

Multiple paternity occurs with low frequency in the territorial roe deer, *Capreolus capreolus*

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An explanation for female multiple mating when males offer no material benefits but sperm remains elusive, largely because of a lack of empirical support for the genetic benefits hypothesis. We used 21 microsatellite markers to test for multiple paternities among 88 litters of roe deer, *Capreolus capreolus*, and to investigate the inbreeding avoidance hypothesis as a potential mechanism for the evolution of female multiple mating. From paternity analyses, we found that 13.5% of polytocous litters were sired by more than one male. We also found that a half-sib relationship was more likely than a full-sib relationship for 20.5% of all litters. This is the first report of multiple paternities in a territorial ungulate species. In support of the inbreeding avoidance hypothesis, we found that parents who were strongly related produced offspring with lower individual heterozygosity that survived less well during their first summer than fawns with unrelated parents. In addition, fawns from multiple paternity litters survived their first summer better than fawns from single paternity litters. However, it remains unclear whether all female multiple paternity events in this species are provoked by an initial consanguineous mating. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society* 2009, 97, 128–139.

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INTRODUCTION

Because of anisogamy and differential parental investment between the sexes, males have the capacity to fertilize a number of females, whereas females usually need only one male to father a particular litter (Bateman, 1948). Hence, to maximize fitness, males are expected to mate with as many females as possible, whereas females are not expected to solicit

multiple mating. Yet, females often do mate multiply within a single reproductive cycle (Arnqvist & Nilsson, 2000), either with the same male (repeated matings), or with different males (polyandry), potentially resulting in multiple paternity (i.e. paternity shared by several males within a single litter or brood; Birdsall & Nash 1973; Gavin & Bollinger 1985; Zane *et al.* 1999). Despite the crucial importance of multiple mating in sexual selection and in the maintenance of genetic variation (Sugg & Chesser, 1994), the reasons why females frequently mate with several males are still subject to controversy (Fedorka & Mousseau, 2002).

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Optimization theory predicts that multiple mating by females should evolve when the direct costs (e.g. increased susceptibility to predation, physical injury, disease, energetic loss; Daly, 1978; Thornhill & Alcock, 1983; Hurst *et al.*, 1995; Blanckenhorn *et al.*, 2002) are offset by benefits (Reynolds, 1996; Newcomer *et al.*, 1999). In some species, costs can be offset by direct benefits provided by males such as nuptial gifts, access to territories, protection against predators, and paternal care of offspring (Arnqvist & Nilsson, 2000; Wiklund *et al.*, 2001). Alternatively, females may mate several times to avoid male harassment (Galimberti *et al.*, 2000) and ensure fertility (Orsetti & Rutowski, 2003). However, in large mammalian herbivores, where males contribute no material resources other than sperm and females do not gain obvious direct mating benefits, females are assumed to obtain some genetic (indirect) benefits from multiple mating.

Potential genetic benefits may involve: (1) gaining 'good genes', either via sperm competition or female choice of sperm (Kempnaers *et al.*, 1992; Otter & Ratcliffe, 1996; Simmons, 2001), which may result in the production of offspring of higher genetic quality (Zeh & Zeh, 2001); (2) genetic incompatibility avoidance (Zeh & Zeh, 1996), exploiting post-copulatory mechanisms in order to minimize the risk and/or cost of fertilization by genetically incompatible sperm; (3) increasing offspring genetic diversity (Madsen *et al.*, 1992; Byrne & Roberts, 2000) by producing offspring sired by different males, potentially reducing sibling competition or serving as a hedge against environmental uncertainty ('genetic bet-hedging'; Zeh & Zeh, 2001); and finally, (4) inbreeding avoidance (Brooked *et al.*, 1990; Stockley *et al.*, 1993). Although high levels of multiple mating can be compatible with all these hypotheses, only the offspring diversity hypothesis predicts high rates of multiple paternities.

Although multiple paternities have been documented in various polytocous taxa, including birds (Gowaty & Karlin, 1984; Gavin & Bollinger, 1985; Petrie & Kempnaers, 1998) and mammals (Birdsall & Nash, 1973; Schenk & Kovacs, 1995; Say *et al.*, 1999; Waser & De Woody, 2006), there is so far little evidence for it in large herbivores [but see, in pronghorn *Antilocapra americana* (Ord 1815): Carling *et al.*, 2003; Soay sheep *Ovies aries* (Linnaeus, 1758): Pemberton *et al.*, 1999; white-tailed deer *Odocoileus virginianus* (Zimmermann, 1780): DeYoung *et al.*, 2002; Sorin, 2004]. Furthermore, multiple paternities have not yet been investigated among territorial ungulates, in which male territoriality is commonly viewed as a mating tactic to ensure paternity by monopolizing oestrous females present within a defined area of dominance (Owen-Smith, 1977). In this case, multiple mating by

females with different males is not expected and, if identified, would require a revised view of territorial mating systems in large herbivores, particularly with regard to female mate choice, party size, and the operational sex ratio.

