorded four times a day from ages 10–15 days. Statistics were performed on the means for each hatchling. 1 = stationary, 2 = slow movement around cage with periods of rest, 3 = slow continuous movement, 4 = attenuating slow and rapid movement, 5 = rapid movement with sporadic attempts to escape. *Drinking speed* (number of swallows/sec) was recorded every 3 days when snakes were given water. We recorded when each hatchling first shed its skin (called *Shedding times*). Pine snake hatchlings do not eat until they have shed their skin for the first time.

For the *3 m timed course* snakes were placed on a track 3 m long and 10 cm wide (rough carpet), and forced to move rapidly by fingers moving behind them. For the *righting response* snakes were placed on their back, and a stop watch was used to measure the time required to turn onto their venters. Snakes were tested at 10–12 days post-hatching, snakes were tested once, and Kruskal-Wallis χ^2 were used to test differences among groups.

In the *incline test* snakes (12–14 days post-hatch) were placed inside a glass tube, and the tube was slowly raised to determine the angle when the snake slipped or fell out of the tube. In the *tube test* each snake (12–14 days post-hatch) was placed over the glass tube 25 cm from the table (1 cm diameter), and I recorded its behaviour. Snakes could fall onto a pillow 1 m below or manipulate themselves so they could crawl back to the table. In the *ability to bridge a gap test* the snake was placed over a tube that was half its body length away from its home cage. The snake could either crawl back to the table, or balance itself while moving to its home cage.

In the *antipreator test*, snakes (14–17 days post-hatch) were acclimated to an aquarium for 5 min and visually isolated from both experimenters. Snakes were placed in the aquarium by an assistant that then disappeared behind a door, the observer was never visible to the snakes. An experimenter then walked from 12 m away directly toward the aquarium, while the second experimenter recorded the initial distance when the snake responded, its initial response, its second response, and its second response distance.

In 1984, the high incubation temperature snakes were prematurely returned to the field and thus only the middle and low incubation temperature snakes were tested. All snakes had fasted for at least three days prior to any testing. The three temperature groups each year were matched across clutches to avoid any bias due to female vigor or fitness. To avoid possible bias due to differential contribution of females in the two years, I analyzed the 1984 and 1985 data separately. This results in some loss of statistical power, but produces a more conservative test of hypotheses. Upon completion of tests all hatchlings were returned to their original nests and allowed to emerge with sufficient time to find winter hibernacula.

There were no differences in primary sex ratios as a function of incubation temperature (Burger and Zappalorti 1988).

Results

Each year there were significant differences in drinking speed, activity levels and shedding times among the temperature groups, despite the fact that snakes were maintained at the same ambient temperature and tested at the same number of days post-hatching (Table 1). Snakes that had been incubated at low temperatures shed earlier and drank more slowly than snakes from eggs incubated at medium (1984, 1985) or high (1985) temperatures. The latter differences persisted throughout the test period of 24 weeks (Fig. 1). Activity scores in 1985 were highest for snakes incubated at medium temperatures compared to those incubated at high or low temperatures (Table 1).

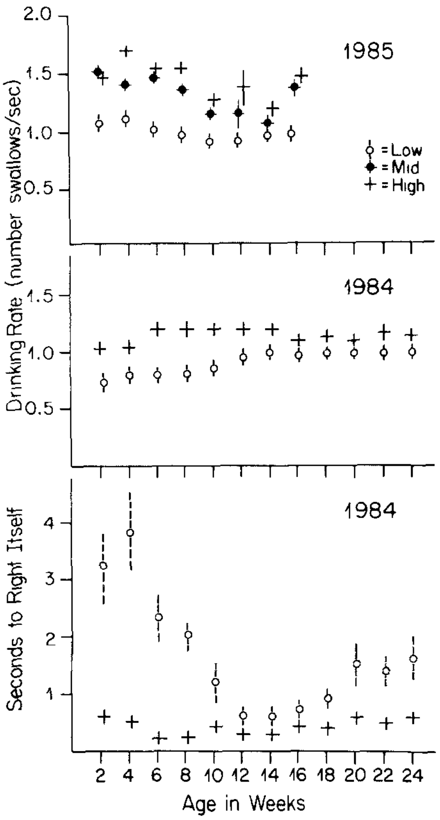

Fig. 1. Righting response and drinking rate as a function of age for 2–24 week old pine snakes (1984–1985)

Snakes from eggs incubated at low (1984, 1985) and high (1985) temperatures moved and righted themselves nore slowly than those from eggs incubated at medium temperatures (Table 1). In the low incubation temperature snakes the righting response improved in the first two months, but was still significantly slower than that of the other group throughout the study (Fig. 1). There was a significant improvement with age for the low temperature group ($\chi^2 = 44.01$, $P < 0.0001$), whereas higher temperature hatchlings maintained a constant level of performance over time.

