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Effects of Temperature and Moisture on Embryonic Diapause of the Veiled Chameleon (*Chamaeleo calyptratus*)

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ABSTRACT The development of lizard embryos is typically initiated at fertilization and continues until birth or hatching. In contrast, embryonic development of some chameleons is arrested at the gastrula stage, and embryos remain at this stage for months after the eggs are laid. Our research tested the hypothesis that increased temperature, moisture, or both, are associated with the resumption of development by diapausing embryos of *Chamaeleo calyptratus*, the veiled chameleon. After 40 days of incubation at 25°C in a relatively dry substrate, eggs were subjected to: 1) no change in temperature or moisture, 2) no change in temperature but change from a dry to a wet substrate, 3) change to a warmer temperature but no change in substrate moisture, or 4) an increase in both temperature and substrate moisture. Overall, embryos initiated development after 50–60 days to 80 or more days of incubation. Neither substrate moisture nor water uptake by eggs was related to the interval when development resumed. In contrast, development was initiated about 10 days earlier for eggs in the high temperature treatment compared to eggs in the low temperature treatment. Our results suggest that neither water availability nor water uptake by eggs affect the length of diapause but that an increase in ambient temperature initiates development of diapausing embryos of *C. calyptratus*. *J. Exp. Zool. 301A:629–635, 2004.*

INTRODUCTION

Development of squamate embryos is typically initiated at fertilization. Development occurs while eggs are in the oviduct and embryos are at limb bud stages at the time of oviposition (Andrews and Mathies, 2000). Development then continues until hatching. In contrast, the embryos of some chameleons are gastrulae at the time of oviposition and embryos remain at this stage for several months after oviposition. Such embryonic diapause ('arrested development when the immediate proximate environment would normally foster active development', Ewert, '91) has been documented by direct observations of embryos of a few species of chameleons (Bons and Bons, '60; Blanc, '70), and is inferred from the long incubation periods of other *Chamaeleo* and *Furcifer* species. Incubation periods of six months to a year or more are typical observations for these taxa (Ferguson, '94; Necas, '99), whereas incubation periods of two to three months are typical of lizards that lay eggs of similar mass to chameleon eggs (1–1.5 g) (Birchard and Marcellini, '96). In the laboratory, diapause occurs regardless of incubation conditions and is thus an obligate part of the life cycle. Nonetheless, diapause in *C. calyptratus* is broken spontaneously without any sort of environmental stimulation. This 'facultative' breaking of diapause contrasts with that of some species of turtles, for which an environmental cue is mandatory (Ewert, '91). The nature of the seasonal cycle and of specific environmental conditions are presumably the ultimate determinate of the length of diapause and the length of incubation. But do environmental factors affect when development is resumed?

Our study was designed to test the hypothesis that exogenous environmental factors affect the length of diapause of chameleon embryos. Our experimental subject was the veiled chameleon, *C. calyptratus*, native to Yemen. *C. calyptratus* is a good model species for this research because its reproductive biology is similar to other *Chamaeleo* that exhibit directly observed embryonic diapause or lengthy incubation periods suggesting embryonic diapause (Bons and Bons, '60; Minton, '66;

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Cuadrado and Loman, '99). Because embryos are in diapause in 'winter' when temperature and moisture are relatively low, we predicted that increased temperature or moisture or both would act as cues to initiate development.

MATERIALS AND METHODS

Source, initial incubation of eggs, and animal care approval

Seventeen eggs from each of four clutches were obtained from a commercial breeder (Davidson Dragons, Lexington, NC) on May 8, 2002. These clutches had been oviposited on April 29 and 30 and on May 4 (2 clutches). They were transferred to Andrews' laboratory on May 9 and eggs were weighed and numbered with a fine India Ink pen according to clutch and sequential number within each clutch. Eggs were placed in small plastic containers partially filled with moistened vermiculite (dry treatment, see below). Containers were placed in an environmental chamber at a mean temperature of 25°C.

The Virginia Tech Animal Care Committee approved the Research Protocol for this study in April 2001.

Experimental design and incubation protocols

The experiment was started 40 days after oviposition when sub-sets of eggs from each clutch were shifted to new combinations of temperature and moisture. We selected a 40 d pre-experimental period based on preliminary observations on two clutches of *C. calyptratus* that were incubated at a constant 28°C in the laboratory. Eggs from these two clutches were sampled at approximately monthly intervals to establish how long the embryos were in diapause. For these eggs, diapause lasted at least two months, but not more than three months, after oviposition.