In roe deer, *Capreolus capreolus* (Linnaeus, 1758), male territoriality occurs from late March to early September (Andersen *et al.*, 1998), females are polytocous (up to four fawns, most often two; Hewison, 1996; Andersen *et al.*, 1998), and the polygyny level is quite low (males sire from 0 to 15 fawns during their lifetime; Vanpé *et al.*, 2008a). During the mid-summer rut, oestrous females are courted and mated repeatedly and frequently (Danilkin & Hewison, 1996), making them likely candidates for multiple paternities. However, there is only one very short annual oestrous (24–36 h; Hoffmann *et al.*, 1978) and it is still unclear whether a female mates repeatedly with the same partner or with different males. Indeed, no study has yet shown evidence that multiple mating in roe deer leads to multiple fertilizations or paternities.

In the present study, we used polymorphic microsatellite markers (Vanpé *et al.*, 2008a) and two maximum likelihood methods, namely parentage assignments (implemented in CERVUS 3.0 software; Kalinowski *et al.*, 2007) and tests of half-sib versus full-sib relationship categories (implemented in ML-Relate software; Kalinowski *et al.*, 2006), to quantify the occurrence of multiple paternities in an intensively monitored roe deer population and to investigate the potential benefits of multiple mating to females. In roe deer, females do not gain obvious direct benefits from mating repeatedly, although they may mate with different males to guard against male infertility or sperm depletion. Male dominance is site-specific and females do not appear to be passive during the rut, frequently soliciting specific males (Danilkin & Hewison, 1996; Börger, 2006) or rejecting nonterritorial males (Liberg *et al.*, 1998; Börger, 2006). Hence, female harassment by males is unlikely to explain female multiple mating in roe deer. We therefore focussed our study on genetic benefits and tested for the potential of inbreeding avoidance to explain the possible occurrence of multiple mating in roe deer, although other hypotheses are also considered (e.g. fertilization insurance). Male roe deer are generally faithful to their rutting territories for the whole of their reproductive life span, while adult females are also highly sedentary (Hewison *et al.*, 1998). In addition, although natal dispersal occurs to a variable degree (Gaillard *et al.*, 2008), it is not sex-biased (Coulon *et al.*, 2006); (Gaillard *et al.*, 2008); hence, a large proportion of young of both sexes is philopatric, remaining on, or adjacent to, their mother's range for the rest of their reproductive lives. This 'budding' system of socio-spatial organization leads to

a local reproductive unit composed of family groups where the level of genetic relatedness is high and hence incestuous mating and associated elevated levels of inbreeding are potentially common (Kurt *et al.*, 1993) although behavioural mechanisms, such as the recently identified rut excursions (Lovari *et al.*, 2008; Richard *et al.*, 2008), may allow females to avoid such matings. Hence, if females are able to distinguish close relatives but cannot avoid mating with them at the beginning of their oestrous, and if this is disadvantageous, the inbreeding avoidance hypothesis predicts that females should attempt to re-mate with genetically dissimilar males to replace the sperm of their first partner (via post-copulatory mate choice) or to reduce the risk of being fertilized only by a close relative (via multiple paternities). If females cannot distinguish close kin, they should copulate with several different males to achieve multiple paternities, reducing the risk that all their offspring will be sired by a close relative. This should enhance the fitness of some (or all) of their offspring because offspring resulting from outbred matings often survive better than those from inbred ones (Ralls *et al.*, 2005). Hence, under the inbreeding avoidance hypothesis: (1) a lower degree of relatedness between parents should result in more highly heterozygous offspring and, as a result, higher offspring survival (DiBattista *et al.*, 2008) and (2) fawns from multiple paternity litters should survive better than fawns from single paternity litters.

MATERIAL AND METHODS

STUDY POPULATION AND TISSUE SAMPLING

Bogesund is a 2600 ha area of fragmented habitat in central eastern Sweden (59°23'N, 18°15'E; for further details, see Vanpé *et al.*, 2007). This population has been intensively monitored by capture–mark–recapture since 1988 (Kjellander *et al.*, 2006). Subsequent to 1997, new-borns have also been marked just after birth. Mother–offspring relationships are assessed by direct observation of fawns with their mothers just after birth or during autumn. Tissue samples were collected for DNA genotyping from all individuals caught for the first time from 1988 to 2006. We usually removed a small piece of ear skin tissue (although note that only hair samples were taken from 1997 to 2003 on new-born fawns; $N = 146$). We sampled a total of 1757 individuals, of which 634 were fawns. However, because only a small proportion of the fawns were sampled from the 1988 to 1991 cohorts, we excluded these from subsequent paternity analyses (Vanpé *et al.*, 2008a). Thus, we sampled 606 fawns from 1992 to 2006, of which 288 had a known mother.

