The effect of constant and fluctuating incubation temperatures on the phenotype of black ratsnakes (*Elaphe obsoleta*)

L.D. Patterson and G. Blouin-Demers

**Abstract:** According to the maternal manipulation hypothesis, females manipulate the phenotypes of their offspring by selecting favourable incubation conditions. In oviparous black ratsnakes (*Elaphe obsoleta* Say in James, 1823), females manipulate offspring phenotype through nest-site selection. This study aimed to determine whether the thermal mean and variance of the incubation regime affect fitness-related traits in hatchlings. We incubated 136 eggs in a split-clutch design at two thermal means (26 and 29 °C) and variances (constant and ±3 °C). Hatchlings incubated at higher temperatures hatched earlier, were longer, faster, and less defensive. Hatchlings incubated at constant temperatures hatched earlier and were longer. For athletic performance, there was a significant interaction between temperature mean and variance: hatchlings incubated at 29 °C swam faster, had a lower muscular strength, and righted themselves equally fast when incubated at constant temperatures, whereas hatchlings incubated at 26 °C were stronger, swam faster, and righted themselves more slowly. Overall, constant incubation temperatures produced hatchlings with phenotypes favouring higher survival than fluctuating temperatures, but the effect of thermal variance was not as pronounced as the effect of thermal mean. Therefore, we found some support for the hypothesis that black ratsnakes prefer communal over single-female nests because communal nests have higher, more constant temperatures.

**Introduction**

The phenotype of an organism is the result of an interaction between genetic and environmental factors. The environmental conditions under which an embryo develops affect not only the rate of embryogenesis, but also its trajectory (e.g., Shine and Harlow 1993). Even small environmental changes during embryogenesis can result in major phenotypic modifications (Brown and Shine 2004). In oviparous reptiles, the temperature and soil water content of the nest have been found to affect the incubation period, growth rate, morphology, behaviour, and sexual differentiation of hatchlings (Hare et al. 2004; Brown and Shine 2005a; Ji et al. 2006). Moreover, the phenotypic differences among neonates induced by incubation conditions may persist for many months after hatching (Shine and Harlow 1996) and affect juvenile survival (Jayne and Bennett 1990; Qualls and Andrews 1999; Hare et al. 2004). According to the maternal manipulation hypothesis (Shine 1995), female ectotherms can manipulate the phenotypes of their offspring through the selection of an appropriate incubation environment. When a female chooses to expose her offspring to incubation conditions that produce phenotypic traits that maximize offspring growth and survival, she enhances her
own fitness. If adaptive modifications to offspring phenotype are achieved primarily through female choice of incubation conditions, natural selection should act on female behaviour rather than on alleles for particular offspring traits. Examples of female behaviour that control the thermal conditions under which their young develop include behavioural thermoregulation during pregnancy in viviparous reptiles, egg-brooding, and nest-site selection (Shine 2003). In the oviparous scincid lizard *Bassiana superperi* (Gray, 1838) (eastern three-lined skink), for instance, females consistently prefer to lay their eggs in nests with high and variable temperatures (Shine and Harlow 1996). In this species, incubation temperatures account for approximately half as much variation in hatching phenotype as do “nest-of-origin” (including genetic) effects (Shine et al. 1997).

Black ratsnakes (*Elaphe obsoleta* Say in James, 1823) are oviparous and females control offspring phenotypes primarily through nest-site selection (Blouin-Demers et al. 2004). The majority of female black ratsnakes lay their eggs in communal nests, but occasionally a female may choose to lay eggs in a solitary nest (Blouin-Demers and Weatherhead 2000; Blouin-Demers et al. 2004). The temperature in communal nests is higher and less variable than in single-female nests (Blouin-Demers et al. 2004). Females prefer to nest at mean temperatures characteristic of communal nests, and these higher temperatures produce hatchlings with phenotypes likely to enhance their survival (Blouin-Demers et al. 2004). However, the effects of temperature variance on female nest-site selection and hatching phenotype have not been explored. Since communal and single-female nests differ in thermal variance and thermal mean, it is possible that female ratsnakes prefer communal nests not only because these offer higher mean temperatures, but also because they offer more constant temperatures. Our goal was to test the prediction that higher, more constant incubation temperatures produce offspring phenotypes that enhance fitness.

