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Plasticity and limitations of extended egg retention in oviparous *Zootoca vivipara* (Reptilia: Lacertidae)

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The transition between oviparity and viviparity in reptiles is generally accepted to be a gradual process, the result of selection for increasingly prolonged egg retention within the oviduct. We examined egg retention plasticity in an oviparous strain of the lacertid lizard *Zootoca vivipara*, a species having both oviparous and viviparous populations. We forced a group of female *Z. vivipara* to retain their clutch *in utero* by keeping them in dry substrata, and assessed the effect on embryonic development and hatching success, along with offspring phenotype and locomotor performance. Forced egg retention for one additional week affected the developmental stage of embryos at oviposition, as well as hatchling robustness and locomotor performance. However, embryos from forced clutch retention treatment reached one stage unit more than control embryos at oviposition time. Embryos from control eggs were more developed than embryos from experimental eggs after approximately the same period of external incubation, showing that embryonic development is retarded during the period of extended egg retention, despite the high temperature inside the mother's body. Significant differences in external incubation time were only found in one of the two years of study. Hatching success was much lower in the experimental group with forced egg retention (21.1%) than in the control group (95.4%). Therefore, we conclude that there are limitations that hinder the advance of intrauterine embryonic development beyond the normal time of oviposition, and that extended egg retention does not represent clear advantages in this population of *Z. vivipara*. Nevertheless, the fact that some eggs are successful after forced egg retention could be advantageous for the females that are able to retain their clutch under unfavourable climatic conditions. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 75–82.

ADDITIONAL KEYWORDS: developmental constraints – embryonic development – evolution of viviparity – hatching success – hatchling phenotype.

INTRODUCTION

It is generally accepted that viviparity in reptiles has evolved from oviparity, and that such an evolutionary transition has come about gradually through selection for increasingly prolonged egg retention within the oviducts, with its implied progressive increase in the degree of intrauterine embryonic development (Tinkle & Gibbons, 1977; Shine, 1983; Andrews, 2002). For this reason, egg retention is considered to be a key component of the evolutionary progression to viviparity in reptiles, and several studies have focused on the constraints and selective pressures that operate on the time that eggs are retained *in utero* before oviposition (Andrews & Rose, 1994; Mathies & Andrews, 1996; Warner & Andrews, 2003; Braña & Ji, 2007). Consequently, extended egg retention has achieved great importance in the current models of the evolution of viviparity, particularly in the 'cold-climate' model (Shine, 1985; Rodríguez-Díaz *et al.*, 2010). In this model, retaining eggs in cold environments is thought to be selectively advantageous, as retained eggs are generally subjected to warmer temperatures than those in nests, and therefore embryonic development proceeds faster *in utero*.

Considerable variation in the stage of development of the embryo at oviposition has been documented among Squamate reptiles, from chamaleonid lizards in which embryos are diapausing gastrulae at the

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time of oviposition (Andrews *et al*., 2008), to viviparous lizards and snakes that bear fully developed hatchlings (Blackburn, 1995; Lourdais *et al*., 2004). Between these extremes, the majority of oviparous squamates oviposit eggs with embryos at early limb-bud stages (Shine, 1983; Andrews & Mathies, 2000). In addition to some intraspecific variability in the duration of egg retention under normal conditions (see, e.g. Braña, Bea & Arrayago, 1991; Calderón-Espinosa, Andrews & Méndez de la Cruz, 2006), many species of squamates are able to extend the period that eggs are retained in the oviduct beyond the normal time of oviposition in response to unfavourable (e.g. dry) environmental conditions (Stamps, 1976; Warner & Andrews, 2003). The consequences of extending egg retention vary greatly between species: in some species, embryonic development continues almost to the point of hatching during the extended time of retention (e.g. *Sceloporus scalaris*; Mathies & Andrews, 1996), whereas in other species development progresses by only one or two additional stages (e.g. *Sceloporus undulatus*; Warner & Andrews, 2003) or is completely arrested (e.g. *Urosaurus ornatus*, Mathies & Andrews, 1999; *Calotes versicolor*, Shanbhag, Saidapur & Radder, 2003).

