Microsatellite markers reveal multiple paternity and sperm storage in the Mediterranean spur-thighed tortoise, *Testudo graeca*

S. Roques, C. Díaz-Paniagua, and A.C. Andreu

Abstract: The spur-thighed tortoise (*Testudo graeca*) is a terrestrial species in which multiple mating is frequently observed. We assessed the probability of multiple paternity in clutches (n = 15) laid by eight females, including successive clutches. Paternity was determined by microsatellite analyses at three loci. A large number of alleles per locus (n = 15–22) provided high probabilities of detecting multiple paternity, particularly at all loci combined (P = 0.989). Multiple paternity was found in 20% of the clutches in which offspring displayed more than two paternal alleles. However, this frequency may have been underestimated, given the small clutch sizes and the few loci used. Also, *T. graeca* is able to store sperm from single or multiple matings and can use it to fertilize subsequent clutches of eggs, as indicated by the fact that the second clutch of a captive female was sired by a different male and that clutches of another female were multiply sired by the same males. These results confirm that multiple paternity exists in *T. graeca* and that sperm storage in this species may be an important reproductive strategy to fertilize multiple clutches per year.

Résumé : La tortue grecque (*Testudo graeca*) est une espèce terrestre chez laquelle il y a souvent des accouplements multiples. Nous avons évalué la probabilité de paternités multiples dans 15 couvées déposées par huit femelles, y compris des pontes successives. La paternité a été déterminée par l'analyse de trois locus microsatellites. Un nombre élevé d'allèles par locus (n = 15–22) permettait la détection de paternités multiples avec une probabilité élevée (P = 0,989), particulièrement par l'analyse combinée de l'ensemble des locus. Nous avons observé une paternité multiple dans 20 % des couvées pour lesquelles la progéniture possède plus de deux allèles d'origine paternelle. Par ailleurs, cette fréquence est probablement sous-estimée, étant donné le faible nombre de petits par couvée et le nombre peu élevé de locus étudiés. Également, la seconde ponte d'une femelle en captivité a été fécondée par un mâle différent de la première et les couvées successives d'une autre femelle ont été fécondées conjointement par les mêmes mâles; ces observations confirment que *T. graeca* est capable de stocker le sperme d'un ou plusieurs accouplements et de l'utiliser par la suite pour féconder ses pontes successives. Nos résultats démontrent l'existence de la paternité multiple chez cette espèce et de la mise en réserve du sperme comme stratégie reproductive importante pour pouvoir féconder plusieurs pontes au cours de l'année.

Introduction

Genetic parentage has been investigated in many taxa to understand mating strategies and to test hypotheses regarding mating behaviour and reproductive systems. Recent studies of the mating systems of poikilothermic vertebrates, particularly reptiles, have assessed paternity and internesting strategy (Höggren and Tegelström 1995; Gullberg et al. 1997; McCracken et al. 1999; Davis et al. 2001; Morrison et al. 2002). Studies of these strategies in turtles have mainly focussed on aquatic species with large clutches (Galbraith et al. 1993; Fitzsimmons 1998; Bollmer et al. 1999; Kichler et al. 1999; Valenzuela 2000; Pearse et al. 2001; Crim et al. 2002; Hoekert et al. 2002; Moore and Ball 2002). Assessing multiple paternity in tortoises is impeded by small clutch sizes and the lack of molecular markers for these species. Only a single example of multiple paternity has been documented in tortoises (Palmer et al. 1998, *Gopherus agassizzii*). Most genetic studies have documented multiple paternity in both marine and freshwater turtles (see review in Pearse and Avise 2001), suggesting that this strategy may be an adaptive feature of the mating system of turtles (Galbraith 1993).

Sperm storage is known to occur in terrestrial turtle species, enabling the females to fertilize their ova several months or even years after insemination (Gist and Jones 1987, 1989). For example, in the desert tortoise, *G. agassizii*, allozyme analyses demonstrated that 42% of the clutches resulted from fertilization with sperm stored from matings that took place at least 2 years before egg laying (Palmer et al. 1998). Sperm storage, along with frequent matings during a long reproductive season, may therefore promote multiple paternity in tortoises.