**Materials and methods**

**Study site**

We studied ratsnakes in a 10 km × 3 km area surrounding the Queen’s University Biological Station, approximately 100 km south of Ottawa (Ontario, Canada). In this area, ratsnakes nest in large piles of decomposing vegetation that are loose enough to allow the snakes to burrow to depths of up to 75 cm (Blouin-Demers et al. 2004). Nests include large leaf piles, compost piles, and hollow tree stumps.

**Egg collection and incubation**

In July 2006, we collected eight clutches of eggs (*n* = 100) from known communal nests. Clutches are generally laid far enough apart in communal nests that they are easily distinguishable. We obtained an additional three clutches of eggs (*n* = 36) from gravid females caught opportunistically in the field and that subsequently laid in the laboratory. We equally and randomly divided each clutch among four treatments (*n* = 34 eggs in each treatment): two constant-temperature treatments (26 and 29 °C) and two treatments with means of 26 and 29 °C but fluctuating on a diurnal cycle (±3 °C). These temperatures were chosen to approximate those in communal and single-female nests; the diurnal fluctuations in single-female nests are approximately ±3 °C (Fig. 1b in Blouin-Demers et al. 2004), whereas communal nests do not fluctuate diurnally (Fig. 1a in Blouin-Demers et al. 2004).

This split-clutch design was used to control for genetic and maternal factors that affect offspring phenotype. We placed the eggs in plastic containers so that they were half-buried in moist vermiculite (1:2 ratio by mass of vermiculite and water). The constant-temperature incubators were constructed from plastic containers insulated with closed-cell foam boards. Temperature was controlled with thermostats and heat was provided by 100 W light bulbs. Small fans ran continuously in each incubator to prevent any thermal gradients from forming. The fluctuating-temperature incubators were commercially made (Model LBC700, Constant Temperature Control Limited, Aurora, Ontario). We placed miniature temperature dataloggers (iButtons, Dallas Semiconductors Inc., Dallas, Texas) in the incubators to monitor the temperature. The mean temperatures (±95% CI) over the incubation period were 25.7 ± 0.035 and 29.5 ± 0.035 °C for the constant-temperature incubators, and 26.0 and 29.0 °C (precisely) for the fluctuating-temperature incubators (Fig. 1). Every 2nd day, we added water to each egg container to return it to its initial mass, which compensated for water loss via evaporation. We also shuffled the containers when replacing them in the incubators to compensate for any possible positional effects. Beginning in mid-August, we checked the eggs daily for signs of hatching, removed hatched neonates, and placed them in individually labelled containers.

**Morphological and behavioural measurements**

Morphological and behavioural measurements were made within 24 h of hatching. First, we assessed hatchlings for defensive behaviour upon first handling. We scored each individual on a scale from 1 to 5 (1, fleeing; 2, rattling tail; 3, gaping; 4, striking; 5, biting). Sex was assessed by eversion of the hemipenes. We measured snout–vent length (SVL) and tail length (TL) to the nearest millimetre using a ruler taped to the table and used the mean of two measurements in our analyses (Blouin-Demers 2003). We measured mass to the nearest 0.1 g on an electronic scale.