Four eggs were allocated to each of four treatments from each clutch (total of 16 eggs per clutch). The treatments were: 1) a control, with no change in temperature or moisture (25°C/dry), 2) no change in temperature, but a change from a dry to a wet substrate (25°C/wet), 3) a change to a warmer temperature, but no change in moisture (28°C/dry), and 4) an increase in both temperature and moisture (28°C/wet). The *a priori* prediction was that development would be initiated earlier in at least one of treatments 2–4 than in the control where neither moisture nor temperature was altered.

Nest temperature in nature is not known for any chameleon species. Moreover, *C. calyptratus* is found in hot and dry habitats as well as in relatively cool and moist habitats (Schmidt, 2001). Therefore, temperature regimes were selected to fall within natural conditions for lizard eggs and that were also known to result in high survival of *C. calyptratus* eggs in captivity (Andrews et al., '99; Schmidt, 2001). Environmental chambers (Percival Scientific I-30BLL series) were programmed to fluctuate ± 3°C around means of 25 or 28°C. For the former, the daytime temperature was 28°C (8 h) and nighttime temperature was 22°C (8 h), and the temperature ramped linearly for four hours between these temperatures in the morning and the evening (mean = 25°C). For the latter, the daytime temperature was 31°C (8 h) and nighttime temperature was 25°C (8 h), and the temperature ramped linearly for four hours between these temperatures in the morning and the evening (mean = 28°C). Temperatures within the environmental chambers and within the egg containers were monitored hourly for 24 hours at roughly weekly intervals with Omega data loggers; chamber temperatures were adjusted if necessary to maintain targeted temperature regimes. Means recorded within the egg containers averaged 0.3°C higher than in the chambers themselves and actual mean incubation temperatures were 25.4 and 28.3°C.

Eggs were buried in vermiculite leaving roughly one-quarter of their top surface exposed so that they could be monitored without disturbance. Water potentials of the vermiculite were based on a standard curve established by vapor pressure psychometry that related water content to water potential (Andrews, unpublished data). Vermiculite in the dry treatment (50 g of water per 100 g of vermiculite) had an initial water potential of −280 kPa and vermiculite in the wet treatment (80 g of water per 100 g of vermiculite) had an initial water potential of −175 kPa. Vermiculite was changed monthly. Over the month, water potentials decreased as a result of evaporation from the egg containers and water uptake by eggs. In the dry and wet treatments, water potentials decreased to about −350 kPa and −200 kPa (averaging 40 and 68 g of water per 100 g of vermiculite), respectively. While water potential was thus not constant during the observations, the low moisture treatment was always drier than the high moisture treatment.

Eggs were weighed every 10 d during incubation to assess net water uptake. Reptile eggs take up water during development and the net increase in egg mass is largely a function of moisture availability, incubation temperature, and the metabolic rate of the embryo (Ackerman et al., '85; Packard and Packard, '88; Ackerman, '94). The increase in egg mass thus indicates net water uptake. Because, however, the mass of the egg also reflects its initial size, we used the mass of eggs at day 40 (when the experiment was initiated) as a covariate in statistical analyses.

Egg sampling protocols

One egg per clutch was sampled on day 40 to assess the embryonic stage of development when the experiment began. Eggs from each clutch-treatment combination were randomly assigned to be sampled on day 50, 60, 70, or 80. The mass of the egg was recorded and then the egg was opened and the embryo preserved in alcohol for later staging. Staging was conducted by RMA at the end of the experiment under conditions such that the clutch and treatment of embryos were not known. In a parallel experiment we determined that development among eggs within clutches is usually synchronized. Because we sampled only one egg per clutch per sampling period for the experiment reported in this paper, the stages reported could have some error but these errors would make it less rather than more likely to find significant differences among treatments.

Embryos were staged using figures in Dufaure and Hubert ('61) for *Zootoca (Lacerta) vivipara*. This staging table was compared with one for *Furcifer lateralis* (Blanc, '74). The two tables provide an identical sequence of stages (although not always the same stage numbers); the Dufaure and Hubert ('61) table was used because it is the most commonly used table for lizard embryos. In the Dufaure and Hubert ('61) scheme, embryonic development starts at stage 1 with the initiation of cell division (cleavage) and ends at stage 40 when hatching occurs. Cleavage occurs during stages 1–4, and the embryonic bud/shield is distinguished at stage 5. Stages 5–9 are associated with gastrulation. Stages 10–20 are associated with neurulation; organogenesis commences at stage 21, and limb buds are first seen at stage 27. Gastrulae were recorded as such. Embryos with stages of 10 or more were considered to have initiated development. The earliest neurula we observed (stage 17) had four somites and was clearly distinguished from gastrulae.