The range of the spur-thighed tortoise, *Testudo graeca*, is mainly north Africa but extends to south and east Europe and west Asia, and it usually consists of open scrubland habitats (Iverson 1992). This endangered species is of medium size and has a long annual reproductive period. First courtships are observed in autumn and, after a hibernation period,

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the main courting period occurs in spring, from February to June. Females lay one to four clutches (mean clutch size = 3.4 eggs) between April and July, with approximately 21-day internesting intervals (Díaz-Paniagua et al. 1996, 2001). During the mating season, males are very active, moving distances of up to 1012 m daily (Díaz-Paniagua et al. 1995). Because multiple matings are frequently observed during this active season, including matings with egg-bearing females, multiple paternity was suspected.

Microsatellite markers have provided a new approach to study mating systems in many taxa (Queller et al. 1993). They have became a useful tool to study both multiple paternity and internesting strategy, and provide an alternative to controlled-mating experiments. As they allow identification of individuals with great confidence, microsatellites can provide valuable information such as the number of males fathering offspring and the identity of those males. Microsatellites may, therefore, be sufficiently informative for inferring several important relationships, such as those between multiple paternity and either male or female fitness or between multiple paternity and clutch size.

Our aim was to assess the paternity of hatchlings from 15 different clutches of eight *T. graeca* females using three microsatellites markers and to test the hypothesis of sperm storage in this species.

Materials and methods

Sampling

Eight egg-bearing females from an area of 59 ha inside Doñana National Park (SW Spain) were monitored from April to June 2001. In addition, 55 individuals (44 males and 11 females) from the same zone were caught to calculate allelic frequencies and to genotype candidate fathers in the adult population. The 44 males represent approximately 46% of the total number of males in the sampled area (according to the density of individuals estimated in Andreu et al. 2000). All tortoises were individually marked by notches in the shell. Gravid females were placed in separate enclosures within their natural area to locate their respective nests. Once they had laid their first clutch (egg deposition detected by means of X-rays, see details in Díaz-Paniagua et al. 1996), females were released in the field. After 15–20 days, females were captured again to monitor their second clutches. When shelled eggs were detected, females were placed again in the enclosures to locate their second nests. All the eggs were transferred to the laboratory for incubation until hatching. In total, 15 clutches were monitored during the nesting season: two successive clutches of seven females and the single clutch of one female. Among the seven females with successive clutches, recent deposition of the first clutch was detected in two females (1172, 1362), which were immediately released in the field. The other females were released later (6–10 days) after egg laying and one was not released (female 1448) during the internesting period.

Microsatellite analysis

Blood samples were obtained from both adults and live hatchlings (over 4 months old) by nail-clipping and were preserved in 100% ethanol. Genomic DNA was extracted using a lithium chloride method adapted from Gemmell and Akiyama (1996). For unhatched eggs, DNA was extracted from a piece of tissue using a standard phenol–chloroform method (Sambrook et al. 1989). Genotypes were determined at three microsatellite loci, CmuB08, CmuD16, and CmuD51, specifically designed for the bog turtle, *Clemmys muhlenbergii* (unpublished). Polymerase chain reaction (PCR) was performed in 20-μL reaction volumes (3 mM $MgCl_2$, 0.25 μM of each primer, 1× *Taq* buffer, 0.75 U (1 U ≈ 16.67 nkat) of *Taq* polymerase, 0.25 mM of each dNTP, and 20–50 ng of DNA template). The second primer of each pair was end labelled with one of three fluorescent labels: yellow (HEX) for CmuB08, green (TET) for CmuD16, and blue (6-FAM) for CmuD51. PCR cycling conditions were as follows: a denaturing step of 2 min at 94 °C, 10 cycles of 30 s at 92 °C, 30 s at 60 °C with a 1 °C decrease at each cycle, 30 s at 72 °C, 24 cycles of 30 s at 92 °C, 30 s at 50 °C, 30 s at 72 °C, and a final step of 5 min at 72 °C. PCR products were analysed on an automatic ABI PRISM® 310 DNA sequencer (Applied Biosystems, Foster City, Calif.). For each sample, 3–4 μL of PCR product was diluted in 75 μL of water. A 2.5-μL aliquot of the dilution was mixed with 12 μL of formamide plus 0.4 μL of internal size standard (red color (TAMRA), 500 bp) and denatured for 3 min at 95 °C. Data collection and analysis, as well as automated scoring of the alleles for each sample, were performed using Genescan software (Applied Biosystems). Tabulation of data for each locus was conducted with Genotyper software (Applied Biosystems).