MOLECULAR ANALYSIS

Genotyping was performed using 21 microsatellite markers (Galan *et al.*, 2003; Vanpé *et al.*, 2008a). The total exclusion power (i.e. the average probability of excluding a single randomly-chosen unrelated individual from parentage at one or more loci; Marshall *et al.* (1998) was 0.9997 when one parent was known, and 0.9850 when both parents were unknown. The probability of detecting multiple paternities with a single locus was in the range 0.006–0.397 and was 0.985 when all 21 loci were used.

Based on independent repeat-genotyping, the estimation of the typing error rate was 3.81% per locus or 2.51% per allele (Vanpé *et al.*, 2008a). We also used the CERVUS 3.0 software to check known mother–fawn pairs to identify genotyping errors from mismatches between mothers and their offspring. Of the 101 independent known mother–fawn pairs, a total of 26 pairs (25.7%) mismatched, with the number of mismatches per pair being in the range 1–6 (mean \pm SD = 2.11 ± 1.42). Several mother–offspring pairs with mismatches at up to three loci could clearly be ascribed to scoring or typographical error and so we had an a priori expectation that these pairs ($N = 21$) represented cases of typing errors. Mother–offspring pairs with mismatches at more than three loci ($N = 5$) probably represented cases where the fawn did not belong to the supposed mother, and hence these were removed from the data bases of known mother–fawn pairs. When we considered only mother–offspring pairs with mismatches at up to three loci, the genotyping error rate was 3.7% per locus.

PATERNITY ASSIGNMENT AND IDENTIFICATION OF LITTERS WITH MULTIPLE PATERNITIES

Parentage was assessed using a likelihood-based approach implemented in CERVUS 3.0 (Kalinowski *et al.*, 2007). The values for user-defined input parameters incorporated in CERVUS 3.0 are provided in Vanpé *et al.* (2008a), except that, in the present study, we fixed the error rate to the true error rate estimate (3.7% per locus). We also incorporated some information on relatedness among candidate mothers and fathers for each cohort in the parentage simulation when estimating critical logarithm of the odds ratio (LOD) or Delta scores prior to parentage analysis [i.e. the mean relatedness between candidate mothers or fathers (the probability that each allele belonging to a relative is identical by descent to the corresponding allele carried by the individual to which they are related) and the average proportion of all candidate parents that are relatives (Kalinowski *et al.*, 2007)]. Relatedness measures were estimated using the ML-Relate software (Kalinowski *et al.*, 2006). We successfully assigned a father to 278 fawns (45.9%) at the

80% confidence level using CERVUS 3.0, of which 138 (22.8%) were also assigned at the 95% confidence level. We investigated 79 twin litters and nine triplet litters, involving 185 fawns (the remaining 93 fawns did not have any known sampled sib-ships). We considered that multiple paternities only occurred when at least two of the fawns from a given litter were assigned a different father with 80% confidence by CERVUS 3.0. Note that we chose a conservative criteria and did not consider litters for which one fawn was assigned a given father with 80% confidence and the other could not be assigned to any sampled father with 80% confidence as multiple paternity cases ($N = 12$ twin litters and two triplet litters).

Although parentage analysis allowed us to identify father–offspring relationships with 80% confidence, it did not allow us to identify full- or half-sibling relationships unless the fathers were included in our sample. Hence, we also used an additional means of determining multiple paternities by performing tests of relationship categories for the siblings in each litter implemented in the ‘specific hypothesis test module’ of the ML-Relate software (Kalinowski *et al.*, 2006). This software uses simulation (100 random genotype pairs were simulated) to determine which out of the putative relationship (here half-sib) and the alternative relationship (here full-sib) is more consistent with offspring genotype data. Note that, for triplet litters, there are three possible tests that can be performed, whereas only two tests are independent. We therefore decided to randomly select two of the three possible tests. In addition, because performing multiple tests tends to increase type I errors, we implemented the false discovery rate (Benjamini & Hochberg, 1995; Storey, 2002), using the GeneTS package in the R 2.6.0 software, to account for the large number of half-sib versus full-sib relationship tests we performed using ML-Relate.

