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Incubation temperature modulates post-hatching thermoregulatory behavior in the Madagascar ground gecko, *Paroedura pictus*

Mark S. Blumberg*, Sean J. Lewis and Greta Sokoloff†

Program in Behavioral and Cognitive Neuroscience, Department of Psychology, University of Iowa, Iowa City, IA 52242, USA

*Author for correspondence (e-mail: mark-blumberg@uiowa.edu)
†Present address: Department of Psychology, Indiana University, Bloomington, IN 47405, USA

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Summary

All vertebrates regulate body temperature within narrow limits, regardless of their physiological capabilities. When do these limits develop, and can they be modified by manipulations of the developmental thermal environment? We addressed these questions by incubating the eggs of the Madagascar ground gecko, *Paroedura pictus*, at three temperatures and by assessing thermoregulatory behavior in hatchlings. Thermoregulatory behavior was assessed using a two-choice shuttle paradigm, and skin temperatures were measured non-invasively using infrared thermography. The shuttling behavior of hatchlings was systematically affected by the temperature at which they were incubated, and follow-up tests suggested that this effect persisted for at least three weeks post-hatching. The body temperature data from the shuttling experiment were used to model thermoregulatory behavior in a complex thermal environment; the model predicted systematic effects of incubation temperature on thermal preference. The specificity of the alteration in thermoregulatory behavior by incubation temperature is compelling and provides evidence for powerful pre-hatching influences on a fundamental, life-sustaining behavioral process.

Key words: thermoregulation, Madagascar ground gecko, *Paroedura pictus*, incubation temperature.

Introduction

Animals inhabit and thrive in an extraordinary range of terrestrial and aquatic thermal environments; from Arctic polar bears and Antarctic fish to the many organisms that have adapted to life around superheated deep-ocean thermal vents (Blumberg, 2002). The adaptation of species to such a diversity of thermal environments has required the alteration and coordination of many enzymatic systems. Yet, other processes that contribute to these remarkable adaptations have essentially been ignored. The possible contribution of developmental processes to the setting of thermal regulatory ranges is one such example. Therefore, in the present experiment, we manipulate the thermal environment of an egg-laying reptilian species during incubation and assess the thermoregulatory behavior of hatchlings. We show that incubation temperature plays a significant role in shaping the thermoregulatory behavior of hatchlings.

The use of behavior to maintain thermal homeostasis is a vital thermoregulatory component in all animals, regardless of their physiological capabilities (Satinoff, 1978). Many reptiles, including lizards, regularly shuttle between sun and shade (or other warm and cool microenvironments) as a means of regulating body temperature within a relatively narrow range (Heath, 1970). The body temperatures that trigger heat-seeking and heat-avoiding behaviors form lower and upper thresholds that define the range of body temperatures within which these ectotherms can tend to their non-thermoregulatory needs (Barber and Crawford, 1977). Interestingly, the factors that establish these thresholds have yet to be identified.

Temperature is a critically important factor during development (Satinoff, 1991). In reptiles, eggs must be incubated within a narrow range of temperatures (approx. 10°C) to remain viable (Deeming and Ferguson, 1991a). Within this range of viability, however, it is known that the thermal environment modulates a variety of anatomical, physiological and behavioral characteristics, including sex, growth rate, size, pigmentation, anti-predator behavior and running speed (Burger, 1998a; Crews et al., 1998; Deeming and Ferguson, 1991a; Gutzke and Crews, 1988) Only three studies, however, have examined the influence of incubation temperature on thermoregulatory behavior in young reptiles (Lang, 1987; O'Steen, 1998; Rhen and Lang, 1999a), and only one of these examined the behavior of hatchlings. In the study of Lang (1987), Siamese crocodile (*Crocodylus siamensis*) eggs from a single clutch were incubated either at 32.5–33.5°C or at 27.5–28°C, and subjects were then raised on thermal gradients. (A thermal gradient is a surface that is heated at one end and cooled at the other, thus establishing a continuous distribution of temperatures.) Because crocodiles exhibit

temperature-dependent sex determination (TSD), only males were produced at the high incubation temperature and only females were produced at the low incubation temperature. Of the hatchlings, Lang assessed the thermal preference of four males and two females on the thermal gradients. His results suggested that the hatchlings incubated at the high temperature (i.e. males) preferred warmer temperatures than the hatchlings incubated at the low temperature (i.e. females). Moreover, this apparent difference in thermal preference persisted through at least 60 days post-hatching.