The aim of this study was to determine egg retention plasticity and its consequences on incubation time, hatching success of eggs, hatchling locomotor performance, and several hatchling phenotype traits that probably influence fitness, with the aim of evaluating the implications for the evolution of viviparity. To do this, we used *Zootoca vivipara* (Jacquin, 1797) (Squamata: Lacertidae) as the model organism, because it is one of the very few amniote species in which there are viviparous and oviparous populations, with each population showing a characteristic and stable mode of reproduction (Braña, 1986). These organisms are ideal models in which to study the evolution of viviparity, and hence variation in egg retention: phylogenetic differences that could lead to misinterpretations are minimized, as oviparous and viviparous individuals are the same species, with otherwise similar morphological, physiological, and ecological adaptations. In this study, we worked with individuals from a Cantabrian (North Spain) population of *Z. vivipara*, which lay eggs with embryos at quite advanced developmental stages (31–34, according to Dufaure & Hubert's 1961 developmental scale; see Braña, 1986; Braña *et al*., 1991). Most oviparous Squamate reptiles lay eggs with embryos at around stage 30 (Shine, 1983), few species oviposit at stages 31–34, and very few species oviposit at stages 35–39 (Dufaure & Hubert's 1961 stage 40 represents full development, as attained in live-bearing species). This suggests that some constraints must exist that make it difficult for embryonic development to progress inside the mother's body much beyond stage 30 (Andrews & Mathies, 2000).

MATERIAL AND METHODS

We collected gravid females from an oviparous population located in Las Señales (León, northern Spain), between 1650 and 1750 m a.s.l., during the spring–summers of 2006 and 2007. These individuals were transported to the Zoology laboratory of the University of Oviedo, and were housed in terraria exposed to the natural photoperiod. Terraria were also illuminated for 7 h a day with 60-W light bulbs that facilitated behavioural thermoregulation. Refuges, dishes of water, and food (mealworms) were also provided. After egg-laying, all of the adults that were collected, as well as the hatchlings from experimental incubations, were released into the wild at their places of capture.

The plasticity of egg retention was assessed by maintaining a group of females ($n = 29$; experimental group) in terraria with dry soil as a substrate, which forces them to delay oviposition in response to drought conditions (Andrews & Rose, 1994). Another group of gravid females ($n = 29$; control group) was kept in terraria with moist soil, an appropriate substrate for oviposition to occur at the normal time. Both groups were balanced as regards to female size and reproductive state.

One week after females from the control group started to lay their eggs, we placed moist peat in the experimental terraria and experimental females started oviposition at that time. Immediately following oviposition, clutches were collected from terraria and weighed. Then, one egg was dissected to determine the embryonic stage at oviposition according to the developmental scale of Dufaure & Hubert (1961). Embryos with traits intermediate between two stages of this scale were designated as half stages. The remaining eggs of each clutch were weighed and incubated individually at 25 °C in plastic containers with vermiculite and distilled water (proportion 1/1 by mass) as substrate. Containers were periodically checked to verify that the vermiculite stayed moist and they were rotated daily inside the incubators to minimize possible effects of temperature gradients on development.

At the same time, we determined the developmental stage and dry mass of a set of control embryos sampled at 4-day intervals from oviposition to hatching, for use as a reference to compare with embryo stage after extended egg retention. These eggs were incubated at 25 °C, a temperature that is considered to be close to the mean temperature experienced by eggs inside their mother's body, as the temperature selected in a laboratory thermal gradient by gravid females from the population used in this study is 32.33 ± 1.27 °C (Rodríguez-Díaz *et al*., 2010), and their body temperature is much lower than this temperature during the period of time (17 h a day) in which they do not thermoregulate (laboratory temperature, mean minimum temperature ± SD, 18.56 ± 2.75 °C, $n = 25$; mean maximum temperature ± SD, 26.68 ± 4.53 °C, $n = 25$). In order to assess the relationship between embryo dry mass and incubation time, the curve was fitted to an exponential function by log-transforming the dependent variable (see Andrews, 2004).

We studied the effect of forced egg retention on clutch mass, stage of development at oviposition, incubation time, hatching success, and hatchling morphology (head, abdomen, and tail lengths, and robustness). A number of clutches ($n = 8$) from the experimental group were not included in the final analyses (except for clutch size and developmental stage of embryos at oviposition) because they were laid in dry substrate and thus became highly dehydrated, so that no embryo from those clutches survived until hatching. The morphological traits were measured *in vivo*, using a digital caliper (Vogel, Germany). We took only one hatchling from each clutch to avoid pseudoreplication problems, and balanced the number of males and females to minimize the effects of sexual dimorphism in the analyses. Sex determination by eversion of hemipenes in males (Harlow, 1996) was carried out after locomotor performance tests, so as to avoid the excessive manipulation of hatchlings before conducting these tests.