**Performance measurements**

We assessed three measures of performance: swimming speed, righting time, and muscular strength. Each measure of performance was assessed at both 29 and 26 °C in an environmental chamber to determine whether individuals performed best at their mean incubation temperature. We equilibrated the hatchlings at the appropriate temperature for at least 20 min prior to performance trials. In each case, we counted the better of two trials. Swimming speed was assessed rather than crawling speed because the latter typically provoked a defensive response. We ran the swimming trials in a 2 m long trough filled with water, timing the swimming speed over the middle 1.5 m with a stopwatch. To encourage the hatchlings to swim at maximum speed, we gently prodded their tails. For righting time, hatchlings were smoothed out until straight, placed on their backs, and allowed to flip. Righting time was determined using the playback mode of a digital video camcorder (Canon...
ER600). Muscular strength was measured with a 100 g spring scale (Shine and Brown 2002). We used surgical tape to affix the tails of hatchlings to the end of the spring scale that was taped to the table. Hatchlings were smoothed out and gently tapped on the tail to encourage muscular contractions. We noted the strongest of the first five rapid contractions, allowed hatchlings to rest for 10 s, and repeated the procedure a second time.

All experimental procedures were in accordance with guidelines from the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Statistical analyses

The purpose of all statistical analyses was to determine if temperature mean, temperature variance, or their interaction affected hatching date and neonate traits.

For hatching success, we pooled the data by temperature mean and then by temperature variance, and analyzed each data set with a $\chi^2$ test. Hatching date was analyzed with a multiway ANOVA. Independent factors were temperature mean (TM) and temperature variance (TV). For defensive behaviour, we used a nonparametric Kruskal–Wallis test. Independent factors were TM, TV, clutch, sex, TM × TV, and TM × sex. Morphological differences were tested with a MANOVA followed with univariate ANOVAs for SVL, TL, and mass. Independent factors were TM, TV, clutch, sex, TM × TV, and TM × sex. Performance differences were tested with a MANOVA with TM, TV, clutch, sex, the temperature at which the hatchling was tested (TT), TM × TV, TM × sex, and TM × TT as independent factors. The TM × TT interaction was used as an independent factor to determine whether hatchlings incubated at different mean temperatures performed better at their own incubation temperature. Since there was a marginally significant TM × TV interaction for performance, we conducted separate MANOVAs for each mean temperature and each temperature variance. Multivariate analyses were followed with separate ANOVAs for swimming speed, righting time, and muscular strength. All univariate $F$ tests (for morphology and performance) were mixed-model ANOVAs, with thermal mean and (or) variance as fixed effects and clutch as a random effect. The analysis of performance analysis was a repeated-measures design because there were two measurements per hatchling (one at each temperature tested). Clutch was included as a control variable in all analyses because maternal and genetic effects influence phenotypic traits in rat snakes (Blouin-Demers et al. 2004).

Assumptions of multivariate normality and homoscedasticity for the MANOVAs were assessed following the methods suggested by Stevens (1996). All nonsignificant interactions that were not of interest were removed from the final models. All terms of interest that were nonsignificant were removed from subsequent analyses (e.g., in the univariate $F$ tests following a MANOVA). All statistical analyses were conducted with JMP version 6 (SAS Institute Inc., Cary, North Carolina). All marginally significant (0.05 ≤ $P$ < 0.1) results were considered because of our modest sample size (Nakagawa 2004).

Results

All full models were significant except for the $\chi^2$ tests. There were strong clutch effects ($P < 0.001$) in all analyses except for defensive behaviour ($P = 0.36$). There were no significant interactions between temperature mean and sex (males and females were affected equally by the incubation treatments). There was no interaction between mean incubation temperature and temperature tested, indicating that hatchlings did not perform better at the mean temperature at which they were incubated. Thus, we arbitrarily chose to present data for performance tested at 29 °C only.

Hatching success and date

Hatching success was 85% for the 136 eggs we incubated. There was no significant effect of temperature mean ($\chi^2_1 = 0.433$, $P > 0.5$; Fig. 2) or variance ($\chi^2_1 = 0.325$, $P > 0.5$; Fig. 2) on hatching success. There was a significant effect of temperature mean ($R^2 = 0.55$, $F_{1,25} = 560.99$, $P < 0.001$) and variance ($R^2 = 0.023$, $F_{1,25} = 23.53$, $P < 0.001$) on hatching date. On average, eggs incubated at 29 °C hatched 14 days earlier than those incubated at 26 °C and...
eggs incubated at constant temperatures hatched 3 days earlier than those incubated at fluctuating temperatures (Fig. 2).

