

# Varying Hydric Conditions during Incubation Influence Egg Water Exchange and Hatchling Phenotype in the Red-Eared Slider Turtle

Virginie Delmas<sup>1,\*</sup>

Xavier Bonnet<sup>2</sup>

Marc Girondot<sup>1</sup>

Anne-Caroline Prévot-Julliard<sup>1</sup>

<sup>1</sup>Université Paris-Sud, Laboratoire Ecologie, Systématique et Evolution, Unité Mixte de Recherche 8079, Orsay F-91405, France; and Centre National de la Recherche Scientifique, Orsay F-91405, France; <sup>2</sup>Centre d'Etudes Biologiques de Chizé, Centre National de la Recherche Scientifique–Unité Propre de Recherche 1934, Villiers en Bois, Beauvoir sur Niort F-79360, France

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## ABSTRACT

Environmental conditions within the nest, notably temperature and moisture of substrate, exert a powerful influence during embryogenesis in oviparous reptiles. The influence of fluctuating nest temperatures has been experimentally examined in different reptile species; however, similar experiments using moisture as the key variable are lacking. In this article, we examine the effect of various substrate moisture regimes during incubation on different traits (egg mass, incubation length, and hatchling mass) in a chelonian species with flexible-shelled eggs, the red-eared slider turtle (*Trachemys scripta elegans*). Our results show that the rate of water uptake by the eggs was higher in wet than in dry substrate and varied across development. More important, during the first third of development, the egg mass changes were relatively independent of the soil moisture level; they became very sensitive to moisture levels during the other two-thirds. Moreover, hydric conditions exerted a strong influence on the eggs' long-term sensitivity to the moisture of the substrate. Even short-term episodes of high or low levels of moisture modified permanently their water sensitivity, notably through modification of eggshell shape and volume, and in turn entailed significant effects on hatchling mass (and hence offspring quality). Such complex influences of fluctuating moisture levels at various incubation stages on hatchling phenotype

better reflect the natural situation, compared to experiments based on stable, albeit different, moisture levels.

## Introduction

In oviparous reptiles, females generally abandon their eggs in a subterranean nest cavity. Thermal and hydric conditions within the nest exert powerful effects on embryogenesis. Hatching success and neonate phenotype are sensitive to ambient conditions during incubation, and long-term posthatching effects have also been documented (Deeming 2004; Rhen and Lang 2004). The influence of environmental temperature on reptile embryos and hatchlings has been well established for a wide array of species. Thermal environment can strongly influence embryonic growth rate (Deeming and Ferguson 1989; Georges et al. 2005), incubation length (Yntema 1978), embryo survival (Brooks et al. 1991), the sex of the hatchlings in species with temperature-dependent sex determination (Bull 1980), and a wide range of traits, such as neonate morphology (Brana and Ji 2000; Ji and Du 2001), behavior (Burger 1998), and survival rates (Brooks et al. 1991; Andrews et al. 2000). Therefore, thermal selection of the optimal nest site by females is a critical factor for their reproductive success (Warner and Andrews 2002a; Brown 2004).

Fewer studies have addressed the effects of specific substrate moisture during incubation. Water availability in the nest can fluctuate greatly and suddenly during the incubation period, owing to changing atmospheric humidity and temperature (Packard et al. 1985). The hydric environment can have important consequences for embryonic development (Packard 1991). Sufficient water availability within the nest is needed to support embryogenesis in several reptile species (Packard 1999), and unsuitable hydric conditions may result in embryo death or hatching failure (Gettinger et al. 1984; Tucker and Paukstis 2000). Several studies have reported that flooding might be a significant factor in nest failure (Moll and Legler 1971; Janzen 1994), increasing mortality being associated with increasing immersion intervals (Plummer 1976; Kam 1994). Embryo death results from the influx of water into the egg, which provokes egg swelling and the rupture of the shell and membranes. Extremely moist conditions can also increase fungal infections and invasion by other microorganisms (Tracy 1980), which can reduce gas exchange (Packard and Packard 1984). Conversely, extreme dry conditions lead to important water loss and lethal desiccation of the egg because the embryos need a minimal quantity of water to complete development

\* Corresponding author. Present address: Centre National de la Recherche Scientifique–Unité Propre de Recherche 1934, Villiers en Bois, Beauvoir sur Niort F-79360, France; e-mail: virginie.delmas@u-psud.fr.

(Muth 1980; Packard et al. 2000). Water availability within the nest also influences embryo growth rate, metabolism, and incubation length in some species (Morris et al. 1983; Miller and Packard 1992; Janzen et al. 1995). On wet substrate, embryos mobilize yolk reserves more efficiently, incubate longer, and yield larger hatchlings than embryos incubated on dry substrate (Gutzke and Packard 1987; Finkler 1999). Therefore, water availability during incubation influences posthatching traits such as locomotor performance, growth, and survival of neonates (Finkler 2001; Packard and Packard 2001).

