

Further evidence of the existence of oviparous populations of *Lacerta (Zootoca) vivipara* in the NW of the Balkan Peninsula

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Abstract – The lizard *Lacerta (Zootoca) vivipara*, which is viviparous in the greatest part of its distribution range, has however some oviparous populations on the southern margin of its range. The present study aimed at determining the reproductive mode and the *ATA* (aspartate transaminase) enzyme characteristics of four populations in Slovenia and one population in Croatia. The Slovenian females studied here presented an oviparous reproductive mode which strongly resembled those observed in the oviparous populations of south-western France and north-western Spain. Our electrophoresis analyses revealed the existence of two distinct alleles, *ATA*⁻¹⁵⁰ and *ATA*⁻²⁰⁰, in the oviparous populations of Slovenia. These alleles were identical to those observed in the French and Spanish oviparous group and were distinct from the allele *ATA*⁻¹⁰⁰ characterizing the viviparous populations that we had previously studied. Although we did not study the reproductive mode of Croatian females, the allele *ATA*⁻²⁰⁰ observed in one population of Croatia strongly suggested that this population might also be oviparous. © 2000 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

***Lacerta (Zootoca) vivipara* / Squamata / Lacertidae / reproductive modes / biogeography**

Résumé – **Données nouvelles sur l'existence de populations ovipares de *Lacerta (Zootoca) vivipara* au nord-ouest de la péninsule balkanique.** *Lacerta (Zootoca) vivipara* est une espèce de lézard qui, bien qu'habituellement vivipare, présente cependant quelques populations à reproduction ovipare en bordure méridionale de son aire de répartition. L'étude présentée ici a pour but de préciser le mode de reproduction et les phénotypes enzymatiques de l'aspartate transaminase (*ATA*), pour quatre populations de Slovénie et une population de Croatie. Les femelles provenant des quatre populations slovènes présentent un mode de reproduction ovipare comparable à celui précédemment mis en évidence dans les populations du sud-ouest de la France et du

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nord-ouest de l'Espagne. Les populations ovipares de Slovénie, tout comme les populations ovipares de France et d'Espagne précédemment étudiées, portent exclusivement les allèles ATA^{-150} et ATA^{-200} . Cela les distingue des populations vivipares précédemment étudiées, qui sont exclusivement porteuses de l'allèle ATA^{-100} . La présence de l'allèle ATA^{-200} dans une population de Croatie dont nous n'avons pas pu étudier les caractères de reproduction, suggèrent fortement que cette population pourrait également être ovipare. © 2000 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

***Lacerta (Zootoca) vivipara* / *Lacerta* / *Lacertidae* / modes de reproduction / biogéographie**

Version abrégée

Lacerta (Zootoca) vivipara est une espèce de lézard qui présente à la fois des populations ovipares et des populations vivipares. Les populations vivipares, largement majoritaires, sont distribuées depuis le Massif central en France et les îles Britanniques jusqu'en Scandinavie et jusqu'en Russie orientale. Des populations ovipares de cette espèce ont tout d'abord été indentifiées à l'extrême sud-ouest de son aire de répartition, dans une zone englobant les monts Cantabriques, le Pays Basque, les Pyrénées et l'Aquitaine. L'importante extension septentrionale de la souche vivipare de *Lacerta (Zootoca) vivipara* et la localisation de la souche ovipare de cette espèce en marge méridionale de l'aire de répartition apparaissent conformes à l'une des prédictions adaptatives de la théorie des « climats froids ». On pouvait donc, en se référant à cette théorie, suggérer que d'autres populations ovipares de *Lacerta (Zootoca) vivipara*, distinctes de celles du sud-ouest de la France et du nord-ouest de l'Espagne, pourraient éventuellement exister dans les parties les plus méridionales de l'aire de répartition de cette espèce, notamment dans la péninsule Balkanique. Les investigations réalisées en Bulgarie montrèrent en fait qu'en dépit de leur localisation à des altitudes et des latitudes comparables à celles des populations ovipares pyrénéennes toutes les populations de ce pays présentaient un mode de reproduction vivipare. Plus récemment, une observation ponctuelle (basée sur l'examen d'une seule femelle reproductrice) démontra l'existence d'un mode de reproduction ovipare dans une population (mont Sneznik) de Slovénie. L'étude présentée ici a eu pour but de déterminer le mode de reproduction et les phénotypes enzymatiques de l'aspartate transaminase (*ATA*), pour quatre autres populations de Slovénie et pour une population de Croatie. Ces caractéristiques ont été comparées à celles des populations ovipares et vivipares précédemment étudiées.