Statistical analyses

We conducted three statistical analyses. To assess the pattern of water uptake by eggs, we conducted a three-factor ANCOVA in which the dependent variable was egg mass, the class variables were temperature, moisture level, and day of sample (50, 60, 70, and 80 days), and the covariate was egg mass at day 40. Clutch was treated as a blocking factor because differences among females (joint effects of parental genotypes plus any additional contributions due to maternal effects) can contribute to variation in incubation length, hatchling size, etc. (Andrews et al., 2000). By considering it as part of the design, we account for variability in the data associated with differences among females, and thus avoid confounding treatment effects with maternal effects. The analysis thus involved 64 eggs (4 clutches, 4 treatments, 4 days). Dummy variables were used to account for the increase in slope of the regression between the dependant variable and the covariate through time.

To determine whether treatment affected the order in which embryos began to develop, we used a Friedman two-way ANOVA based on ranks (Siegel, '56) in which the ranks were the treatment order in which embryos initiated development within each clutch and the two factors were clutch and treatment. The first embryo to develop (or that exhibited the most advanced stage) was assigned a rank of one, and so forth. This analysis involved ranking 16 eggs (one egg per clutch per treatment).

We also determined if the actual amount of water taken up by eggs was related to the sample day when development was first noted. This analysis used the 16 eggs ranked in Table 1. For these 16 eggs, we conducted a four-factor ANCOVA in which the dependent variable was egg mass on day 70, the class variables were temperature, moisture level, clutch, and developmental group, and the covariate was egg mass on day 40. Eggs were assigned to one of two developmental groups: development initiated by day 70 or first observed on day 80 or later. Egg masses on day 70 were not available for two ranked eggs from clutch D because they were sampled on day 60 and their masses were thus not available for day 70. The mean mass of the two remaining eggs from the same clutch and treatment group on day 70 was

TABLE 1. Stages of C. calyptratus embryos from four temperature and moisture treatments during incubation[1]

	Treatments			
Clutch	25°C/dry 60, 70, 80 d	25°C/wet 60, 70, 80 d	28°C/dry 60, 70, 80 d	28°C/wet 60, 70, 80 d
A	G, G, G(4)	G, G, 17(3)	G, G, 20(2)	G, G, 25(1)
B	G, 20(2), 28	G, G, 25(4)	G, 18(3), 33	NF, 26(1), 28
C	G, G, 25(3)	G, G, 21(4)	G, G, 26(2)	G, 20(1), 28
D	G, 19(3), 31	NF, G, 23(4)	28(1), 30, 32	21(2), 31, 30

[1]All embryos from eggs sampled at 40 and 50 d were gastrulae. Numerical stages are not given for gastrulae (G) and for eggs that were not fertile (NF). Stages are given for embryos sampled at 60, 70, and 80 d, respectively, within each clutch/treatment combination. The rank order in which development was initiated among treatments within clutches is given in parentheses (1 = 1st to develop, etc.).

used instead. Egg mass on day 80 was not used as the dependent variable because the masses of five additional ranked eggs would not have been available because of previous sampling.

All parametric analyses were conducted with SAS software (SAS, '97). Least Squares Means procedures were used for *a posteriori* tests. Significance was at $P < 0.05$.

RESULTS

The mean mass of eggs in the four clutches ranged from 1.4 to 1.7 g (overall mean = 1.6 g) at the time of oviposition and from 1.9 to 2.3 g on day 40 (overall mean = 2.2 g). The amount of water uptake by eggs prior to the imposition of experimental conditions thus averaged 0.6 g. The mass of eggs continued to increase during the experimental period with both incubation temperature and moisture affecting mass (Fig. 1, Table 2). The significant temperature*water and temperature*-days interactions were associated with greater water uptake at high than low water at 28°C than at 25°C and with greater water uptake through time at 28°C than at 25°C, respectively. Overall, eggs took up more water when incubated at 28°C than at 25°C, and eggs incubated at 28°C took up more water at 80% water than at 50% water (P's < 0.001, least squares means comparisons). Eggs incubated at 25°C did not differ in water uptake at 80% and 50% water ($P = 0.12$).

When the experiment was initiated at 40 days of incubation, all embryos sampled were at the gastrula stage. At 50 days, embryos from all clutch and treatment combinations were still gastrulae.