Statistical analysis

Allelic frequencies, as well as expected and observed heterozygosities, were calculated using GENETIX version 4.03 (Belkhir et al. 1996–2001). Hardy-Weinberg (HW) equilibrium and heterozygote deficiency were tested, using GENEPOP version 3.1 (Raymond and Rousset 1995), in 63 individuals including the 8 females and the 55 individuals from the adult population. Tests for linkage disequilibrium were also performed, using GENEPOP, at all pairs of loci. Null allele frequencies were estimated with CERVUS version 2.0 (Marshall et al. 1998) using an iterative algorithm based on the difference between observed and expected frequencies of homozygotes (Summers and Amos 1997).

Paternity assessment

Paternal alleles were deduced from the comparison of both maternal and offspring genotypes. We assumed that clutches with more than two paternal alleles were fathered by more than one male. However, a third paternal allele appearing in only one offspring at only one locus was classified as a result of allelic mutation.

The process of exclusion consisted of comparing the genotypes of candidate parents with that of the offspring (taking account of the other parent's genotype, if available), with candidates excluded as parents if a mismatch occurred at one or more loci. Two exclusion probabilities (EP) were calculated using CERVUS (Marshall et al. 1998), considering each locus separately or all loci combined: EP1, the probability of excluding a random male from paternity when only offspring are sampled and EP2, the probability of excluding

Table 1. Statistics for three microsatellite loci in 63 *Testudo graeca* from Doñana National Park (Spain), including sample size (*N*), number of alleles per locus (*n*), observed (H_O) and expected (H_E) heterozygosity, and exclusion probabilities with and without one parent already known.

					Exclusionary power	
Locus	*N*	*n*	H_O	H_E	Neither parent known	One parent known
CmuB08	62	15	0.839	0.841	0.558	0.719
CmuD16	63	22	0.905	0.920	0.697	0.821
CmuD51	63	16	0.905	0.867	0.633	0.776
Overall		53	0.882	0.876	0.951	0.989

a random male from paternity when both offspring and the mother are sampled. The parentage analysis module available in CERVUS was used to calculate an assignment success of candidate parents to the offspring. For each offspring tested, parentage is assigned to the most likely candidate parent at two predetermined confidence levels: relaxed (80%) and strict (95%).

Results

Population genetic analysis

High genetic variability was observed among the 63 individuals, with an average number of 18 alleles per locus, varying from 15 to 22 alleles (Table 1, Fig. 1). Expected heterozygosity varied between 0.841 and 0.920 with a mean of 0.876 (Table 1). In the adult population, the hypothesis of the population being in HW equilibrium could not be rejected for any of the three loci (CmuB08, $P = 0.838$; CmuD16, $P = 0.333$; CmuD51, $P = 0.238$), and the probability of heterozygote deficiency was not significant (CmuB08, $P = 0.551$; CmuD16, $P = 0.108$; CmuD51, $P = 0.875$). Non-amplifying alleles could lead to mistaken conclusions; this is particularly important in parentage analyses (Pemberton et al. 1995). The null allele frequencies calculated with CERVUS were relatively low in all loci (CmuB08, 0.31%; CmuD16, 1.03%; CmuD51, 0%). The null hypothesis of no linkage disequilibrium could not be rejected for any of the three pairs (CmuB08/CmuD16, $P = 0.172$; CmuB08/CmuD51, $P = 0.252$; CmuD16/CmuD51, $P = 0.069$).