Les femelles provenant des quatre populations slovènes sont indubitablement ovipares: elles ont pondu des œufs dont la coquille, blanche et parcheminée, est tout à fait similaire à celle des œufs observés dans les

populations ovipares françaises et espagnoles. Cette coquille est en revanche totalement différente de la membrane coquillière transparente enveloppant l'embryon de la forme vivipare jusqu'à la parturition. Deux des femelles slovènes étudiées ont pondu une deuxième ponte, 3 à 4 semaines après leur première ponte. Cette aptitude à réaliser deux pontes successives, qui a précédemment été observée dans des populations ovipares pyrénéennes, n'existe pas chez la forme vivipare de l'espèce.

Les femelles de l'échantillon slovène étudié se caractérisent par une taille corporelle moyenne et par une fécondité moyenne (nombre d'œufs dans la première ponte) significativement plus grandes que celles observées dans un échantillon de femelles ovipares des populations françaises. Il n'existe pas en revanche de différence significative de la relation entre fécondité et taille corporelle, ni de différence significative de fécondité ajustée (nombre d'œufs par ponte corrigé pour les différences de taille corporelle) entre les femelles de ces deux échantillons. Le poids moyens des œufs au moment de la ponte et la taille moyenne des nouveau-nés à l'éclosion ne diffèrent pas significativement entre les échantillons ovipares slovène et français comparés. Comparativement aux œufs de la souche ovipare française, les œufs de la souche ovipare slovène contiennent des embryons significativement moins développés au moment de leur ponte, nécessitent une période d'incubation externe significativement plus longue et donnent naissance à des jeunes significativement plus lourds. Compte tenu des faibles effectifs analysés (huit femelles de Slovénie), de nouvelles investigations seront nécessaires pour vérifier si les différences observées entre les échantillons ovipares français et slovènes sont bien réelles.

Du point de vue enzymatique, les populations ovipares de Slovénie, tout comme les populations ovipares de France et d'Espagne précédemment étudiées, portent exclusivement les allèles ATA^{-150} et ATA^{-200} . Cela les distingue des populations vivipares précédemment étudiées, qui sont exclusivement porteuses de l'allèle ATA^{-100} . La présence de l'allèle ATA^{-200} dans une population de Croatie dont nous n'avons pas pu étudier les caractères de reproduction suggère cepen-

dant fortement que cette population pourrait également être ovipare.

De nouvelles recherches, visant à préciser l'extension géographique du groupe ovipare identifié au nord-ouest de la péninsule Balkanique, semblent indis-

pensables. Elles devraient permettre de déterminer s'il existe – ou non – des zones de contact entre ce groupe ovipare et les populations vivipares des Alpes, des Carpathes, et des Massifs montagneux Bulgares.

1. Introduction

The lizard *Lacerta (Zootoca) vivipara* (Jacquin, 1787 [1]) has both oviparous (egg-laying + incubation in natura) and viviparous (gestation + parturition) populations. The viviparous populations are widely distributed from central France and the British Isles up to Scandinavia and eastern Russia. Oviparous populations of *Lacerta (Zootoca) vivipara* were first identified in the extreme south-western part of the species' range, from the Cantabric mountains in Spain up to the Pyrénées and Aquitaine in France. No contact zones have been found between oviparous and viviparous populations in southern France (*figure 1.A*) [2–4]. Ecological and morphological resemblance, successful experimental (laboratory) hybridization and short genetic distance calculated from allozymes and mtDNA studies, all indicate that oviparous and viviparous strains of *Lacerta (Zootoca) vivipara* can be considered as belonging to a single species [4–9]. Studies of allozymes showed that, despite the small genetic differentiation (Nei's genetic distance $D = 0.102$, for 13 polymorphic loci) of the oviparous and viviparous forms of *Lacerta (Zootoca) vivipara*, the alleles of the ATA enzyme (aspartate transaminase) could be used as a convenient genetic marker for the recognition of the two reproductive forms of this species: all oviparous lizards from south-west France and north-west Spain exhibit the fast migrating alleles ATA^{-150} or ATA^{-200} of the enzyme, whereas all viviparous lizards from France and Bulgaria exhibit the slow migrating allele ATA^{-100} of this enzyme [6, 7].