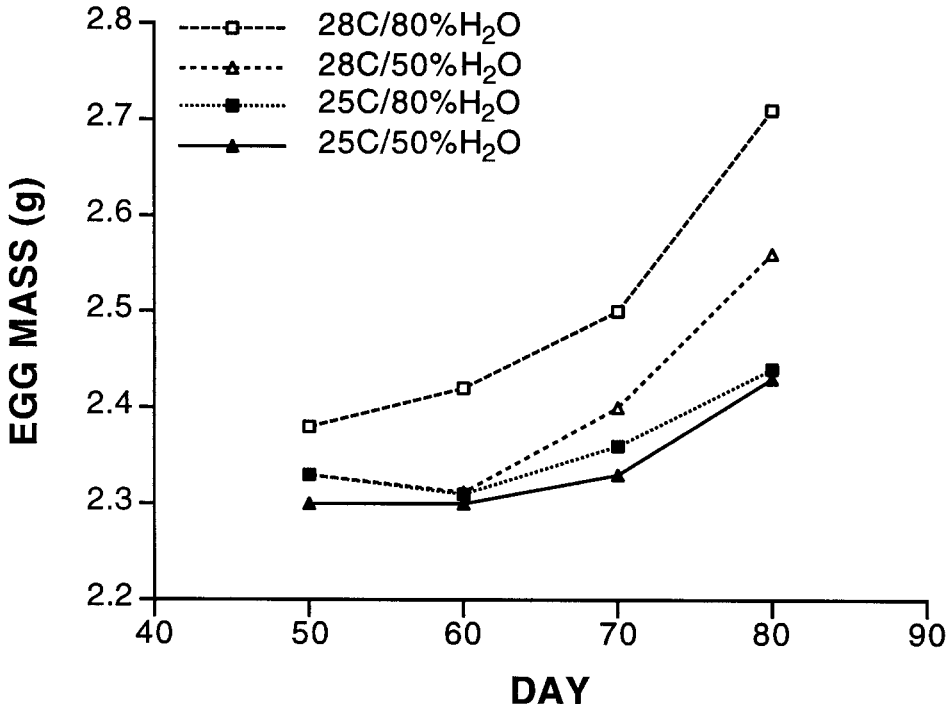

Fig. 1. Mean mass of eggs in the four treatments plotted as a function of treatment and day that eggs were sampled. Values have been adjusted for the mass of eggs on day 40 (least-squared means from ANCOVA's for each of the four days).

TABLE 2. F-ratios and P values for treatment effects (temperature, water, and day of sample, and their interactions) in analysis of covariance on the mass of eggs[1]

Source of variation	d.f.	F	*P*
Clutch	3,41	6.1	= 0.002
EG1–EG4	1,41	268–349	< 0.001
Temperature	1,41	118.9	< 0.001
Water	1,41	43.3	< 0.001
Day	3,41	1.2	= 0.314
Temperature * Day	3,41	12.5	< 0.001
Temperature * Water	1,41	18.5	= 0.001
Water * Day	3,41	0.7	= 0.536
Temperature * Water * Day	3,41	1.2	= 0.321

[1]The covariate was egg mass at day 40 (when experiments were initiated). EG1-EG4 are tests of the covariate on each of the four days that eggs were sampled; different slopes were fitted on each day because slopes increased through time.

By 60 days, two of the 16 embryos sampled had initiated development, and by 80 days, only one of the embryos sampled had not initiated development (Table 1).

Development was initiated non-randomly among treatments ($P = 0.014$, Friedman two-way ANOVA). The distribution of developmental stages among treatments indicates earlier initiation of development in the 28°C treatments than the 25°C treatments, regardless of moisture. Development was initiated first in the 28°/80% water treatment in clutches A, B, and C and first in the 28°/50% water in clutch D (Table 1). The first and second embryos within clutches to initiate development were in the two 28°C treatments in 7 of 8 cases. Moreover, the stage when development was first noted was similar in the 28°C and 25°C treatments, 22.6 and 21.4, respectively ($P >> 0.05$, Fisher Exact Test). Earlier development in the 28°C treatments thus reflects the time when development was initiated, not simply that the rate of development was greater in the 28°C than the 25°C treatments.

We also examined whether water uptake of the eggs ranked in Table 1 was related to when development was initiated. Mass (water uptake) on day 70 was related only to temperature ($F_{1,8} = 10.8$, $P = 0.011$). Moisture level, clutch, developmental group (development initiated by day 70 versus later than day 70), and the covariate (egg mass on day 40) did not contribute to the overall model ($F_{1,8} = 3.5$, $P = 0.10$; $F_{3,8} = 1.12$, $P = 0.40$; $F_{1,8} = 0.9$, $P = 0.37$; $F_{1,8} = 4.9$, $P = 0.06$, respectively).