ESTIMATION OF FAWN SURVIVAL, OFFSPRING HETEROZYGOSITY, AND (GENETIC) RELATEDNESS BETWEEN INDIVIDUALS

Because most fawn mortality occurs within the first weeks of life in roe deer and other large herbivores (Gaillard *et al.*, 2000), we investigated the effect of offspring individual heterozygosity and parental relatedness on fawn summer survival. We considered that fawns survived (did not survive) their first summer if they were known to be still alive after (to be dead before the end of) weaning (end of August). We determined fawn summer survival based on regular observations of animals in the field, annual winter captures, and the mortality signal of radio-collars for radio-tracked individuals. We radio-tracked and observed neonates (equipped with a collar) every

day up to a fawn age of 4–6 weeks and thereafter one to three times a week up to an age of 8 weeks (for a similar approach, see Jarnemo, 2002; Jarnemo *et al.*, 2004). In our analysis, we did not include fawns that died from human causes (either killed by hay mowing machines or hunted) during their first summer ($N = 2$) and fawns for which the survival status could not be assessed with certainty ($N = 16$; i.e. mainly fawns that were never observed after being marked as neonates). When the cause of mortality was not obvious, fawns were autopsied to check for fox bites, potential evidence of starvation/hypothermia/diseases, etc.

We calculated three microsatellite measures of genetic diversity (individual heterozygosity) aimed at reflecting levels of individual inbreeding [standardized multilocus heterozygosity (std H), internal relatedness (IR) and homozygosity by locus (HL); Coltman *et al.*, 1999; Pemberton *et al.*, 1999; Amos *et al.*, 2001; Aparicio *et al.*, 2006 for all fawns using the IR macroN3 excel macro developed by W. Amos’ group (<http://www.zoo.cam.ac.uk/zoostaff/amos/#Computer%20Programs>). The three measures were highly inter-correlated (Pearson’s correlation coefficient: $|r| > 0.95$, d.f. = 187, $P < 2.2 \times 10^{-16}$, in all cases). Although Aparicio *et al.* (2006) suggested that of the three measures HL performed best, we chose to retain all three to facilitate comparison with other studies. Note that allele frequencies were estimated and standardization was performed based on a subset of the whole sample (individuals born in 1992) to reduce multigenerational effects.

We also estimated the relatedness between parents of each multiple litter, as well as mean relatedness among candidate mothers and fathers for each cohort, using the ML-Relate software (Kalinowski *et al.*, 2006). ML-Relate calculates maximum likelihood estimates of pairwise relatedness such as that of Thompson (1991) and Milligan (2003), which are more accurate than other estimators (Milligan, 2003). Likelihood calculations have been described in detail elsewhere (Wagner *et al.*, 2006).

STATISTICAL ANALYSIS

All statistical analyses were performed using R 2.6.0 software (R Development Core Team, 2004). To investigate whether offspring individual heterozygosity and parental relatedness differed between fawns that survived and fawns that died during their first summer, we modelled fawn survival as a binomial response (survived = 1, did not survive = 0) using a logistic regression with either std H, HL, or IR (including those fawns for which we had survival data, $N = 167$) or parental relatedness (including those fawns for which we had both survival data and

successfully assigned a mother and a father, $N = 60$) fitted as an explanatory variable. As the year of birth (i.e. cohort) of the fawns strongly influences their survival (cohort effect; Gaillard *et al.*, 1997; Pettorelli *et al.*, 2005), we included it in a generalized linear model implemented in the glm module in the stats package of R software. We did not include the possibility of either between-sex differences (i.e. previous studies repeatedly show that no difference occurs; Gaillard *et al.*, 1997; Pettorelli *et al.*, 2005) or effects of body mass, although heavier fawns likely do survive better (Da Silva *et al.*, 2009), because fawns were marked at different ages and we did not have accurate information on early growth patterns in the studied population. We compared the best nongenetic model with the model including each of the genetic diversity measures (Da Silva *et al.*, 2009) for a similar approach). Model selection was performed using the small sample corrected Akaike Information Criterion (AIC_c) as recommended by Burnham & Anderson (2002). The best model was taken as the one with the smallest AIC_c value. However, when the difference between AIC_c values (ΔAIC_c) was less than 2, we considered that both models had similar support and relied on parsimony criterion to select the best model (Burnham & Anderson, 2002).

We finally compared the proportion of fawns that died during their first summer between multiple paternity litters and single paternity (but polytocous) litters (as indicated using CERVUS 3.0) using a Pearson's chi-squared test with Yates' continuity correction. We also compared the heterozygosity of fawns from multiple paternity litters and single polytocous paternity litters using a Wilcoxon rank sum test.

