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Comparative Biochemistry and Physiology Part A 124 (1999) 205–213

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The influence of thermal and hydric environments on embryonic use of energy and nutrients, and hatchling traits, in the wall lizards (*Podarcis muralis*)

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Received 6 May 1999; received in revised form 28 July 1999; accepted 17 August 1999

Abstract

Among (different) clutch variation in egg composition, influence of thermal and hydric environments on incubating eggs, embryonic use of energy and nutrients, and hatchling traits were studied in the wall lizard *Podarcis muralis* from a lowland population of Northern Spain. When initial egg mass was kept constant, we found that some measured egg variables such as water, lipid-free organic material, ash, calcium and magnesium in egg contents, and ash and magnesium in eggshell, remained remarkably constant, whereas other variables differed considerably among clutches. All viable eggs increased in mass over the course of incubation due to absorption of water, and mass gain during incubation was dependent on initial egg mass, temperature and substrate water potential. Variations in the wet mass of hatchlings among treatments stemmed mainly from variations in water content. Hatching success, embryonic use of energy and nutrients, and sex, size and mass of hatchlings were unaffected over a wide range of substrate moisture. The incubation length decreased as temperature increased. However, the effect of substrate moisture on duration of incubation varied with temperature. The influence of incubation temperature on the snout-vent length of hatchlings, if present, was very weak. Incubation temperatures did not affect the sex ratio and carcass dry mass of hatchlings, but significantly affected the tail length of hatchlings, with individuals from the highest temperature having the shortest tails. The energy expenditure of embryogenesis during incubation remained remarkably constant among treatments, and energy reserves in the hatchling were largely dependent on allocation of energy materials in eggs. A high incidence of dead-in-shell embryos occurred in eggs that were laid in June and, thereafter, when ambient temperatures were high. Deformed hatchlings were distributed nearly equally among treatments.

Keywords: Reptilia; Lacertidae; *Podarcis muralis*; Egg; Incubation; Calorimetry; Calcium; Magnesium; Lipids; Hatchling

1. Introduction

An embryo's internal and external environments have been known to influence its survival and development [10]. Of all external factors that may potentially influence embryonic development, temperature and moisture are undoubtedly the most important factors. Numerous studies indicate that both factors may profoundly influence hatching success, hatching time and size, behavior, skin-shedding and post-hatching growth of hatchlings [2,9,13,27,30]. In species with environmental sex determination, the two factors, temperature in particular, may also influence the sex of hatchlings [8,11,19,25].

Earlier work on the wall lizard *Podarcis muralis* also showed an influence of incubation temperature on hatching success, hatching time and size, mass, running performance and post-hatching growth rate of hatchlings [41]. However, the eggs were incubated at just one level of substrate moisture, so the extent to which moisture influences incubating eggs and resultant hatchlings remains unknown. In addition, because *P. muralis* is a multiple-clutched lizard and lays more and larger eggs in first clutches than in subsequent clutches [21], we focused here on the composition of freshly laid

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1095-6433/99/$ - see front matter
PII: S1095-6433(99)00111-7

eggs to show its influence on hatchling composition. Working in this direction could be very important in explaining variations in hatchling size and mass, because the variations might partly stem from seasonal variations in egg composition in lizards that lay multiple clutches.

The experimental protocol outlined was established to show [1] among clutch variation, by which we mean between different clutch variation, in egg composition, and [2] the influence of thermal and hydric environments on the water budget of incubating eggs, embryonic use of energy and nutrients and hatchling traits. Our purpose was to test the subtle influence of thermal and hydric environments on incubating eggs and resultant hatchlings.

2. Materials and methods

Fifty-three adult females and 10 adult males were collected on 13–25 April 1996 from a lowland population in Oviedo, Northern Spain. The captured lizards subsequently were transported to the University of Oviedo, where they were individually marked (toe-clipped) for future identification. Males, and females with various sized yolked follicles, were housed in a $2.2 \times 1.6 \times 0.5$ m^3 (length × width × height) plastic enclosure. Females bearing oviductal eggs were randomly assigned to terraria (400×500 mm^2) placed in the laboratory. Lizards were exposed to a natural light cycle and some direct sunlight. Supplementary heating with suspended lamps, dishes of water and hiding places were provided so that lizards had ample opportunities for behavioral thermoregulation during the photophase [4]. Each terrarium contained a rock-covered wooden box (100×150 mm^2) which served as the egg-laying site, filled with moist soil and vermiculite. We fed lizards with mealworms (larvae of *Tenebrio molitor*), crickets and water containing mixed vitamins and minerals. Lizards in the enclosure completed an oviposition cycle similar to the cycle described for neighboring populations in the field [3,7]. Oviposition occurred between 22 April and 7 July. Detailed data on reproductive female, clutches and oviposition cycle were reported elsewhere [21]. All eggs were measured and weighed promptly so as to avoid any uncertainty about initial mass due to loss or gain of water.

2.1. Composition of eggs

One or two eggs from each of the clutches with more than three eggs were opened at laying. Egg contents (yolk plus embryo, about stage 27 or 28 [6,14]) were removed and weighed. Shells were rinsed in distilled water, dried by blotting with a paper towel, and weighed. Egg contents and shells were then oven dried to a constant mass at 60°C, weighed, and stored frozen until they could be processed for determination of composition.