**Defensive behaviour**

There was a significant effect of temperature mean ($H_{[1,102]} = 7.93$, $P = 0.005$), but not of temperature variance ($H_{[1,102]} = 0.12$, $P = 0.731$) or sex ($H_{[1,102]} = 2.51$, $P = 0.11$), on defensive score. Hatchlings incubated at a mean temperature of 26 °C were more defensive (mean = 3.85, 95% CI = 3.53–4.17) than those incubated at 29 °C (mean = 2.80, 95% CI = 2.36–3.24).

**Morphology**

Temperature mean (Wilks’ $\lambda = 0.62$, $F_{[3,296.87]} = 20.66$, $P < 0.001$), temperature variance (Wilks’ $\lambda = 0.15$, $F_{[3,296.87]} = 4.90$, $P = 0.003$), and sex (Wilks’ $\lambda = 1.22$, $F_{[3,296.87]} = 40.81$, $P < 0.001$) all affected morphology. Differences in morphology between the mean temperature treatments were due to SVL ($R^2 = 0.18$, $F_{[1,104]} = 37.13$, $P < 0.001$) and mass ($R^2 = 0.01$, $F_{[1,104]} = 4.84$, $P = 0.03$), but not tail length ($R^2 = 0.004$, $F_{[1,104]} = 1.52$, $P = 0.22$). Hatchlings incubated at 29 °C were longer than those incubated at 26 °C, whereas those incubated at 26 °C were heavier than those incubated at 29 °C (Fig. 3). Differences in morphology between temperature variance treatments were

---

**Fig. 2.** Effect of thermal mean (26 or 29 °C) and variance (constant and ±3 °C daily cycle) of the incubation treatment on the (A) hatching success and (B) hatching date of neonate black ratsnakes (*Elaphe obsoleta*). Values are means ± 95% CI ($n = 9$ clutches per treatment for both A and B).

**Fig. 3.** Effect of thermal mean (26 or 29 °C) and variance (constant and ±3 °C daily cycle) of the incubation treatment on the (A) snout–vent length, (B) mass, and (C) tail length of neonate black ratsnakes (*Elaphe obsoleta*). Values are means ± 95% CI ($n = 29, 26, 29$, and 32 for the 26, 26 ± 3, 29, and 29 ± 3 °C treatments, respectively.)
also due to SVL ($R^2 = 0.05$, $F_{[1,104]} = 10.45$, $P = 0.002$) and mass ($R^2 = 0.02$, $F_{[1,104]} = 9.99$, $P = 0.002$), and only marginally to tail length ($R^2 = 0.009$, $F_{[1,104]} = 3.28$, $P = 0.07$). Hatchlings incubated at constant temperatures were longer and heavier than those incubated at fluctuating temperatures (Fig. 3). The morphological difference between the sexes was due to tail length ($R^2 = 0.32$, $F_{[1,104]} = 110.06$, $P < 0.001$), with males having longer tails than females.

**Performance**

In the full model, both temperature mean (Wilks’ $\lambda = 0.07$, $F_{[3,564.13]} = 4.82$, $P = 0.003$) and temperature variance (Wilks’ $\lambda = 0.35$, $F_{[3,564.13]} = 22.09$, $P < 0.001$) affected performance. Temperature tested (Wilks’ $\lambda = 0.03$, $F_{[3,564.13]} = 1.96$, $P = 0.12$), sex (Wilks’ $\lambda = 0.007$, $F_{[3,564.13]} = 0.41$, $P = 0.74$), the interaction between temperature mean and temperature tested (Wilks’ $\lambda = 0.006$, $F_{[3,564.13]} = 0.40$, $P = 0.75$), and the interaction between temperature mean and sex (Wilks’ $\lambda = 0.004$, $F_{[3,564.13]} = 0.26$, $P = 0.85$) were not significant. Because there was a marginally significant interaction between temperature mean and variance (Wilks’ $\lambda = 0.04$, $F_{[3,564.13]} = 2.49$, $P = 0.06$), however, the main effects of each could not be interpreted meaningfully. The data were separated by mean temperature, and then by temperature variance, and analyzed separately.