Although Lang's results are intriguing, the use of a small number of subjects from a single clutch of eggs of a TSD species presents obvious interpretational difficulties. Nonetheless, despite the methodological problems with Lang's experiment, Deeming and Ferguson (1991b) remarked that his experiment "may indicate that differences in preferred body temperatures between individuals, and between species… are not solely genetic traits but may be physiologically acquired traits established during incubation… These experiments need repeating on a larger scale with a full range of incubation temperatures, including those that produce both males and females" (pp. 162–163).

The present experiment is in part a response to Deeming and Ferguson's call for a more thorough and systematic investigation of the effect of incubation temperature on the establishment of thermal regulatory ranges. The initial step was to identify a reptilian species that satisfied a number of criteria that allow us to avoid the methodological shortcomings of Lang's experiment. Based on these criteria, we chose the Madagascar ground gecko (*Paroedura pictus*), a nocturnal species that exhibits genetic sex determination (GSD; L. Talent and B. E. Viets, unpublished data). *P. pictus* is, as its name suggests, a ground-dwelling species that inhabits the dry forests, savannas and semi-desert areas of southern Madagascar (Henkel and Schmidt, 1995). Moreover, it breeds easily and rapidly in captivity, with females producing a clutch of two eggs every 3–4 weeks. Importantly, the embryo tolerates a wide range of incubation temperatures (22–32°C). In addition, because hatchlings weigh less than 1 g and, consequently, have little thermal inertia, infrared thermography (IR thermography) can be used to measure dorsal skin temperature noninvasively and thereby provide a reliable estimate of core body temperature.

Materials and methods

Subjects

Adult Madagascar ground geckos (*Paroedura pictus*) (L.) were obtained from a commercial breeder (Glades Herp, Inc., Fort Myers, FL, USA), and a breeding colony of 10 females and five males was established. A pair of females and a male were housed in an approximately 38 l aquarium equipped with a source of heat and hide boxes. Each aquarium was checked twice daily for the presence of eggs. Once laid, each egg was transferred to an incubator inside a small plastic container filled with moistened vermiculite; eggs were misted with water three times a week to maintain adequate humidity. If multiple eggs were found in the same aquarium, they were always placed in different incubators. The adult geckos were fed crickets, coated with a calcium and vitamin D dietary supplement, three times a week, and water was available *ad libitum*. Geckos were maintained on a 12 h:12 h L:D cycle (lights on at 06.00 h).

Procedure

Once laid, each egg was transferred to one of three incubators at temperatures of 22–24°C (hereafter designated as 23°C), 26°C or 30°C. The eggs remained in the incubators undisturbed until hatching. Of the 67 hatchlings for which data are reported here, 78% were tested on the night after hatching, 13% on the second night, and 9% on the third night (subjects not tested on the first night post-hatching were distributed evenly across conditions). Hatchlings remained in the incubator until testing and were not fed until after the first test was completed.

The test began by placing the hatchling on the shuttle apparatus at 18.00 h. This apparatus consisted of a ceramic surface, comprised of Peltier diodes, enclosed by a Plexiglas cylinder (radius=6 cm); the temperature of each 4 cm × 4 cm Peltier diode was manipulated using a custom-designed computerized system that allows for accurate and stable delivery of current. The temperature of one half of the surface within the cylinder was maintained at 41°C, while the temperature of the other half was maintained at 16°C. These temperatures are above and below the range of body temperatures tolerated by other nocturnal lizards and are similar to those used in other shuttle experiments (Hammel et al., 1967; Templeton, 1970).

The IR thermography system consists of a thermoelectrically cooled scanner, computer interface hardware, and acquisition and analysis software (FLIR Systems, Portland, OR, USA). To accurately measure absolute skin and diode temperatures using IR thermography, it was first necessary to measure the emissivity of the skin. (Emissivity is the ratio of the radiant energy emitted by a surface to the energy emitted at the same temperature by a black body radiator.) To accomplish this, the skin of hatchlings was heated to at least 40°C, and an emissivity value was obtained. Across a range of skin temperatures, values acquired using IR thermography were compared with those acquired using a reference thermocouple attached to the skin. Finally, average emissivity values were obtained and a regression equation was derived with the thermocouple temperature as the independent variable and the IR temperature as the dependent variable. The equation was then used to adjust the dorsal skin temperature values obtained using IR thermography. The same process was used for measurement of diode surface temperature.

