Molecular Ecology (2004) **13**, 719–723 doi: 10.1046/j.1365-294X.2004.02102.x

SHORT COMMUNICATION

Multiple paternity in clutches of common lizard *Lacerta vivipara*: data from microsatellite markers

D. LALOI,* M. RICHARD,* J. LECOMTE,† M. MASSOT* and J. CLOBERT*

**Laboratoire d'Ecologie, UMR 7625, Université Pierre et Marie Curie, 7 quai Saint Bernard, case 237, 75252 Paris cedex 05, France; †Laboratoire Ecologie, Systématique et Evolution, UMR 8079, bât. 362, Université Paris Sud, 91405 Orsay cedex, France*

Abstract

The common lizard (*Lacerta vivipara*) is a small live-bearing lacertid that reproduces once a year. In order to document the poorly known mating system of this species, we present here an assessment of multiple paternity using microsatellite markers. Paternities were established within 122 clutches belonging to two wild populations from contrasted areas and to four seminatural enclosed populations. The proportion of multiply sired clutches was found to be very high (between 50.0% and 68.2%) and similar among populations, which suggests that the mating system of this species may be insensitive to environmental and population conditions.

Keywords: *Lacerta vivipara*, Lizards, mating system, microsatellites, multiple paternity

Received 23 September 2003; revision received 20 November 2003; accepted 20 November 2003

Introduction

Multiple mating in female animals constitutes one of the challenging questions about mating systems. In this broad field of investigation, studies in vertebrates have been dominated for a long time by works on birds and mammals (e.g. Birkhead & Møller 1992; Smuts & Smuts 1993; Reynolds 1996; Jennions & Petrie 2000) while reptiles, and especially lizards, have been understudied until recently. In an extensive review, Olsson & Madsen (1998) found evidence of multiple copulations in 12 out of 20 lizard species studied for their mating systems. Nevertheless, most of these data are based on behavioural observations or on counting of mating scars resulting from the male's mouthgrip on the female's abdomen during copulation. Such data generally do not indicate if multiple copulations come from the same male or from different partners, nor if they lead to effective multiple paternity. Indeed, recent lizard studies using molecular markers have revealed that effective paternity can contrast with the estimation given by matings observations (Olsson *et al*. 1994, 1996b; Gullberg *et al*. 1997; LeBas 2001). Some of these studies also provided original data concerning mating systems, for instance on mate choice and sperm competition (Olsson & Madsen 1998).

Lacerta vivipara is a small lacertid (adult snout-vent length = 50–70 mm, females on average larger than males) living in peatbogs and moist heathlands, widely distributed throughout Eurasia. This live-bearing lizard reproduces once a year and has a short mating period in April–May. With regard to mating system in natural populations, Bauwens & Verheyen (1985) have detected multiple (two to three) mating scars in females and they have suggested that different scars may be from different partners as they could occur within one to three days. Nevertheless, we assume that mating scars cannot be used to assess accurately the numbers of partners in this species, at least because a male can sometimes inflict multiple scars during a single copulation (personal observation). Behavioural observations on captive individuals by Heulin (1988) have proved that females can copulate with more than one male. However, as the experimental situation (three males and three females in a small enclosure) may enhance the opportunity for copulation, this author suggested that polyandry might be weaker in natural populations. Such a divergence was observed in the closely related species *Lacerta agilis* where females may mate with more than 15 males in few days in captivity while they rarely mate more than five to six times in the wild (Olsson *et al*. 1996a; Olsson & Madsen 1998). Finally, even if multiple matings occur in *L. vivipara*, there is no evidence that they lead to effective multiple paternity within clutches. Here, we present an assessment of multiple

Correspondence: D. Laloi. Fax: (+ 33) (0)1 44 27 35 16; E-mail: dlaloi@snv.jussieu.fr

paternity using microsatellite DNA loci. In order to investigate variations of the level of multiple paternity, we checked families from three populations with different environmental conditions: one natural population from a high altitude area, one natural population from a low altitude area, and four experimental populations maintained in seminatural enclosures. This experimental population was located at low altitude but it was originally constituted with lizards coming from the same area as the first studied wild population.