Paternity analysis

The combined paternity exclusion probability with one locus and one parent known varied between 0.719 and 0.821; the probability was 0.989 when all loci were used (Table 1). Parentage assignation success was 62% and 87% at 80% and 95% confidence levels, respectively, at all loci combined.

Multiple paternity was found in 3 of the 15 clutches (20%); in these 3 clutches, offspring displayed more than two paternal alleles at more than one locus (see Table 2). Three other clutches displayed more than two non-maternal alleles at only one locus, which could result from allelic mutations. Therefore, single paternity was assumed in the nine remaining clutches. Among the 44 father candidates, only 2 matched the inferred paternal genotypes (clutch E and clutch O). However, in several cases in which the offspring genotype was similar to that of its mother (clutch P, female 1172; clutches D and O, female 1362), it was not possible to deduce which one of the alleles was the paternal one. We also found two offspring lacking maternal alleles at loci CmuD16 (E3, female 1370) and CmuD51 (J4, female 1313) (Table 2). These results may be due to a mutation of one of the maternal alleles, as these offspring could not be from a different nest. Moreover, we identified the potential father of clutch E (CmuB08, 203/227; CmuD16, 195/215; CmuD51, 207/219), supporting the idea that the mutant allele of offspring E3 should be allele 227.

The mutation rate ranged from 11.1×10^{-3} (calculation based solely on maternal mutations) to 27.3×10^{-3} (calcula-

Fig. 1. Allelic frequencies for three microsatellite loci observed in 63 adult *Testudo graeca* from Doñana National Park (SW Spain).

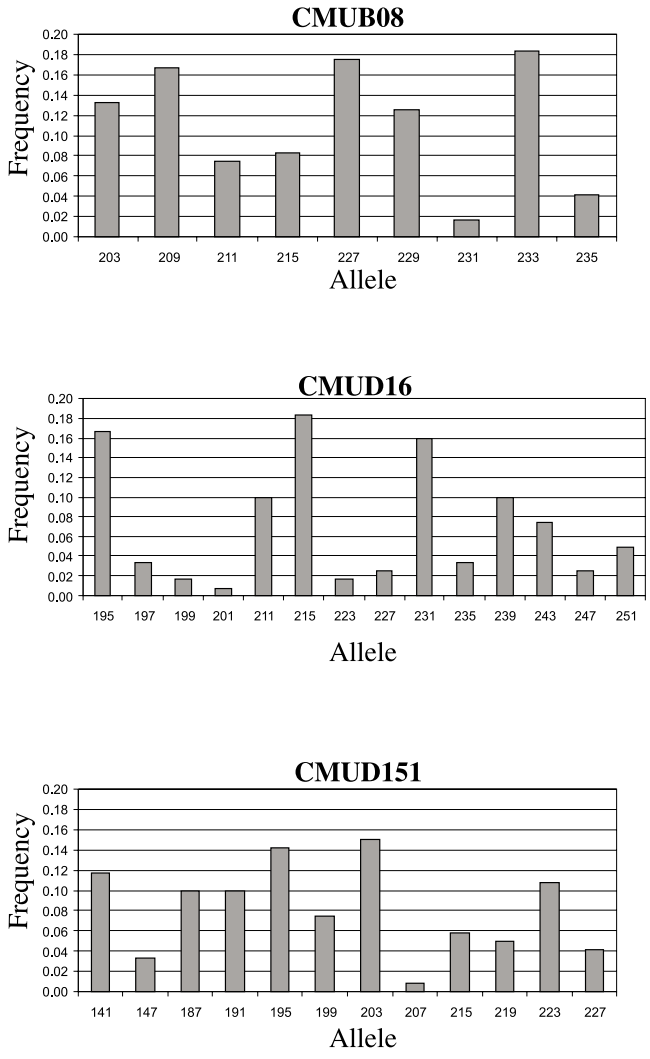

Table 2. Multilocus paternity data.