Finally, the IR system was programmed to record an image to disk every five seconds beginning at 21.00 h and ending 6 h later at 03.00 h; thus, data were recorded exactly midway through the lights-off period. The following morning, the animal was removed from the apparatus, weighed, and body length (from tip of snout to tip of tail) was measured.

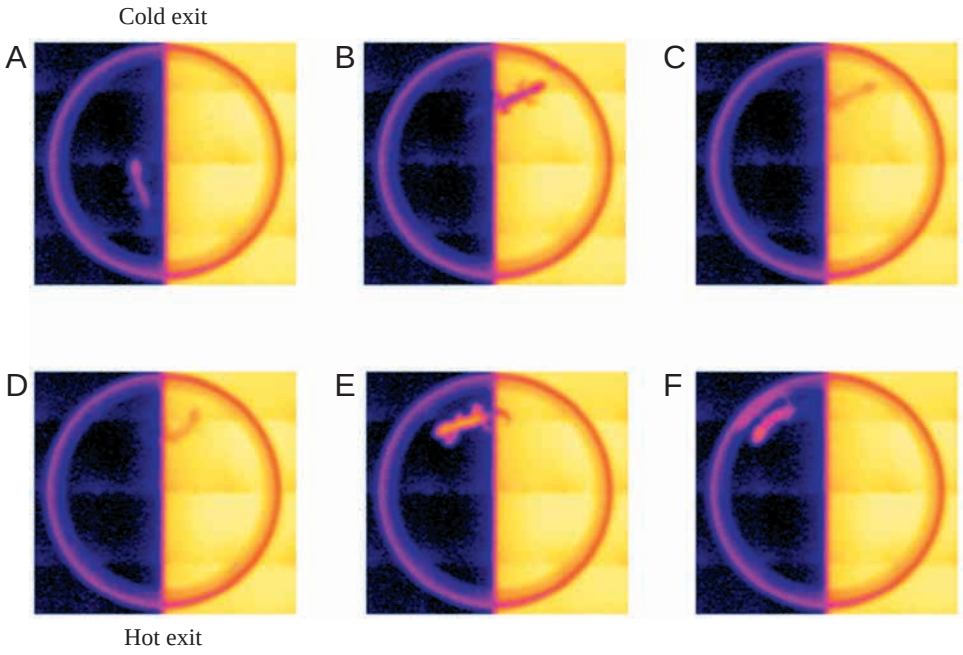

Fig. 1. Infrared thermographs of a newly hatched Madagascar ground gecko *Paroedura pictus* exhibiting shuttling behavior. The subject is confined to a Plexiglas cylinder and chooses between a surface temperature of 16°C on the left (blue surface) and 41°C on the right (yellow surface). In this 2-min sequence, the hatchling begins on the cold side of the apparatus (A). In the next frame (B), it has crossed over to the hot side where it remains stationary and gains heat from the hot floor (C). Eventually, it begins to move again (D) and crosses back over to the cold side of the apparatus (E) where it gradually loses heat to the cold surface (F). As indicated, the labels 'cold exit' and 'hot exit' denote the frames preceding a crossover to the hot and cold side of the apparatus, respectively.

Data analysis

Data were analyzed for each run by reviewing all 4320 images and determining when crossovers occurred. A 'crossover' was defined as the movement of three-quarters of the subject's body (defined as the region from the snout to pelvic girdle) across the dividing line between the hot and cold regions within a 30-s period. (The use of a very conservative definition of crossover excluded many crossing events but was necessary to standardize the measurement procedure across subjects and experimental conditions.) The time of a 'cold exit' was defined as the last image in which the hatchling was located on the cold side of the apparatus before a crossover began, and the time of a 'hot exit' was defined as the last image in which the hatchling was located on the hot side of the apparatus before a crossover began (Fig. 1). Then, using the data analysis functions of the IR system, the temperature in the mid-back region of a hatchling was measured for each cold and hot exit. The mean and standard deviation of these values were calculated for each subject and used for subsequent analyses. For each subject, mean exit temperatures were excluded from the analyses when they were derived from fewer than eight crossovers; eight hot exit temperatures and five cold exit temperatures, evenly distributed across experimental conditions, were excluded for this reason. In addition, for each incubation temperature, individual values that exceeded the mean ± 1.96 S.D. were excluded as statistical outliers; for the analysis of first-night data, only two cold-exit data points and three hot-exit data points were excluded as outliers.