RESULTS

MULTIPLE PATERNITY OCCURRENCE AND FREQUENCY

Of the 79 twin litters and nine triplet litters studied, 31 and two of the twin and triplet litters, respectively, involving a total of 37 sib relationships, were successfully assigned a father for all fawns by CERVUS 3.0, whereas, for 12 twin and two triplet litters, a father was assigned to only some of the fawns of that litter (Table 1). Of those litters where all fawns were assigned a father, 26 twin and two triplet litters had a unique father for all fawns, whereas the five remaining twin litters (13.5% of the 37 sib relationships) revealed multiple paternities (Table 1). Of these five twin litters, only one was assigned a different father for both fawns with at least 95% confidence. For the others, different fathers were assigned for the two fawns with between 80% and 95% confidence ($N = 1$) or with at least 95% confidence for one father and between 80% and 95% confidence for the other ($N = 3$; Table 1). Note that with paternities assigned at 80% and 95% confidence, there is a probability of at least 0.8 and 0.95, respectively, that the paternity is assigned to the correct male, and a probability of 0.2 and 0.05, respectively (or less), that paternity is assigned to an incorrect male. Hence, the probability that all of the five cases of multiple paternity revealed by CERVUS may be due to misassignment was only 2.6% [probability = $(1 \times [1 - (0.8^2)]) \times ((3 \times [1 - (0.8 \times 0.95)]) \times (1 \times [1 - (0.95^2)]))$]; T. Marshall, pers. comm.].

Based on the tests of half-sib versus full-sib relationship categories implemented in the ML-Relate software, 15 of the 79 studied twin litters revealed a half-sib relationship and three of the nine studied triplet litters revealed a half-sib relationship for at

Table 1. Details of paternity assignment for the polytocous litters for which CERVUS 3.0 could assign a father for all fawns of a given litter

	Total number of litters	Number of twin litters	Number of triplet litters	Number of litters for which all fathers were assigned at > 95% confidence	Number of litters for which all fathers were assigned with between 80% and 95% confidence	Number of litters for which one or more fathers were assigned at > 95% confidence and the remaining father(s) were assigned with between 80% and 95% confidence
Single paternity	28	26	2	8	9	12
Multiple paternity	5	5	0	1	1	3
Total	33	31	2	9	10	15

least two of its sib-ships (17.0% of the 106 tested sib relationships or 20.5% of the tested litters). The tests of the other 64 twin and six triplet litters indicated full-sib relationships. Note that when we implemented the false discovery rate correction for multiple testing (corrected $P = 0.001$), a half-sib relationship was indicated in only 6.8% of the tested litters (five of the 79 twin litters and one of the nine triplet litters).

Only two of the five twin litters indicated as cases of multiple paternities by CERVUS were confirmed by the ML-Relate tests of half-sib versus full-sib relationship categories. For the three other litters, ML-Relate indicated that a full-sib relationship was more likely than a half-sib relationship. Interestingly, of the 14 litters for which at least one fawn was assigned a given father with 80% confidence by CERVUS whereas the other remained unassigned (cases that could have been considered as multiple paternities; see discussion), three revealed a half-sib relationship based on the ML-Relate analyses.

RELATIONSHIP BETWEEN PARENTAL RELATEDNESS, OFFSPRING HETEROZYGOSITY, AND OFFSPRING SURVIVAL

The model including the effect of cohort only (best nongenetic model) had a lower AICc than the models including both an effect of fawn cohort and an effect of

offspring individual heterozygosity (either std H, HL or IR) on fawn survival (Table 2). Therefore, we did not find any evidence for an influence of genetic factors on fawn survival (slight positive trend for std H: mean \pm SE slope = 0.933 ± 1.105 , and slight negative trends for HL and IR: -1.706 ± 1.936 and -0.862 ± 1.253 , respectively). By contrast, the model including an effect of parental relatedness on fawn survival had a lower AICc than the model including only cohort effects (Table 2). Parental relatedness negatively influenced fawn survival (mean \pm SE slope = -6.536 ± 3.480), in support of the inbreeding avoidance hypothesis (Fig. 1). It is noteworthy that parental relatedness was also correlated with offspring heterozygosity measures ($r = 0.44$, for IR; $r = 0.52$, for HL; $r = -0.51$, for std H).

In addition, there was a tendency for fawns from multiple paternity litters to survive their first summer better (13.3% mortality) than fawns from single paternity litters (20.5% mortality; $\chi^2 = 0.69$, d.f. = 1, $P = 0.41$; Fig. 2). Note, however, that fawns from multiple paternity litters survived their first year significantly better (33.3% mortality) than fawns from single paternity litters (57.4% mortality; $\chi^2 = 5.29$, d.f. = 1, $P = 0.02$; Fig. 2). Finally, females that produced litters sired by different males did not have offspring of higher genetic diversity than females that produced litters sired by only one male

Table 2. Model selection for the effects of individual offspring heterozygosity [standardized heterozygosity (std H), internal relatedness (IR) or homozygosity by loci (HL)] and for the effects of parental relatedness on fawn survival