	Female genotypes				Offspring genotypes			Inferred paternal alleles		
Female	CmuB08	CmuD16	CmuD51	Clutch	CmuB08	CmuD16	CmuD51	CmuB08	CmuD16	CmuD51
1494	203/209	197/243	141/227	A1	**209**/235	215/**243**	195/**227**	211, 235	211, 215, 239	195, 223
				A2	**203**/235	**197**/215	**141**/223			
				A3	**203**/211	**197**/211	**141**/195			
				A4	**209**/235	239/**243**	223/**227**			
				K1	**209**/235	239/**243**	195/**227**	235	215, 239	195, 223
				K2	**203**/235	**197**/215	223/**227**			
1313	209/229	211/239	199/215	B1	**209**/231	**239**/251	195/**199**	215, 227, 231, 233	211, 215, 251	187, 195
				B2	**209**/227	215/**239**	195/**215**			
				B3	227/**229**	**239**/251	195/**215**			
				B4	**209**/233	**239**/251	187/**215**			
				B5	**209**/215	**211**/211	0			
				J1	**229**/231	215/**239**	195/**199**	215, 227, 231, 233	211, 215, 251	187, *191*, 195
				J2	227/**229**	**211**/251	187/**215**			
				J3	**209**/227	**239**/251	195/**215**			
				J4	**209**/233	**239**/251	187/191			
				J5	215/**229**	**211**/211	187/**215**			
1172	203/233	195/231	191/203	C1	227/**233**	**195**/215	187/**203**	227, 229	199, 215	187, 199
				C3	**203**/229	**195**/199	**191**/199			
				P1	209/**233**	215/**231**	187/**203**	209, 215, 227, 203/233	199, 215, 195/231	187, 199, 223, 191/203
				P2	227/**233**	**195**/215	**191**/**203**			
				P3	215/**233**	199/**231**	199/**203**			
				P4	**203**/**233**	**195**/**231**	**191**/223			
1362	227/233	231/235	191/199	D1	211/**233**	231/**231**	147/**191**	211, 227/233	215, 231	147, 191
				D2	**227**/**233**	215/**235**	**191**/191			
				D3	211/**233**	231/**231**	147/**199**			
				O1	**227**/**233**	231/**231**	187/**199**	211, 229, 227/233	231	147, 187
				O2	**227**/229	231/**231**	147/**199**			
				O3	211/**227**	231/**235**	147/**191**			
1370	211/215	195/247	141/203	E1	203/**211**	**195**/195	**141**/219	203, 227	195, 215, *227*	207, 219
				E2	**211**/227	195/**247**	**203**/219			
				E3	203/**215**	215/227	**203**/207			
				M1	203/**215**	**195**/215	**203**/219	203, 227	195, 215	219
				M2	203/**215**	**195**/195	**141**/219			
				M3	**211**/227	215/**247**	**203**/219			
				M4	203/**215**	**195**/195	**141**/219			
1363	229/233	215/243	195/203	F1	**229**/233	**215**/215	191/**195**	209, 233	215, 231	191, 195
				F2	209/**233**	**215**/231	195/**203**			
				F3	**233**/233	**215**/215	195/**203**			
				L1	209/**233**	**215**/215	**195**/195	209, 233	215, 231	191, 195
				L2	209/**229**	**215**/231	195/**203**			
				L3	209/**233**	231/**243**	191/**195**			
				L4	233/**233**	215/**231**	195/**203**			
1448	227/229	195/243	203/223	N1	209/**227**	**195**/211	187/**223**	209, 215	211, 223	141, 187
				N2	209/**227**	**195**/211	141/**223**			
				N3	215/**229**	211/**243**	141/**223**			
				N4	209/**229**	223/**243**	187/**223**			
				Q1	209/229	**195**/223	187/**223**	209, 215	223, 239	141, 187
				Q2	209/**229**	**195**/239	141/**223**			
				Q3	215/**227**	**195**/239	141/**223**			

Table 2 (*concluded*).