In order to extrapolate from the two-choice shuttle data to the behavior of hatchlings in a more complex thermal environment, a dual-limit stochastic model (Barber and Crawford, 1977) was implemented using Mathematica (Version 4.0, Wolfram Research, Inc., Champaign, IL, USA). This model assumes the presence of upper and lower threshold detectors with stochastic response characteristics defined by a mean and standard deviation and also uses these response characteristics to predict how an animal would behave in an environment where many thermal choices are available (e.g. a thermal gradient). Thus, this model can provide an estimate of an animal's 'thermal preference' (Fraenkel and Gunn, 1961).

Results

As expected, incubation temperature had a profound impact on the growth and development of the geckos (Fig. 2). With increasing incubation temperature, incubation time decreased substantially ($F_{2,64}$=265.4, P<0.0001). In addition, both body length ($F_{2,64}$=8.0, P<0.001) and body mass ($F_{2,64}$=6.9, P<0.002) increased as incubation temperature increased. It is clear from the proportional changes in incubation time and hatchling body length and body mass that the growth rate of the embryos was accelerated at the higher incubation temperatures, which is consistent with previous research on other reptiles (Deeming and Ferguson, 1991a). Thus, these results confirm the efficacy of the independent variable, incubation temperature, in modulating at least some basic developmental processes in our subjects.

Although incubation temperature did not have a significant effect on hot exit temperature ($F_{2,53}$=1.4), its effect on cold exit temperature was highly significant ($F_{2,57}$=8.7, P<0.001; Fig. 3). *Post-hoc* analyses revealed that each step-wise increase in incubation temperature resulted in a significant increase in cold exit temperature (P<0.05), from an average of 23.9±0.3°C at the lowest incubation temperature to an average of 25.6±0.3°C at the highest incubation temperature. These differences in thermoregulatory behavior cannot be accounted for by differences in overall activity, as there were no

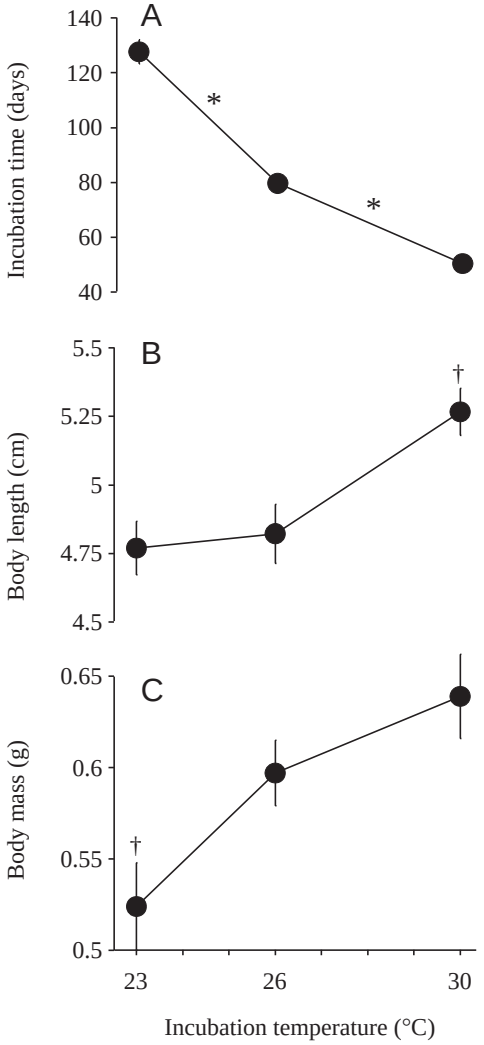

Fig. 2. The effect of incubation temperature on (A) incubation time, (B) body length and (C) body mass of newly hatched Madagascar ground geckos *Paroedura pictus*. Body length and body mass were measured within two days of hatching. For all measurements, the number of subjects per group was 20 (at 23°C), 23 (at 26°C) and 24 (at 30°C). *Significant difference between adjacent points. †Significantly different from the other two points.