In the incline test there was a significant difference in the angle at which snakes began to slip and the percent that slid out of the tube (Table 1). Fewer mid and high temperature snakes slid down compared to low temperature snakes, and they were able to climb farther up the vertical tube than the low temperature snakes in 1984, but not in 1985 (Table 1).

In the tube test in both years more of the low temperature snakes fell off the glass tube, fewer reached the table (in 1984 only), and they moved a shorter distance along the top of the tube than did snakes hatched from eggs incubated at higher temperature (Table 1).

In the gap test, snakes could either go back to the table (through or over the tube), cross the gap to their home cage, or remain motionless. More low temperature snakes fell off and only 6% (1984) and 27% (1985) of the low temperature snakes reached the table or home cage compared to 39% (1985) and 75% (1984) of those hatched from eggs incubated at higher temperatures.

Most of the 1985 behavioral tests showed a tendency for the hatchlings from the high temperature incubation group (32°) to perform less well than the mid-temperature group (28°, Table 1). This suggests that there may be selection against eggs from nests exposed to prolonged periods of high incubation temperatures.

In the antipredator tests (1985), low temperature snakes responded less appropriately than the middle and high incubation temperature snakes. Snakes reponded to the approaching person by becoming motionless, attempting escape by rapid movement in the opposite direction, or by continuing their previous activity (no response). Most snakes responded initially by becoming motionless, and secondly by rapid escape movement. A higher proportion of the snakes in the low temperature group did not respond to the approaching person (Table 2). Further, fewer low temperature snakes tried to escape. The response distance also varied significantly for the initial and secondary responses. Low temperature snakes let the "predator" approach closer before responding. These differences were not due to overall failure to move in the test situation since there were no significant differences among the three temperature groups in the mean latency to explore initially when first introduced to the test aquarium ($\chi^2 = 2.31$, $df = 2$).

Discussion

In the present study pine snakes from eggs incubated at low temperatures performed less well than those from eggs incubated at medium or high temperatures. The tests performed relate to survival skills required in nature. Snakes moving over rough terrain or logs sometimes fall, and an ability to right themselves quickly results in their being exposed to predators for less time. Similarly, hatchlings move up inclines (los, hills, sand banks) in search of hibernacula. Low-incubation temperature hatchlings were less able to go up steep inclines than those incubated at a higher temperature.

These tests measured ability to perform a given task. However the ability to reach their home cage (gap test) examined both their ability to move, and

Table 1. Comparison of behaviour of hatchling pine snakes as a function of incubation temperature. Given are means ± SE

Test	Temperature			Kruskal-Wallis
	Low	Middle	High	χ^2 (*P*)
1984 Experiment				
Temperatures (° C)	21	26		
Number of snakes	9	12		
Physiology				
Shedding age (days[a])	10.6 ± 0.9	11.4 ± 0.2		6.81 (0.04)
Drinking speed (number swallows/s)	0.92 ± 0.11	1.45 ± 0.32		13.25 (0.0001)
Behavioural tests				
Righting response (s)	1.8 ± 0.2	0.4 ± 0.03		77.9 (0.0001)
Incline[b]				
angle slid down	42.7 ± 2.8	86.9 ± 2.2		52.3 (0.0001)
angle fell	65.8 ± 3.4	none fell		77.4 (0.0001)
percent slid	89	33		48.5 (0.0001)
distance moved up (cm)	16.4 ± 6.6	140.8 ± 75		69.8 (0.0001)
Tube test[c]				
percent fell off	87	37		40.9 (0.0001)
time to fall off (s)	190 ±30	153 ±170		NS
percent reach table	0	35		45.3 (0.0001)
distance moved toward table (cm)	1.41 ± 0.5	19.1 ± 17.7		85.0 (0.0001)
Ability to bridge gap to home cage				
time to fall off (s)	74 ±11	49 ± 54		NS
percent to reach cage or table	6	75		45.3 (0.0001)
1985 Experiment				
1985 Temperatures (° C)	23	28	32	
Number of snakes	48	74	47	
Physiology				
Shedding time (day[a])	9.5 ± 0.3	11.6 ± 0.1	12.6 ± 0.5	38.6 (0.001)
Drinking speed (swallows/s)	1.02 ± 0.14	1.41 ± 0.22	1.55 ± 0.12	181.1 (0.0001)
Mean activity score	3.2 ± 0.1	3.9 ± 0.08	3.1 ± 0.09	6.1 (0.0001)
Behavioural tests				
3 m timed course (s)[d]	8.0 ± 0.4	5.4 ± 0.1	6.3 ± 0.4	6.6 (0.0001)
Righting response (s)	0.70 ± 0.02	0.58 ± 0.02	0.67 ± 0.03	15.7 (0.001)
Incline				
angle slid down	44.7 ± 5	46.2 ± 7	37.4 ± 5	6.29 (0.04)
percent slid	26	12	17	28.8 (0.001)
distance moved up (cm)	196 ± 2	200 ± 0	186 ± 5	NS
Tube test[c]				
percent fell off	77	63	78	12.3 (0.001)
time to fall (s)	27 ± 4	35 ± 4	40 ± 5	NS
percent reach table	14	29	18	NS
distance moved toward table (cm)	22.9 ± 2	24.1 ± 1	15.9 ± 2	20.2 (0.0001)
Ability to bridge gap to home cage				
time to fall off (s)	89 ±11	49 ± 9	47 ±18	8.60 (0.01)
percent to reach cage or table	27	39	40	NS