The locomotive performance of hatchlings was evaluated, as it provides data on a complex, integrative, and ecologically important variable that is likely to influence fitness (Van Damme *et al*., 1992; Garland & Losos, 1994; Braña & Ji, 2000; but see Le Galliard, Clobert & Ferrière, 2004). Maximal sprint speed is thought to be selectively important in Squamates because, although this feature is seldom used in the lizard's everyday activities, it is of great importance, e.g. in avoiding predators, and hence the faster the lizard runs, the higher its probability of surviving to breed (Braña, 2003). Sprint speed was tested within 48 h of hatching and hatchlings were not fed until after the test. They were kept at 32 °C for 30 min prior to testing as a way to ensure that all hatchlings were at the same temperature prior to running. The test was carried out in a 1-m-length corridor, where the hatchling lizards were encouraged to run by tapping them lightly with a paintbrush. Races were recorded with a video camera at 30 frames per second. From the recording, we calculated: (1) the speed in the fastest five consecutive frames (sprint speed); (2) the number of stops; and (3) the maximum distance covered between stops.

STATISTICAL ANALYSIS

All data were checked for normality and homocedasticity (through Kolgomorov–Smirnov and Levene's tests, respectively) prior to further statistical analysis. A Mann–Whitney *U*-test was carried out to analyse stage of embryonic development at oviposition, because this variable did not meet the assumptions needed to conduct parametric tests. χ^2 tests between control and experimental groups were conducted to assess hatching success. Some clutches were not included in these analyses because they were extremely dehydrated at oviposition because they had been laid in dry substrate. Incubation time, abdomen length, and number of stops were log-transformed to approximate normality.

One-factor analyses of variance (ANOVA) were carried out to analyse incubation time, head and abdomen lengths, and number of stops during trials. We also performed a number of ANCOVAs using the following covariates: female snout–vent length or SVL (analysis of clutch size); female mass (analysis of clutch mass); and hatchling SVL (analyses of tail length, hatchling mass, sprint speed, and the longest distance covered between stops). The significance level for all tests was set at $\alpha = 0.05$.

RESULTS

Significant differences in the stage of development of embryos at oviposition were found between groups (Mann–Whitney *U*-Tests, 2006, Z-adjusted = –3.869, $P < 0.001$; 2007, Z-adjusted = –3.300, $P < 0.001$; Fig. 1). Females in the control group laid eggs containing embryos at stages 32–34, according to Dufaure & Hubert's (1961) staging system, as previously found in the same population (Rodríguez-Díaz *et al*., 2010), whereas the maximum stage attained by embryos from the experimental group was 35. In spite of this difference, we did not find a significant effect of forced egg retention on incubation time from oviposition to hatching (ANOVA, $F_{1,45} = 1.727$, $P = 0.195$). We did, however, find significant differences in incubation time between years (ANOVA, $F_{1,45} = 31.946$, $P < 0.001$), and for this reason we analysed the effects of treatment on incubation time separately in 2006 and 2007. Forced egg retention affected incubation time in 2006 (control group, 20.44 ± 1.04 days, $n = 9$; forced egg retention group, 17.80 ± 0.97 days, $n = 5$; ANOVA, $F_{1,12} = 22.83$, $P < 0.001$), but not in 2007 (ANOVA, $F_{1,31} = 0.777$, $P > 0.05$), possibly because of the larger overlap of embryo stages between groups in 2007 (see Fig. 1). Hatching success was much lower in the experimental group (32.39%) than in the control group (95.37%; $\chi^2 = 81.53$; $P < 0.001$). This result was not just explained by differences in developmental