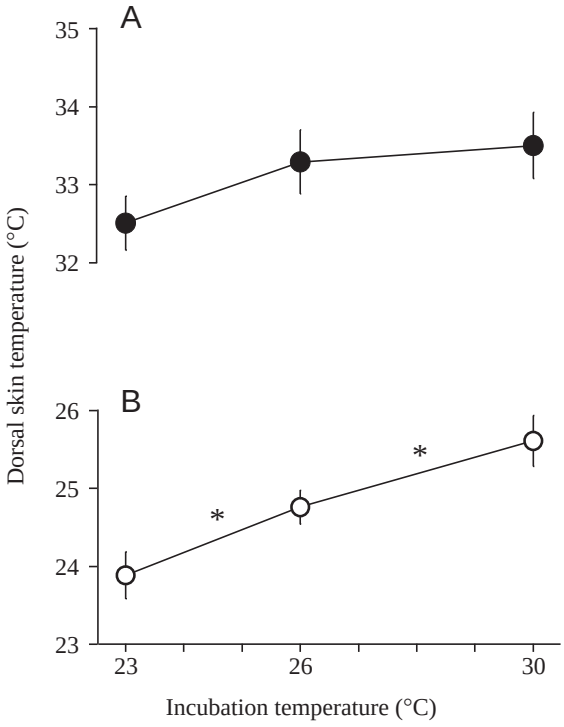

Fig. 3. The effect of incubation temperature on dorsal skin temperatures of newly hatched Madagascar ground geckos immediately prior to crossovers to the (A) cold (hot exit) and (B) hot (cold exit) sides of the apparatus. For cold exit temperatures, the number of subjects per group was 17 (at 23°C), 22 (at 26°C) and 21 (at 30°C); the respective numbers for hot exit temperatures were 15, 21, and 20. *Significant difference between adjacent points.

significant differences between groups in the number of crossovers performed during the 6 h tests (cold exit: $F_{2,64}$=1.2; hot exit: $F_{2,64}$=1.2).

Of the five breeding trios of two females and one male, four trios each contributed 10–12 eggs to the study and one trio contributed 22 eggs. For this last trio alone, incubation temperature had a significant effect on cold exit temperature ($F_{2,18}$=6.8, P<0.01), with cold exit temperature increasing from an average of 24.1±0.5°C at the lowest incubation temperature to an average of 26.5±0.6°C at the highest incubation temperature. Despite the relatively small number of subjects in the other trios, incubation temperature had a statistically significant effect on cold exit temperature for one of them ($F_{2,8}$=14.2, P<0.005), with cold exit temperature increasing from an average of 22.9±0.3°C at the lowest incubation temperature to an average of 26.0±0.5°C at the highest incubation temperature.

It is possible that body size or body length, both of which increased with increasing incubation temperature (see Fig. 2), mediated the effects of incubation temperature on our measures of thermoregulatory behavior. There was, however, no effect of these body size measures on cold exit temperatures. Specifically, neither body length (r^2=0.02, N=56) nor body weight (r^2=0.03, N=60) accounted for significant proportions of the variance in cold exit temperature.

To examine the stability of the effect of incubation temperature on thermoregulatory behavior, a subset of hatchlings from each condition was tested twice more, at 7–15 and 14–24 days post-hatching. These subjects were housed in aquaria similar to those used to house the adults. Most importantly, the aquaria were heated at one end, thus allowing hatchlings to thermoregulate behaviorally throughout the day and night between tests. Although the number of subjects tested more than once in the 23°C (N=5), 26°C (N=7) and 30°C (N=7) conditions is small (in part owing to mortality), the pattern observed in these follow-up tests is similar to that seen in Fig. 3. Specifically, for this subset of subjects incubated at 23°C and 30°C, mean cold exit temperatures were,

respectively, 24.1±0.5°C and 24.8±0.7°C on the first test night, 24.1±0.3°C and 25.0±0.4°C on the second test night, and 23.8±0.5°C and 25.3±1.2°C on the third test night. The consistency of this finding is particularly surprising given the extended acclimation period outside of the incubator and the lack of experimental control over the time when feeding last occurred (Lang, 1987). Thus, these data provide preliminary but suggestive evidence that the differences in thermoregulatory behavior induced by differences in incubation temperature remain stable beyond the first few days post-hatching.