Not all the above effects have been observed in all reptile species; some effects have even been refuted by several authors (Marco et al. 2004; Brown and Shine 2005). Such controversies highlight the difficulty in generalizing results regarding the effects of water availability on developing reptile embryos and hatchlings. They also suggest that the influence of moisture on incubating eggs differs between reptile species (Tucker and Paukstis 2000). The amount of water present in the substrate and required for successful development of the embryos varies considerably, even among closely related species, and hatching success can be compromised by either deficit or excess of humidity. It is therefore crucial to increase the diversity of detailed studies on a wide range of species from a wide range of environments in order to assess the validity of current hypotheses concerning the effects of moisture during incubation or to propose reliable conclusions about the water requirements of reptile embryos. Unfortunately, the majority of previous studies have been conducted under constant and extreme hydric conditions that are not ecologically realistic when compared to the fluctuations of substrate moisture recorded in natural nests (Packard et al. 1985; Booth 2002; Shine and Brown 2002; Brown and Shine 2005). It is more likely that eggs in natural nests experience somewhat unpredictable fluctuations in water availability. Both wetness and dryness events may occur at different times during the incubation period. Studies performed under ecologically realistic scenarios (i.e., fluctuating hydric regimes) are therefore required. Fluctuating water availability within the nest may be particularly important for species that dig shallow nests, where the eggs may encounter several drought and flooding events during a given incubation period, which in turn can affect the hydric environment inside the egg (Plummer 1976; Cagle et al. 1993; Kam 1994). The water potential (i.e., the retention force of water by the substrate) partly controls water exchanges between the eggs and the nesting environment (Packard 1999). Other factors, such as egg size or the structure and thickness of the eggshell, can also play a role (Booth 2002; Marco et al. 2004). Response of eggs to substrate moisture seems to be very important in reptile species that lay eggs with a flexible shell compared to species that lay rigid-shelled eggs (Packard 1999). Flexible-shelled eggs desiccate rapidly when they are exposed to dry conditions but absorb large quantities of water when they are exposed to wet environments (Feder et al. 1982; Ackerman et al. 1985). In comparison, rigid-shelled eggs appear to be less affected by hydric conditions within the nest during incubation (Packard 1999; Booth 2002).

In this study, we examined the effects of fluctuating substrate moisture during incubation, independent of incubation temperature, on various traits: egg mass changes, incubation length, hatching success, and hatchling mass. We imposed various experimental shift combinations of the substrate water potential on a large number of eggs (188 eggs from 26 clutches) in a species with flexible-shelled eggs, the red-eared slider turtle (*Trachemys scripta elegans*). Over the entire period of embryonic development, we manipulated both the timing and the number of the hydric shifts imposed on the eggs as well as the proportion of time the eggs spent on a dry versus a wet substrate.

We emphasize that a somewhat complex experimental design was required to examine the response of eggs and embryos to fluctuating substrate moisture representing more realistic conditions than those used in previous studies. Notably, consecutive brief shifts of water potential likely represent the alternation of rainfalls and hot and dry days better than conditions maintained at constant levels over prolonged periods.

The main question addressed in this study was whether brief exposures to a dry or a wet substrate can influence subsequent long-term water uptake or water loss by the eggs. Our experimental design also enabled us to examine the effect of the proportion of time exposed to different substrate moistures. Finally, we had the opportunity to explore the consequences to the hatchlings of various hydric treatments during incubation. Such study of the effects of varying water potential on neonate characteristics may thus be crucial to understanding the pattern of selection on life-history traits that are likely to influence individual fitness. It may also be crucial in conservation biology, because effects of water potential on incubation length, hatching success, and hatchling phenotype partly determine the conditions in which a species could acclimate in a new environment.

## Material and Methods

### Egg Collection

Twenty-six gravid female slider turtles *Trachemys scripta elegans* (Wied, 1839) were collected between June 1 and June 4, 2003, from the zoological "Association Tortues Passion" facility (Vergèze, France). To facilitate clutch identification, each female was isolated in a plastic box with some water. Oviposition was induced by an intramuscular injection of 1 mL (i.e., approx. 4 IU) of oxytocin (Ewert and Legler 1978). The eggs were referenced by a code number on the eggshell and weighed to the nearest 0.001 g with a Sartorius electronic balance. The eggs were placed on wet cotton in a cool box to prevent desiccation and shocks during transfer to the laboratory, which lasted approximately 48 h.

### Incubation of the Eggs under Laboratory Conditions

On June 7, 188 fertilized eggs, recognizable by a white spot on the eggshell, were selected from 26 clutches and distributed equally among 19 plastic boxes covered to prevent water evap-

oration (i.e., each box contained roughly 10 eggs). Eggs from the same clutch were randomly distributed to different boxes to prevent the confounding box and clutch effects. Eggs were totally buried in moistened vermiculite and incubated in two programmable incubators (IPP 200-400, Memmert, Schwabach, Germany) at a constant temperature of  $28.9^{\circ} \pm 0.1^{\circ}\text{C}$ , which is estimated to yield both sexes in equivalent proportions for this species (Godfrey et al. 2003). Every other day, the boxes were rotated to control for possible local variations of temperature within the incubators. In natural nests, thermal and hydric conditions probably fluctuate in partial synchrony (dry and hot vs. wet and cold periods). Under experimental conditions, it would be logistically difficult to tease apart the respective effects of temperature and moisture if both fluctuate during incubation, so here we tested the influence of the shifts in moisture independent of temperature fluctuations.