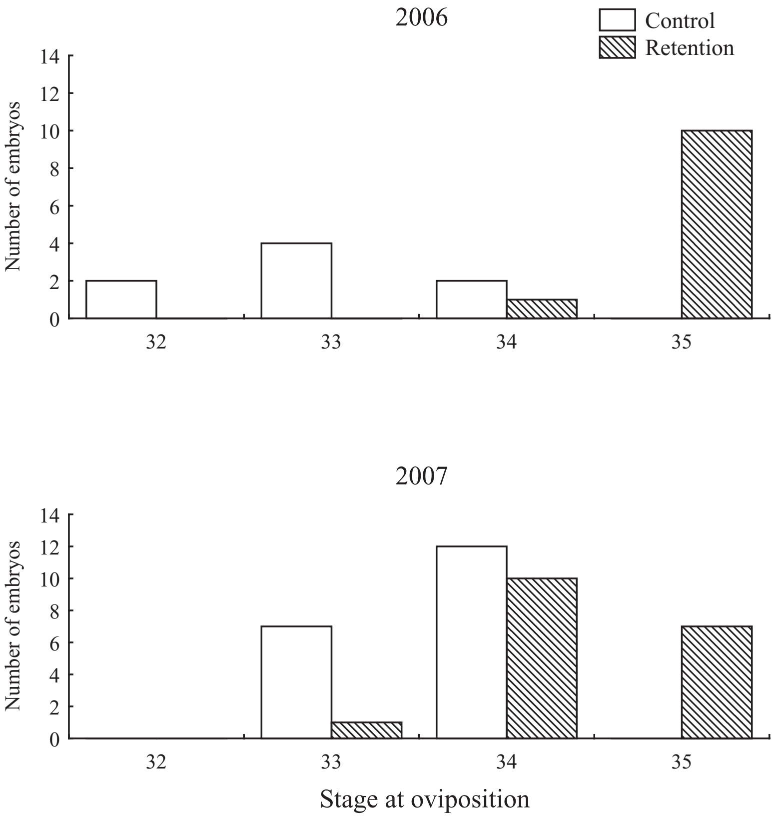

Figure 1. Development of embryos *Zootoca vivipara* from control and forced retention groups at oviposition in 2006 and 2007, according to the stages described by Dufaure & Hubert (1961).

stage at oviposition: although hatching success was particularly low in clutches at stage 35 (17.78%), differences for clutches at stage 34 in both treatments were also significant (experimental group, 58.33%; control group, 94.12%; $\chi^2 = 14.58$; $P < 0.001$). We found differences between years regarding clutch size (ANCOVA, $F_{1,42} = 31.680$, $P < 0.001$), but not in relation to clutch mass (ANCOVA, $F_{1,38} = 1.456$, $P > 0.05$). Clutch sizes were larger in 2007 than in 2006, which presumably reflects differences in the field conditions between years and different reproductive investment by females. However, differences between treatments were not found for any of these variables (for clutch size, ANCOVA 2006, $F_{1,18} = 0.518$, $P > 0.05$; 2007, $F_{1,21} = 2.222$, $P > 0.05$; for clutch mass, ANCOVA, $F_{1,38} = 0.477$, $P > 0.05$).

Preliminary analyses showed that the morphology and locomotor performance of hatchlings did not differ between years. For this reason, only treatment was used as a factor in the analyses of these variables. Experimental manipulation had no significant effects on most morphological traits (head, abdomen, and tail length), but hatchlings from the control group were more robust (size-corrected mass). With regard to locomotor performance, control hatchlings stopped less often during trials than those from the retention group (Table 1), and no significant differences were found in sprint speed or maximum distance covered without stopping during trials.

Embryos of the subsample of eggs used to assess variation of embryo dry mass and developmental stage along the external incubation, were at stages 33–34 ($n = 14$) at oviposition, and reached stages 34.5–36 ($n = 9$) after 4 days, and stages 37–37.5 ($n = 9$) after 8 days of external incubation at 25 °C. After that, embryonic development reached stages 38.5–39.5 ($n = 9$) by day 12, and stage 40 ($n = 9$) by day 16. On the other hand, after 7 days of forced egg retention, eggs contained embryos at stages 33–35 ($n = 29$), which were less developed than those from control eggs after 4 days (Mann–Whitney *U*-test adjusted $Z = 3.317$; $P < 0.001$), and after 8 days of external incubation (adjusted $Z = 4.783$; $P < 0.001$). The dry mass of embryos increased exponentially with incubation time (Fig. 2). In the first 4 days following oviposition, embryo mass increased slowly, after which the increase in mass proceeded at a much faster rate.