We extracted nonpolar lipids from dried samples of egg contents in a Soxhlet apparatus for a minimum of 5.5 h using absolute ether as solvent. The amount of lipids in a sample was determined by subtracting the lipid-free dry mass from the total sample dry mass. We determined ash and energy in egg contents using a GR-3500 adiabatic bomb calorimeter (Changsha Instruments, People's Republic of China). We determined the inorganic content of eggshells by burning samples in a muffle furnace at 600°C for a minimum of 12 h and weighing the remaining ash. Samples for calcium and magnesium determinations were digested completely in hot concentrated nitric acid. Digestates were brought to volume in volumetric glassware and stored in a refrigerator for later determination of calcium and magnesium. Concentrations of the two elements in digestates were determined using a WFX-1B atomic absorption spectrophotometer (Beijing 2nd Optical Instruments, People's Republic of China).

2.2. Incubation of eggs

We incubated eggs at 26, 29 and 32°C ($\pm$ 0.3°C). All eggs incubated at 26°C were from the first clutches because of the long incubation period at low temperature [41]. Eggs incubated at the other two temperatures were from both the first and the subsequent two clutches. Eggs were incubated individually in covered plastic jars (100 ml) that contained known amounts of vermiculite and distilled water to produce approximately -220 kPa (1 g water/1 g vermiculite), -12 kPa (2 g water/1 g vermiculite) and 0 kPa (3 g water/1 g vermiculite) water potentials [22]. Because of the in limited availability, eggs at 26°C were incubated only at -12 kPa water potential. Eggs from single clutches were distributed as equally as possible among treatments, and were half-buried in the substrate, with the surface near the embryo exposed to air inside the jar. We moved jars among shelves in the incubators daily according to a predetermined schedule to minimize any effects of thermal gradients inside the incubator. Incubation temperatures in close proximity to eggs were monitored twice daily using a digital thermometer. Eggs were weighed (to the nearest 0.001 g) at 5-day intervals. The final egg mass was taken 1 day prior to hatching. Jars were weighed daily and, if necessary, distilled water was mixed evenly into substrates to compensate for water absorbed by the eggs and for the small loses due to evaporation. Duration of incubation was defined as the elapsed time from egg laying to hatchling emergence.

2.3. Composition, size and mass of hatchlings

Upon emergence, each hatchling was measured and weighed. Pertinent body measurements included snout-vent length (SVL), tail length (TL) and body mass. Deformed hatchlings were excluded from further analyses. A total of 111 hatchlings were frozen after measuring and weighing. The frozen hatchlings were later thawed for data collection. We separated each killed hatchling into carcass, yolk sac and fat bodies. The three components were oven dried to constant mass at 60°C, weighed, and preserved frozen for later determination of composition, following the procedures already described for eggs. The sex of hatchlings was determined by pressing on both sides of the tail base using forceps for the presence or absence of hemipenes. Whereas presence of hemipenes allowed unequivocal sex assignation of males, their apparent absence could also be caused by failure of evagination in some cases. Therefore, to reinforce our judgment of sex assignation, we made counts of rows of ventral scales, revealing that ventral scales of females clearly outnumbered those of males (female, 27.8 ± 0.2, $N = 60$; male, 24.6 ± 0.2, $N = 62$; $F_{1, 120} = 112.15$, $P < 0.0001$), as previously reported for adult *P. muralis* [15].

2.4. Statistical analysis

All variables were tested for normality, using the Kolmogorov–Smirnov test, and for homogeneity of variance using Bartlett's test, prior to further statistical analysis, and no transformations were needed. We used one- and two-way analyses of variance (ANOVA), regression statistics and analysis of covariance (ANCOVA), when the assumptions of parametric analyses were met. Nonparametric analyses were used when these assumptions were violated. Significance level was set at $\alpha = 0.05$. Prior to testing for differences in adjusted means, the homogeneity of slopes was checked. Throughout this paper, values are presented as mean $\pm$ one standard error.

3. Results

3.1. Among clutch variation in egg composition

Total egg wet mass accounts for a significant part of the variation in all of the measured egg variables. Thus, the covariate was used to adjust values presented so as to control for variation in data stemming from variation in egg size. Adjusted means for water, lipid-free organic material, ash, calcium and magnesium in egg contents, and ash and magnesium in eggshell, remained remarkably constant, whereas those for other egg variables varied considerably, among clutches (Table 1). Eggs of the subsequent two clutches contained noticeably higher quantities of lipids and energy in egg contents and thinner shells than did eggs of the first clutch (Table 1). A two-way ANOVA (with female and clutch as factors) on the females that laid three clutches did not reveal maternal effects on the composition of eggs (all $P > 0.05$).