At a mean incubation temperature of 26°C, temperature variance (Wilks’ $\lambda = 0.62$, $F_{[3,225.41]} = 15.60$, $P < 0.001$) affected performance. To determine the relative contribution of righting time, swimming speed, and muscular strength to the multivariate effect, separate univariate ANOVAs were conducted. The significant effect of temperature variance was due to swimming speed, muscular strength, and marginally to righting time (Table 1).

At a mean incubation temperature of 29°C, temperature variance (Wilks’ $\lambda = 0.40$, $F_{[3,225.38]} = 11.24$, $P < 0.001$) affected performance. The significant multivariate effect of temperature variance was due to swimming speed and muscular strength but not to righting time (Table 1).

Overall, when incubated at 26°C, hatchlings righted themselves faster when incubated at fluctuating temperatures, but they were stronger and swim faster when incubated at constant temperatures. At 29°C, hatchlings swim faster when incubated at constant temperatures, but they were stronger when incubated at fluctuating temperatures (Fig. 4).

Temperature mean (Wilks’ $\lambda = 0.17$, $F_{[3,246.28]} = 4.52$, $P = 0.006$) affected performance in constant incubation treatments. All three measures of performance contributed to the multivariate significance (Table 1). Temperature mean (Wilks’ $\lambda = 0.07$, $F_{[3,243.37]} = 1.82$, $P = 0.15$) did not affect performance in fluctuating incubation treatments.

Overall, when incubated at constant incubation temperatures, hatchlings swim and righted themselves faster when incubated at 29°C, but they were stronger at 26°C.

**Discussion**

Our results show that both the mean and the variance of the incubation temperature experienced by black ratsnake embryos affect fitness-related phenotypic traits of hatchlings. Specifically, the results suggest that high and constant
incubation temperatures improve the likelihood of survival of hatchlings. Because high and constant temperatures are characteristic of the communal nests preferred by female black ratsnakes (Blouin-Demers and Weatherhead 2000), the results are consistent with the hypothesis that females select the thermal incubation environment (in terms of temperature mean and variance) that produce the most favourable offspring phenotypes (Shine 1995).

First, the incubation period of black ratsnake eggs was significantly shorter at higher and more constant temperatures. Early hatching in temperate species minimizes the risk of embryo mortality owing to freezing, maximizes the chances of finding a suitable hibernaculum before the onset of winter, and gives neonates a head-start on growth (Webb et al. 2001). Precocious hatchlings would be at an advantage in our population because hatching occurs in September, a few weeks prior to the first frost.

Second, higher temperatures produced longer (but lighter) hatchlings, whereas constant temperatures produced longer and heavier hatchlings than fluctuating temperatures. Tail length, however, was unaffected by temperature mean or variance. Large body size has been found to improve survival in reptiles (e.g., Fox 1975; Ferguson and Fox 1984; Jayne and Bennett 1990; Brown and Shine 2005). Larger hatchlings exhibit enhanced locomotory performance, more successful handling of prey items (Booth et al. 2004), and are more likely to survive hibernation (Kissner and Weatherhead 2005). Hatchlings incubated at constant temperatures were heavier than those incubated at fluctuating temperatures. A greater mass may indicate more residual yolk and, therefore, greater energy reserves (Booth 2006). However, mass is a poorer predictor of survival in neonate snakes than SVL (Jayne and Bennett 1990; Brown and Shine 2005). Longer tails enhance locomotion in some snakes (Shine and Shetty 2001), but even tail loss may not compromise survival in others (Aubret et al. 2005). Moreover, the difference in tail length between males and females was much greater than the difference among incubation treatments, suggesting that the effects of incubation-induced tail-length differences on survival may be negligible.

