

Influence of egg aggregation and soil moisture on incubation of flexible-shelled lacertid lizard eggs

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Abstract: Many oviparous terrestrial species deposit flexible-shelled eggs into the soil. These eggs are sensitive to the hydration level of the nest environment. Among other factors, water exchange of eggs during incubation may be affected by the soil water potential. To evaluate whether egg aggregation influences embryonic development, we incubated flexible-shelled Schreiber's green lizard (*Lacerta schreiberi*) eggs under three levels of soil water potential (wet: -150 kPa; intermediate: -650 kPa; dry: -1150 kPa) and under two levels of aggregation (aggregated: in groups of six eggs with physical contact among them; isolated: groups of six eggs each 1 cm apart). The availability of water during egg incubation influenced egg mass and hatchling size. Eggs incubated in dry soils absorbed less water and produced smaller hatchlings. The selected levels of soil water potential did not influence incubation duration or hatching success. When soil was wet or dry, we did not find any effect of egg aggregation in embryonic development. However, when soil water potential was intermediate, aggregated eggs absorbed less water and their embryos hatched at smaller sizes compared with isolated eggs. Moreover, variability and range of egg water absorption and hatchling size were higher among aggregated eggs than among solitary ones when access to water was restricted. In these cases, eggs competed with different success for water, a limited resource in the nest environment.

Résumé : Plusieurs espèces terrestres ovipares déposent des oeufs à coquille flexible dans le sol. Ces oeufs sont sensibles au degré d'hydratation de l'environnement du nid. L'échange d'eau par les oeufs durant l'incubation peut être affecté, entre autres facteurs, par le potentiel hydrique du sol. Afin d'évaluer si la répartition contagieuse des oeufs influence le développement embryonnaire, nous avons incubé des oeufs à coquille flexible de lézards de Schreiber (*Lacerta schreiberi*) à trois degrés de potentiel hydrique (humide : -150 kPa; moyen : -650 kPa; sec : -1150 kPa) et à deux niveaux de contagion (contagion : groupes de six oeufs ayant un contact physique entre eux; isolement : groupes de six oeufs distants de 1 cm les uns des autres). La disponibilité de l'eau durant l'incubation des oeufs affecte la masse des oeufs et la taille des nouveau-nés. Les oeufs incubés dans les sols secs absorbent moins d'eau et produisent des nouveau-nés plus petits. Les degrés choisis de potentiel hydrique du sol n'influencent pas la durée de l'incubation, ni le succès de l'éclosion. Dans les sols humides ou secs, il n'y a pas d'effet de la répartition contagieuse des oeufs sur le développement embryonnaire. En revanche, au niveau moyen de potentiel hydrique du sol, les oeufs à répartition contagieuse absorbent moins d'eau et leurs embryons éclosent à des tailles plus petites que les oeufs isolés. De plus, lorsque l'accès à l'eau est réduit, la variabilité et l'étendue de l'absorption d'eau par les oeufs et de la taille des nouveau-nés sont plus élevées dans le cas des oeufs à répartition contagieuse que dans celui des oeufs isolés. Dans cette situation, les oeufs font compétition pour l'eau, une ressource limitée dans l'environnement du nid, avec des succès variables.

[Traduit par la Rédaction]

Introduction

Many oviparous reptiles deposit flexible-shelled eggs that are permeable to water and sensitive to the water potential of the oviposition site (Thompson 1987; Packard and Packard 1988). These eggs usually develop in physical contact with the surrounding soil. During incubation, eggs can absorb water from the soil, increasing their mass and volume up to three or four times during development (Packard and

Packard 1988; Overall 1994). For many species, absorption of water is necessary to successfully complete the embryonic development (Morris et al. 1983; Packard and Packard 1988; Packard 1991). The eggs of many species of reptiles develop during the dry season when nests often become exposed to low water potentials. Dry environmental conditions can cause substantial water losses in eggs and they commonly succumb during incubation (Muth 1980; Packard 1991). Embryos incubated in wet environments hatch at larger sizes than embryos in eggs from dry environments. The amount of water absorbed may decisively influence hatching success or hatchling survival and fitness (Gutzke et al. 1987; Janzen et al. 1995; Finkler 1999).