The shuttling behavior of lizards has been modeled as comprising upper and lower thresholds that govern the timing of crossovers during shuttling behavior (Barber and Crawford, 1977). In addition, these thresholds are stochastic rather than absolute, exhibiting normal frequency distributions with characteristic means and standard deviations. When these threshold distributions are sufficiently non-overlapping and the body temperature of the lizard lies between the two thresholds, the model predicts that the lizard's behavior will be largely non-thermoregulatory, thus freeing the animal to engage in other behaviors. The shuttle apparatus compels a choice between hot and cold temperatures (unless the animal straddles the two temperature zones, as occasionally happens), thereby forcing the body temperature of the subject beyond each threshold and allowing the experimenter to collect statistically meaningful threshold temperature data.

To justify the assumption of normality, the six frequency distributions (cold exit and hot exit distributions at each of the three incubation temperatures) were tested using the Kolmogorov–Smirnov normality test. Although one of the six distributions deviated significantly from normality (hot exit, 23°C: χ^2=10.5, d.f.=2, N=641, P=0.01), the remaining five distributions did not (1.2<χ^2<5.6, d.f.=2, 410<N<736, P>0.10).

Thus, from the present data, the means and standard deviations of cold and hot exit temperatures were entered into the stochastic model. First, as expected, the frequency distributions of the cold exit temperatures exhibit an orderly progression with increasing incubation temperature (Fig. 4A); the hot exit temperatures also exhibit an orderly progression although, as described above, this effect was not significant. Next, the model uses the threshold information provided by the two-choice temperature selection experiment employed here to predict the behavior of animals on a continuous thermal gradient. Specifically, as shown in Fig. 4B, the curves for 'heating transitions' indicate the probability that a hatchling with a specific dorsal skin temperature will move toward a hotter region of the environment; similarly, the curves for 'cooling transitions' indicate the probability that a hatchling with a specific dorsal skin temperature will move toward a cooler region of the environment. These two curves intersect at the point where a hatchling is equally likely to move toward hot or cold. As shown in the insert in Fig. 4, this point of intersection, which can be conceptualized as the 'temperature preferendum', increases systematically with incubation temperature.

Discussion

The aim of this experiment was to investigate the influence of incubation temperature on post-hatching thermoregulatory behavior using a reptilian species that breeds easily in captivity and that could be readily tested using our experimental procedure. The results clearly and reliably demonstrate for the first time in a GSD reptile a direct effect of incubation temperature on thermoregulatory behavior that is present at hatching and that may persist through at least the first three weeks post-hatching. The use of a GSD species is particularly significant because, with only a few exceptions, investigators have focused on epigenetic processes in reptilian species that exhibit TSD; in turn, this focus has fostered the view that epigenetic processes are perhaps most salient in TSD species. In contrast, incubation temperature has been shown to influence a variety of post-hatching behaviors in the pine snake (*Pituophis melanoleucus*), a GSD species (Burger, 1998a,b; Burger and Zappalorti, 1988). Thus, the present results provide additional support for considering the importance of the developmental thermal environment in GSD reptiles and open up a wide range of important questions concerning the development and evolution of homeostatic systems in a variety of vertebrate species.

There are several methodological features of this experiment that deserve some comment. First, by testing hatchlings, it was expected that the assessment of cold and hot exit temperatures would be relatively uncontaminated by possible effects of post-hatching thermal acclimation and other possible influences of the rearing environment. This is not to say, however, that this focus on hatchlings could not have introduced other issues that may have a bearing on the present results, including the differential effects of incubation temperature on the size and hormonal composition of the yolk (Deeming and Ferguson, 1989; Rhen and Lang, 1999b).