The latitudinal distribution of the two reproductive forms is well fitted to the 'cold climate' theory, predicting that viviparity is a biological feature allowing populations of lizards to colonize regions of high latitude, whereas oviparity might remain advantageous at lower latitude [10, 11]. According to this, one could not exclude that, in addition to the south-western area, oviparous populations of *Lacerta (Zootoca) vivipara* might also persist elsewhere on the southern limit of the species range. In particular, the Balkan Peninsula, which marks the southernmost extent of the distribution area (*figure 1.A*), was likely to contain such oviparous populations. However, investigations in Bulgaria showed that, despite their location at a latitude comparable – or even lower – than those of the south-western oviparous group, all populations in this region are indeed viviparous [6].

Finally, a recent observation provided the first evidence of the existence of an oviparous reproductive mode in one population of the Sneznik Mountain (*figure 1.B*) in Slovenia. However, this observation was based on a single

female which, moreover, died at the onset of oviposition and failed to expulse half of her clutch [12]. In order to verify – and to extend – this first observation, we attempted to determine the reproductive mode and the allozyme characteristics (ATA alleles) of several populations of the north-west of the Balkan Peninsula, during the course of 1999.

2. Materials and methods

We analysed the ATA enzyme characteristics of 15 Slovenian individuals captured in 1999: one adult male, one adult female and two subadults came from a wet meadow at Medvedce (altitude 245 m, latitude 46°22' N, longitude 15°41' E), two adult females and one adult male came from a peatbog at Kot in the Pohorje Mountains (altitude 1 040m, latitude 46°26' N, longitude 15°26' E), two adult females, one adult male and two subadults came from a wet meadow at Zelenci (altitude 840 m, latitude 46°30', longitude 13°44' E), one adult male and one adult female came from a wet meadow on the banks of Cerknisko Lake (altitude 480 m, latitude 45°46' N, longitude 14°22' E). In addition to this Slovenian sample we also analysed the ATA characteristics of three collection specimens (one male, two females) from Plitvitce (altitude 1 100 m, latitude 44°52' N, longitude 15°36' E) in Croatia, which had been kept in our freezer since 1989. The location of these sites is presented in *figure 1.B*.

Enzyme extraction and starch gel electrophoresis techniques were identical to those used in our previous studies [6, 7]. For each starch gel electrophoresis performed, we determined the ATA enzyme phenotypes of Slovenian and Croatian individuals by comparing the migration of their enzyme extracts to those of the enzyme extracts of oviparous and viviparous French individuals whose ATA enzyme phenotypes had previously been identified [6, 7].

We studied the reproductive mode of eight Slovenian females captured in 1999: one female from Medvedce, three females from the Pohorje Mountains, two females from Zelenci, and two females from the Cerknisko Lake. The female from Medvedce was captured in early April, whereas all other females were caught in early May. The females were transported to the French laboratory on 12 May where they were reared separately in plastic terraria until egg-laying. Each terrarium was equipped with a shelter, dishes of food and water and a 40-W bulb that provided heat for 6 h/d. We checked terraria for clutches four times a day. Clutches were removed from the terraria. The presence of sterile (i.e. unfertilized) eggs, which are