#### Experimental Design

The hydric treatments imposed on the eggs aimed to simulate fluctuations of substrate moisture during incubation likely encountered by the clutches within natural nests. We defined three water potentials of substrate for the experiment: (i) a “wet substrate” ( $-10$  kPa: 2 g of sterilized water/g of vermiculite) that represented soil after rainfalls, (ii) an “intermediate substrate” ( $-398$  kPa: 0.44 g of sterilized water/g of vermiculite), and (iii) a “dry substrate” ( $-1,000$  kPa: 0.09 g of sterilized water/g of vermiculite) that represented soil during a drought event. These values of water potential were obtained from the establishment of the function linking the volumetric water content and the water potential of the medium used (i.e., vermiculite) in our experiment. This relation was obtained by a centrifugation method and from weights of water-saturated medium samplings after successive centrifugation speeds (B. Verdier, personal communication).

At the laboratory, the eggs were exposed during the first few days (i.e.,  $3 \pm 2$  d) to a substrate that was intermediate in terms of water potential, until the beginning of embryogenesis. This procedure ensured that all eggs began embryogenesis under favorable conditions. Afterward, among all the hydric treatments (i.e., boxes), one box of eggs remained in the intermediate substrate during the entire course of incubation. For the other boxes, one or two shifts of the substrate moisture level during incubation generated various hydric perturbations, as described in Figure 1. These shifts were imposed at different times during the incubation period, but with a time period of 10 d between shifts. For example, some eggs were first exposed to a brief wet or dry episode during early development and then remained in the intermediate substrate for the rest of incubation, while other eggs experienced either prolonged wet or dry conditions during the entire incubation period. For the statistical analyses, we divided the whole incubation duration (i.e., approx.  $63 \pm 2$  d at  $28.9^{\circ}\text{C}$ ; V. Delmas, unpublished data; also see “Results”) into three equal periods (P1, P2, and P3) of 20 d (Fig. 1). The shifts in substrate moisture were organized

as follows: at the beginning of each period, two sets of three boxes were transferred from intermediate substrate to either wet or dry substrates. Within each period, two of the three boxes were finally transferred again to a different moisture substrate, while the third box remained at the same moisture until hatching (Fig. 1). This multitude of trajectories enabled us to obtain a variety of hydric treatments during incubation (a total of 19 different treatments), in terms of both timing and duration of the perturbations. The succession of the intermediate, wet, and dry episodes was therefore variable among all 19 treatments. Nevertheless, regardless of the timing of the shifts, the total duration spent in each moisture substrate was similar for several treatments (Fig. 1). Moreover, for each incubation period (P1, P2, and P3), a minimum of two boxes (i.e., approx. 20 eggs) experienced the same moisture level (Fig. 1). Overall, the broad effects of dry versus wet substrates were replicated at two levels: first, each box contained the eggs from roughly 10 different females; second, several boxes experienced comparable broad wet versus dry conditions over incubation. These two levels of investigation (broad substrate moisture effects and specific trajectory of each of the 19 treatments) were taken into account in the statistical analyses.

#### Data Collection

Every other day, from the beginning of embryogenesis until hatching, the eggs were individually weighed with a Sartorius electronic balance (to the nearest 0.001 g). Before replacing the eggs, the boxes were weighed to readjust the water potential by replacing the water evaporated and/or absorbed by the eggs or by replacing the totality of the substrate if necessary. Eggs ( $N = 12$ ) covered by fungus or other aborted eggs were removed and opened to examine their contents. Among them, nine eggs with no viable embryos were excluded from the statistical analyses investigating the changes in egg mass during incubation. After pipping and before hatching, each egg was placed on wet cotton in an individual plastic cup. The duration of incubation was defined as the elapsed time between the appearance of the white spot on the eggshell and hatching. After emergence, the turtles were maintained in the humidified cup until total absorption of the yolk (i.e.,  $3 \pm 2$  d). The hatchlings were then weighed to the nearest 0.001 g with an electronic Sartorius balance.

#### Statistical Analyses

All data were tested for normality (Kolmogorov-Smirnov) and for homogeneity (Bartlett’s test) before statistical analysis. We used a mixed linear model to analyze normally distributed data and a generalized mixed model for the data with a nonnormal distribution. Statistical analyses were performed using SAS for Windows, version 9.1 (SAS Institute 2003). A backward stepwise procedure was used to select minimal models, according to the Akaike Information Criterion (AIC) for model comparison (Burnham and Anderson 1998).



dividual covariate), hydric treatment during incubation, and all two-way interactions were defined as fixed factors. Hydric treatment was represented by the hydric stress, measured as the proportion of time spent by each egg in a dry substrate during the course of incubation.