Second, the use of IR thermography was significant for providing an accurate measure of thermoregulatory behavior and its consequences without the need to use probes that can interfere with behavioral expression. Although direct and simultaneous measures of core temperatures would perhaps have been ideal, we chose a species that is particularly small at hatching to minimize differences between core and skin temperatures. Specifically, given their small size (<1 g) and low thermal inertia, it is reasonable to assume that our IR measurements provided reliable estimates of core body temperature, even during rapid changes in temperature. This assumption was borne out by measuring changes in cloacal temperature (using a thermocouple) and skin temperature (using IR thermography) in a dead hatchling during a series of cooling tests. As expected, IR thermography recorded changes in dorsal skin temperature that were at least as rapid as those recorded using the thermocouple.

Given that *P. pictus* is classified as a nocturnal species, one might wonder whether the two thermal choices used in the shuttle apparatus (i.e. 16°C and 41°C) were appropriate. First, our subjects exhibited systematic shuttling between the two sides of the apparatus and rarely indicated through their behavior that the

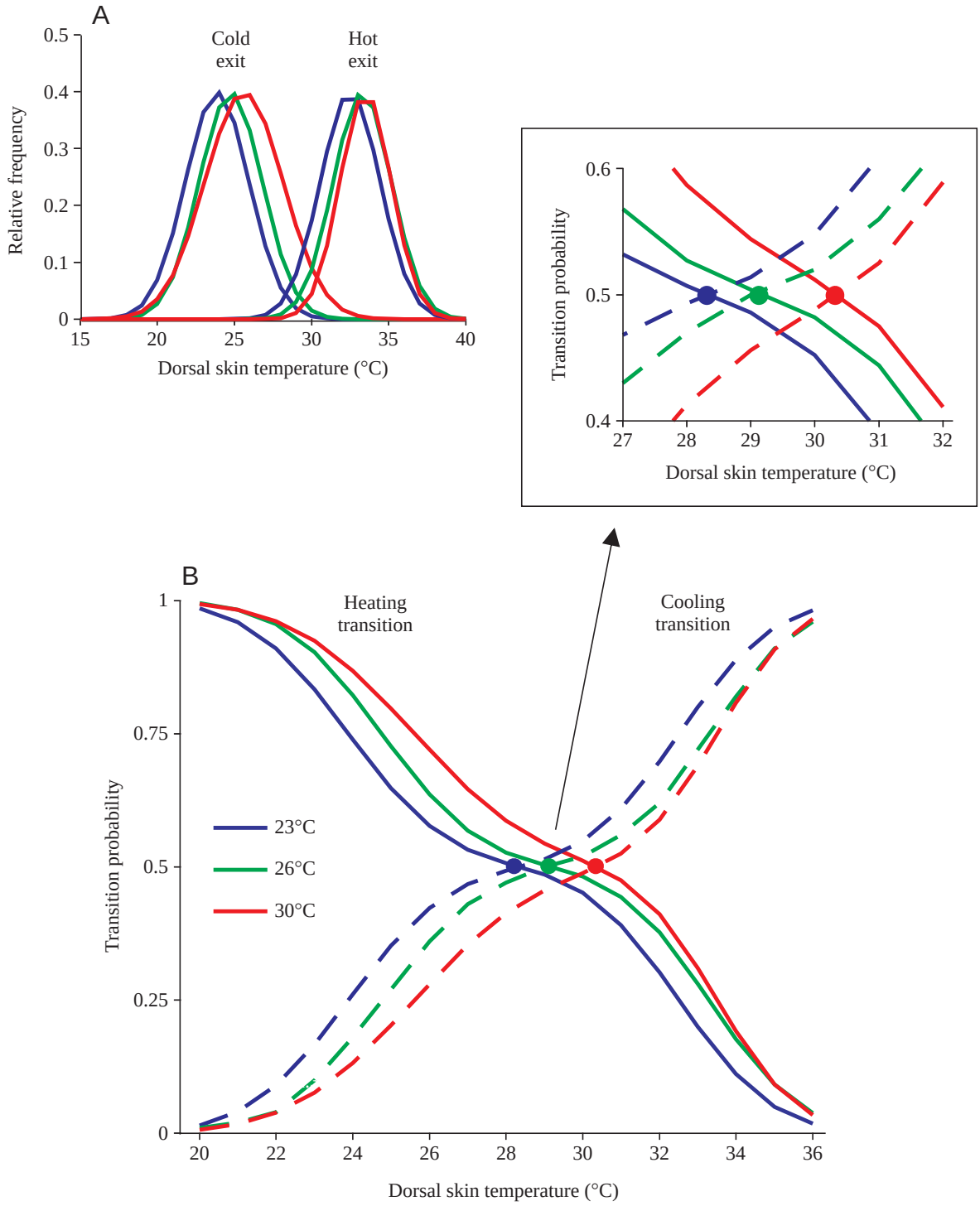

Fig. 4. (A) Frequency distributions of dorsal skin temperatures for cold and hot exit temperatures based on the means and standard deviations averaged across hatchlings. Distributions are presented separately for hatchlings incubated at 23°C (blue), 26°C (green) and 30°C (red). (B) Transition probabilities calculated using the dual-limit stochastic model of Barber and Crawford (1977). The heating transition curves (solid lines) indicate the probability that a hatchling, behaving in a complex thermal environment, would move toward heat; the cooling transition curves (broken lines) indicate the probability that a hatchling would move away from heat. The point of intersection of the two curves indicates an equal likelihood of moving toward or away from heat. As shown most clearly in the insert, the dorsal skin temperature at which this equilibrium point occurs increases systematically with incubation temperature.

two surfaces were either too hot or too cold. Second, although we have no information on the natural thermal microenvironment of *P. pictus*, the body temperatures of geckos in general, and at least two nocturnal reptiles (the night lizard *Klauberina riversiana* and the shovel-nosed snake *Chionactis occipitalis*), range from the mid-teens to the mid-thirties (Brattstrom, 1965). Finally, it should be stressed that the classification of a reptile as nocturnal can foster the mistaken impression that thermoregulatory shuttling is a less important feature of its daily activity. Indeed, some geckos and lizards that have been classified as nocturnal have nonetheless been observed basking in direct sunlight (Brattstrom, 1965; Templeton, 1970).