DISCUSSION

Embryos were in diapause (i.e., recorded as gastrulae) at 40 and 50 days of incubation. Two embryos initiated development between 50 and 60 d and only one had not done so by 80 days. This means that development was initiated during a roughly one-month period. Given an incubation period of about 6 months for *C. calyptratus* eggs, embryos were in diapause for about one-third or more of the total incubation period. Because sampling was terminal, we could not determine if embryos that initiated development earlier also hatched earlier than embryos that initiated development later. While manipulations of temperature regimes affect the total length of incubation for *Furcifer pardalis* eggs (e.g., Ferguson, '94), the association between the length of incubation and the length of diapause has not been determined.

C. calyptratus eggs took up more water when incubated at 28°C than at 25°C, and eggs incubated at 28°C took up more water in the wet than the dry treatment. Greater net water uptake by reptile eggs under wet conditions than under dry conditions is typical (Packard and Packard, '88). Water uptake, however, is often greater at low than high temperatures (Gutzke and Packard, '87; Packard and Packard, '87, '88; Alberts et al., '97). This pattern, in part, is the result of the greater metabolic heat production by developing embryos at high than low temperature. As a consequence of temperature differentials between eggs and their substrate, the evaporation of water vapor from eggs is greater at a high than a low temperature. As a consequence, eggs incubated at a high temperature have a lower net water uptake than do eggs incubated at a low temperature (Ackerman et al., '85; Ackerman, '94). The difference in water uptake between eggs incubated at low relative to high temperature may be especially pronounced towards the latter part of the developmental period (Packard and Packard, '87) when embryos are the largest and have relatively high metabolic rates. In contrast, during the period that we observed eggs of *C. calyptratus*, embryos were at very early developmental stages; embryonic metabolism would thus have had a negligible to small effect on water balance of the eggs. Under these conditions, water balance would have been dominated by the difference between the water vapor pressure of the egg and the water vapor pressure of the medium (Ackerman, '94). Because this difference increases with temperature, net water uptake by eggs should have been, and was,

greater at the high experimental temperature than the low one.

We tested the hypothesis that increases in temperature or moisture or both are associated with the initiation of development by diapausing embryos of *C. calyptratus*. Our results do not support the hypothesis that moisture acts as a cue for the termination of diapause. Neither water availability in the substrate itself nor net water uptake by eggs was related to the time when development was initiated. Water uptake was higher in the 28°C/80% water treatment than the other treatments, although development was initiated at the same time in both the 28°C/80% water and the 28°C/50% water treatments. Moreover, contrasts of the water uptake of eggs in which embryos had initiated development by day 70 or earlier and eggs in which embryos had initiated development later than 70 d support the conclusion that water availability to embryos does not affect the time when development is initiated. While water uptake is necessary for the normal development of lizard eggs (Packard and Packard, '88), the actual amount of water taken up by *C. calyptratus* eggs was not related to the length of embryonic diapause. An increase in incubation temperature, however, did shorten the period of diapause. Eggs that were shifted to the two 28°C treatments initiated development about 10 days before eggs in the two 25°C treatments. Our results thus indicate that an increase in ambient temperature decreases the length of diapause of *C. calyptratus* embryos and that the amount of water uptake by eggs is not related to the length of diapause.

Embryonic diapause in chameleons has a restricted taxonomic distribution. Chameleons with known embryonic diapause or that have lengthy incubation periods are members of the subgenus *Chamaeleo* or the genus *Furcifer* (Necas, '99). Monophyly of these two taxa is supported by phylogenetic analyses of Raxworthy et al. (2002) and Townsend and Larson (2001). These two lineages thus represent independent radiations in Africa, the Indian subcontinent, and the Mediterranean region versus Madagascar, respectively (Raxworthy et al., 2002). Diapause may thus represent an ancestral trait for both lineages. But why does diapause characterize these groups of chameleons? Eggs of the temperate and subtropical *C. chamaeleon* and *C. zeylanicus* are laid in the fall and hatching occurs the following summer or fall; embryos are in diapause during winter months (Bons and Bons, '60; Minton, '66; Cuadrado and Loman, '99). Eggs of the tropical *F. lateralis* and *F. pardalis* are laid during the wet (warm) season and hatching does not occur until the following wet season; embryos are in diapause during the dry (cool) season (Schmidt, '86; Blanc, '70; Bourgat, '70). Diapause may thus reflect adaptation to climatic seasonality. Diapausing gastrulae may be better able to withstand cold or dry conditions than more developed embryos, or a delay in the initiation of development may ensure that hatching occurs at a time of year favorable for the growth and survival of neonates, or both.

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