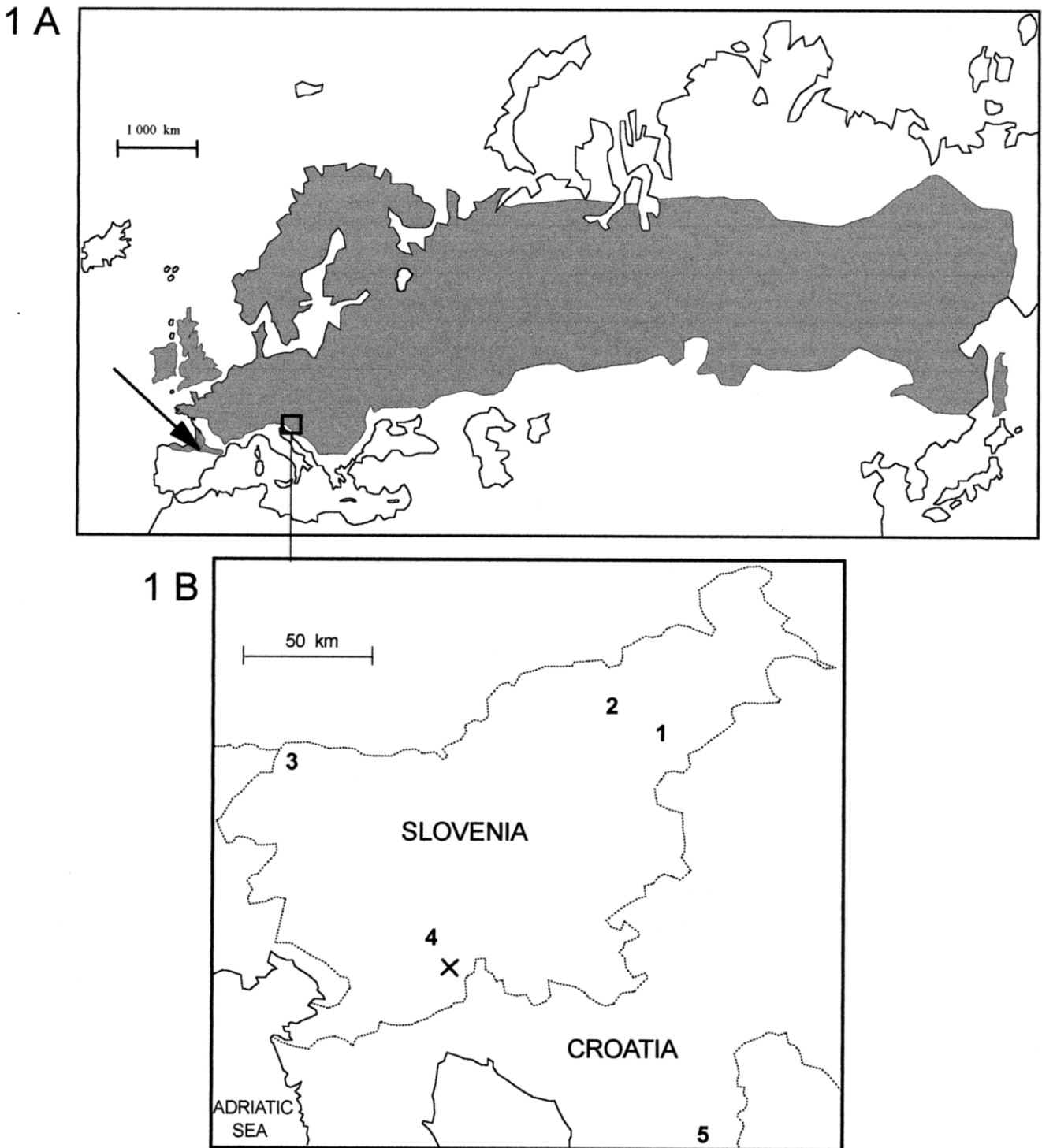


Figure 1. Distribution range of *Lacerta (Zootoca) vivipara* (1A) and location of the populations sampled in Slovenia and Croatia (1B).
A. The arrow indicates the location of the oviparous group isolated in south-western France and north-western Spain.
B. The Slovenian sampled sites are Medvedce (1), Kot-Pohorje (2), Zelenci (3), Cerknisko Lake (4) and the Croatian site is Plitvitce (5). The cross X indicates the localization of the oviparous population previously identified by Bohme et al. [12] in the Sneznik Mountain.

easily identifiable by their smaller size and by their flattened and folded appearance [3], was noted. One fertilized egg from each clutch was dissected to determine the developmental stage of the embryo according to the ter-

minology of Dufaure and Hubert [13]. Two to three fertilized eggs from each clutch were kept under nearly 100 % relative humidity in small containers with damp sand and were incubated in a temperature chamber which provided

Table 1. Reproductive characteristics of eight Slovenian females of *Lacerta (Zootoca) vivipara*.*

Female code	Origin	SVL (mm)	Clutch	Egg-laying date	EMBR	NE	fEW (mg)	stEW (mg)
M1	Medvedce	67	first	May 24	–	9st	–	167 ± 9
Z1	Zelenci	62	first	May 30	32	8	225 ± 4	–
P1	Pohorje	68	first	June 3	31	9	189 ± 6	–
P2	Pohorje	62	first	June 5	30	6	214 ± 12	–
C1	Cerknisko	63	first	June 5	31	7	256 ± 7	–
C2	Cerknisko	71	first	June 6	31	12	247 ± 17	–
Z2	Zelenci	74	first	June 6	32	10	251 ± 12	–
P3	Pohorje	65	first	June 12	32	8	238 ± 10	–
P1	Pohorje	68	second	July 3	31	5	273 ± 9	–
Z2	Zelenci	74	second	July 11	32	3 + 5st	276 ± 8	165 ± 5