We chose to model the behavior of hatchlings to predict thermal gradient behavior rather than simply measure thermal gradient behavior directly. We made this choice because the behavior of a reptile on a thermal gradient is shaped by its upper and lower thresholds and that, between these thresholds, behavior is highly variable and probabilistic. As a result, many days of observation are required to gather reliable data using a thermal gradient (Barber and Crawford, 1977). This requirement did not seem practical given (1) the age and fragility of our subjects and (2) that the primary goal in this experiment was to define the characteristics of the upper and lower thresholds of our subjects, a goal that is best accomplished using a shuttle paradigm.

The mechanism by which incubation temperature influences post-hatching thermoregulatory behavior is unknown. Incubation temperatures could influence the course of thermoregulatory development through a process of 'thermal imprinting'. Such imprinting may be irreversible, even after acclimation to different environments (Winkler, 1985). In addition, because shuttling behavior in lizards is modulated by a combination of brain, core and skin temperatures (Hammel et al., 1967), incubation temperature may exert its effects by altering the development of thermosensitive neurons. It is equally plausible, however, that incubation effects are mediated by differences in metabolic rate or a related variable (O'Steen and Janzen, 1999). A better understanding of the mechanisms that underlie this phenomenon will be one necessary step in understanding the ecological significance of variations in incubation temperature.

There have been remarkably few studies concerning the role of epigenetic processes in the development of homeostatic regulatory ranges, including those concerned with temperature regulation. For example, there is intriguing evidence that cultivation temperature shapes thermoregulatory behavior in the nematode *Caenorhabditis elegans* (Hedgecock and Russell, 1975; Mori and Ohshima, 1995), and that the developmental thermal environment irreversibly modifies thermoregulatory behavior in fish (Winkler, 1985). These findings on the development of thermoregulatory behavior in worms, fish and reptiles might prove to be of broader significance for the development of thermoregulatory processes in birds and mammals, including humans. Although one might suppose that genetic influences on thermoregulatory development would be paramount in homeothermic avian and mammalian species, there is little empirical basis for such a supposition. Indeed, it appears that incubation temperature can modify some aspects of post-hatching thermoregulation in an endotherm, the Muscovy duck *Cairina moschata* (Nichelmann and Tzschentke, 1997). Finally, it should also be stressed that such developmental effects are not likely to be restricted to the thermal domain; the regulatory ranges of other homeostatic systems may also be established early in development (Blumberg, 2001).

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