Table 1. Effects of forced egg retention on morphology and locomotor performance of hatchling *Zootoca vivipara* (mean ± SD)†

Hatchling traits		Control group	Retention group	Treatment effects
Head	M	7.16 ± 0.49	7.44 ± 0.45	$F_{1,43}$ = 0.592 NS
(mm)	F	7.24 ± 0.53	6.79 ± 0.40	
Abdomen	M	13.41 ± 0.39	13.36 ± 0.72	$F_{1,44}$ = 1.512 NS
(mm)	F	14.96 ± 0.74	15.00 ± 0.58	
Tail	M	26.85 ± 1.25	26.63 ± 1.65	$F_{1,43}$ = 2.133 NS
(mm)	F	26.52 ± 1.82	25.89 ± 0.82	
Robustness	M	0.229 ± 0.019	0.216 ± 0.010	**$F_{1,43}$ = 7.505****
(g)	F	0.231 ± 0.023	0.222 ± 0.013	**C > T**
Sprint speed	M	40.96 ± 6.50	36.00 ± 7.75	$F_{1,43}$ = 1.963 NS
(cm s^{-1})	F	36.60 ± 7.19	36.33 ± 5.07	
Number of stops	M	0.088 ± 0.048	0.151 ± 0.096	**$F_{1,45}$ = 3.927***
	F	0.118 ± 0.070	0.138 ± 0.072	**C < T**
Distance	M	18.58 ± 5.57	17.43 ± 10.42	$F_{1,42}$ = 0.046 NS
(cm)	F	16.27 ± 8.46	16.89 ± 7.43	

Symbols immediately after F values represent significant levels: NS $P > 0.05$; *$P < 0.05$; **$P < 0.01$.

†F ratios correspond to the single effects of treatment in ANOVAs (head and abdomen length, number of stops) or ANCOVAs (tail, robustness, sprint speed, and maximum distance covered during trials with snout–vent length as a covariate).

Descriptive statistics for abdomen length and number of stops are presented as direct values, but analyses were carried out on log-transformed variables; M, male; F, female; C, control; T, treatment. Significant treatment effects are in bold type.

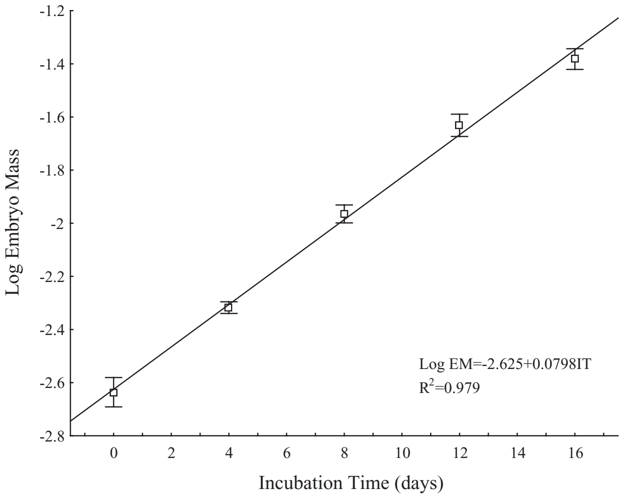

Figure 2. Advancement of embryo dry mass during external incubation at 25 °C in control eggs of *Zootoca vivipara*; EM, embryo mass; IT, incubation time.

DISCUSSION

After 1 week of forced egg retention, most female *Z. vivipara* laid eggs containing embryos at stages 34 and 35, i.e. one stage unit beyond the maximum stage reached by embryos from females allowed to lay their eggs at the normal time of oviposition (stages 32–34 according to Dufaure & Hubert's 1961 developmental scale; Rodríguez-Díaz *et al*., 2010; this study), with this upper limit in embryonic stage at oviposition being very marked, as no female produced eggs with embryos beyond stage 35.

In *Z. vivipara*, as in many other lizard species, embryonic development proceeds faster at higher incubation temperatures (*Z. vivipara*; Rodríguez-Díaz *et al*., 2010; this population). However, in spite of the fact that thermoregulating pregnant females maintain a body temperature higher than the environmental temperature, and higher than our experimental incubation temperature (25 °C), intrauterine embryogenesis is retarded during the retention period in this species. This is evidenced by the high degree of development attained during the first week of external incubation in control eggs (stages 37–37.5), which contrasts with the maximal embryo stages attained *in utero* by experimental embryos (stages 33–35). A similar retardation in embryogenesis during extended egg retention occurs in the sceloporine lizard *S. undulatus* (Mathies, 1998; Warner & Andrews, 2003).