*Hatchling Mass.* We used a mixed linear model. Clutch identity and its interactions were defined as random factors. Initial egg mass (as individual covariate) and the proportion of time elapsed in dry substrate during incubation period, in addition to the two-way interactions, were defined as fixed factors.

## Results

### *Changes in Egg Mass during the Course of Incubation*

We found a significant clutch (=maternal) effect on the changes in egg mass during incubation (model 1 in Table 1). All the eggs exhibited marked changes in mass over incubation time (Fig. 2). Such changes were systematically positive under wet or intermediate conditions. They tended to be negative in dry substrates, although not systematically, notably toward the end of incubation. As expected, we observed a significant effect

of the current water potential of the substrate, with a greater increase of egg mass in the wet substrate than in the intermediate and dry substrates (Fig. 2). The rates of the egg mass changes, in interaction with the different substrates, varied significantly according to the incubation period (Table 1). All the effects were more pronounced in the last two-thirds of incubation (Fig. 2). Interestingly, we also found significant effects of the incubation period and of the substrate previously encountered by the eggs (i.e., the memory effect). The memory effect also varied significantly according to the incubation period and with the current water potential of substrate. There was no other significant effect (model 1 in Table 1). The analysis performed using the second model (model 2 in Table 1) confirmed the significant effect of clutch identity. It revealed a significant effect of the initial egg mass, in addition to a wide-ranging clutch effect, on the changes in egg mass over time. The effect of the hydric treatment remained significant only during the two last periods of incubation (Fig. 2).

### *Hatching Success*

The vast majority (93%) of the eggs developed successfully: 176 healthy hatchlings emerged from the 188 incubated eggs. Mor-

Table 1: Significant factors that influenced changes in egg mass during incubation in the red-eared slider turtle

	Model 1		Model 2	
	Tests	<i>P</i>	Tests	<i>P</i>
Random effects:				
Clutch	$Z = 2.25$	.0122	$Z = 2.19$	.0141
Clutch $\times t$	$Z = 2.37$	.0090	$Z = 2.16$	.0152
Fixed effects:				
$t$	$F_{1, 24} = 15.58$	.0006	$F_{1, 24} = 6.99$	.0142
Initial egg mass	$F_{1, 4,383} = 1,115.5$	<.0001	$F_{1, 4,387} = 1,135.2$	<.0001
Substrate	$F_{2, 4,383} = 232.86$	<.0001	...	...
Period (P1, P2, P3)	$F_{2, 4,383} = 6.83$	.0011	...	...
Memory	$F_{2, 4,383} = 42.52$	<.0001	...	...
Substrates in P1	...	...	$F_{2, 4,387} = .42$	.6574
Substrates in P2	...	...	$F_{8, 4,387} = 1.13$	.3380
Substrates in P3	...	...	$F_{6, 4,387} = 2.46$	.0225
Initial egg mass $\times t$	$F_{1, 4,383} = 1.33$	.2486	$F_{1, 4,387} = 5.04$	.0248
Substrate $\times t$	$F_{2, 4,383} = 93.32$	<.0001	...	...
Period $\times t$	$F_{2, 4,383} = 7.00$	.0009	...	...
Memory $\times t$	$F_{2, 4,383} = 18.39$	<.0001	...	...
Period $\times$ substrate $\times t$	$F_{4, 4,383} = 139.02$	<.0001	...	...
Period $\times$ memory $\times t$	$F_{4, 4,383} = 25.87$	<.0001	...	...
Substrate $\times$ memory $\times t$	$F_{4, 4,383} = 117.21$	<.0001	...	...
Substrates in P1 $\times t$	...	...	$F_{2, 4,387} = 3.63$	.0366
Substrates in P2 $\times t$	...	...	$F_{8, 4,387} = 6.76$	<.0001
Substrates in P3 $\times t$	...	...	$F_{6, 4,387} = 23.36$	<.0001

Note. In model 1, we tested separately the influence of the incubation periods (P1, P2, and P3) and of the hydric treatment (moisture of the substrate). In model 2, we examined the influence of the hydric treatment within each incubation period (moisture of substrates in P1, P2, and P3). Only results with main factors and significant interactions with time  $t$  are reported. Ellipses indicate that there is no tested effect in the corresponding model.