[a] From hatching to shedding. All snakes maintained at constant temperature;

[b] Snake inside glass tube that is gradually raised to a 90° angle. Chi Square for percents computed on raw data. In 1985 no snakes fell out of the tube;

[c] Snake balanced over glass tube, suspended 0.25 m from table;

[d] Time to move over a 3 m distance when pursued by fingers

their ability to perceive the situation. Snakes either started to choose one path and continued it, chose one path but aborted it in favor of another, or remained motionless. More of the low temperature snakes remained motionless rather than take a particular course (as did those incubated at higher temperatures).

In the predator test, most snakes initial response was to cease movement and remain motionless. These tests suggest that in nature the initial response is to freeze. Their coloration makes them particularly cryptic on the pine needles and oak leaves. If a predator continues approaching, the snakes move away rapidly in the opposite direc-

Table 2. Response of hatchling pine snakes to an approaching predator as a function of incubation temperature. Given are percent responding

Response	Incubation Temperature			$\chi^2(P)$
	Low	Medium	High	
Number of snakes	48	74	47	
Anti-predator behaviour[a]				
Initial response				
no response	21	4	3	
motionless	68	86	88	
rapid movement	9	10	9	38.7 (0.001)[b]
Second response (of initial responses)				
motionless	55	6	6	
rapid movement	45	94	95	125.8 (0.001)
Distance of initial response (m)	1.8 ± 0.3	3.0 ± 0.3	3.1 ± 0.2	26.2 (0.001)
Distance of second response (m)	0.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	18.5 (0.001)

[a] tested with an approaching person;
[b] *df* = 4. Goodness of Fit χ^2-test on raw data

tion. Snakes from eggs incubated at medium and high temperatures thus would have avoided predators. However the low incubation temperature hatchlings often did not respond initially, and many that responded initially continued to remain motionless until the person picked them up. This might result in lower survival in nature.

The adaptive significance of the physiological responses (drinking speed, shedding times) are more difficult to interpret, and I can offer no explanation for them.

Taken altogether, the results indicated that incubation temperature affects the physiology and behavior of pine snakes. The differences persisted even though the snakes were maintained at the same temperature following hatching, and the differences persisted for at least 24 weeks following hatching. These differences were not a function of thermal acclimation because they were not reversible. Further, they are likely not an artifact of constant incubation temperature, since in those species where sex is determined by incubation temperature a constant incubation temperature does not affect the outcome (Bull and Vogt 1979). These behavioural differences were not due to age since all tests were performed the same number of days after hatching. Previous studies have noted structural, thermal preference, and sex-determination differences as a function of incubation temperatures (see Introduction). In most studies the young were never observed post-hatching. In reptiles, nest humidity can affect sex and development, but in this case sand was maintained at a similar dampness, and sphagnum was always saturated with water. Nest humidity should, however, be examined as a variable affecting hatchling behaviour and physiology.

The results of the present experiments are broad-based, including shedding time, drinking speeds, movement and behaviour, and suggest that a snake's future physiology and behaviour is being influenced by incubation temperature. Sex-determination in some reptiles may be part of this general phenomenon, or even a consequence of the phenomenon rather than a factor which itself is under direct selection, accounting for our inability to find an adaptive reason for temperature-dependent sex determination (Pieau 1971; Yntema 1976; Bull 1985a; Huey 1982). Some of the effects may represent behavioural deficits or abnormalities produced by non-optimum incubation conditions. Morphological differences (body length, occipital scale length) were statistical, rather than obvious, and the experimenters could not pick out the temperature group of the hatchlings by watching them (Burger et al. 1987). Snakes incubated at low temperatures (21° and 23°) were not lighter in weight than other snakes, although they were shorter in length (Burger et al., 1987). However snakes of similar length incubated at low temperatures (21° and 23°) performed behavioural tests less well than those incubated at higher temperatures (χ^2-tests).

Incubation temperature determination of a snakes' physiology and behaviour could result in low temperature hatchlings being slow, mid temperature hatchlings being faster, and high incubation temperature hatchlings again functioning less optimally. Clearly eggs from nests exposed to prolonged periods of extreme temperature would have lower hatchability, more behavioural abnormalities, and presumably lower fitness. These features potentially influence distribution. Range extensions without climate moderations would not be successful. The ramifications of incubation temperature influencing subsequent physiology and behaviour are numerous and could include: selection for optimal nest sites, selection for optimal timing of egg-laying (to avoid lethal or detrimental temperatures), and limiting extreme Northern and Southern range extensions.

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