Materials and Methods

Collection of samples in natural populations

We collected samples in two wild populations in 2000. The first population is located in mountains of southern France (Mont Lozère, Lozère, 44°30′ N, 3°45′ E) at an altitude of 1420 m. It has been part of an ongoing demographic and behavioural study for the past 15 years (Massot & Clobert 2000). The second population is located in northern France (Forêt d'Orient, Aube, 48°20′ N, 4°25′ E) at an altitude of about 110–180 m.

To obtain clutches, pregnant females were captured in early July and kept in the laboratory until parturition. This period corresponds to the second month of gestation, parturition occurring generally in July or early August. Females were housed in individual terraria with damp soil and a shelter. To facilitate thermoregulation, we provided an incandescent lamp as a heat source for 6 h per day. Each female was also supplied with water and *Pyralis* larvae. After birth, tissue samples were obtained by cutting 2–3 mm tail tip of each female and their offspring, a non-destructive technique since lizards practice natural tail autotomy to escape predators. In clutches with incomplete hatching success, we also took tissue samples from dead born embryos. Then, all females and their viable hatchlings were released at the place where the female was captured within five days after birth, i.e. before juvenile dispersal (Clobert *et al*. 1994). The mother's capture point is likely to be close to the offspring natal site as females are highly sedentary during gestation (Bauwens & Thoen 1981).

Semi-natural enclosed population

We analysed multiple paternity in seminatural enclosed populations which has been part of a long-term experimental study of demography and dispersal (Boudjemadi *et al*. 1999a). These populations were originally constituted with lizards collected during July 1995 from Mont Lozère and brought to the Ecological Research Center of Foljuif near Paris, France (42°16′ N, 2°42′ E, altitude 200 m). They were randomly distributed into four seminatural enclosures (10 × 10 m) covered with nets to exclude avian predators, each enclosure receiving six postgravid females and their litter, four adults males, and five yearlings of each sex (age and sex structure similar to natural populations). During spring 1997 and 1998, gravid females were captured and kept in the laboratory until they gave birth. The rearing conditions were similar to those applied for the study of wild populations. Regular monitoring allowed us to obtain tissue samples from all males (i.e. putative fathers) in addition to those of the females and their offspring.

Microsatellite amplification and analysis

Genomic DNA was extracted from ethanol-preserved samples using Perfect gDNA Blood Mini Isolation kit for animal blood (Eppendorf). We applied the manufacturer's protocol except for the use of a small piece of tissue instead of blood. To obtain genotypic profiles, we used six highly polymorphic microsatellite DNA loci characterized in *Lacerta vivipara*: Lv-3–19, Lv-4–72, Lv-4-alpha, Lv-2–145, Lv-4-X and Lv-4–115 (Boudjemadi *et al*. 1999b). Primer sets were redesigned for multiplexing purposes. Microsatellite polymorphism was analysed using the fluorescent dye detection method. One primer of each locus was labelled, with VIC (green) for Lv-3–19, Lv-4-alpha and Lv-2–145, NED (black) for Lv-4–72 and Lv-4–115, and 6-FAM (blue) for Lv-4-X. Polymerase chain reaction (PCR) was carried out in 10 μL of a mixture containing 15–50 ng of DNA, 50–200 nM of each primer, 300 μM of dNTPs, 1 μL of Qbiogene 10X incubation buffer (50 mM KCl, 10 mM TrisHCl, 1.5 mM $MgCl_2$, 0.1% TritonX-100, pH 9.0) and 0.25 U of *Taq* DNA polymerase (Qbiogene). A PCR multiplex (for Lv-3–19, Lv-4–72, Lv-4-X and Lv-4–115) and simplex (for Lv-4-alpha and Lv-2–145) was performed in a GeneAmp PCR System 9700 thermocycler, using the following profile: an initial denaturing step of 12 min at 94 °C; followed by 30 cycles of 15 s at 94 °C; 15 s at 53 °C (56 °C for loci Lv-2–145 and Lv-4-alpha) and 30 s at 72 °C, a final extension step at 72 °C for 10 min. Electrophoreses were performed with an ABI 310 automated sequencer. Allelic size was determined using GENESCAN software version 2.1 by reference to the GENESCAN ROX 400HD size standard and by comparison to previously scored samples.