	Female genotypes				Offspring genotypes			Inferred paternal alleles		
Female	CmuB08	CmuD16	CmuD51	Clutch	CmuB08	CmuD16	CmuD51	CmuB08	CmuD16	CmuD51
1497	203/227	211/227	141/203	G1	**203**/227	**227**/235	187/**203**	211, 227	199, 231, 235	187, 223
				G2	**203**/211	**211**/231	**141**/223			
				G3	**227**/227	199/**211**	**141**/187			

Note: Maternal alleles are in boldface type. Among the inferred paternal alleles, potential mutant alleles are underlined and maternal ones are in italic type.

tion based on all mutations), based on two and five mutation events, respectively, in 180 genotypes (Table 2). These rates are higher than those in the range of 5.7×10^{-4} to 9.6×10^{-3} reported for other turtle species (Fitzsimmons 1998; Pearse et al. 2001; Crim et al. 2002) and may suggest that the events found in paternal alleles are the result of undetected fathers rather than mutations.

Analysis of successive clutches

Successive clutches were obtained for seven of the eight females. The same paternal alleles were observed at all three loci in both clutches of two females (1313 and 1363) (Table 2). This suggests that the male (clutches of female 1363) or males (clutches of female 1313) that sired the first clutch also sired the second one. Successive clutches of two other females (1370 and 1494) may have also been sired by the same male. However, in these cases, comparison of clutches of the same female was difficult owing to mutations and also given the low probability of detecting all paternal alleles among four or fewer offspring. For example, the first and second clutches of female 1370 shared the same paternal alleles at two loci (CmuB08 and CmuD16), while the second clutch lacked a paternal allele at the third locus (CmuD51) (allele 207). It could be that those clutches were sired by different fathers, but the occurrence of the same paternal alleles at both loci CmuB08 and CmuD16 best suggests that allele 207 was not detected in the second clutch because of small clutch size. It is important to note that these four females (1313, 1363, 1370, 1494) were released more than 5 days after the first nesting and probably did not mate with new males before egg shelling. Their second clutch was thus fertilized by stored sperm.

On the other hand, the two clutches of each of three females (1172, 1362, and 1448) had different paternal alleles. One of these females (1448) was not released during the internesting period and so paternal alleles of the second clutch certainly came from stored sperm. In female 1172, the second clutch could have been sired either by the father of the first clutch and an additional male or by other males. As this female was released immediately after nesting, she had opportunities to mate again with the same male or with new males. Similarly, the second clutch of female 1362 (released immediately after the first nesting) had a different single father, which could have resulted from mating again before shelling the eggs of the second clutch.

Discussion

Multiple paternity

Our genetic data provided evidence of the occurrence of multiple paternity (MP) in clutches of *T. graeca*. More than two paternal alleles were present at more than one locus in 20% of the clutches analysed. The incidence of such a strategy in the Mediterranean spur-thighed tortoise is similar to that found in *Lepidochelys kempii* (27%) (Kichler et al. 1999), but much higher than in *Chelonia mydas* (9%) (Fitzsimmons 1998). However, on the whole, single paternity was found to be more frequent than MP, with 80% of clutches possessing alleles of only one male.

The incidence of MP in wild populations of turtles has been generally poorly assessed. However, among the few existing studies (only in five of them was the sample >20 clutches), low incidence of MP was found in both species with relatively large clutches and species with relatively small clutches. Also, some marine turtles (*L. kempii*, Kichler et al. 1999; *Caretta caretta*, Moore and Ball 2002) have been found to have high frequencies of MP, leading the authors to conclude that males were not a limiting resource. In contrast, in other reptiles such as squamates, MP was found at much higher frequencies than single paternity (Barry et al. 1992; Höggren 1995; Gullberg et al. 1997; McCracken et al. 1999; Morrison et al. 2002; Stapley et al. 2003).

In our study, however, MP may have been underestimated. First, the probability of detecting MP increases with the number of alleles present at a locus and with the number of loci used. Therefore, the analysis of more loci in this study might have increased the number of observed multiply sired clutches.