Several factors such as soil water potential, temperature, egg size, and shell porosity influence egg water absorption (Gutzke and Packard 1987; Ackerman 1991; Packard 1991). Another important factor affecting water exchange is the proportion of eggshell contacting the substrate (Packard et al. 1980; Tracy and Snell 1985). In wet environments, the

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capacity of absorbing water by eggs is related to the proportion of eggshell in contact with substrates, while in dry substrates, the same proportion is related to the water loss (Packard 1999).

Besides the substrate and the air chamber that usually persist in the nest cavity during incubation, eggs can be in contact with other eggs. Some species or small individuals of some other species can lay single eggs in exclusive chambers. However, most species lay several eggs together (reviewed in Packard and Packard 1988), and in some cases, eggs are firmly attached to each other forming a compact mass (A. Marco, personal observations). Moreover, different females, even of different species, may concentrate several clutches in the same cavity, increasing egg aggregation (Graves and Duvall 1995). The eggshells of aggregated eggs are partially or completely limiting with other eggshells, which could act as a barrier to the ambient moisture and decrease their capability of exchanging water.

We hypothesized that physical contact among eggs may affect water absorption by flexible-shelled eggs and thus embryonic development and hatchling size. To test this hypothesis, we incubated either aggregated (groups of six eggs having physical contact among them) or isolated flexible-shelled lizard eggs under different levels of soil water potential. Isolated eggs were separated from each other by a distance of 1 cm to minimize the possible influence of thermal or water potential gradients within each incubation chamber not caused by the presence of the eggs. For the study, we selected the Schreiber's green lizard (*Lacerta schreiberi*), a species from the lacertid family that includes a wide range of clutch sizes, from 1 to >25 eggs. Specifically, the selected species can lay from 4 to 24 eggs (Marco et al. 1994). This clutch size permitted the distribution of siblings in all of the treatments and thus minimized the influence of genetic and maternal effects. Schreiber's green lizard inhabits the mountain and coastal areas of Portugal and northwestern Spain. Egg laying takes place at the end of May through June. Embryos hatch and emerge from soil during August, rarely later (Marco et al. 1994).

Materials and methods

Egg collection

We collected six gravid Schreiber's green lizard females in Sierra de Candelario (Salamanca, Spain) on 17 June 2000. They were housed in 1 m × 0.35 m × 0.3 m glass terraria in the laboratory at approximately 26 °C under natural daylight giving some exposure to UV radiation. Females were always fed live larvae of the genus *Tenebrio* and crickets (*Achaeta domestica*) ad libitum and had dechlorinated tap water available. The floor of the containers had 12 cm of wet soil that we watered regularly. Egg laying took place after a maximum of 6 days of captivity. Females laid a mean clutch size of 12.7 eggs per clutch. Immediately after egg laying, we extracted the eggs from the soil. We cleaned the eggs with a soft brush and weighed (± 0.01 g) and measured (± 0.1 mm) them. Before the beginning of the experiment, we incubated the eggs at 26 °C in wet sand for a maximum of 5 days. After egg laying, females were immediately released to the same places from where they were collected.