Third, hatchlings incubated at high mean temperatures swam and righted themselves faster than those incubated at low mean temperatures. The effect of temperature variance, however, was dependent on the mean temperature. At 29 °C, hatchlings swam faster, righted themselves equally fast, and had lower muscular strength when incubated at constant temperatures. At 26 °C, hatchlings also swam faster, but they were also stronger and righted themselves more slowly when incubated at constant temperatures. Locomotor performance has been found to be a good predictor of early survival in garter snakes (Jayne and Bennett 1990). Although muscular strength has been assumed to affect the fitness of snakes because of its putative link to constriction of prey (Shine and Brown 2002; Brown and Shine 2004), we are unaware of empirical data linking strength and fitness in snakes. Interestingly, for muscular strength and righting time, fluctuating temperatures seemed to equalize the performance of hatchlings incubated at different mean temperatures: the performance difference between hatchlings incubated at mean temperatures of 26 and 29 °C was much greater when temperature was constant than when tempera-

![Fig. 4](https://example.com/figure4.png)
ture fluctuated. Thermal variance thus appears to promote performance uniformity. Hatchlings in the 26 and 29 °C fluctuating temperature treatments experienced considerable overlap in incubation temperatures (Fig. 1B). This overlap in incubation temperatures may be responsible for the similarity in their performance. Temperature fluctuations have been found to increase morphological uniformity in smooth softshell turtles, *Apalone mutica* (Lesueur, 1827) (Ashmore and Janzen 2003).

Finally, defensive behaviour was affected by temperature mean but not by temperature variance. Hatchlings incubated at high temperatures were less reliant on aggressive defence than those incubated at lower temperatures. Aggressiveness is presumed to be detrimental to neonate survival because it is a fallback strategy adopted chiefly when unable to flee from danger (Hertz et al. 1982). Because neonates from low incubation temperatures are slower, they have to be more aggressive.

In previous studies, the effects of temperature variance during incubation have been mixed. Temperature variance has been found to have no effect on hatching success (Ashmore and Janzen 2003; Mullins and Janzen 2006). Body size tends to decrease with thermal variance (Webb et al. 2001; Webb et al. 2006; Du and Ji 2006), while mass increases (Webb et al. 2001). Incubation period at fluctuating incubation temperatures has been found to be longer (Webb et al. 2006) or equal (Webb et al. 2001) to that at constant temperatures, or to be minimized at mean variance (Ashmore and Janzen 2003). Temperature fluctuations during incubation have also been found both to increase (Ashmore and Janzen 2003; Du and Ji 2006) and to have no effect (Mullins and Janzen 2006; Demuth 2001) on locomotory performance. Clearly, the effects of incubation temperature variance on hatchling phenotype and performance are less ubiquitous than the effects of mean incubation temperature.

In our experiment, the effects of temperature variance during incubation on ratsnake hatchling phenotype and performance are also mixed. Although constant incubation temperatures provided no clear benefit for hatching success, defensive behaviour, righting time, or muscular strength, constant incubation temperatures did shorten incubation period and produced longer, faster hatchlings. There are more empirical data linking hatchling fitness to size, speed, and hatchling date than linking hatchling fitness to strength, righting time, and defensive behaviour. Therefore, there is support for the idea that female black ratsnakes prefer to lay eggs in communal nests because these have higher, more constant temperatures than single-female nests. We found that mean incubation temperature, however, had stronger phenotypic effects than incubation temperature variance. To assess further the role of thermal variance in nest-site selection by female ratsnakes, it would be useful to determine experimentally whether females prefer to lay their eggs in substrates at constant temperatures rather than in substrates with fluctuating temperatures.

**Acknowledgements**

Funding for this study was provided by grants from the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, the Ontario Innovation Trust, and Parks Canada to G.B.D., as well as an Undergraduate Summer Research Award from the Natural Sciences and Engineering Research Council of Canada to L.D.P.

**References**


Jayne, B.C., and Bennett, A.F. 1990. Selection on locomotor per-