Assessment of paternity and analyses

In natural populations, mothers and their offspring were genotyped for five microsatellite loci (all but Lv-2–145). Because actual genotypes of putative fathers remained unknown, the extent of multiple paternity was inferred from the genotypes of juveniles after subtraction of maternal alleles. Multiple paternity was first detected by the presence of three or more paternal alleles per locus. Then, the minimum number of fathers to explain genetic composition of each clutch was estimated applying a conservative reconstruction of the possible paternal genotypes. In the

Table 1 Extent of multiple paternity in clutches of common lizard. Number of fathers were obtained after inference of paternal haplotypes from hatchlings genotypes in wild populations and after paternity assignment in the seminatural enclosed populations

	Number (%) of clutches with		
	1 father	2 fathers	3–5 fathers
Population from Mont Lozère	14 (*31.8*)	28 (*63.6*)	2(*4.6*)*
Population from Forêt d'Orient	7 (*50.0*)	6 (*42.9*)	1 (*7.1*)*
Enclosed population — 1997	11 (*42.3*)	9 (*34.6*)	6 (*23.1*)
Enclosed population — 1998	12 (*33.3*)	14 (*38.9*)	10 (*27.8*)

*In natural populations, the proportion of litters with 3–5 fathers is probably underestimated as a result of the conservative method applied for detection of paternity.

enclosed populations, all individuals including potential fathers were genotyped for the six microsatellite loci. Paternity assignments were then carried out by a likelihood approach using the CERVUS software version 2.0 (Marshall *et al*. 1998). To prevent false identification of father due to genotyping or reading errors, particularly in natural populations where offspring's genotypes could not be compared to putative fathers, we ignored additional fathers when the detection relied on a single locus in a single hatchling. Conversely, such a precaution may lead to some underestimation of the number of fathers. This, however, only occurred in one clutch from the Mont Lozère population.

Results and Discussion

Forty-five females and their 256 offspring from the Mont Lozère, as well as 15 females and their 79 offspring from the Forêt d'Orient, were genotyped for five loci. Considering all loci, both populations do not deviate significantly from Hardy–Weinberg equilibrium. Among the 45 clutches from the Mont Lozère, one clutch with only two hatchlings was excluded from the estimation of multiple paternity as it was not possible to detect more than two paternal alleles in such a situation. Multiple paternity was detected in 30 (68.2%) of the 44 remaining clutches, 28 clutches (63.6%) being sired by at least two males and two clutches (4.6%) by at least three males (Table 1). Among the 15 clutches from the Forêt d'Orient, one clutch with only two hatchlings was excluded from the analysis of paternity. Multiple paternity was detected in seven clutches (50.0%), six clutches (42.9%) being sired by at least two males and one clutch (7.1%) by at least three males (Table 1). In both populations, clutch size does not differ between singly sired and multiply sired clutches ($t = 0.39$, $P > 0.10$ for the Mont Lozère population; $t = 0.13$, $P > 0.10$ for the Forêt d'Orient population; see Table 2 for details on clutches' characteristics).

From the semi-natural enclosed populations, a total of 670 individuals were genotyped for six loci, including 26 clutches in 1997, 36 clutches in 1998 and all adults. The data from the four enclosures were pooled since the respective populations did not differ according to the number of mates ($\chi^2 = 3.88$, $P > 0.10$). However, data from 1997 and 1998 are presented separately as the population size increased significantly between these two years, which might affect the pattern of matings. Considering all loci, the population does not deviate significantly from Hardy–Weinberg equilibrium. The exclusionary power of paternity assignments vary between 0.937 and 0.999 according to enclosure and year. Multiple paternity was detected in 15 out of 26 clutches in 1997 (57.7%) and in 24 out of 36 clutches in 1998 (66.7%), with, respectively, six clutches in 1997 (23.1%) and 10 clutches in 1998 (27.8%) sired by more than two males (Table 1). Extreme cases of multiple paternity were two clutches sired by four males (one clutch of six hatchlings in 1997 and one clutch of 13 hatchlings in 1998) while up to five males were found in one clutch of 11 hatchlings obtained in 1998. Clutch size does not differ between singly sired and multiply sired clutches ($t = 0.37$, $P > 0.10$ in 1997; $t = 0.06$, $P > 0.10$ in 1998; see Table 2 for details on clutches' characteristics).