Experimental procedures

The experiment was conducted from 23 June until the end of hatching on 20 August 2000 in two incubation chambers (one for each replicate) at 26 °C (Jaeger-Pfrommer, Wächtersbach, Germany). We incubated 72 Schreiber's green lizard eggs in 12 plastic containers filled with wet vermiculite that had been assigned to one of six experimental treatments (two replicates). Each container had six eggs, one from each clutch. We used cylindrical containers 15 cm in diameter and 8 cm tall. We considered two factors of variation, soil water potential and level of aggregation, with two replicates for each treatment. We selected three levels of soil water potential, -150, -650, and -1150 kPa, and four containers corresponded to each of these treatments. The selected values for temperature and moisture fall within the ranges of values for natural nests at our study site (A. Marco, personal observations). To obtain the selected soil water potentials, we added distilled water to clean vermiculite (previously dried up at 80 °C in a stove until constant mass) following the methodology proposed by Packard et al. (1987). After water addition, filled containers were weighed on a digital balance to the nearest of 0.1 g. During the incubation, the container mass was regularly checked to detect any water loss, adding distilled water when necessary. The possible slight impact of water absorption by eggs during incubation on the substrate water potential was corrected at days 12, 28, and 40, replacing the substrates. To evaluate the influence of aggregation on embryonic development, in two containers of each water potential level, eggs were located in aggregation and in the other two containers, all eggs were separated. The six aggregated eggs were located together, with all eggs having physical contact in two layers. Alternatively, the isolated eggs were located 1 cm apart from each other. One egg of each clutch was randomly assigned to each container, minimizing the effect of intraclutch variability (maternal and genetic effects). The eggs were completely covered by the substrate and containers were closed to reduce water evaporation.

At the beginning of the experiment, at the third day and every time we replaced the substrate (days 12, 28, and 40), we recorded the survival and the external shape and aspect of eggs and weighed them (± 0.01 g). The rate of egg mass increase was the increase in egg mass from the beginning of the experiment divided by the egg mass at the beginning of the experiment (day 0). Forty days elapsed between the first and last egg mass measurement. Immediately after hatching, we measured hatchling snout-vent length (SVL) (± 0.1 mm) and mass (± 0.01 g). We also checked for morphological or behavioral alterations. Hatchlings were housed in 30-L plastic containers in the laboratory at approximately 26 °C under natural light and were fed crickets ad libitum.

To determine whether treatments had an effect on hatchling locomotor performance, we measured the running speed of each individual within 24 h of hatching. All hatchlings had the tail intact. Hatchlings were forced to run 1 m (following Huey et al. 1981). The track was constructed of cardboard and was 120 cm long with vertical walls 30 cm high positioned 20 cm apart; the floor was lined with filter paper. To calculate the running speed, we considered the time that hatchlings took to run 100 cm, excluding the first and last 10 cm of the track. Running times were recorded with a

Table 1. Results of the repeated measures two-way ANOVA and the five univariate two-way ANOVAs that analyse the influence of the substrate water potential and the level of egg aggregation on egg mass during incubation of Schreiber's green lizard (*Lacerta schreiberi*) eggs.

Factor	Water potential			Aggregation			Interaction		
	Wilks' λ	$F_{[2,6]}$	P	Wilks' λ	$F_{[1,6]}$	P	Wilks' λ	$F_{[2,6]}$	P
Overall	0.001	24.07	0.001	0.070	9.93	0.045	0.009	7.14	0.014
Day 0		0.32	0.74		0.61	0.46		0.82	0.48
Day 3		21.22	0.002		12.76	0.012		2.16	0.197
Day 12		24.12	0.001		15.63	0.008		4.55	0.063
Day 28		96.11	<0.001		2.89	0.140		7.82	0.021
Day 40		132.90	<0.001		24.5	0.003		15.4	0.004

stopwatch to the nearest of 0.1 s. We ran each hatchling twice and considered the mean running speed for each individual. Between each trial, hatchlings were allowed to rest for 2 min. At the end of the experiment, hatchlings were released in the area where females had been collected.

All animals were collected and cared for in accordance with the principles and guidelines of Spanish laws and the Canadian Council on Animal Care.