	<i>k</i>	AIC	AIC _c	ΔAIC _c	W
cohort	15	154.50	157.68	0.00	0.65
IR + cohort	16	156.02	159.65	1.97	0.24
constant	1	162.26	162.28	4.60	0.07
IR	2	163.39	163.46	5.78	0.04
IR * cohort	30	173.63	187.31	29.63	0.00
cohort	15	154.50	157.68	0.00	0.62
HL + cohort	16	155.72	159.34	1.66	0.27
constant	1	162.26	162.28	4.60	0.06
HL	2	162.93	163.00	5.32	0.04
HL * cohort	30	175.55	189.23	31.54	0.00
cohort	15	154.50	157.68	0.00	0.63
stdH + cohort	16	155.78	159.41	1.73	0.27
constant	1	162.26	162.28	4.60	0.06
stdH	2	163.04	163.11	5.43	0.04
stdH * cohort	29	171.92	184.62	26.94	0.00
relatedness + cohort	11	58.88	62.76	0.00	0.65
cohort	10	60.89	63.97	1.21	0.35
relatedness * cohort	18	68.15	81.24	18.48	0.00

k, number of parameters; AICc, Akaike Information Criterion corrected for small sample size; ΔAICc, difference in AICc between the given model and the selected model; W, Akaike weight, representing the ratio of ΔAICc values for each model relative to the set of candidate models. The selected model (smallest AICc value) is shown in bold.

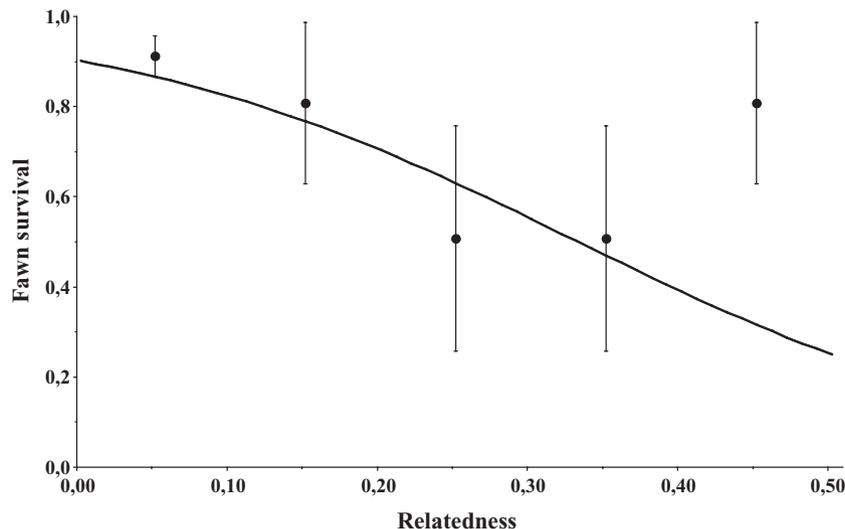


Figure 1. Variation of fawn survival as a function of parental relatedness. The line corresponds to the best-fitted curve and the filled circles correspond to the observed survival values (\pm SE). Observed survival was calculated for successive 0.1 intervals of parental relatedness and centred at the mid-point of the interval.

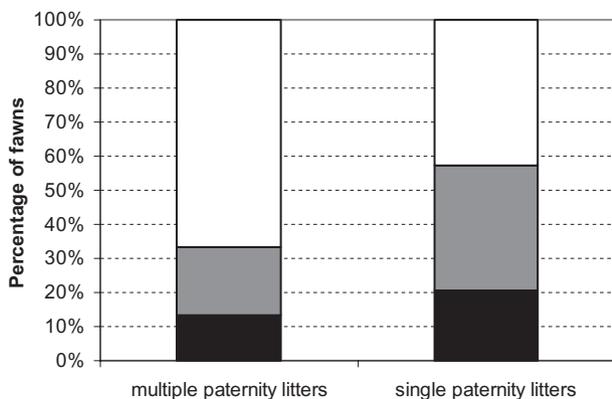


Figure 2. Proportion of fawns from multiple and single paternity litters that died during their first summer (in black), survived their first summer but died during their first winter (in grey) and survived to their first birthday (in white).

($W = 236.5$ and $P = 0.83$ for IR; $W = 317$ and $P = 0.32$ for HL; $W = 296.5$, $P = 0.55$ for std H; Fig. 3).

DISCUSSION

In the present study, we provide the first report of the occurrence of multiple paternity in a territorial ungulate and hence clear evidence that the previously reported multiple mating in female roe deer can lead to multiple fertilizations and multiple paternities. In support of the inbreeding avoidance hypothesis, we found that mating with close kin is disadvantageous

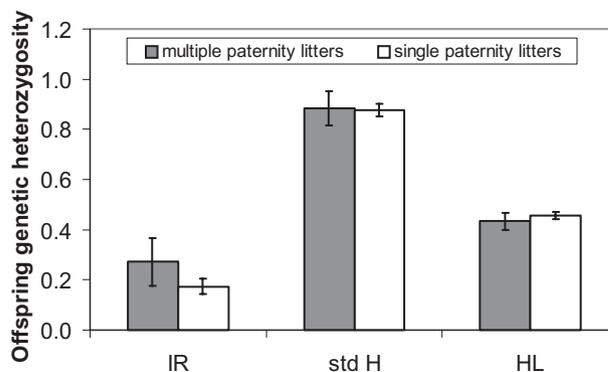


Figure 3. Mean \pm SE genetic heterozygosity of offspring from litters sired by different males (in grey) and litters sired by only one male (in white), either measured as internal relatedness (IR), standardized heterozygosity (std H) or homozygosity by loci (HL).

for females because their fawns appeared to survive their first summer less well than fawns of mothers that mated with an unrelated male.

