Developmental Arrest during Embryonic Development of the Common Chameleon (*Chamaeleo chamaeleon*) in Spain

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ABSTRACT

Embryonic development of the common chameleon, *Chamaeleo chamaeleon*, was monitored from oviposition to hatching at a field site in southwestern Spain and in the laboratory under five experimental temperature regimes. Embryos were diapausing gastrulae at the time of oviposition; developmental arrest in the field continued as cold torpor during winter. Post-arrest development in the field commenced in April, and hatching occurred in August, for a total incubation period of 10.5 mo. In the laboratory, one group of eggs was incubated at a constant warm (26°C) temperature. The remaining treatments simulated field conditions and consisted of initial periods of warm temperature of 0, 27, 46, and 71 d, a subsequent 4-mo period of cold winter (16°C) temperature, and a final period of warm (26°C) temperature. Embryos in the constant warm temperature treatment were in diapause an average of 3 mo, with clutch means ranging from 2 to 4 mo. Hatching among clutches occurred over 2 mo. In contrast, for field and experimental eggs that experienced cold winter conditions, hatching within treatments occurred over 2–14 d; "winter" conditions synchronized development. The length of time between the end of cold conditions and hatching did not differ among treatments; development thus resumed as soon as temperature was suitable regardless of the initial period of warm temperature. Diapause in nature thus insures that embryos remain gastrulae after oviposition despite nest temperatures that may be warm enough to support development.

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Introduction

The life cycle of many reptiles includes a period in which embryonic development is temporarily arrested (Ewert 1985, 1991). Ecologically, developmental arrest serves two general and nonexclusive functions. One is to avoid development during periods that would be unfavorable to normal embryonic development, such as seasonal drought or cold. A second function is to establish seasonal timing of later life-history events (e.g., hatching or reproduction). Arrest can be under endogenous control and occur at the same stage of development regardless of environmental conditions; this form of arrest is called embryonic diapause (Ewert 1985, 1991). Arrest may also be a direct facultative response to environmental conditions that would not support development. For example, cold temperature or hypoxic conditions may temporarily arrest development (Ewert 1985; Kennett et al. 1993, 1998; Parker and Andrews 2006).

Several lineages of chamaeleonid lizards are unique among squamate reptiles in that embryos are diapausing gastrulae at the time of oviposition and development remains arrested for several months (Bons and Bons 1960; Blanc 1974; Andrews and Donoghue 2004). During diapause, gastrulation proceeds but extremely slowly regardless of temperature (Ewert 1985, 1991). Eggs of the tropical *Furcifer pardalis*, *Furcifer lateralis*, and related species in Madagascar are laid before the winter dry season, and eggs of the temperate and subtropical *Chamaeleo calyptratus*, *Chamaeleo chamaeleon*, and related species in northern Africa, the Mediterranean region, and India are laid before winter, the cool season. Diapause thus prevents rapid development during a time of year that is unfavorable to embryos or hatchlings. Of this latter group, *C. chamaeleon*, at least, also exhibits cold torpor, a form of developmental arrest in which development is suspended or greatly slowed by low temperature (Ewert 1991). Embryos of *C. chamaeleon* are in diapause when oviposition occurs in the fall, and diapause is succeeded by cold torpor during winter months (Díaz-Paniagua 2007). While the general pattern of developmental arrest in nature is thus known, the relationship between the stages of embryonic development and the two types of developmental arrest is not. Our objectives were (1) to determine the progression of the stages of embryonic development during developmental arrest and during subsequent development through hatching and (2) to relate the pattern of embryonic development to seasonal changes in nest temperature.

Chamaeleo chamaeleon is widely distributed in northern Af-

rica, with isolated populations in Spain, Portugal, and some Mediterranean islands. In Spain, the species is restricted to discontinuous patches of native Mediterranean scrub along the southern coast (Mellado et al. 2001). Its life history and reproductive biology are well known (e.g., Bons and Bons 1960; Blasco et al. 1985; Cuadrado and Loman 1999; Díaz-Paniagua et al. 2002; Díaz-Paniagua and Cuadrado 2003); only features relevant to this research are discussed here. Mating occurs from mid-July to mid-September; oviposition is concentrated in October. Females lay eggs at the end of long and deep burrows that they excavate in sandy soil. Burrows averaged lengths of 39 and 52 cm (2 yr, range 20–87 cm), and nest chambers averaged depths of 32 and 36 cm (2 yr, range 15–60 cm). After oviposition, females pack the external part of tunnels with soil and conceal the entrance. In nature, the length of incubation is about 10 mo. Hatching occurs in August; hatchlings escape from the nest by digging a vertical tunnel above the nest chamber.