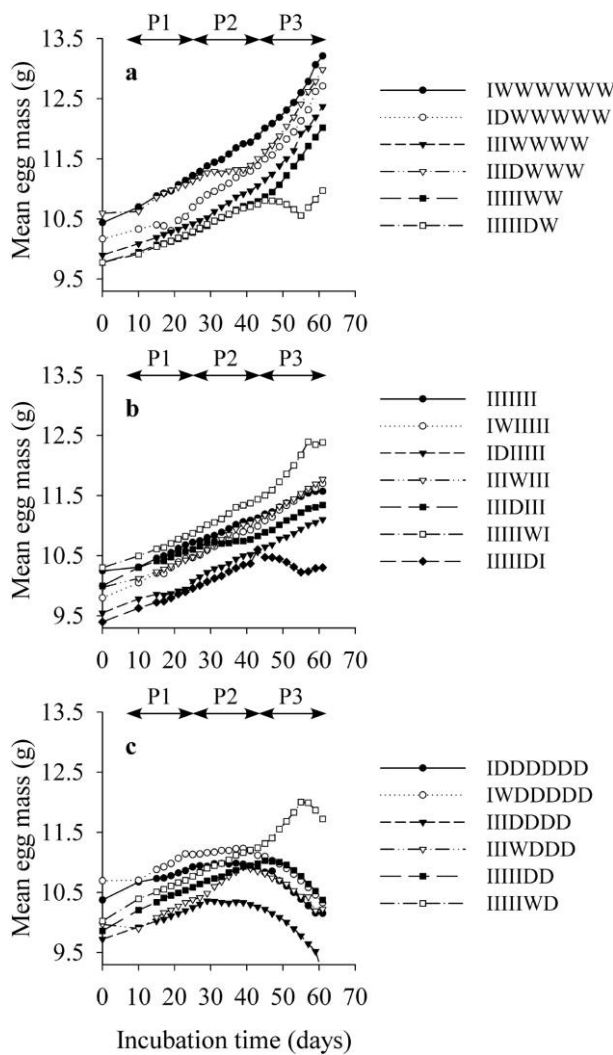


Figure 2. Profiles of the mean changes in egg mass during incubation. Each point represents the mean value calculated on  $\approx 10$  eggs; the standard variations were always small and are not reported, for ease of reading. The 19 trajectories are grouped as follows: (a) eggs that completed incubation in a wet substrate, (b) eggs that completed incubation in an intermediate substrate, and (c) eggs that completed incubation in a dry substrate.

tality was limited to eight eggs aborted at an early stage of embryogenesis (perhaps because of fungal infections) and four eggs aborted after rupture of the eggshell during the last incubation period. Owing to the very low mortality, we were unable to perform a statistical analysis concerning hatching success as a function of hydric treatment of the eggs during incubation.

*Incubation Length*

According to the best model, there was a significant effect of clutch identity (Table 2), in addition to a positive effect of initial egg mass (Fig. 3a), on incubation length. We also found a

negative effect of the proportion of time that the eggs spent in dry substrate on the duration of the incubation (Fig. 3b). There was no significant effect of the two-way interactions.

*Hatchling Mass*

The selected model suggested that there was a significant effect of clutch identity, as well as a marked positive effect of initial egg mass, on hatchling mass (Table 3; Fig. 4a). The proportion of time that the eggs spent in dry substrate during incubation was also significantly and negatively correlated with hatchling mass (Table 3; Fig. 4b). The eggs that spent more time in dry substrate produced lighter hatchlings. Finally, no effect of the interactions was detected.

**Discussion**

*Changes in Egg Mass during Incubation*

Some of our results were expected, notably, the fact that the changes in egg mass all the way through the incubation period were positively influenced by the moisture level of the substrate (Ackerman et al. 1985; Cagle et al. 1993). The eggs maintained in a wet substrate gained significantly more water than the eggs incubated in intermediate and dry substrates (Gutzke and Packard 1986; Booth 2002); the latter condition even entailed a marked mass loss when applied for long time periods (Fig. 2c). Under wet conditions, less water is retained from capillarity and other forces generated by the substrate, and water is more likely to cross the shell to penetrate into the egg. Conversely, a dry substrate can provoke desiccation of the eggs.

Thanks to a relatively comprehensive experimental design, our data provided new insights. First, the alternation of dry (i.e., mimicking drought) and wet (i.e., rainfalls) substrates to incubate the eggs over the whole incubation period (2 mo) was more realistic than constant hydric conditions commonly used in previous studies. This novel approach allowed examining the specific effects of different moisture levels and their interactions with various successive changes in the moisture level throughout incubation. At the end of the experiment, we had 19 different hydric treatments; a situation more representative of the variety of the natural conditions experienced by clutches in the field. Because we maintained a constant temperature, we can focus on the effect of moisture per se. Besides the relative complexity of our design, we had several replicates at any time (i.e., more than one box of 10 eggs belonging to different fe-

Table 2: Main factors that influenced incubation duration

	Tests	P
Random effects:		
Clutch	Z = 2.19	.0302
Fixed effects:		
Initial egg mass	$F_{1, 136} = 4.80$	.0308
Time in dry substrate	$F_{1, 136} = 17.46$	<.0001