Table 1
Composition and F values of ANCOVA for freshly laid *P. muralis* eggs[a]

	First clutch ($N = 37$)	Second clutch ($N = 25$)	Third clutch ($N = 10$)	F
Egg contents				
Wet mass (mg)	$289.1^{c} \pm 0.7$ (281.0–296.8)	$292.2^{b} \pm 0.7$ (283.1–298.3)	$296.3^{a} \pm 0.9$ (292.6–303.6)	18.69***
Dry mass (mg)	$93.4^{b} \pm 1.1$ (72.8–104.1)	$96.8^{b} \pm 1.4$ (87.3–111.1)	$103.7^{a} \pm 2.9$ (83.7–119.6)	8.25***
Water (mg)	195.7 ± 1.4 (180.9–216.2)	196.1 ± 1.4 (182.5–207.1)	192.6 ± 3.1 (174.3–208.9)	0.72^{NS}
Organic mass (mg)	$87.9^{b} \pm 1.0$ (67.7–97.9)	$91.7^{b} \pm 1.4$ (82.6–105.1)	$98.3^{a} \pm 2.8$ (79.3–114.1)	9.56***
Nonpolar lipids (mg)	$31.7^{c} \pm 0.5$ (23.3–36.7)	$34.9^{b} \pm 0.8$ (27.8–43.8)	$39.0^{a} \pm 1.4$ (31.4–49.2)	18.39***
Lipid-free organic mass (mg)	56.2 ± 0.7 (44.4–63.1)	56.8 ± 0.9 (50.2–66.8)	59.4 ± 1.6 (48.0–64.6)	1.93^{NS}
Ash mass (mg)	5.47 ± 0.11 (4.00–6.55)	5.11 ± 0.16 (3.66–7.03)	5.35 ± 0.18 (4.38–6.22)	1.83^{NS}
Calcium (mg)	0.64 ± 0.01 (0.48–0.89)	0.65 ± 0.02 (0.47–0.87)	0.59 ± 0.02 (0.48–0.68)	1.79^{NS}
Magnesium (μg)	78.4 ± 1.6 (59.9–103.4)	84.8 ± 2.4 (63.9–114.0)	82.1 ± 2.8 (67.6–95.9)	2.94^{NS}
Energy (kJ)	$2.47^{c} \pm 0.03$ (1.90–2.81)	$2.61^{b} \pm 0.04$ (2.32–3.01)	$2.82^{a} \pm 0.08$ (2.28–3.32)	12.68***
Eggshell				
Dry mass (mg)	$12.6^{a} \pm 0.3$ (8.1–17.2)	$11.6^{b} \pm 0.1$ (10.4–12.9)	$10.8^{b} \pm 0.5$ (7.6–13.7)	7.07**
Organic mass (mg)	$10.1^{a} \pm 0.3$ (5.8–14.1)	$9.2^{b} \pm 0.1$ (8.0–10.2)	$8.4^{b} \pm 0.5$ (5.5–10.9)	6.93**
Ash mass (mg)	2.51 ± 0.04 (2.04–3.11)	2.39 ± 0.03 (2.21–2.74)	2.40 ± 0.08 (1.96–2.82)	2.57^{NS}
Calcium (mg)	$1.26^{b} \pm 0.03$ (0.95–1.61)	$1.43^{a} \pm 0.03$ (1.25–1.72)	$1.28^{b} \pm 0.05$ (1.04–1.54)	9.55***
Magnesium (μg)	18.4 ± 0.3 (14.4–22.3)	18.8 ± 0.3 (16.8–22.5)	18.1 ± 0.6 (14.9–21.0)	0.59^{NS}

[a] Data are expressed as adjusted means $\pm$ one S.E. (range), with total egg wet mass (set at 320 mg) as the covariate. Symbols after F values represent significance levels: NS, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Means with different lettered superscripts differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

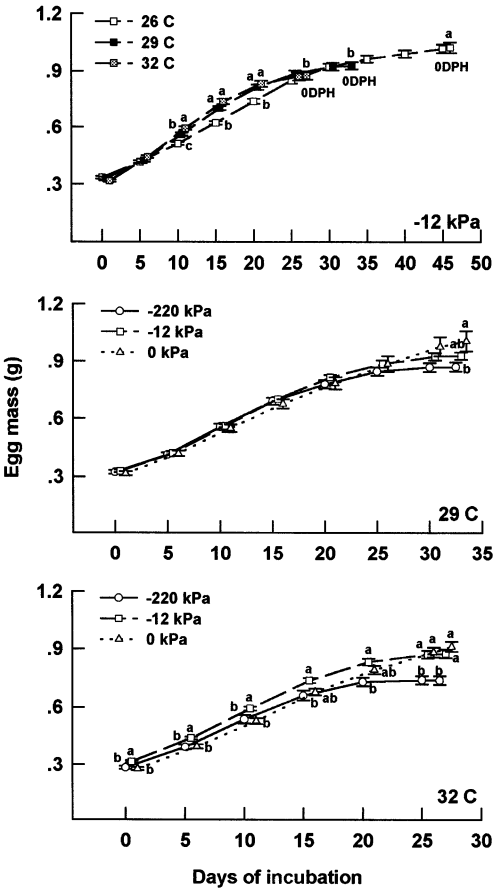

Fig. 1. Temporal changes in mass of *P. muralis* eggs incubated in different thermal and hydric environments. Data are expressed as means ± one standard error. Egg masses were compared at 5-day intervals, and final egg masses were compared 1 day prior to hatching (0DPH). Means with different lettered superscripts differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

3.2. Change in egg mass

Water uptake is obligatory for flexible-shelled eggs of *P. muralis* (Fig. 1). At an early stage of incubation, losing even a small amount of water, as indicated by the shrinkage in shell, may have a lethal effect on egg survivorship.