FREQUENCY OF MULTIPLE PATERNITY

Multiple paternities were indicated in our roe deer population by both of the methods we employed: 17.0% of the sib relationships tested by ML-Relate revealed half-sibs and 13.5% of multiple litters were assigned paternity to more than one father with at least an 80% confidence by CERVUS 3.0. However, in only one litter (2.7%) of suspected multiple paterni-

ties were different fathers assigned to the two fawns with at least 95% confidence by CERVUS.

By incorporating a potentially overestimated error rate in the simulation module of CERVUS 3.0, we may have made the software too permissive in its paternity assignments for cases with father–offspring mismatches at one or more loci. However, of the ten fathers involved in multiple paternity litters revealed by CERVUS, seven had no locus mismatch with their fawns and three had one locus mismatch. Hence, it is unlikely that all, or even most, of our multiple paternity litters revealed by CERVUS are due to genotyping error. This suggests that multiple paternities likely do occur, but at a low frequency.

Note that we did not consider litters for which one fawn was assigned a given father with 80% confidence and the other could not be assigned to any sampled father as cases of multiple paternities ($N = 12$ twin litters and two triplet litters; see Material and Methods). If all these litters were considered as potential multiple paternity cases, the frequency of multiple paternities would be 21.7% (17/79 twin litters + 2/9 triplet litters, i.e. 23 of the 106 sib relationships). In addition, when we looked at average relatedness values among littermates to quantify the rate of multiple paternity, based on the following formula (S. Kalinowski, pers. comm.): $\text{Avg}(r) = p(0.25) + (1 - p)(0.5)$ where $\text{Avg}(r)$ is the average relatedness among littermates (here 0.45), and 0.25 and 0.5 are the values of relatedness between half-sibs and between full-sibs, respectively, we found that p , the proportion of twin litters that have two fathers, was 20%, which was similar to the frequency of multiple paternities suggested by both CERVUS and ML-Relate.

Surprisingly, three of the five litters revealed as cases of multiple paternities by CERVUS were not confirmed by the ML-Relate tests of half-sib versus full-sib relationship categories. This could be either because of genotyping error leading to mismatches between the true father and the fawn and/or because the two fathers of the litter were related (e.g. full or half sibs, parent–offspring) so that the two fawns (in reality, half-sibs) had genotypes which were compatible with a full-sib relationship. We therefore examined the relationship between the two fathers of each litter revealed as cases of multiple paternities by CERVUS using the ML-Relate software. We found that the fathers of two of these three litters were unrelated, but the fathers of one litter were half-sibs. Hence, paternal sib-ship explains only part of the discrepancy in results between the two approaches. It seems that the discrimination by ML-Relate between full- and half-sibs is not clear-cut, probably because of overlap in the distribution of relatedness values between them.

COMPARISON WITH OTHER SPECIES

In comparison with our results, Carling *et al.* (2003) reported multiple paternities in 44% of polytocous litters assigned with 80% confidence in a free-ranging population of pronghorn where males defend harems, but only one case where the two offspring were assigned different fathers with 95% confidence. In two enclosed populations of white-tailed deer, with a mating system based on dominance hierarchy, DeYoung *et al.* (2002) and Sorin (2004) reported multiple paternity in 26% and 22% of polytocous litters assigned with 95% confidence respectively, although the former study concerned a captive population at unusually high density. Finally, in an insular population of roving Soay sheep, of 80 sets of twins for which both lambs had an assigned father, only 21 (26%) had the same father, according to Pemberton *et al.* (1999). Hence, the frequency of multiple paternities in roe deer appears to be amongst the lowest yet found for an ungulate species for which multiple paternities have been quantified using genetic analysis.