Data analysis

To determine whether the substrate water potential or the level of egg aggregation had an overall effect on egg mass during incubation, we used a repeated measures two-way analysis of variance (ANOVA). We also used a multivariate two-way analysis of variance (MANOVA) to determine the influence of egg aggregation and soil water potential on egg incubation and hatchling size, considering as dependent variables the rate of egg mass increase at day 40, time to hatching, and hatchling mass and SVL. In the statistical analysis we used only the mean values of each container. We used univariate two-way ANOVAs to determine the effect of each selected factor and the interaction between them on each dependent variable used in the MANOVA. We also used Tukey's honest significance difference (HSD) for post-hoc comparisons of means of ANOVAs. To determine the effect of experimental treatments on embryo survival, we used the χ^2 test. To determine whether the substrate water potential or the level of egg aggregation influenced the variability of measured variables among eggs within each container, we used two-way ANOVAs where the dependent variables were the standard deviations of hatchling mass and SVL of each experimental container.

Results

At the beginning of the experiment, mean egg mass did not differ among treatments (Table 1). For this reason, we did not use this variable as a covariate in the rest of the statistical analysis. The repeated measures ANOVA indicated an overall effect of soil water potential and egg aggregation on egg mass variation during incubation (Table 1). After 72 h of exposure to the experimental conditions, eggs from all treatments had absorbed water (Fig. 1), increasing their mass, on average, by 27.4%. At this moment, the effects of aggregation and water potential treatments were already significant (Table 1). Eggs exposed to the highest water potential absorbed more water than eggs exposed to the other two dryer treatments (Fig. 1). At the wettest treatment, the level

of egg aggregation did not influence the rate of water absorption, but at -650 and -1150 kPa, single eggs absorbed more water than aggregated ones (Fig. 1). Similar results were obtained when we analysed the egg mass after 12, 28, and 40 days of exposure to experimental treatments (Fig. 1). At day 40, when embryos were almost fully developed, there were differences in water absorption rate among the three levels of soil moisture (Table 2, Fig. 2). A post-hoc Tukey's HSD test revealed differences in egg mass among isolated and grouped eggs only at -650 kPa.

Two eggs died during the experiment, but there was no influence of treatments on hatching success ($\chi^2 = 4.041$, $df = 5$, $P < 0.544$). Hatching, on average, took place after 56.3 days of incubation and 53.6 days of exposure to the experimental treatments. We did not find any external developmental abnormalities in eggs and hatchlings. The two-way MANOVA showed an overall effect of the substrate water potential, level of aggregation, and the interaction between the two factors on embryonic development and hatchling characteristics (Table 2). Mean hatchling size increased with soil water potential (Figs. 3 and 4). At -150 and -1150 kPa, aggregation had no effect on hatchling size. However, at -650 kPa, hatchlings from aggregated eggs were significantly smaller in body length (6%) and mass (12%) than those from solitary ones (Figs. 3 and 4). At this intermediate water potential, egg aggregation had the same effect on embryos as the driest treatment, and solitary embryos hatched at a size similar that of embryos incubated at the wettest treatment.

Mean variability on hatchling SVL within each container was significantly higher for aggregated eggs than for solitary eggs (ANOVA, $F_{[1,6]} = 7.541$, $P = 0.033$). The mean values of the standard deviation of hatchling SVL within each container were 0.789 (mean range = 2.5 mm) for solitary eggs and 1.155 (mean range = 4 mm) for aggregated eggs. The substrate water potential had no effect on variability among individual hatchlings (ANOVA, $F_{[2,6]} = 0.436$, $P = 0.665$).

Discussion

Several studies have already demonstrated the benefits of wet substrates for embryonic development in different reptiles such as chelonians in the families Emydidae and Chelydridae (Gutzke et al. 1987; Packard et al. 1987; Janzen et al. 1995) and (or) snakes (Black et al. 1984; Gutzke and Packard 1987; Ji and Du 2001). In most of cases, the relationship between egg incubation and soil water potential was detected in species that had flexible-shelled eggs. However,

Fig. 1. Influence of substrate water potential and two levels of aggregation on mass of Schreiber’s green lizard (*Lacerta schreiberi*) eggs during an experimental incubation of 40 days (wet: -150 kPa; intermediate: -650 kPa; dry: -1150 kPa).