All viable eggs gained mass throughout incubation due to absorption of water (Fig. 1A–C). Mass gains were generally dependent on initial egg mass. A single exception was for eggs incubated at 32°C and −220 kPa water potential ($r^2 = 0.04$, $F_{1,6} = 0.25$, $P < 0.64$). Mass gains were also dependent on temperature (Fig. 1A) and water potential (Fig. 1B,C). Eggs incubated in wetter substrates gained more water than those in drier substrates (Fig. 1B,C), and eggs incubated at higher temperatures gained water faster than those at lower temperatures but at the same water potential (Fig. 1A). A comparison of pre-pip egg masses showed that eggs incubated at lower temperatures gained more water than those at higher temperatures but at the same water potential (Fig. 1A).

3.3. Duration of incubation, hatching success and sex ratio of hatchlings

Duration of incubation was not affected by initial egg mass. Therefore, the covariate was excluded from consideration and an ANOVA was employed. Duration of incubation varied considerably among treatments ($F_{6,171} = 2754.71$, $P < 0.0001$), and decreased as temperature increased, with a small range within each treatment (Table 2). However, the effect of water potential on duration of incubation varied with temperature. A two-way ANOVA (with temperature and water potential as factors) on the eggs at 29 and 32°C showed the interaction of temperature and water potential to be a significant source of variation in incubation length ($F_{2,134} = 12.85$, $P < 0.001$). At 32°C, water potential did not affect duration of incubation ($F_{2,50} = 2.94$, $P > 0.05$) but at 29°C, incubation length varied slightly among moisture treatments ($F_{2,85} = 3.68$, $P < 0.06$), with eggs in −12 kPa substrates taking less time to complete development.

Survival by embryos to hatching was affected by incubation temperature (G-test: $G = 9.78$, degrees of freedom (d.f.) = 2, $P < 0.025$), but not by water potential (29°C, $G = 0.52$, d.f. = 2, $P > 0.75$; 32°C, $G = 1.74$, d.f. = 2, $P > 0.25$) (Table 2). Hatching success was greatest for eggs incubated at 26°C and decreased as incubation temperature increased. Neither incubation

Table 2
Effects of thermal and hydric environments on duration of incubation, hatching success, and sex ratio in the wall lizard, *P. muralis*[a]

Treatment (°C/kPa)	Number of incubated eggs	Duration of incubation (days)	Hatching success (%)	Sex ratio (females/males)
26/−12	42	46.8 ± 0.2 (44.9–48.9)	90.5 (38/42)	10/9
29/−220	25	33.3 ± 0.2 (32.2–35.0)	76.0 (19/25)	5/9
29/−12	59	33.8 ± 0.1 (32.5–35.5)	83.1 (49/59)	13/17
29/0	29	33.5 ± 0.1 (32.9–34.6)	69.0 (20/29)	6/8
32/−200	21	27.7 ± 0.2 (27.2–29.2)	38.1 (8/21)	7/4
32/−12	64	27.4 ± 0.1 (26.6–28.5)	57.8 (37/64)	15/11
32/0	24	27.8 ± 0.2 (26.9–29.0)	41.7 (10/24)	8/10

[a] Data on incubation duration are expressed as means ± one S.E. (range), and data on sex ratio are based on the dissected hatchlings.

Table 3
Body mass, snout-vent length, tail length and F values (final row) of ANCOVA for *P. muralis* hatchlings incubated in different thermal and hydric environments[a]

Treatment (°C/kPa)	N	Body mass (mg)	Snout-vent length (mm)	Tail length (mm)
26/−12	38+0	$378.9^{a} \pm 5.2$ (313.4–437.1)	$25.7^{ab} \pm 0.1$ (23.5–27.1)	$39.4^{ab} \pm 0.4$ (23.5–27.1)
29/−220	11+8	$372.2^{ab} \pm 4.8$ (325.3–401.6)	$25.5^{ab} \pm 0.1$ (23.8–26.3)	$39.9^{a} \pm 0.4$ (23.5–27.1)
29/−12	41+8	$364.6^{abc} \pm 3.3$ (325.6–408.0)	$25.7^{a} \pm 0.1$ (24.5–26.9)	$39.5^{ab} \pm 0.3$ (23.5–27.1)
29/0	12+8	$356.6^{bcd} \pm 6.0$ (292.4–424.4)	$25.3^{ab} \pm 0.2$ (23.7–26.7)	$39.5^{ab} \pm 0.4$ (23.5–27.1)
32/−200	2+6	$351.5^{abc} \pm 7.9$ (321.2–389.5)	$25.2^{ab} \pm 0.3$ (23.9–26.6)	$36.7^{c} \pm 1.0$ (23.5–27.1)
32/−12	31+5	$346.2^{cd} \pm 4.3$ (303.0–390.6)	$25.5^{ab} \pm 0.2$ (23.3–27.3)	$37.5^{c} \pm 0.4$ (23.5–27.1)
32/0	2+7	$339.9^{d} \pm 8.1$ (310.6–380.4)	$24.9^{b} \pm 0.2$ (24.2–26.3)	$37.3^{bc} \pm 0.6$ (23.5–27.1)
		$F_{6, 171} = 6.82$***	$F_{6, 166} = 2.28$*	$F_{6, 152} = 3.94$***

[a] Data are expressed as adjusted means ± one S.E. (range), with initial egg mass (set at 320 mg) as the covariate. Sample sizes (N) are shown as the number of first clutch eggs plus the number of second clutch eggs. Symbols after F values represent significance levels: *, $P<0.05$; ***, $P<0.001$. Adjusted means with different lettered superscripts differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

temperature ($G = 1.12$, d.f. = 2, $P > 0.50$) nor water potential ($G = 0.24$, d.f. = 2, $P > 0.75$) influence the sex ratio of hatchlings, and the overall sex ratio (females/males = 0.94) did not differ from equality ($G = 0.28$, d.f. = 1, $P > 0.75$) (Table 2).