Material and Methods

Collection of Eggs

Clutches were obtained in 2005 at the Jardín Botánico at Rota and at the Centro de Recuperación de Especies Amenazadas facility at El Puerto de Santa María, Cádiz Province, Spain, under scientific research permits from the Consejería de Medio Ambiente de la Junta de Andalucía. Clutches for field studies were collected from September 27 to October 3 (mean, September 30). Clutches for laboratory treatments 1–4 were collected October 3–9 (mean, October 6) and for treatment 5 from September 27 to October 7 (mean, October 1). To find nests, we searched the study areas for gravid females and monitored them daily until they were observed digging nesting burrows. We removed eggs after females had oviposited and filled their burrows. A few females were located after they had oviposited, and their nests were detected from characteristic "scrapes" around recently completed burrows (Fernández 1989). Eggs were weighed immediately, numbered consecutively within clutches with a soft graphite pencil, and placed individually in sealed 100-mL plastic containers. Eggs were completely buried in vermiculite moistened with distilled water at a ratio of 1.0 g vermiculite to 0.7 g water. Females from which we obtained eggs averaged 105 mm snout-vent length (range 87–135 mm) with average postoviposition masses of 22 g (range 17–30 g). Mean clutch size was 12 eggs (range 6–28), and mean egg mass was 1.2 g (range of clutch means 1.0–1.9 g).

Staging and Development

We characterized embryonic development using the normal staging table for *Zootoca* (*Lacerta*) *vivipara* (Dufaure and Hubert 1961, as presented by Porter [1972]), with specific modifications for use with chameleon embryos (Andrews 2007). In this table, embryonic development starts at stage 1 with the initiation of cell division (cleavage) and ends at stage 40 with hatching. Stages 5–9 are associated with gastrulation and stages 10–20 with neurulation, and organogenesis commences at stage 21. Embryos were assigned half-stages if they were intermediate in stage between two designated stages.

For most eggs, embryo stages were determined indirectly from the area of the yolk sac and chorioallantoic membrane (YS-CAM) and their precursor, the area opaca vasculosa (AV; Patten 1951). These vascularized extraembryonic membranes are readily observed when eggs are candled. For *Chamaeleo calyptratus*, a close relative of *Chamaeleo chamaeleon*, the area of these membranes is related to embryo stage and the associated three major phases of development (Andrews 2007). During phase 1, the AV is a small red disk that increases slowly in area during gastrulation, neurulation, and embryogenesis up to stage 27, when the AV covers about 10% of the surface area of the egg. Embryos are in diapause only during gastrulation. The transition to the substantially more rapid process of neurulation thus occurs during phase 1. During phase 2, the AV is replaced by the YS-CAM; these membranes grow rapidly and synchronously such that they cover the inner surface of the egg in 2–3 wk. Differentiation is correspondingly rapid, and embryos reach stages 32–34 by the time the YS-CAM covers 100% of the egg surface. During phase 3, when development is completed, the YS-CAM covers the entire inner surface of the egg and is no longer useful as an index of embryo stage. To test the assumption that the relationships between AV-YS-CAM areas and stage of *C. chamaeleon* and of *C. calyptratus* were similar, eggs of *C. chamaeleon* were sampled and staged at various times during development.

Observations of Development in the Field

Ten eggs from each of the first five clutches collected were allocated to the field study. Each clutch was placed in a plastic "shoe" box. To expose eggs to natural changes in temperature during incubation, boxes were buried at a typical nest location—that is, on the south side of a shrub, where the nest would not be shaded by vegetation (Fernández 1989)—at the El Puerto site. Eggs were at a depth of 35–40 cm. Temperature within three of the boxes was recorded every 4 h with Hobo data loggers. Beginning in the first week in March and at monthly intervals thereafter, boxes were dug up, eggs were weighed, and linear measurements of their major and minor axes were made with a dial caliper. When AVs were small (<5 mm mean diameter), their linear dimensions were measured. When AV-YS-CAMs were larger (5–15 mm mean diameter), their linear dimensions were measured, and their surface areas were estimated as a percentage of the total surface area of the egg. To do this, we compared the vascularized area of the AV-YS-CAM to scale diagrams of eggs indicating coverage of 5%, 10%, 15%, 20%, 25%, 40%, and 50% (areas greater than 50% coverage were estimated from the abembryonic side of the egg by subtracting the area yet to be covered by the YS-CAM from 100%). When YS-CAMs covered about 20% of the egg surface, we estimated percent coverage only; linear measurements

would be biased by curvature of the egg. When the AV reached about 10 mm in diameter (May 2006), one egg from each clutch was preserved in formalin for later staging. Additional eggs were sampled (one per clutch per month) thereafter until hatching. After observations were made, boxes were replaced in the ground. In early July, eggs were moved to the Estación Biológica de Doñana in Sevilla, where hatching could be monitored daily. Egg boxes were placed in a shaded outdoor patio where the temperature was similar to temperatures in nests in July and August (Díaz-Paniagua 2007). Hatchlings were weighed on the day they hatched. They were later released at field sites.