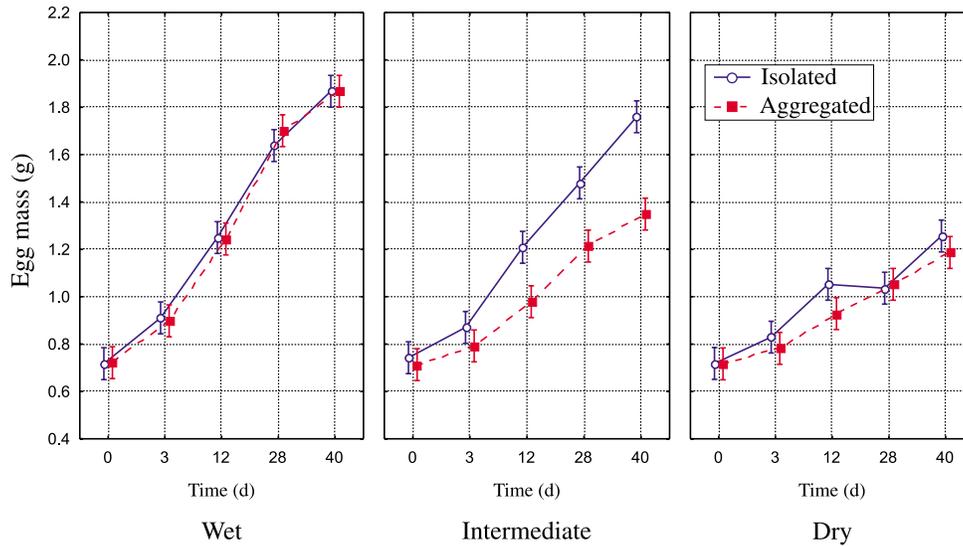


Table 2. Results of the two-way MANOVA and post-hoc two-way ANOVAs that analyse the influence of the substrate water potential and the level of egg aggregation on egg incubation and hatchling mass of Schreiber’s green lizard.

	Overall				Day 40			Duration			Hatchling mass		
	Wilks’ λ	F	df	P	F	df	P	F	df	P	F	df	P
Water potential	0.005	17.6	2, 6	<0.001	210.4	2, 6	<0.001	0.3	2, 6	0.782	39.1	2, 6	<0.001
Aggregation	0.067	18.5	1, 6	0.008	30.6	1, 6	0.001	<0.1	1, 6	0.968	15.5	1, 6	0.008
Interaction	0.060	4.11	2, 6	0.035	16.3	2, 6	0.004	1.6	2, 6	0.279	8.21	2, 6	0.019

Fig. 2. Influence of substrate water potential and egg aggregation (○, isolated; ■, aggregated) after 40 days of exposure to experimental conditions on egg mass of Schreiber’s green lizard eggs.

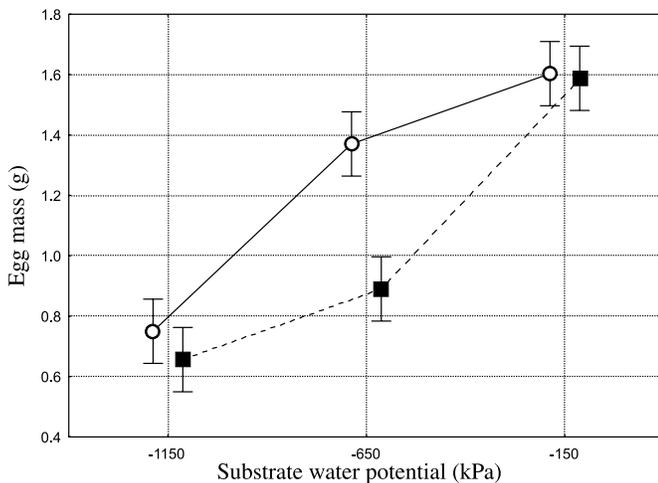
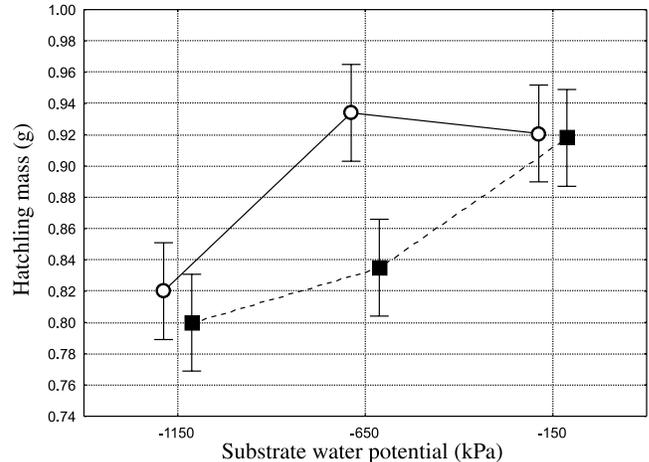