A high incidence of dead-in-shell embryos occurred in eggs that were laid in June and thereafter, and none of the eggs from third clutches survived to hatching. Of the 83 fertile eggs that died during incubation, 40 died at an early stage (within 5 days after laying), and 43 died at a later stage (1–5 days prior to hatching, with yolk sac either absorbed or unabsorbed). A total of 19 hatchlings exhibited apparent trunk and/or tail malformations. Deformed hatchlings were found in each treatment, the frequency being independent of treatment (G-test: $G = 4.19$, d.f. = 6, $P > 0.50$).

3.4. Size of hatchlings

We adjusted means for body mass, SVL and TL of hatchlings by ANCOVA, using initial egg mass as the covariate followed by the Tukey's post hoc test, so as to control for variation in data stemming from variation in egg size. There were no significant differences in adjusted means between male and female hatchlings (all $P > 0.05$), so we pooled data for both sexes in subsequent analyses.

Table 3 shows adjusted means of body mass, SVL and TL of hatchlings. Adjusted mean body mass of hatchlings varied considerably among treatments and decreased as temperature increased. Adjusted mean TL also varied considerably among treatments, with hatchlings from the eggs incubated at 32°C having the shortest tails. Adjusted mean SVL of hatchlings varied slightly, but significantly, among treatments, with hatchlings from eggs incubated at 32°C and 0 kPa water potential having relatively shorter SVLs. At 29 and 32°C, substrate moisture apparently did not influence body mass, SVL and TL of hatchlings.

An ANCOVA on hatchlings from eggs incubated at −12 kPa water potential confirmed the conclusion that incubation temperature affected body mass ($F_{2, 119} = 12.93$, $P < 0.0001$) and TL ($F_{2, 107} = 9.89$, $P < 0.001$) of hatchlings. However, the analysis did not show influence of incubation temperature on SVL of hatchlings ($F_{2, 115} = 1.12$, $P > 0.33$).

3.5. Composition of hatchlings

We adjusted means for composition of hatchlings by ANCOVA, using initial egg mass as the covariate. Adjusted means for dry, organic and lipid-free organic materials remained nearly constant among treatments (Table 4). Hatchlings from eggs incubated at 32°C and −220 and 0 kPa water potentials contained noticeably lower quantities of calcium and magnesium but higher quantities of lipids and energy than did hatchlings from other incubation conditions (Table 4). Adjusted means for dry and organic materials in shells from hatched eggs remained nearly constant, whereas those for other shell variables varied considerably among treatments (Table 4).

Table 5 shows dry masses of carcass, post-hatching yolk and fat bodies of hatchlings. Initial egg mass affected carcass dry mass in all treatments. Therefore, we adjusted means for carcass dry mass by ANCOVA using initial egg mass as the covariate. There were no significant differences in adjust mean carcass dry mass among treatments ($F_{6, 104} = 0.26$, $P = 0.956$). When setting initial egg mass at 320 mg (the overall mean egg mass in this study), we found that the values varied within a very narrow range from 67.4 to 68.8 mg. Dry masses of post-hatching yolk and fat bodies of hatchlings were not affected by initial egg mass. Therefore, the covariate was eliminated from consideration and an ANOVA was performed. This analysis showed that maximum post-hatching yolk and fat bodies occurred in hatchlings from eggs incubated at 32°C, and −220 and 0 kPa water potentials (Table 5).

Table 4
Composition and F values of ANCOVA for *P. muralis* hatchlings[a]