Experimental Design and Observations of Development in the Laboratory

Experimental treatments assessed the duration of developmental events at (1) the mean warm temperature during which active development normally takes place and (2) simulated field conditions in which warm temperature was interrupted by a 4-mo cold winter. Treatments that simulated field conditions determined whether the length of warm temperature exposure immediately after oviposition in the fall would affect subsequent development (Table 1). On the one hand, a long warm period in the fall could accelerate development such that embryos would be relatively advanced at the end of winter. On the other hand, development during embryonic diapause could be independent of temperature; all embryos would then be at the same developmental stage at the end of winter.

The two experimental temperatures (warm = 26°C, cold = 16°C) and the length of "winter" were based on previous observations of seasonal variation in nest temperature of *C. chamaeleon* (Díaz-Paniagua 2007) and on temperatures known to arrest development of lizard embryos (Shine and Harlow 1996; Andrews et al. 1997). Eggs from each of seven clutches were allocated among the four treatments to minimize maternal effects; each treatment received from one to four eggs per clutch depending on clutch size. The treatments were as follows: T1, eggs incubated at a constant 26°C; T2, eggs incubated at 26°C for 1.5 mo before winter (November 22–March 22); T3, eggs incubated at 26°C for 1 mo before winter (November 3–March 3); and T4, eggs incubated at 16°C for 4 mo (October 6–February 2) starting at oviposition. Eggs were returned to 26°C for the remainder of incubation after "winter."

We also exposed some eggs to warm conditions for 71 d before shifting them to the cold temperature (T5). Eggs were shifted to 16°C on December 11 and returned to 26°C on April 12. Eggs ($n = 12$) allocated to this treatment were from five clutches. Five eggs were leftovers from three clutches used for field observations, six were from an entire clutch that was too small to be used in T1–T4, and one was from a clutch used in the laboratory experiments. Four of the five clutches in this treatment thus did not correspond to those clutches distributed among T1–T4. Because of the potential for bias in statistical comparisons, observations for T5 are reported for comparative purposes only.

For all treatments except T1, egg boxes were placed in a common constant temperature incubator (mean temperature 16.3°C) during the 4 mo of cold exposure. During periods of warm temperature, individual egg containers were allocated among five Jaeger Bruttechnik reptile egg incubators (FB 50-Rep). Temperatures in each incubator were recorded every 4 h with Hobo data loggers and adjusted weekly to biweekly as required to maintain a mean temperature of 26°C. When eggs were shifted between 16° and 26°C, they were exposed to 20°C during a 1-d transition period.

Dimensions of AV-YS-CAMs and eggs in T1–T5 were measured or estimated as described for field eggs. For T1, AV-YS-CAMs were measured on November 4 and 24 and at weekly intervals thereafter until the YS-CAM covered 100% of the egg surface. For T2–T5, AV-YS-CAMs were measured on the day eggs were placed in the cold incubator, on the day eggs were returned to warm conditions, and at weekly intervals thereafter until YS-CAM coverage was 100%. For T4, AVs were also measured on November 23 because an AV was not visible when they were placed in the cold chamber. Four eggs from T1 and T2 were sampled on November 23, and 12 eggs from T1, T4, and T5 were sampled between January 19 and May 6 to assess the relationship between AV-YS-CAM area and embryo stage. Vermiculite was replaced every 2 mo, excluding the cold period. Incubators were checked daily for hatchlings. Hatchlings were weighed on the day they hatched and released at field sites within a week or so of hatching.

Table 1: Temporal sequence of temperatures experienced by experimental eggs

Treatment	October	November	December	January	February	March	April	May–August
T1	W	W	W	W	W	W	W	W
T4	C	C	C	C	W	W	W	W
T3	W	C	C	C	C	W	W	W
T2	W	W/C	C	C	C	C	W	W
T5	W	W	W/C	C	C	C	C	W

Note. Eggs were incubated at a constant 26°C (warm [W]) or 16°C (cold [C]), as indicated. W/C indicates warm for the first half of the month and cold for the last half. T1–T4 involved seven clutches, each with eggs evenly allocated among the four treatments. T5 involved an independent set of eggs. In this and the following tables, T1, the constant warm regime, is listed first, and the remaining treatments are listed in order of increasing length of the initial warm period.