Also, the probability of detecting three or more paternal alleles among four or fewer offspring is very low. For example, McCracken et al. (1999) showed that the correlation of litter size with the number of loci at which multiple paternity was detected was highly significant. Most female tortoises in this population of *T. graeca* lay clutches of three or four eggs, while only 10% of the females lay larger clutches (Díaz-Paniagua et al. 2001). Only one female in this study (1313) laid clutches with more than four offspring, and it was in these offspring that we detected the highest number of paternal alleles. Several hypotheses may explain the higher incidence of MP in larger clutches. First, the probability of detecting more paternal alleles is higher in larger clutches simply because detecting multiple sires with a smaller number of offspring is more difficult, as discussed above. Also, more sperm may be required to fertilize larger clutches. Alternatively, it is likely that females laying large clutches (usually big females) may be able to store more sperm than other females. These questions cannot be resolved with our data, and a larger sample would be necessary to infer relationships between clutch size and MP in *T. graeca*.

Finally, mutations were suspected when a third paternal allele was found at only one locus in a single offspring (three cases). However, in clutch 1 (female 1494) for example, a probable mutant allele was detected in the population at high frequency (allele 211 at CmuD16 with a frequency of 8.33%) and may have come from undetected fathers.

Internesting strategy: sperm storage

The evidence of MP, the finding that the second clutch of the non-released female (1448) was sired by a different male, and the finding that clutches of female 1313 were multiply sired by the same males (Table 2) all confirmed that *T. graeca* is able to store sperm from single or multiple matings prior to nesting and use it to fertilize all subsequent clutches of eggs, without additional internesting mating (see Table 2). In addition, these results show that *T. graeca* may use several internesting strategies. Among all females, four different cases of father coincidences between successive clutches were brought to the fore: (1) a clutch was sired by the same single male as the previous clutch (females 1363, 1370, and 1494); (2) a clutch was sired by a different male than the previous clutch (females 1448 and 1362); (3) the second clutch was sired either by the father of the previous clutch and an additional male or by other males (female 1172); and (4) the same males sired both clutches (female 1313). The time elapsed from nesting to the release date did not enable a new male to fertilize the next clutches in five females (1448, 1363, 1370, 1313, 1494) (shelled eggs are detected in oviduct ca. 1 week after previous nesting, our own observations), supporting the hypotheses that second clutches were inseminated by stored sperm. On the other hand, in the cases of females 1362 and 1172, which were released immediately after nesting, sperm of the second male may have come from remating after first nesting.

Sperm storage is considered to play an important role in reproduction of turtle species in which male and female cycles do not coincide. In *T. graeca*, although sperm storage was assumed to occur because of the asynchrony of autumnal mating with spring egg maturation (Devine 1984) (although mating also occurs in spring), it was never demonstrated. Sperm storage is also thought to increase the probability of fertilizing clutches, particularly in species in which males are a limiting resource or in populations of low density (see Galbraith et al. 1993). *Testudo graeca* is a species with small clutches and frequent mating and remating during the annual cycle. In this species, the advantages of storing sperm would be mainly related to selection; for example, there would be the possibility of maturing eggs earlier in spring by using sperm stored from fall matings. The number of clutches that a female may lay per season is limited by the length of the nesting season (Díaz-Paniagua et al. 1996), and the possibility of starting egg maturation earlier would enable a female to lay a higher number of clutches within a season. The use of stored sperm from a previous mating would then contribute indirectly to an increase in the number of clutches.

Monitoring of the same female tortoises and their hatchlings across successive years should give us deeper insight into sperm storage strategy in *T. graeca*. Also, clutch sizes were too small to provide a rigorous conclusion of internesting mating. To compare both clutches of a female, it would be necessary to know fathers' genotypes to distinguish paternal alleles that appear for the first time in a clutch from those that simply are not found in the clutch because of a small number of eggs. Further studies on the mating system of *T. graeca* would be improved by controlling the identity of different males inseminating females, both before their first annual nesting and in internesting intervals, as in a previous study of a desert tortoise (Palmer et al. 1998).

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