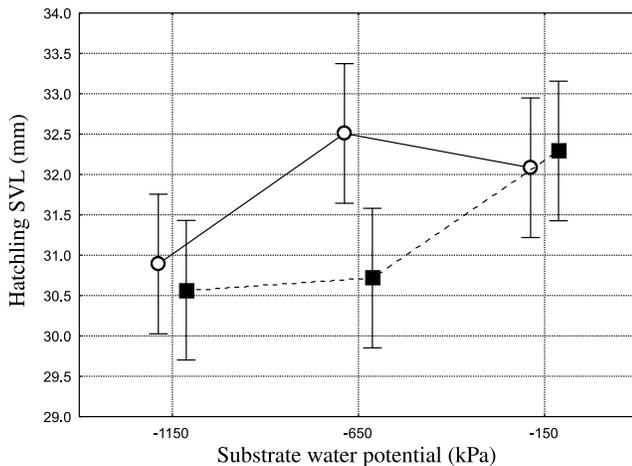
Fig. 3. Influence of substrate water potential and egg aggregation (○, isolated; ■, aggregated) during incubation on mass of Schreiber’s green lizard hatchlings.



Booth (2002) also documented beneficial effects of wet substrates in rigid-shelled eggs. There is also evidence of this relationship in lizards such as those from the families Iguanidae (Muth 1980; Tracy and Snell 1985; Phillips et al. 1990) and Phrynosomatidae (Packard et al. 1980; Tracy 1980). Wetter substrates increase egg water absorption, hatchling size, and even egg survival more so than dryer substrates. The results of our study demonstrate the influ-

ence of soil moisture on flexible-shelled eggs and embryonic development in a lacertid species. Incubation in wetter substrates resulted in higher water uptake by the eggs and larger hatchlings. Eggs incubated at -1150 kPa suffered a significant water loss, but embryos survived until hatching and hatchlings did not show alterations or abnormalities. The eggs are thus very tolerant to very dry soil conditions. An earlier study on the lacertid *Podarcis muralis* did not find

Fig. 4. Influence of substrate water potential and egg aggregation (○, isolated; ■, aggregated) during incubation on snout–vent length (SVL) of Schreiber's green lizard hatchlings.



beneficial effects of high soil water potential on embryonic development (Ji and Braña 1999).

Hatching at a larger body size may be very advantageous to reptiles. Large or well-hydrated hatchlings usually survive better than small ones during the neonatal period (Ferguson and Fox 1984; Vleck 1991). For example, large hatchlings may be better than small ones emerging from the dry nests at avoiding predators or capturing larger prey (Packard 1991; Janzen et al. 2000). Hatchlings with higher water contents at hatching would also be able to survive longer and to lose more water before physiological performance is adversely affected by desiccation (Finkler 1999). Although there are many reasons to expect it, bigger is not always better (Congdon et al. 1999).