At first sight, the pattern of multiple paternity seems to differ between surveyed populations with respect to the number of fathers: clutches sired by more than two

Table 2 Summary of clutches' characteristics

		Clutch size*			
	N	Mean ± SE	min	max	% multiply sired clutches**
Population from Mont Lozère	45	5.7 ± 1.6	2	9	68.2
Population from Forêt d'Orient	15	5.4 ± 2.0	2	10	50.0
Enclosed population — 1997	26	6.2 ± 2.6	2	13	57.7
Enclosed population — 1998	36	4.9 ± 2.7	1	12	66.6

*Including dead born embryos. **Clutches with only two juveniles were excluded from the estimation of multiple paternity.

males appear quite rare in natural populations while they constitute about a quarter of the clutches in the semi-natural populations. Nevertheless, since the males (i.e. the putative fathers) remained unknown in natural populations, the conservative method applied for detection of paternity may have led to some underestimation of the actual number of fathers. Indirect evidence for this underestimation can be given applying the inference of paternity from juveniles' genotypes, when males are unknown, to our data set from enclosed populations. This leads to an identical estimation of the number of multiply sired clutches, but to fewer fathers in these clutches than the number obtained by paternity assignment to known males (only 4.3% of 1997 clutches and 3.6% of 1998 clutches appeared to be sired by more than two males when paternity was inferred from hatchlings' genotypes, while 23.1% and 27.8% were, respectively, found by paternity assignment). Given that this methodological bias can explain differences in paternity estimation between natural and enclosed populations, we suspect that the pattern of paternity does not differ markedly between the different populations studied. Indeed, when we pooled the clutches in two classes, singly sired vs. multiply sired clutches, the level of multiple paternity did not differ significantly between populations ($\chi^2 = 2.05$, $P > 0.10$). This constancy of multiple paternity level among various sites suggests that the mating system of *Lacerta vivipara* may be insensitive to environmental and population conditions.

In lacertids, multiple paternity often relate to the coexistence of conflicting male mating strategies. Most cases refer to territorial species where territory-holding males sired the greater part of the hatchlings while floaters may achieve some matings, leading to multiply sired clutches. For example, 23–62% of clutches are multiply sired in *Scelophorus virgatus* (Abell 1997), 25% in *Ctenophorus ornatus* (LeBas 2001), 65–82% in *Eulamprus heatwolei* (Morrison *et al*. 2002). Cases of multiple paternity in nonterritorial lizards are more scarce. In *Lacerta agilis*, females have been found to accept courtship by several males (Olsson *et al*. 1994, 1996a) and four out of five clutches (80%) were sired by more than one male (Gullberg *et al*. 1997). Males of this species guard their present female between several hours to several days following copulation, which is an uncommon behaviour in lizards but can be expected in such a competitive situation. In *Lacerta vivipara*, males do not form territory (Avery 1976), they do not exhibit any type of mate guarding and, until now, there has been no evidence of various male mating strategies. Further studies should thus address other possible reasons that could lead to an almost constant pattern of single vs. multiple paternity in this species. This might include studies on the benefits of multiple mating, especially for females, as well as on the possible existence of more than one male strategy.

Acknowledgements

We thank Sandrine Meylan and Jean-François Le Galliard for encouraging discussions, and three anonymous referees for helpful comments on a previous version of the manuscript.

Supplementary material

The following material is avaialbale from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2102/MEC2102sm.htm

References

Abell AJ (1997) Estimating paternity with spatial behaviour and DNA fingerprinting in the striped plateau lizard, *Sceloporus virgatus* (Phrynosomatidae). *Behavioural Ecology And. Sociobiology*, **41**, 217–246.