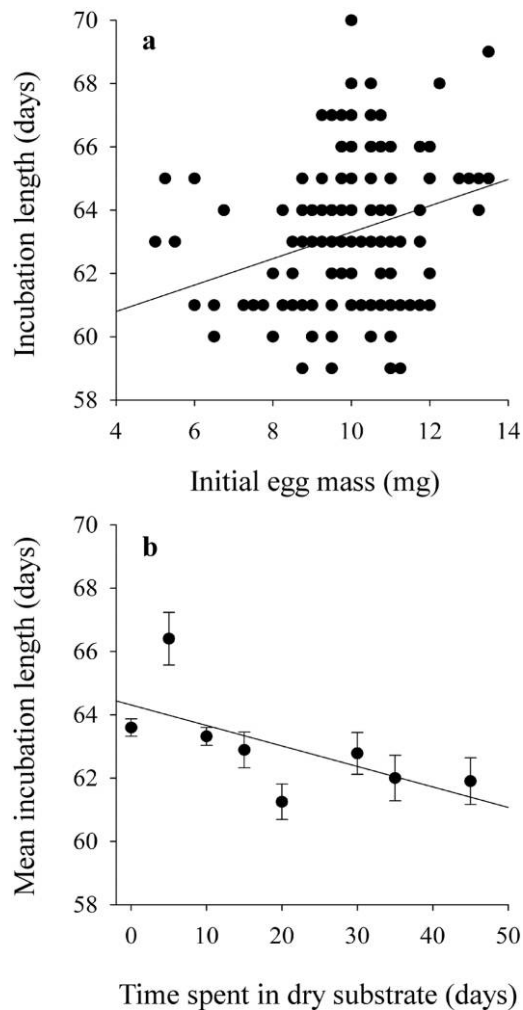


Figure 3. Relationships between incubation length and initial egg mass or time spent in dry substrate. *a*, Incubation length as a function of initial mass of the egg. *b*, Mean incubation length ( $\pm$  SE) as a function of the amount of time spent by the eggs in dry substrate throughout incubation period.

males) for each moisture level. Similarly, several boxes spent the same total amount of time within each moisture level. Overall, although the design generated somewhat complex results (Fig. 2), broad patterns emerged.

The repeated measures throughout incubation revealed important variations in the sensitivity of the eggs in relation to different hydric conditions. First, eggs' water exchanges were not critically affected by the different water potentials of the substrate we imposed during the first third of incubation (Fig. 2). In wet and intermediate substrates, all eggs increased in mass at a similar rate (Table 1). In dry substrates, none of the eggs lost water; a slight increase in mass was instead observed in most cases (Fig. 2). Second, the ability displayed by eggs during early incubation (i.e., the first third) to extract water from the soil changed over time. Notably, after 40 d of incubation, dry conditions systematically resulted in a marked loss

of water (Fig. 2c). By contrast, the eggs that completed the incubation in a wet substrate continued to absorb significant amounts of water (Fig. 2a), with a significant acceleration of this process; such an effect was also apparent, although less marked, in an intermediate substrate (Fig. 2b). Overall, during the first third of development, water uptake by the eggs was relatively independent of soil moisture, but later, it became very sensitive to this key parameter, especially during the last third of incubation (Table 1; Fig. 2). These results are in agreement with previous work on other species with flexible-shelled eggs that reported (i) substantial gains of water by eggs during the first third of incubation, even in dry substrates (Packard 1999; Rimkus et al. 2002), and (ii) a significant influence of substrate moisture on egg water exchange during the final two-thirds of incubation (Gutzke and Packard 1986). Water exchange patterns of the eggs are affected by water potential of the soil but also by other physical factors, such as temperature, the proportion of the eggshell making contact with the substrate (the eggs were totally buried in our experiment), and the thermal conductivity of the medium. Peculiar interactions between these factors have been proposed to explain phases of water uptake by the eggs in very dry substrate (Packard 1999; Rimkus et al. 2002).

Previous studies suggested that the early gains of water, during the first third of incubation, modified the osmotic concentration inside the eggs, as well as their shape, volume, and external shell layer (M. J. Packard 1980; Packard and Packard 1989; G. C. Packard 1999). The surface area of the eggshell in contact with the substrate increases with the quantity of water absorbed; a positive relationship between water uptake and egg size (i.e., surface area) has been documented in turtles (Ackerman et al. 1985; Packard 1999; Marco et al. 2004). Moreover, when eggs swell, there is an acute alteration in the arrangement of the adjacent mineral shell units that compose the eggshell, which, in turn, modify conductance of the eggshell (Feder et al. 1982). Finally, a gradual exfoliation of the external layer, with a concomitant decrease in the resistance to water movement through the egg layers, has been observed toward the last third of incubation (Feder et al. 1982; Packard and Packard 1989). Such changes in the structure of the shell affect water conductance across the eggshell. At the same time, the changing osmotic concentration likely modifies the water potential difference across the eggshell, which, in turn, alters the driving force involved in water exchange. The growing embryo modifies the chemical composition, structure, and functioning of the