Statistical Analyses

The duration of developmental events was determined from observations on the mean day of oviposition (day 0), the day that diapause ended for each egg (T1), the day that eggs in T2–T5 were shifted from 16° to 26°C, and the day of hatching for each egg. To assess when rapid YS-CAM growth was initiated and ended, that is, the beginning and end of phase 2, we used regression analyses that related YS-CAM coverage to the day of observation. Percent YS-CAM was natural log transformed to linearize the relationship between YS-CAM and day. We excluded observations of less than 10% coverage and 100% coverage to eliminate data for portions of the curve where the YS-CAM had not started rapid growth or was fixed in size, respectively (Fig. 1). Resultant regression equations were then used to predict the days when YS-CAM areas were 10% and 100%. The onset of phase 2 varied substantially among clutches for T1. Regressions were therefore determined independently for each clutch; values in analyses were measurements for individual eggs. AVs from some eggs in three of the seven clutches had been difficult to see throughout development, and the regressions between percent YS-CAM and date were not significant; predictions were not made for these clutches. Regressions for the remaining clutches were significant at $P \leq 0.01$. Because YS-CAM development was highly synchronized within treatments for T2–T5, values used in analyses were clutch means of percent coverage. All regressions were significant ($P \leq 0.001$). The length of phase 2 is reported as the difference between the predicted days of 10% and 100% YS-CAM coverage. Lack of replication of the estimates of 10% and 100% YS-CAM coverage precludes statistical comparisons among clutches or treatments for durations of developmental events that incorporated these values. Before ANCOVA, the assumption of homogeneity of slopes was satisfied by testing for the significance of the interaction of the covariate with treatment. Comparisons of survival among treatments were based on observations of individual eggs. Parametric analyses were conducted with SAS software (SAS Institute 2003). Least squares means procedures were used for a posteriori tests. Nonparametric analyses were from Siegel (1956), and variance component calculations for Model II ANOVAs were from Sokal and Rohlf (1981). Significance was at $P < 0.05$.

Results

AV-YS-CAM Area and Embryo Stage

The relationships between AV-YS-CAM area and embryo stage for the 36 embryos of *Chamaeleo chamaeleon* that we sampled and for a larger sample of *Chamaeleo calyptratus* embryos (Andrews 2007) were very similar (Fig. 1). Observations on *C. chamaeleon* will therefore be presented in the context of the three phases of development described for *C. calyptratus*.

During phase 1, the size of the AV of the *C. chamaeleon* eggs increased gradually relative to the progression of embryonic differentiation (stage). An AV was not visible on eggs candled at the time of oviposition. Our earliest estimate of the initial size of the AV was from eggs in T1 on November 4, 26 d after oviposition. Overall mean diameter was 3.9 mm, with clutch means ranging from 3.5 to 4.2 mm. These diameters are comparable to the 3–4-mm diameters associated with gastrulae of *C. calyptratus* shortly after oviposition (Andrews 2007). Three embryos from T1 and T2 staged at 47 d were gastrulae; AV diameters were 5.2, 5.7, and 7.2 mm. AVs increased slightly in size as embryos progressed through neurulation and early embryogenesis; AV diameters of stage 20, 25, 25, and 27 embryos were 6.8, 7.2, 7.2, and 9.2 mm (5.7%, 6.5%, 6.5%, and 10.4% AV coverage of egg), respectively. During phase 2, the size of the YS-CAM increased rapidly relative to the embryo stage. YS-CAM coverage exceeded 10% for all but one of nine *C. chamaeleon* embryos at stages 28–30. The stages of two embryos for which YS-CAM coverage was close to or had just reached 100% were 32.5 and 33. Of the 18 embryos sampled after YS-CAM coverage reached 100% (phase 3), all had stages of 34–40.