* Origin: see figure 1.B for location of the sites. SVL: snout-vent length of the female; Clutch: first or second clutch of the female, NE: number of fertilized + sterile (st) eggs in the clutch, fEW: mean weight (\pm standard deviation) of the fertilized eggs, stEW: mean weight (\pm standard deviation) of the sterile eggs, EMBR: embryo stage (Dufaure and Hubert's terminology [13]) observed in one egg dissected after being laid.

a constant temperature of 22.5 °C (precision \pm 0.5 °C). The remaining eggs were fixed in alcohol for other purposes. The incubation containers were checked every day for hatchlings.

The number of eggs, egg weights and embryo stages at egg-laying, characterizing the Slovenian clutches were compared to those of 44 clutches of French oviparous females previously studied in our laboratory [3, 4]. These French oviparous females came from 16 distinct localities distributed all over the Pyrénées and the Aquitaine regions at altitudes ranging from 40 to 2 020 m: Louvie, Palomnières, Benou, Aubisque, Tourmalet, Saint-Raphael, Saint-Symphorien, marais de l'Anguille, Bélesta, Moulis, Estats, Bouillouses, col de Pause, col de Port, Gabas, Pourtalet (for a localization map: see [3, 4, 7]). The duration of incubation (i.e. the interval between oviposition and egg-hatching) of the Slovenian eggs was compared to that of a subset of 23 eggs (one to three eggs from nine clutches) of the French oviparous sample, which had been incubated under the same temperature condition (22.5 °C) in our laboratory.

All the mean values are presented \pm their standard deviation. Statistical tests were performed using BIOM software [14].

3. Results/Discussion

3.1. ATA alleles characteristics

Our electrophoretic analyses revealed the existence of two distinct alleles, ATA^{-150} and ATA^{-200} , in the sample studied. These fast migrating alleles ATA^{-150} and ATA^{-200} were identical to those observed in the French and Spanish oviparous group and were distinct from the slow migrating allele ATA^{-100} characterizing the viviparous populations that we had previously studied [6, 7].

The ATA enzyme phenotypes observed in the sample studied were: the homozygous phenotype $ATA^{-150/-150}$ for nine Slovenian specimens, the heterozygous phenotype $ATA^{-150/-200}$ for five Slovenian specimens and the homozy-

gous phenotype $ATA^{-200/-200}$ for one Slovenian specimen and for the three Croatian specimens. Hence, the relative frequencies of the alleles ATA^{-150} and ATA^{-200} were, respectively, 0.64 and 0.36 in the sample studied.

These allelic frequencies were not significantly different (Fisher exact probability $P = 0.35$) from those (0.47 for the ATA^{-150} , 0.53 for the ATA^{-200}) characterizing a sample of 89 individuals coming from oviparous populations in south-western France and north-western Spain [6, 7].

3.2. Reproductive characteristics of the Slovenian females

The eight Slovenian females whose reproductive characteristics were studied had a mean snout-vent length (SVL) of 66.5 ± 4.4 mm. All these females laid a first clutch of eggs between May 24 and June 12 (table 1). The mean number of eggs (NE) in these eight clutches was 8.6 ± 2 eggs. The number of eggs per clutch was positively correlated to the females' snout-vent length: the regression line was $NE = 0.35 SVL - 15$ (correlation coefficient $r = 0.84$, $P < 0.01$). The clutch of the female from Medvedce was completely sterile. This is possibly because she was captured earlier in the reproductive season (see Materials and methods) and probably did not mate before being captured. In fact, no mark of copulation (due to male bite during mating) was observed on the belly of this female, whereas all other females presented such a mark when captured.