Some factors such as egg surface area, characteristics of eggshell, the proportion of eggshell contacting the substrate, soil water potential, and temperature affect the exchange of water between a reptilian egg and its environment (Packard and Packard 1988; Ackerman 1991). In this study, we demonstrate that the number of eggs which develop together can also influence water exchange. Eggs that develop together have less contact with the substrate than solitary eggs. The probable immediate effect is that aggregated eggs have a lower capacity of water uptake through the eggshell. This phenomenon was evidenced early in the incubation process, when all aggregated eggs had lower mass than isolated ones. However, this initial difference in water uptake was smoothed throughout the incubation period in the extreme moisture treatments. At the end of the experiment, there were no differences in egg or hatchling size between isolated and aggregated eggs in the driest and wettest conditions. Only in the intermediate moisture treatment did isolated eggs have a larger egg mass, which finally favored embryo development and bigger hatchlings. Substrate moisture clearly affected the influence of aggregation on water absorption and embryonic development.

In the wettest treatment, there were no differences between aggregated and solitary eggs. Perhaps, when eggs are in wet environments, there is enough water available for all of the eggs. However, when water is relatively scarce, aggregated eggs can quickly deplete the water available in their

surroundings. In this situation, aggregated eggs would compete among themselves for water, a vital and limited resource. A different ability to absorb the scarce water among the aggregated eggs could explain the higher variability in egg mass increase and hatchling size that we detected in the intermediate moisture treatment. However, solitary eggs would not have competitors for the scarce available water in the immediate neighborhood, and net water exchange would be higher and variability among eggs would be lower. In the driest treatment, eggs absorbed little water and hatchlings were very small, but there were no differences between aggregated and solitary eggs. We believe that the available water was so scarce that water absorption by both solitary and aggregated eggs was very low and it was very difficult to detect significant differences in water exchange between solitary and aggregated eggs.

Aggregated eggs were incubated in groups of six, and no eggs were totally isolated from the nest environment. Clutch size in large reptiles is usually larger than six. For example, the mean clutch size for the selected species for this study is 14 eggs, and there are usually egg masses with internal eggs that have no physical contact with the nest substrate (Marco et al. 1994). In large egg masses, aggregation may have different consequences on egg water uptake depending on their position in the nest, as many of them do not contact the substrate. The absence of contact with the substrate could be complete for eggs that develop inside a large egg mass. For that reason, we believe that the influence of egg aggregation on water uptake and embryonic development in larger egg masses could be stronger than we detected in our study. One centimetre of separation among eggs has been enough to detect an effect of aggregation on egg mass. Nests of single eggs are usually separated from adjacent nests by much more than 1 cm. The selected distance among isolated eggs avoided physical contact among eggs but could have permitted an interaction among the water potentials of the surroundings of each egg. If this is the case, greater distances among eggs probably would have produced stronger effects of aggregation.

We have found aggregation to be detrimental to egg metabolism efficiency in relatively dry substrates. The scarcity of water in the soil is a very common environmental condition during the incubation period of many reptile species with soft-shelled eggs (Packard and Packard 1988). However, many reptiles from arid zones lay large egg masses in a single nest. No dispersion of the eggs of a given clutch has been reported for any reptile species. Only species that lay more than one annual clutch may disperse their annual output in successive and separated nests. In addition to the concentration of eggs in a given clutch in a single nest, communal egg laying has been reported in which several females of the same or different species share the same nest or alternatively use the same gallery and bury their eggs in connected nests (Graves and Duvall 1995). Benefits associated with egg aggregation should be more important than the cost of competition among eggs for soil water. For a single female, the costs of nest site excavation in time, energy, or predation risk are probably very high. Also, other females may save considerable time, energy, or predation risk using the nest excavated by another female (Bock and Rand 1989; Burger and Zappalorti 1991) or may burrow a deeper nest

with the same effort using the gallery of a previous nest (Wiewandt 1982). Egg aggregation could also be related to the female attraction to restricted sites of superior quality for nesting (reviewed in Graves and Duvall 1995). Egg aggregation may also benefit hatchling emergence by several individuals using the same gallery or hatching survival by reducing predation risk.

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