	26°C		29°C			32°C		F
	−12 kPa ($N = 19+0$)	−220 kPa ($N = 8+6$)	−12 kPa ($N = 25+5$)	0 kPa ($N = 8+5$)	−220 kPa ($N = 1+6$)	−12 kPa ($N = 17+5$)	0 kPa ($N = 1+5$)	
Hatchling								
Wet mass (mg)	$371.1^{ab} \pm 5.6$ (325.4–421.7)	$371.9^{a} \pm 5.8$ (325.8–400.5)	$366.1^{ab} \pm 4.7$ (325.5–409.2)	$358.2^{ab} \pm 8.3$ (296.3–421.0)	$350.1^{ab} \pm 8.5$ (322.1–385.6)	$348.8^{ab} \pm 5.4$ (307.1–392.7)	$346.7^{b} \pm 9.9$ (312.3–377.3)	2.36*
Dry mass (mg)	69.2 ± 1.2 (59.3–77.2)	70.7 ± 1.1 (63.4–77.1)	70.7 ± 1.0 (61.6–79.6)	70.9 ± 1.6 (55.4–77.3)	72.5 ± 1.4 (67.5–79.2)	70.1 ± 1.1 (56.3–82.6)	72.6 ± 1.3 (68.8–76.3)	0.61^{NS}
Water (mg)	$302.0^{a} \pm 4.7$ (266.3–346.2)	$301.1^{ab} \pm 5.4$ (259.3–331.3)	$295.3^{ab} \pm 4.9$ (259.9–372.8)	$287.4^{ab} \pm 7.1$ (239.9–347.5)	$277.1^{ab} \pm 7.7$ (251.7–305.8)	$278.6^{b} \pm 4.8$ (239.5–310.7)	$273.4^{ab} \pm 9.9$ (235.9–304.4)	3.34**
Organic mass (mg)	62.3 ± 1.1 (53.6–69.5)	63.8 ± 1.1 (56.6–70.1)	62.9 ± 0.9 (55.1 –70.6)	62.9 ± 1.6 (48.6–69.2)	65.5 ± 1.3 (60.7–71.7)	62.6 ± 1.0 (50.5–74.2)	65.7 ± 1.1 (62.2–68.8)	0.82^{NS}
Nonpolar lipids (mg)	$13.5^{c} \pm 0.3$ (10.6–15.8)	$15.8^{abc} \pm 0.7$ (11.2–19.4)	$14.3^{c} \pm 0.5$ (9.1–19.2)	$15.0^{bc} \pm 0.9$ (8.7–19.0)	$19.2^{a} \pm 0.8$ (15.4–21.6)	$13.9^{c} \pm 0.7$ (8.5–22.2)	$17.9^{ab} \pm 1.0$ (14.8–21.8)	6.24***
Lipid-free organic mass (mg)	48.8 ± 0.9 (40.9–55.1)	48.0 ± 0.9 (41.6–53.1)	48.6 ± 0.8 (41.4–56.1)	47.9 ± 1.1 (39.6–52.0)	46.3 ± 0.9 (43.8–50.1)	48.7 ± 0.7 (42.0–60.0)	47.8 ± 1.3 (43.0–52.0)	0.78^{NS}
Ash mass (mg)	$6.86^{d} \pm 0.13$ (5.79–7.74)	$6.96^{cd} \pm 0.14$ (5.66–7.80)	$7.78^{ab} \pm 0.12$ (5.90–8.97)	$8.04^{a} \pm 0.18$ (6.71–8.89)	$7.00^{bcd} \pm 0.14$ (6.65–7.54)	$7.52^{abc} \pm 0.17$ (5.78–8.60)	$6.91^{cd} \pm 0.20$ (6.21–7.51)	8.23***
Calcium (mg)	$1.43^{a} \pm 0.03$ (1.25–1.66)	$1.40^{a} \pm 0.04$ (1.09–1.61)	$1.39^{a} \pm 0.02$ (1.10–1.63)	$1.33^{ab} \pm 0.03$ (1.09–1.47)	$1.23^{bc} \pm 0.02$ (1.16–1.33)	$1.44^{a} \pm 0.03$ (1.17–1.69)	$1.05^{c} \pm 0.03$ (0.48–0.68)	10.53***
Magnesium (μg)	$104.5^{ab} \pm 2.0$ (88.5–118.4)	$97.1^{ab} \pm 2.9$ (81.4–114.1)	$102.9^{ab} \pm 1.8$ (76.8–122.4)	$106.6^{a} \pm 2.1$ (89.0–112.6)	$94.7^{ab} \pm 1.8$ (90.0–102.1)	$96.8^{b} \pm 1.8$ (87.2–128.3)	$92.5^{b} \pm 2.7$ (82.8–100.6)	4.35***
Energy (kJ)	$1.49^{b} \pm 0.03$ (1.28–1.68)	$1.52^{ab} \pm 0.03$ (1.30–1.70)	$1.53^{ab} \pm 0.02$ (1.31–1.71)	$1.55^{ab} \pm 0.04$ (1.16–1.72)	$1.64^{ab} \pm 0.04$ (1.48–1.81)	$1.54^{ab} \pm 0.03$ (1.18–1.81)	$1.67^{a} \pm 0.03$ (1.57–1.78)	2.27**
Eggshell								
Dry mass (mg)	10.4 ± 0.4 (7.0–13.6)	9.8 ± 0.1 (8.9–10.8)	10.0 ± 0.3 (7.5–14.1)	9.4 ± 0.3 (6.8–11.0)	9.6 ± 0.2 (8.1–17.2)	9.9 ± 0.4 (7.7–13.5)	9.8 ± 0.4 (8.3–10.9)	0.61^{NS}
Organic mass mg)	9.3 ± 0.4 (6.3–12.0)	8.7 ± 0.1 (8.1–9.7)	8.9 ± 0.3 (6.7–12.7)	8.4 ± 0.3 (6.3–9.9)	8.1 ± 0.2 (5.8–14.1)	8.7 ± 0.3 (6.7–11.9)	8.1 ± 0.4 (6.8–9.0)	1.14^{NS}
Ash mass (mg)	$1.15^{bc} \pm 0.05$ (0.72–1.52)	$1.08^{bc} \pm 0.05$ (0.85–1.42)	$1.04^{c} \pm 0.04$ (0.73–1.43)	$1.00^{c} \pm 0.05$ (0.64–1.28)	$1.52^{a} \pm 0.03$ (1.40–1.61)	$1.22^{b} \pm 0.05$ (0.93–1.62)	$1.62^{a} \pm 0.08$ (1.45–1.94)	12.53***
Calcium (mg)	$0.63^{ab} \pm 0.03$ (0.37–0.87)	$0.60^{ab} \pm 0.02$ (0.47–0.73)	$0.58^{b} \pm 0.02$ (0.47–0.84)	$0.57^{b} \pm 0.03$ (0.38–0.73)	$0.69^{ab} \pm 0.01$ (0.64–0.73)	$0.62^{a} \pm 0.02$ (0.48–0.80)	$0.73^{a} \pm 0.04$ (0.64–0.87)	2.67*
Magnesium (μg)	$11.7^{b} \pm 0.5$ (7.4–15.2)	$11.8^{b} \pm 0.2$ (10.0–13.1)	$11.3^{b} \pm 0.4$ (8.0–16.9)	$11.3^{b} \pm 0.4$ (8.4–13.5)	$14.9^{a} \pm 0.3$ (13.8–15.8)	$11.2^{b} \pm 0.6$ (6.5–17.6)	$15.8^{a} \pm 0.8$ (14.3–18.9)	6.20***