Two females (P1 from Pohorje, and Z2 from Zelenci), which were kept with a male in their terraria, mated a second time after having laid their first clutch, and were able to lay a second clutch at the beginning of July. For the second clutch of the female Z2, five eggs out of eight were sterile (table 1). All the eggs laid, even the unfertilized ones, showed a white and opaque parchment-like eggshell (figure 2). At egg-laying, the 68 fertilized eggs had a mean weight of 237 ± 28 mg (range: 180–305 mg). The freshly laid eggs that were dissected contained embryos that had reached developmental stages 30–32 (figure 3). The incubation duration of the 24 eggs kept at 22.5 °C, averaged



Figure 2. Slovenian female of *Lacerta (Zootoca) vivipara* beside her eggs with white and parchment-like eggshells.

34.6 ± 1.3 days (range: 33–37 d). The 24 hatchlings obtained from these eggs had a mean snout-vent length of 21.3 ± 1.2 mm (range 19–24 mm), a mean tail length of 27.8 ± 2.3 mm (range 24–32 mm) and a mean weight of 256 ± 39 mg (range 182–300 mg).

3.3. Comparisons with other oviparous females

The Slovenian females of *Lacerta (Zootoca) vivipara* studied here presented reproductive characteristics that

strongly resembled those observed in the oviparous populations of south-western France and north-western Spain and which were clearly distinct from those observed in the viviparous populations of the species. First, the eggs laid by the Slovenian females had a white and opaque parchment-like eggshell, which is similar to those previously observed in Spanish and French oviparous populations, and which is very different from the transparent eggshell membrane enveloping the developing embryos in the viviparous populations of this species [2, 3, 15]. Second, the potentiality for some females to lay a second annual clutch, which has previously been noted for the French oviparous populations of *Lacerta (Zootoca) vivipara*, strongly contrasted with the fact that viviparous females of this species always lay a single brood per year [3, 11].

Despite the above-mentioned similarities, it was worth examining in more detail whether the Slovenian and the French oviparous females presented some differences in their reproductive characteristics. The data concerning second clutches were not sufficiently numerous to allow any statistical comparisons. Therefore, we limited the comparisons to the data concerning the first clutches. The 44 French oviparous females used for the comparison of reproductive characteristics had a mean snout-vent length (SVL) of 58 ± 4.0 mm (range: 49–67 mm) which was significantly smaller than those of the Slovenian females (*t*-test, $P < 0.05$). These French females produced first clutches whose mean number of eggs (NE), 6.2 ± 1.8 (range: 3–9 eggs), was significantly lower than those of the Slovenian first clutches (*t*-test, $P < 0.01$). The regression line between the French females' snout-vent length and their clutch size was $NE = 0.34 \text{ SVL} - 13$ ($N = 44$, $r = 0.75$, $P < 0.01$) and did not differ significantly from the regression calculated for the Slovenian females (analysis of covariance, $P > 0.05$ both for slopes, intercepts and adjusted means comparisons). Hence, the difference in clutch sizes between French and Slovenian females was only due to their difference in snout-vent length: the adjusted mean of clutch size (i.e. the mean number of eggs per clutch, corrected for the difference in females' snout-vent length) did not differ between French (adjusted mean = 6.7 eggs) and Slovenian (adjusted mean = 6.2 eggs) females. Freshly laid eggs of the French clutches had a mean weight of 238 ± 35 mg ($n = 262$; range: 165–324 mg) which did not differ from those of the eggs of the Slovenian clutches.

The range of embryonic development stages (30–35) observed at egg-laying in the French sample encompassed those of the Slovenian sample (figure 3). However, embryos of the French sample were on average significantly more developed at egg-laying than the Slovenian embryos (Mann Whitney U test, $P = 0.002$).

This might explain that the incubation duration of the French sample of eggs kept at 22.5°C , which averaged 28.6 ± 1.8 days ($n = 23$, range: 27–33 d), was significantly shorter than those of the Slovenian eggs incubated at the same temperature (*t*-test, $P < 0.01$).