Table 3: Main factors that influenced hatchling mass

	Tests	<i>P</i>
Random effects:		
Clutch	$Z = 2.94$	.0017
Fixed effects:		
Initial egg mass	$F_{1,136} = 266.71$	<.0001
Time in dry substrate	$F_{1,136} = 4.63$	.0331

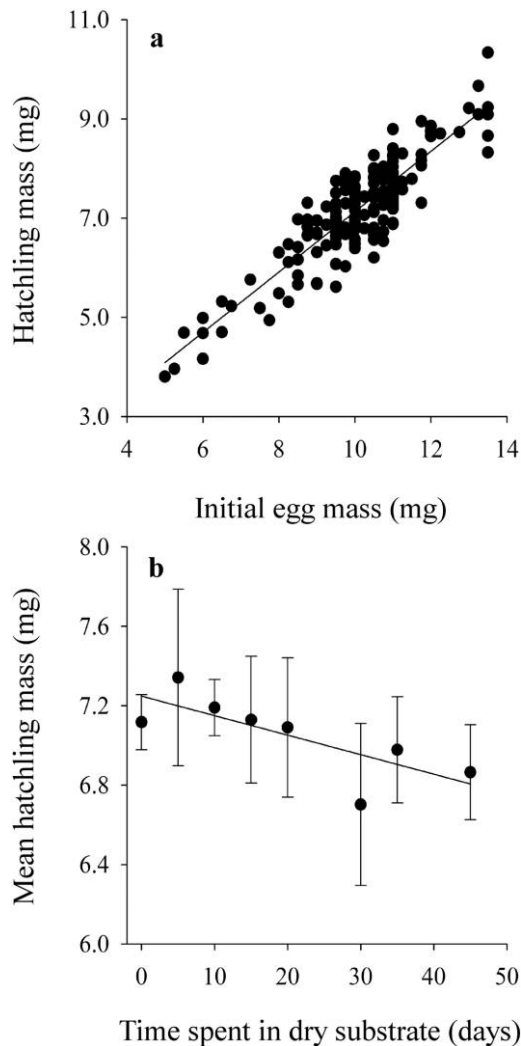


Figure 4. Relationships between hatchling mass and initial egg mass or time spent in dry substrate. *a*, Hatchling mass as a function of the initial mass of the egg. *b*, Mean hatchling mass ( $\pm$  SE) as a function of the amount of time spent by eggs in dry substrate throughout incubation period.

egg (e.g., via an increasing consumption of the lipids of the yolk). At the beginning of incubation, most of the egg is composed of the relatively inert and homogeneous yolk. By contrast, at the end of incubation, most of the yolk has been metabolized and incorporated into embryonic structures, and the embryo itself requires significant exchanges of oxygen and carbon dioxide through the shell (Booth 1998; Thompson and Speake 2003). The blood circulating in the chorioallantois just below the inside surface of the shell, as well as the egg heat production, likely increases the quantities of exchanges through the shell between all parts of the egg and the exterior (Rimkus et al. 2002). Such major changes over time perhaps explain the range of sensitivities of the egg to the moisture of the substrate during incubation.

Besides these general patterns, we found significant effects

associated with the succession of the dry/wet conditions of the substrate. The significant memory effect that we observed implies that the rate of water uptake by the eggs depends not only on the current situation (combination of substrate moisture and embryonic development stage) but also on the hydric conditions previously encountered by the eggs. In this experiment, we observed a short-term memory effect; for instance, the eggs responded differently with regard to the water potential they encountered 5 d before. Importantly, brief wet or dry episodes occurring at any time during incubation influenced significantly the water sensitivity of the eggs. These results complement those of a previous study that documented a long-term memory effect (Gutzke and Packard 1986). In their experiment, Gutzke and Packard (1986) incubated the eggs in either wet or dry environments and later transferred half of them to the other treatment (hence, such a design did not include any fluctuation or brief shift of moisture level). Compensatory water exchanges (e.g., rapid uptake of water for the eggs transferred from dry to wet substrate) were limited to the first two-thirds of incubation. Later, initial wet conditions of incubation generated a high sensitivity of the eggs to water exchanges, similar to what we observed in our study (Gutzke and Packard 1986). Although we cannot tease apart the respective influence of the changes in the shell structure (e.g., modification of the shell permeability) and the changes of the composition of the egg (e.g., development rate of the embryo), our result suggests that even brief episodes of drought or rainfall can significantly influence the development and the porosity of the egg later in incubation. For instance, an early exposure to wet conditions may permanently modify the shell permeability and increase the sensitivity (vulnerability) of the eggs to marked losses of water in the face of a putative subsequent drought (Feder et al. 1982; Packard and Packard 1989). These kinds of results are important in terms of both fundamental ecology and conservation, especially because many freshwater turtles dig shallow nests particularly exposed to precipitation fluctuations. Overall, our results suggest that a flooding or a drought event occurring in the final third of the incubation period may have serious consequences on the developmental trajectory of the embryo, through its water reserves, and consequently on the hatchling mass (Tucker et al. 1997). In addition, these consequences, essentially manifested at the end of incubation (i.e., the last third of incubation), also result from short- and long-term effects of the water flux between the egg and its environment occurring in the first two-thirds of incubation, a far more complex situation than previously thought.