Avery RA (1976) Thermoregulation, metabolism ans social behaviour in Lacertidae. In: *Morphology and Biology of Reptiles* (ed. Barry Cox C), pp. 245–259. Academic Press, London.

Bauwens D, Thoen C (1981) Escape tatics and vulnerability to predation associated with reproduction in the lizard *Lacerta vivipara*. *Journal of Animal Ecology*, **50**, 733–743.

Bauwens D, Verheyen RF (1985) The timing of reproduction in the lizard *Lacerta vivipara*: differences between individual females. *Journal of Herpetology*, **19**, 353–364.

Birkhead TR, Møller AP (1992) *Sperm Competition in Birds: Evolutionary Causes and Consequences*. Academic Press, London.

Boudjemadi K, Lecomte J, Clobert J (1999a) Influence of connectivity on demography and dispersal in two contrasting habitats: an experimental approach. *Journal of Animal Ecology*, **68**, 1207–1224.

Boudjemadi K, Martin O, Simon JC, Estoup A (1999b) Development and cross-species comparison of microsatellite markers in two lizard species, *Lacerta vivipara* and *Podarcis muralis*. *Molecular Ecology*, **8**, 518–520.

Clobert J, Massot M, Lecomte J *et al.* (1994) Determinants of dispersal behavior: the common lizard as a case study. In: *Lizard Ecology. Historical and Experimental Perspectives* (eds Vitt LJ, Pianka ER), pp. 181–206. Princeton University Press, Princeton, New Jersey.

Gullberg A, Olsson M, Tegelström H (1997) Male mating success, reproductive success and multiple paternity in a natural population of sand lizards: behavioural and molecular genetics data. *Molecular Ecology*, **6**, 105–112.

Heulin B (1988) Observations sur l'organisation de la reproduction et sur les comportements sexuels et agonistiques chez Lacerta vivipara. *Vie Milieu*, **38**, 177–187.

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. *Biology Reviews*, **75**, 21–64.

LeBas NR (2001) Microsatellite determination of male reproductive success in a natural population of the territorial ornate dragon lizard, *Ctenophorus ornatus*. *Molecular Ecology*, **10**, 193–203.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Massot M, Clobert J (2000) Processes at the origin of similarities in dispersal behaviour among siblings. *Journal of Evolutionary Biology*, **13**, 707–719.

Morrison SF, Scott Keogh J, Scott AW (2002) Molecular determination of paternity in a natural population of the multiply mating polygynous lizard *Eulamprus heatwolei*. *Molecular Ecology*, **11**, 535–545.

Olsson M, Gullberg A, Tegelström H (1994) Sperm competition in the sand lizard *Lacerta agilis*. *Animal Behaviour*, **48**, 193–200.

Olsson M, Gullberg A, Tegelström H (1996a) Mate guarding in male sand lizards (*Lacerta agilis*). *Behaviour*, **133**, 367–386.

Olsson M, Madsen T (1998) Sexual selection and sperm competition in reptiles. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 503–577. Academic Press, London.

Olsson M, Shine R, Madsen T, Gullberg A, Tegelström H (1996b) Sperm selection by females. *Nature*, **383**, 585.

Reynolds JD (1996) Animal breeding systems. *Trends in Ecology and Evolution*, **11**, 68–72.

Smuts BB, Smuts RW (1993) Male aggression and sexual coercion of females in nonhuman primates and other mammals: evidence and theoretical implications. *Advances in the Study of Behavior*, **22**, 1–63.

David Laloi works on behavioural ecology with a particular interest in the plasticity of behaviour. In parallel with the development of genetic markers for ecological purposes, Murielle Richard works on reproductive behaviour in lizards and birds. Jane Lecomte's research subjects include the dynamics of fragmented animal populations and ecological risk assessment related to transgenic plants cultivation. Manuel Massot studies the evolution of dispersal, phenotypic plasticity, senescence, and consequences of climate's changes, mainly based on a long-term survey of common lizard's populations. Jean Clobert is investigating population dynamics, mating systems, evolution of life history traits and dispersal.