A shortage of oxygen supply inside the oviducts, as a consequence of the increasing demands of growing embryos (Andrews & Mathies, 2000; Parker & Andrews, 2006), could be a reason why embryonic development does not continue beyond stage 35 inside the mother's body, as it has been demonstrated that low oxygen concentrations *in utero* have negative effects on differentiation and growth (Parker, Andrews & Mathies, 2004). Moreover, eggshell should be thin enough to permit gas exchange during prolonged egg retention (Guillette, 1991; Andrews & Rose, 1994). In fact, experimental hybridizations between oviparous and viviparous strains of *Z. vivipara*, in which embryos reach stages 35–36, produce eggs with intermediate eggshell thickness and scarcely mineralized eggshells (Heulin *et al.*, 1992; Arrayago, Bea & Heulin, 1996). Another limitation for intrauterine embryogenesis beyond the normal time of oviposition could be that females might not be able to bear the increase in mass and volume that eggs would undergo if development progressed normally: embryos experience a significant increase in dry mass during the first week of external incubation (see Fig. 2), and also dramatically increase their water uptake during development (Mathies & Andrews, 1996; Andrews & Mathies, 2000).

Females of some lizard species can delay oviposition until the appearance of favourable conditions if nest sites are too dry to lay the eggs (Andrews & Rose, 1994; Mathies & Andrews, 1996), and this plasticity could favour the selection of more advanced developmental stages at oviposition in species in which embryonic development advances during egg retention. Some females of our study population of *Z. vivipara* ($n = 11$) laid their eggs after a few days of forced clutch retention, even when wet substrate was not available, showing that an advance in embryonic development beyond the normal time of oviposition involves some difficulties, as has been previously found in other lizard species (Mathies, 1998). This behaviour decreases a female's reproductive success because clutches laid under drought conditions become dehydrated, and are thus lost (Packard, 1991). The hatching success of normal (i.e. non-dehydrated) clutches was in any case very low for eggs hatched after the forced egg retention time (32.39 versus 95.37% for control clutches), further reinforcing the idea that there is a viability limitation at Dufaure & Hubert's (1961) stage 35 and beyond.

Despite the difference in intrauterine incubation time (1 week longer in the experimental group) and the corresponding difference in embryo developmental stage at oviposition (more advanced in embryos from the forced retention treatment), significant between-treatment differences were not found in external incubation time in 2007. A possible reason explaining this result is the high level of overlap of embryonic stages at oviposition between control and forced retention groups that year: although all experimental females were maintained in dry substrates for one additional week after the control females started to lay eggs, it is possible that some of them might not have actually experienced extended egg retention, with the experimental period being part of the normal retention time. Moreover, hatching success was particularly low in 2007 in clutches with embryos at stage 35 at oviposition, which makes the overlap wider for the remaining clutches over which incubation time was measured.

In addition to the aforementioned effects on embryonic development, forced egg retention has some direct effects on aspects of offspring phenotype that are likely to affect fitness. Hatchlings from the forced retention treatment did not exhibit morphological differences with respect to control individuals, but were less robust, which might make them more vulnerable as prey, less able to get food, and less resistant to prolonged fasting (Ferguson & Bohlen, 1978). Furthermore, although no significant differences were found with respect to sprint speed, experimental hatchlings showed a higher frequency of pauses during the tests. Pausing while fleeing could be connected to a lizard's inclination to run (Sorci *et al.*, 1995; Braña, 2003), such that a high number of pauses would be a behavioural trait with negative consequences on hatchling fitness as regards, for example, their ability to avoid predators.

Extended egg retention is an important component of the evolution of viviparity in Squamate reptiles because the oviparity–viviparity transition is considered to be the result of a progressive increase in the level of intrauterine embryonic development (Tinkle & Gibbons, 1977; Shine, 1985). According to our findings, there is some plasticity in egg retention in the studied population of *Z. vivipara*, but there are also limitations that reduce the extent of intrauterine embryonic development beyond the normal time of oviposition in this species. However, the fact that some eggs are successful after forced egg retention could be advantageous for the females that, under unfavourable climatic conditions, are able to retain their eggs until more suitable conditions. We have not studied the long-term effects of egg retention, and it remains unknown whether, in spite of the low hatching success, retention beyond the normal time of oviposition could have some kind of beneficial effect on survival and reproduction of hatchling *Z. vivipara*, as has been found in other lizard species (Warner & Andrews, 2003).

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