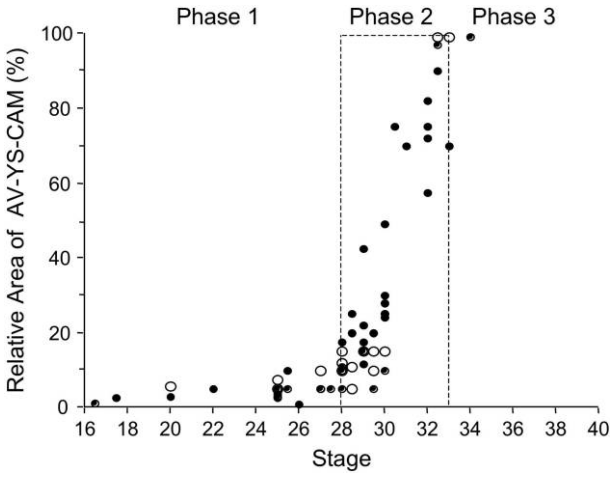

Figure 1. Percent coverage of the surface area of eggs by the AV-YS-CAM as a function of embryo stage for *Chamaeleo chamaeleon* (*open circles*) and for *Chamaeleo calyptratus* (*solid circles*; Andrews 2007). Three gastrulae and 18 embryos at stages 34–40 of *C. chamaeleon* are not shown. The three phases of development based on the size of the AV are indicated.

T1: Eggs Incubated under Warm Conditions

Determination of the length of developmental events for T1 eggs was facilitated by the observation that AV diameter exhibited a small but distinct increase in slope shortly before the end of phase 1. Plots of AV diameter as a function of day of incubation indicated a distinct inflection point for 23 eggs (11 eggs could not be scored because the AV was difficult to see or because independent assessments of the inflection point by R. M. Andrews and C. Díaz-Paniagua did not agree). We associated the inflection point with the end of diapause for two reasons. First, the mean AV diameter of 6.7 mm at the inflection

point corresponded to the size of the AV at the transition between gastrulation and neurulation, as judged by staged embryos; the largest AV diameter of a gastrula was 7.2 mm, and that of a stage 20 neurula was 6.8 mm (from "AV-YS-CAM Area and Embryo Stage"). Second, the day that the inflection occurred was strongly correlated with the day of hatching (Pearson correlation coefficient = 0.93, $P < 0.001$, $n = 14$).

Diapause lasted an average of 90 d (range of clutch means 64–109 d), that is, to January 4 (range of clutch means, December 8–January 23; Table 2), and the length of diapause differed among clutches ($F_{6,15} = 4.5$, $P = 0.009$, one-way Model II ANOVA). Variation in the length of diapause among and within clutches was 52.6% and 47.4% (variance components). Phase 1 averaged 105 d. Embryos were diapausing gastrulae for 90 d (86%) and neurulae through stage 27 for 15 d (14%) of this phase. Phase 2 (10%–100% YS-CAM) averaged 31 d (range of clutch means 25–33 d), and phase 3 (100% YS-CAM to hatching) averaged 80 d (range of clutch means 63–89 d). The period from the end of diapause to hatching averaged 128 d (range of clutch means 111–139 d) and differed among clutches (ED to H, $F_{6,7} = 4.5$, $P = 0.035$, one-way ANOVA). Variation in the length of the period from the end of diapause to hatching among and within clutches was 57.3% and 42.7%.

T2–T5: Eggs Exposed to 4 Mo of Cold Conditions

AV diameter of eggs in T2–T5 increased slowly but consistently during the initial period of warm temperature (Table 3). For example, when embryos were shifted to 16°C, AV diameters of T2 embryos were larger than those of T3 embryos ($F_{1,12} = 36.1$, $P < 0.001$, one-factor ANOVA), with mean AV diameters of 3.6 and 4.8 mm after 27 and 46 d of incubation at 26°C, respectively (Table 3). AVs of T5 eggs that had been exposed to 26°C for an initial 71 d had even larger diameters (mean = 5.6 mm). AV diameters did not increase, however, between the beginning and end of the 4-mo cold period (within-treatment contrasts for T3, T2, T5: $P = 0.34, 0.91, 0.79$, respectively, *t*-tests). Moreover, at the end of the 4-mo cold period, AV diameters of eggs in T4, T3, and T2 did not differ among treatments ($F_{2,45} = 0.48$, $P > 0.50$) or among clutches ($F_{6,33} = 0.26$, $P > 0.50$, two-way mixed-model ANOVA). Nonetheless, while eggs in T4, which were exposed to 16°C immediately after oviposition, did not have a visible AV when checked 48 d after oviposition, AV diameters averaged 4.7 mm at the end of 4 mo of cold exposure.

The total length of phase 1 varied among T2–T4 as a consequence of the variation in the initial length of warm temperature. Otherwise, durations of developmental events were similar among treatments. For example, the period from the end of cold exposure to the end of phase 1 varied only from 16 to 18 d. Phase 2 and phase 3 varied from 13 to 22 d and from 89 to 98 d, respectively. From the end of cold exposure to hatching varied from 127 to 130 d. Durations of developmental events for T5 were comparable to those of T2–T4.