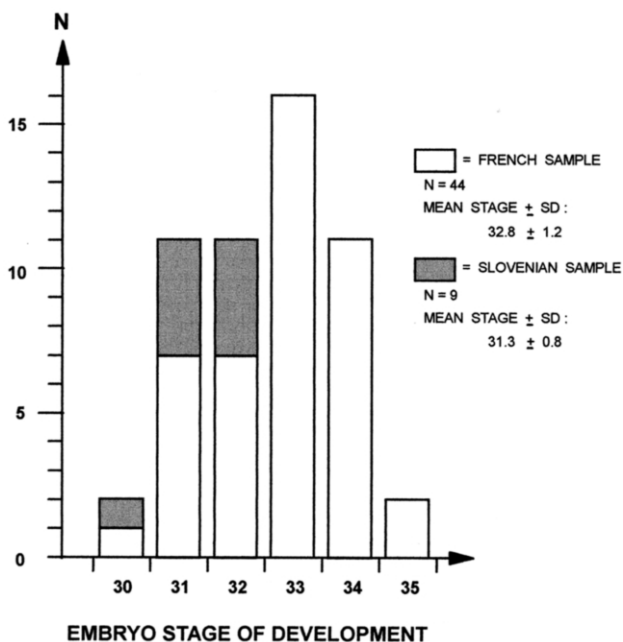


Figure 3. Embryo stages of development observed in the French and Slovenian samples of eggs dissected at egg-laying.

The 23 hatchlings obtained from the French sample of eggs incubated at 22.5 °C had a mean snout-vent length of 20.9 ± 0.8 mm (range: 20–22 mm) which did not differ from those of Slovenian hatchlings. However, their mean weight of 214 ± 23 mg (range: 174–269 mg) and their mean tail length of 25.1 ± 1.4 mm (range: 22–27 mm) were significantly smaller than those of the Slovenian hatchlings (*t*-tests, $P < 0.01$ in both cases).

4. Conclusions/Prospects

The Slovenian populations of *Lacerta (Zootoca) vivipara* studied here undoubtedly present an oviparous reproductive mode that is strongly similar to those characterizing the populations located in south-western France and north-western Spain, and which is distinct from the viviparous reproductive mode observed in the greatest part of the species' range. Because of the small sample examined, further investigations will be necessary to determine whether the small differences in reproductive characteristics observed between French and Slovenian oviparous females are real or are due to a sample bias. The confirmation that Slovenian females lay eggs containing embryos significantly less developed than those observed at oviposition in the eggs of French oviparous females, could provide an interesting intraspecific illustration of the concept of oviparity/viviparity continuum [16, 17]. This concept posits that the evolutionary shift from oviparity to viviparity in Reptiles proceeds through a gradual increase in the length of time eggs are retained in utero prior to oviposition; that is through a gradual increase in the developmental stage reached by the embryos at oviposition, which results in a gradual shortening of the subsequent incubation period of the eggs. Hence, the Slovenian and French oviparous strains of *Lacerta (Zootoca) vivipara* might represent, respectively, an earlier stage and a later stage on this oviparity/viviparity continuum.

From a geographic point of view, our data confirm and significantly extend an earlier report on the existence of an oviparous reproductive mode in Slovenia (figure 1.B). Moreover, the Croatian population studied here presented ATA enzyme characteristics that also strongly suggested that this population might also be oviparous. The fact that

the oviparous populations of *Lacerta (Zootoca) vivipara* appeared to be restricted to regions (NW Spain, SW France, Sloveno-Croatian area) of relatively low latitude as compared to those occupied by most of the viviparous populations of this species, corresponds to one of the predictions of the cold climate theory (see Introduction). However, some viviparous populations, such as those of Bulgaria, exist at latitudes and altitudes comparable to those of the Pyrenean oviparous populations [6]. Therefore, the possibility of a better adaptability to the climatic conditions of the lower latitude, might not be the only factor explaining the persistence of oviparous populations in southern regions. Fortuitous historical events, such as the emergence of local isolation barriers, could also have played a role in preventing contacts between the two reproductive forms, and hence, in favouring the local persistence of isolated oviparous populations in some southern regions. For example, the emergence, during the post-glacial warming period of the Late Pleistocene, of ecological conditions (extension of dry Mediterranean biotops in lowlands) that were unfavourable for *Lacerta (Zootoca) vivipara*, might have prevented a contact between the viviparous populations of the Massif Central and the isolated oviparous populations of the Pyrénées in southern France [8, 18]. This could be the reason why, although we successfully hybridized the two reproductive forms and obtained hybrids showing the heterozygous phenotypes $ATA^{-100/-200}$ or $ATA^{-100/-150}$ in our laboratories [5, 7], such hybrids were not found in natural populations of southern France. Whether similar events could have also led to an isolation of the Slovenian oviparous group is unknown. Hence, future research should also attempt to thoroughly determine the geographic extension of the oviparous form identified in the NW of the Balkan peninsula, and to evaluate whether or not this form has contact and hybridization zones with the viviparous populations of the Alps, Carpaths and Eastern-Balkan ranges.

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