[a] Data are expressed as adjusted means ± one S.E., with initial egg mass (set at 320 mg) as the covariate. Sample sizes (N) are shown as the number of first clutch eggs plus the number of second clutch eggs. Symbols after F values represent significance levels: NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Adjusted means with different lettered superscripts differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

Table 5
Total body dry mass and dry masses of carcass, yolk sac and fat bodies of *P. muralis* hatchlings incubated in different thermal and hydric environments[a]

Treatment (°C/kPa)	*N*	Body dry mass (mg)	Carcass dry mass (mg)	Yolk sac dry mass (mg)	Fat body dry mass (mg)
26/−12	19+0	70.2 ± 1.3 (61.4–86.1)	68.4 ± 1.3 (59.7–84.1)	0.8^{c} ± 0.1 (0.1–2.1)	0.9^{c} ± 0.1 (0.3–1.7)
29/−220	8+6	70.6 ± 1.4 (57.6–77.6)	68.1 ± 1.5 (56.8–76.9)	0.9^{bc} ± 0.2 (0.0–3.1)	1.6^{bc} ± 0.3 (0.4–3.4)
29/−12	25+5	71.4 ± 1.4 (54.9–90.1)	69.6 ± 1.3 (54.5–87.7)	0.6^{c} ± 0.1 (0.1–3.5)	1.1^{c} ± 0.1 (0.3–3.1)
29/0	8+5	72.2 ± 2.9 (47.3–94.8)	69.6 ± 2.7 (46.6–91.1)	1.0^{abc} ± 0.2 (0.1–1.9)	1.6^{bc} ± 0.3 (0.1–3.1)
32/−200	1+6	68.5 ± 2.0 (60.5–74.0)	63.5 ± 1.4 (58.6–67.0)	1.9^{a} ± 0.3 (0.6–3.5)	3.1^{a} ± 0.5 (1.3–5.0)
32/−12	17+5	69.7 ± 1.4 (58.0–87.3)	67.7 ± 1.4 (56.6–85.2)	0.7^{c} ± 0.1 (0.1–2.7)	1.3^{bc} ± 0.2 (0.3–3.7)
32/0	1+5	67.3 ± 1.5 (61.4–71.4)	62.9 ± 1.6 (58.1–69.0)	1.9^{ab} ± 0.4 (0.9–3.8)	2.5^{ab} ± 0.6 (1.1–4.2)
$F_{6, 104}$		0.58^{NS}	1.58^{NS}	5.04***	7.18***

[a] Data are expressed as means ± one S.E. (range). Sample sizes (*N*) are shown as the number of first clutch eggs plus the number of second clutch eggs. Symbols after *F* values represent significance levels: NS, $P>0.05$; ***, $P<0.001$. Means with different lettered superscripts differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

3.6. Embryonic use of energy and nutrients

Because adjusted means for some measured egg variables varied considerably among clutches, we calculated energy expenditure of embryogenesis and conversion efficiencies of energy and nutrients during incubation with sources of eggs taken into account. Energy expenditure of embryogenesis during incubation remained remarkably constant among treatments, and varied within a very narrow range from 0.591 to 0.608 kJ. Overall, approximately 75% dry materials, 71% organic materials, 86% lipid-free organic materials, 46% lipids and 61% energy in egg contents were transferred to the hatchling, and approximately 27% ash, 53% calcium and 20% magnesium were withdrawn from the shell.

4. Discussion

4.1. Composition of eggs

A comparison of the composition of eggs showed among clutch variation in some egg variables, including dry and organic materials, nonpolar lipids and energy in egg contents, and dry and organic materials and calcium in shell. This suggests that seasonal shifts in the composition of eggs occur at least in captive *P. muralis*. Since comparable data on eggs laid in the field were not available to us, we do not know if a similar variation also occurs in nature. When setting the total egg mass constant at 320 mg, we found that the dry mass of the egg contents in eggs of the second and third clutches averaged 5.4 and 10.3 mg, respectively, greater than that in eggs of the first clutch (Table 1). The discrepancies were primarily due to higher proportional allocation of nonpolar lipids in eggs of the subsequent two clutches. Since the maximum energy density usually occurred in the samples with the highest level of lipids [20], it was not surprising that eggs of the subsequent two clutches contained higher energy in the egg contents than those of first clutch. It was worth noting, however, that lipid-free organic and inorganic (including calcium and magnesium) materials in the egg contents remained remarkably constant among clutches. This finding could be interesting, because, for a given sized egg, these variables could be more important than others in determining hatchling size at hatching.