Egg Survival and Hatchling Mass in the Laboratory

Egg survival (survivors/total) during incubation in treatments T1–T4 was relatively high, with respective values of 78% (18/23), 90% (19/21), 95% (21/22), and 80% (12/15). Survival of eggs in T2–T4 pooled (to eliminate cells with values less than 5) did not differ from survival in T1 ($\chi^2 = 1.8$, $P = 0.18$). Seven of the 11 eggs that died, however, were from one clutch. Excluding this clutch, three eggs died in T1, two in T2, and none in T3 or T4. T5 eggs had 82% (9/11) survival, which was comparable to those of T1–T4.

Mass of hatchlings in T1–T4 was related to egg mass, the covariate ($F_{1,23} = 14.7$, $P < 0.001$), but not to treatment ($F_{3,23} = 2.5$, $P = 0.086$, one-factor ANCOVA). Mean masses were as follows: T1, 1.17 g; T2, 1.23 g; T3, 1.26 g; and T4, 1.24 g, at the covariate mean for egg mass of 1.24 g. Hatchlings from T5 had mean masses of 1.09 g and a mean egg mass of 1.18 g.

Field Observations

When eggs were first checked in early March, mean AV diameter was 3.7 mm (range 3.1–4.0), and in early April, mean AV diameter was 4.7 mm (range 4.4–4.8; Fig. 2). Judging by observations on staged embryos, embryos would have been gastrulae in March and April. By May, embryos had a mean stage of 28.6 (range 28–29.5). Development thus resumed in early April when nest temperature averaged 20.4°C. By June, embryos had a mean stage of 34 (range 34–34.5). Rapid growth of the YS-CAM (phase 2) thus occurred during May when nest temperature averaged 24°C. Embryos were at stage 38 in July and stage 40 in August. Hatching occurred August 11–17, 2006. In accord, the first hatchlings of the year were seen at field sites on August 17 (J. J. Gómez, personal communication). The period of incubation following the end of developmental arrest was thus slightly more than 4 mo (early April to mid-August), and the total incubation period was 10.5 mo (early October to mid-August). Thirty eggs (six per five clutches) were incubated to hatching; of these, one egg did not hatch, and one embryo from the 20 eggs sampled was abnormal and would not have survived (96% overall survival). Field hatchlings had mean masses of 1.22 g (initial egg mass averaged 1.15 g). Eggs took up water at roughly the same rate during the entire developmental period (Fig. 2). Egg mass increased by a factor of 1.6 from oviposition in October to the first sample in March, and eggs had doubled their mass shortly before hatching.

Discussion

Diapause and Cold Torpor

Diapause is a process that prepares the embryo for the next stage in its life cycle (Ewert 1985, 1991; Kostál 2006). For example, the very slow increase in AV diameter of *Chamaeleo chamaeleon* at 26°C parallels the protracted pace of gastrulation (Peter 1935; Bons and Bons 1960; Blanc 1974). For eggs in T1,

Table 2: Mean duration (d) of developmental events for *Chamaeleo chamaeleon* embryos

	Phase 1		Phase 2	Phase 3		
Treatment	Ov to ED	ED to 10%	10% to 100%	100% to H	ED to H	Total
T1 overall	90 (22)	15	31	80	128 (14)	218
T1 clutches:						
18	64 (3)	15	33	63	111 (2)	175
9	67 (3)	…	…	…	122 (2)	189
8	89 (2)	…	…	…	133 (2)	222
19	97 (4)	7	33	89	130 (4)	227
5	109 (3)	18	25	82	125 (1)	234
17	101 (4)	18	33	85	136 (2)	237
12	100 (3)	…	…	…	139 (1)	239
	Ov to EC	EC to 10%	10% to 100%	100% to H	EC to H	Total
T4 overall	120	18	20	92	130	250
T3 overall	147	18	13	98	129	276
T2 overall	166	16	22	89	127	293
T5 overall	193	16	24	84	124	317

Note. Durations are from oviposition to the end of diapause (Ov to ED; T1), from oviposition to the end of cold exposure (Ov to EC; T2–T5), from the end of diapause to 10% AV-YS-CAM coverage (ED to 10%; T1), from the end of cold exposure to 10% AV-YS-CAM coverage (EC to 10%; T2–T5), from 10% to 100% AV-YS-CAM coverage (10% to 100%), from 100% AV-YS-CAM coverage to hatching (100% to H), from the end of diapause to hatching (ED to H; T1), and from the end of cold exposure to hatching (EC to H). The length of incubation from oviposition to hatching (total) includes 120 d of cold exposure for T2–T4 (122 d for T5) and the initial number of days at 26°C for T2–T5 (Table 3). For Ov to ED and ED to H, the number of eggs is given in parentheses.