#### *Substrate Moisture, Hatching Success, Incubation Length, and Hatchling Mass*

In our experiment, the vast majority of the eggs produced healthy offspring. Perhaps the eggs of *Trachemys scripta elegans* are extremely resistant to chaotic hydric conditions; we imposed drastic shifts of water potential during incubation. However, other studies reported elevated mortality in various species,

notably under dry conditions (Packard 1999; Ji and Du 2001; Rimkus et al. 2002), including our study model, *T. scripta elegans* (Tucker et al. 1997). In an oviparous snake species, it has been shown that dry conditions cause marked yolk coagulation, the solid mass of yolk directly impairing the hatching process (Aubret et al. 2005). Similarly, an excess of water can entail significant mortality during incubation (Moll and Legler 1971; Janzen 1994). Importantly, these studies were performed under various incubation temperature regimes (constant or fluctuating), a factor that also influences water exchange between the eggs and the environment (Ji and Du 2001). Therefore, the conclusions of these studies are not directly comparable to our results obtained under specific thermal conditions. However, our experimental design, with an incubation temperature set at a noncritical value, enabled us to discard lethal thermal effects per se and to focus on moisture levels. Despite the fact that we exposed the eggs to drastic shifts in moisture level, hatching success and neonate survival were very high. The experimental conditions we employed always remained within a range really acceptable for the embryos. This suggests that, under favorable temperatures, even brief episodes of rain (wet or intermediate substrate) can provide enough water under generally dry conditions (dry substrate) and vice versa under wet conditions. This emphasizes the importance of considering fluctuating and more realistic conditions to interpret otherwise contrasted results.

As observed in many animal species, the duration of incubation was dependent on initial egg mass (Table 2; Fig. 3a), which in turn positively influences the mass of neonates (Brooks et al. 1991; Warner and Andrews 2002b; Aubret et al. 2005). In agreement with previous studies (Packard 1991; Tucker and Paukstis 2000), we also found a significant negative correlation between the proportion of time the eggs spent in dry substrate and incubation duration (Table 2; Fig. 3b). The mechanism involved remains unknown (Packard 1991; Janzen et al. 1995). Moisture levels may affect biochemical reactions or influence local temperature of the substrate (Yntema 1978). We also detected a negative correlation between the time that the eggs spent in dry substrate and hatchling mass (Table 3; Fig. 4b). Very likely, the dry-incubated eggs accumulated less water during incubation (Finkler 1999). Water within the eggs is known to play a key role in the mobilization of yolk reserves by reptile embryos and consequently influences hatchling mass (Packard and Packard 2001). Whatever the factors that influence hatchling mass (body reserves, in combination with water availability), they are critical for hatchling aquatic turtles. During the terrestrial migration from the nest to the aquatic habitat, mortality rates are high and are likely due to predation, desiccation, and starvation (Finkler 2001; Kolbe and Janzen 2002). Previous studies suggest that larger hatchling turtles exhibit greater locomotor performance and higher survival rate than smaller ones (Janzen et al. 2000), but see Congdon et al. (1999).

## Conclusions

Our results show that even short-term changes in nest moisture influence hatchling phenotype through body mass, both immediately through water movement across the eggshell and via potential permanent modifications to the eggshell structure and permeability. Our data clearly highlight the importance of the water potential of the nest during embryonic development in oviparous reptiles. Our complex experimental design provided new insights about substrate moisture effects and partly confirmed some findings obtained under less realistic hydric conditions. We notably found that responses of eggs and embryos to incubation conditions depend on temporal shifts as well as on the absolute amount of time exposed to different soil water contents. The fluctuations of nest moisture and the precise timing during which they occur exert a strong influence on eggs' water exchanges and embryos' development rate via a memory effect of the hydric history previously encountered by the eggs. We also pointed out that the sum of brief shifts of water potential might be less detrimental to hatching success than equivalent amounts of time over prolonged exposures, as commonly used in previous studies. Furthermore, we found correlations between the proportion of time spent in dry substrate and incubation length or hatchling mass similar to those observed under more stable and constant hydric conditions. These latter results suggest that experiments performed under constant and less realistic conditions might strongly underestimate the range of viable conditions for reptile embryos and probably mask mechanisms dependent on moisture substrate that are far more complex than previously thought. Moreover, we found significant maternal effects (i.e., clutch effect, in addition to initial egg mass effect) that play a role in determining the eggs' water exchange profile as well as the phenotype of the hatchlings. Further studies should examine whether reproductive females are able to select nest sites on the basis of their moisture availabilities, as they may do for the thermal characteristics (Warner and Andrews 2002a). A next step should integrate the intermingled effects of temperature and moisture of the substrate (Hewavisenthi and Parmenter 2001; Ji and Du 2001), as well as their respective fluctuations, to yield reliable conclusions.

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