4.2. The influence of substrate moisture

In our study, substrate moisture seemed to affect only the change in the mass of eggs. Hatching success, embryonic use of energy and nutrients, and sex, size, mass and composition of hatchlings were clearly unaffected over a wide range of substrate moistures. These findings contrast with previous reports for some other reptiles [16,17,32–35]. Unlike some embryonic turtles that tend to remain in the egg longer before hatching in wetter than in drier substrates [24,27,28], the length of embryonic development in *P. muralis* was almost unaffected by substrate moisture.

Differences in water uptake by *P. muralis* eggs incubated at different water potentials were not reflected in the size and mass of hatchlings. This is considered to be unusual in reptiles that lay pliable-shelled eggs [17,30,32,37,40], but has been recorded in some reptiles [26,29,36,38,39]. The different conclusions presumably result from differences in the experimental design [27,36]. However, in our study, it was highly unlikely that the lack of an influence of substrate moisture on the size and mass of hatchlings resulted from the experimental design. Therefore, we tend to conclude that eggs of different species do not necessarily respond to differences in substrate moisture in the same manner.

4.3. The influence of incubation temperature

The high embryo mortality at the highest temperature is consistent with the earlier report for *P. muralis* [41], although hatching success in our study was notice-

ably higher (Table 2). However, the actual cause of death still remains unknown, but overheating experienced by the pregnant females in June and thereafter, when ambient temperatures were high, cannot be precluded as the cause. In *P. muralis*, the optimal embryogenesis temperature (near 28°C [41]) is lower than the selected body temperatures (mean = 33.4°C [41]) in the laboratory, and the body temperatures maintained in the field (mean = 33.8°C [4]). These discrepancies may induce pregnant females to shift their thermoregulatory set-points to lower temperatures favoring embryogenesis [5]. In our study, pregnant females were housed in terraria, where the limited space might have prevented them from shifting body temperatures to lower points when room temperatures were higher than their seasonal voluntary range of body temperatures. In fact, no direct evidence, such as the highest frequency of deformed hatchlings, showed a direct association between the highest embryo mortality and the highest temperature.

Significant differences in wet mass were found among hatchlings from eggs incubated at different temperatures, and hatchlings from eggs incubated at lower temperatures were, on average, heavier than their sibs from eggs at higher incubation temperatures (Table 3). However, dry mass remained remarkably constant among hatchlings from different temperatures, suggesting that variations in the wet mass of hatchlings stemmed mainly from variation in water content (Tables 4 and 5).

The influence of incubation temperature on SVL of *P. muralis* hatchlings, if present, was very weak (Table 3). This result contrasts with other reports [17,18,31], including an earlier report for *P. muralis* [41], in that low and/or intermediate temperatures did not maximize hatchling size (SVL). In our study, incubation temperature affected only TL of hatchlings, with hatchlings from eggs incubated at 32°C having the shortest tails (Table 3). Eggs incubated at 32°C contained more eggs from the second clutches (Table 3). However, it was very unlikely that sources of eggs could be responsible for variations in TL of hatchlings, because there were no significant differences in TL between hatchlings from first and second clutches when initial egg mass was kept constant. Given that some traits such as head size and limb lengths, which could be ecologically important, are highly correlated with hatchling SVL, our results may have implications for fitness by keeping SVL of hatchlings unaffected over a wide range of temperatures.

4.4. Embryonic use of energy and nutrients and energy reserve

In our study, the energy expenditure of embryogenesis during incubation was clearly unaffected by incubation thermal and hydric environments. We therefore conclude that incubation of eggs at lower temperatures does not lead to an increased greater energy expenditure due to the consequential increase in incubation time. This conclusion contrasts with that reported for other reptiles [1,2,12,23,30,42–44].

The consistencies in energy expenditure during incubation and carcass dry mass of hatchlings among treatments and the existence of among clutch variation in some egg variables, particularly energy materials, suggest that sources of eggs rather than incubation conditions were responsible for variation in the conversion efficiencies involved. Similarly, the maximum energy reserves (as indicated by the greatest amounts of yolk sac and fat bodies) in the hatchlings from eggs incubated at 32°C, and −220 and 0 kPa water potentials, could be also due to more second clutch eggs incubated in these conditions. It is not surprising that an embryo developing within the egg with a relatively higher proportion of energy materials may have greater energy reserves by the end of embryonic development.

Acknowledgements

Financial support for research expenses were obtained from the European Community contract ER-BCHRX-CT94-0585, the Natural Science Foundation of China NSFC-39270124, and the Zhejiang Provincial Natural Science Foundation. When working in Spain, X.J. was supported by grants from the Universidad de Oviedo (Oficina de Relaciones Internacionales) and the Paos' Foundation that covered his living and trip expenses. We are very grateful to Felipe González de los Reyes and Alfredo Fernández Ojanguren for their help throughout the research.

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