the AV increased gradually in area up to the end of diapause and slightly more rapidly up to the end of phase 1. For eggs in T2, T3, and T5, AVs increased in size until eggs were placed in the 16°C incubator; AV diameter did not change over the subsequent 4 mo of winter temperature. In contrast, at the end of the cold period, AV diameters of T4 eggs that were placed under cold conditions immediately after oviposition did not differ from those of T2 and T3 eggs (Table 3). This observation suggests that AV development could continue at 16°C, but only if the AV had not reached some critical size before cold exposure.

Correspondence between the lengths of developmental events indicates that the end of diapause in T1 was developmentally equivalent to the end of cold exposure in T2–T5. In fact, the mean lengths of time between the end of diapause and the end of phase 1 and the end of diapause and hatching for T1 clutches and between the end of cold exposure and the end of phase 1 and the end of cold exposure and hatching for T2–T5 clutches were almost identical, 15 and 128 d versus 17 and 128 d, respectively. Gastrulation must have been completed during cold exposure in T2–T5 and further development arrested by cold. When eggs were returned to warm conditions, embryos from T2–T5 immediately initiated the rapid development characteristic of neurulae and later stages. As a consequence, durations of subsequent developmental events were highly synchronized within clutches. Such synchrony was not exhibited by T1 clutches. For example, the maximum difference among the mean hatching dates of clutches in T1 was 64 d, while clutches in T2–T5 and in the field group hatched over 2–14 d. Winter conditions thus acted to synchronize development; in the absence of a period of cold torpor, the time when diapause was terminated varied substantially among clutches and, to a lesser extent, within clutches.

Our experimental protocol does not allow us to determine the length of diapause for clutches exposed to 4 mo of cold. We know, however, that development was arrested by cold torpor until the end of the cold period when embryos initiated postarrest development regardless of the length of time they were in diapause at 26°C. Diapause could have been terminated after some fixed length of time at 16°C or at a time proportional to the length of diapause at 26°C. In either case, the outcome would have been the same with regard to when embryos initiated postarrest development. The experimental manipulation necessary to determine the actual length of diapause would be to remove embryos from cold conditions at successively shorter intervals after initial cold exposure. Diapausing embryos would take longer than 15–17 d to reach the end of phase 1; embryos whose development was arrested by cold torpor would reach the end of phase 1 at 15–17 d.

Embryonic diapause in chameleons differs from that in turtles, where a period of low temperature is often necessary for breaking diapause and resumption of development (Ewert 1991; Booth 2002). Diapause in *C. chamaeleon* and other chameleons is terminated endogenously when eggs are incubated at warm temperatures (Ferguson 1994; Andrews and Donoghue 2004). Hatching rates of *C. chamaeleon* are high and compa-

Table 3: AV diameter at the time when T2–T5 eggs were shifted from 26° to 16°C and 4 mo later, when they were returned to 26°C

		26° to 16°C Shift			16° to 26°C Shift		
Treatment	Initial Days at 26°C	Mean Diameter (mm)	SD	*n*	Mean Diameter (mm)	SD	*n*
T4	0	NV	NV	NV	4.7	.22	7
T3	27	3.6	.40	7	4.4	.27	7
T2	46	4.8	.36	7	4.6	.38	7
T5	71	5.6	.56	5	5.1	.64	5

Note. NV indicates not visible.

rable in the field where eggs were protected from predation and desiccation, under simulated field conditions in the laboratory, and under constant warm conditions in the laboratory (Díaz-Paniagua and Cuadrado 2003; Díaz-Paniagua 2007; this study). Nonetheless, incubation at constant warm conditions may not be the best protocol for incubation. Hatchlings from T1 tended to have lower masses than hatchlings from T2–T4. Hatchlings from eggs exposed to periods of cold temperature by Díaz-Paniagua (2007) also had larger body sizes than individuals that were incubated under constant warm conditions. Large hatchling size is sometimes, but not always, associated with enhanced fitness (Sinervo 1990; Warner and Andrews 2002). A period of cold temperature during incubation thus might improve the success of laboratory breeding of chameleons with embryonic diapause.

Developmental Cycle in the Field

During the first 6–7 mo of incubation, embryos of *C. chameleon* in nature are in a state of developmental arrest consisting of an initial period of diapause that is followed by a period of cold torpor, a pattern parallel to that of embryos of many insects (Ando 1983; Sujii et al. 2001). Oviposition by *C. chamaeleon* occurs from late September to early November. An obligate diapause of at least 2 mo precludes developmental progression from gastrulation to neurulation before winter. Embryos were still gastrulae in early April; a nest temperature of 20°C was sufficient to maintain a state of cold torpor (Fig. 2). By early May and early June, embryos were at stages 29 and 34, respectively. Phase 1 of development was thus completed during April and phase 2 during May. Phase 3 lasted slightly longer than 2 mo, with hatching in mid-August. Postarrest development (early April to mid-August) of about 130 d in the field was similar to that observed for experimental treatments (range 111–139 d; Table 2), in accord with mean incubation temperatures of 26°C in both cases.

While diapause in insects can occur at any embryonic stage, diapause for vertebrate embryos occurs only very early in development as blastocysts in mammals (Feldhamer et al. 2004) and as gastrulae in reptiles. This observation suggests that arresting development at other times may be disruptive to embryogenesis. If this explanation is correct, selection would have favored a period of diapause for *C. chamaeleon* sufficiently long to prevent the termination of diapause in a year when temperature was unusually warm in November and December or if oviposition were unusually early in the season. Whether postgastrulae of *C. chamaeleon* would survive winter in Spain or in other parts of their range is not known.

Reproductive Cycles of Chameleons with Embryonic Diapause

The reproductive cycles of *C. chamaeleon*, its close relatives, and other chameleons with embryonic diapause have a number of highly unusual features. (1) Chameleons are the only squamates with embryonic diapause (Ewert 1991). (2) Few, if any, other oviparous squamates oviposit in the fall with incubation extending through winter, although this is a common reproductive strategy of viviparous species (Saint Girons 1985). (3) Incubation is prolonged. Varanids are the only other squamates with incubation periods that extend 6 mo or more (Birchard

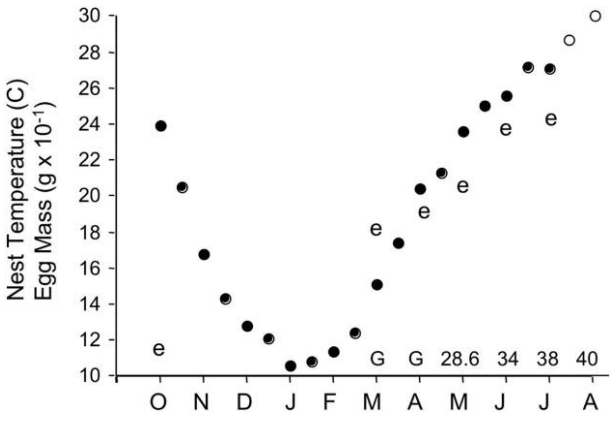

Figure 2. Nest temperature, egg masses, and embryo stages of *Chamaeleo chamaeleon* eggs during incubation in the field. Temperatures in the field are represented by solid circles and those in the "patio" by open circles. Mean temperatures for the first half of the month (day 15 or earlier) are above the hatch mark, and those for the second half of the month (day 16 or later) are between hatch marks. Mean egg masses are indicated by the letter *e*, and mean stages of embryos are indicated above appropriate months. Gastrulae (*G*) were assessed from the size of the AV; the remaining values represent stages of sampled embryos.

and Marcellini 1996). For *C. chamaeleon*, diapause and a subsequent period of cold torpor comprise 6–7 mo of the 10.5-mo incubation period. Even so, the 3–4-mo period of incubation during spring and summer is prolonged as well (phase durations compared with observations for other lizards in Fig. 4.2 in Andrews 2004). Lengthy development after a period of developmental arrest has also been observed for *Chamaeleo calyptratus* and *Furcifer lateralis* and may be typical of chameleons with embryonic diapause (Andrews 2004). This combination of unusual developmental features is associated with Mediterranean and seasonal tropical climates. The association between embryonic diapause and overwinter incubation, as either a cold or a dry period, also characterizes many groups of turtles (Ewert 1991). For example, Australian chelid turtles exhibit two reproductive patterns (Legler 1985). Embryonic diapause occurs during the (winter) dry season for tropical species, while species associated with temperate regions complete their reproductive cycle during the summer. These seasonal reproductive patterns persist even when ranges expand into nontypical climates. Are the evolutionary origins of embryonic diapause in chameleons also in the seasonal tropics? Broadly comparative studies within the Chamaeleonidae would help answer this question. Information on life histories, phylogenetic history, and environments of species that exhibit and do not exhibit embryonic diapause would be particularly helpful in this regard.

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