From the above, although female roe deer commonly mate several times during oestrous, only a few of these multiple matings lead to multiple paternity. This could be either because females typically only mate with one male (albeit repeatedly), or because multiple mating with several males rarely leads to multiple paternities. Unfortunately, because observations of forest roe deer are difficult, no behavioural data are available on the frequency of female multiple mating with different males. However, it is likely that females in territorial species such as roe deer have less opportunity to encounter several males during oestrous compared to females in species where males defend harems, have a dominance hierarchy or a roving mating system. Alternatively, females may exert pre- or post-copulatory choice, preventing the possibility of multiple paternities despite mating with several males. In territorial species, the potential for mate choice by females is theoretically expected to be high because females have unimpeded opportunity to assess either the male and/or his territory (Min, 1997). In addition, in roe deer, a significant proportion of females (up to half) makes rut excursions of short duration outside their normal home range (Richard *et al.*, 2008), crossing the territories of several non-neighbouring males and engaging in courtship activities, potentially mating with one of these non-neighbouring males (Liberg *et al.*, 1998; Lovari *et al.*, 2008). It has also been reported that females frequently solicit courting from specific males (Danilkin & Hewison, 1996; Börger, 2006) or reject courting by nonterritorial and/or young males through avoidance behaviour (Liberg *et al.*, 1998; Börger, 2006). Hence, females appear to play an active role in mate choice.

Finally, the low frequency of multiple paternities that we found may be related to a high turnover of males in this harvested population (see Kjellander *et al.*, 2006), such that females have less need to seek out other non-kin males. Note, however, that the sex ratio in our study population was close to that expected in nonharvested populations because harvest was very low or even zero in some years, and that female rut excursions do occur even in heavily harvested populations (Richard *et al.*, 2008). Further research in other contrasted roe deer populations is required to confirm the generality of our results.

POTENTIAL OF INBREEDING AVOIDANCE TO EXPLAIN FEMALE MULTIPLE MATING

As previously mentioned, an elevated level of inbreeding within population units may be common in roe deer (see Kurt *et al.*, 1993), and especially in our largely isolated population. Indeed, we found that mean relatedness between potential fathers within each cohort was approximately 0.06, and that mean parental relatedness was 0.10 for fawns that survived their first summer and 0.18 for fawns that did not. In comparison, relatedness among first cousins, for example, is $1/8 = 0.125$. This high level of inbreeding could have a negative impact on fawn survival in our population. Indeed, we found that parents that were highly related had offspring with lower individual heterozygosity, which survived less well during their first summer than fawns with unrelated parents (Da Silva *et al.*, 2009). This negative effect of inbreeding on fawn survival (inbreeding depression) should promote the evolution of sex-biased dispersal and/or female mating tactics to avoid consanguinity, such as pre-copulatory mate choice based on cues which indicate genetic relatedness, re-mating (either with genetically dissimilar males when females cannot avoid a first mating with close relatives, or simply with different males when females cannot distinguish close relatives), female sperm selection, and/or multiple paternity.

Gaillard *et al.* (2008) have conclusively shown that dispersal is not sex-biased in roe deer; hence, dispersal is unlikely to be a mechanism for avoiding inbreeding. Instead, the evidence we have presented here for multiple paternities, although not frequent, suggests that females re-mate with different males as a tactic for minimizing the negative effects of inbreeding on their fawns' fitness. Indeed, the results obtained show that fawns from multiple paternity litters tended to survive their first summer better than fawn litters sired by only one male. However, because of small sample size, we were unable to investigate the relationship between the degree of relatedness between partners and the occurrence

of multiple paternities. Note, however, that fathers involved in multiple paternity litters could be either unrelated (e.g. relatedness = 0.00), only weakly related (e.g. 0.21) or highly related (e.g., 0.80) to the mother of the litter, so that females that had litters sired by different males did not have offspring of higher genetic diversity than females that had litters sired by only one male. Further research is therefore needed to investigate whether females attempt to avoid mating with highly related partners and whether multiple paternity is linked to an initial consanguineous mating before we can conclude that the evolution of female multiple mating and multiple paternities in roe deer is linked to inbreeding avoidance.

In this context, it should be noted that our study population is located on a small, largely isolated peninsula, which could explain the unusually high level of inbreeding. Indeed, the level of expected heterozygosity in our population for the 21 microsatellites used was 0.53, which is among the lowest reported values for this species across its range (France: Coulon *et al.*, 2004; Vanpé *et al.*, 2009); Central Europe: Wang & Schreiber (2001); among 36 populations across Europe: Randi *et al.* (2004).

TOWARD ANOTHER EXPLANATION: THE FERTILITY INSURANCE HYPOTHESIS

From the above, it is unlikely that multiple mating is a way for females to increase the genetic diversity or quality of their fawns, and inbreeding avoidance may be one of the most plausible benefits of multiple mating for female roe deer. However, another possibility that concords with the rather low level of multiple paternities in our population involves the fertilization insurance (Olsson *et al.*, 1996; Orsetti & Rutowski, 2003). Roe deer females may mate repeatedly with one or more males to ensure fertilization. In particular, multiple mating with more than one male may provide a female with insurance against the possibility that one of her mates is less than fully fertile (Olsson *et al.*, 1996). This is particularly important in this species, given that females have a single short annual oestrus (whereas all other deer species are polyoestrous). This fertilization insurance mechanism could be behind the fact that adult females have a very high probability of conceiving each year (typically > 0.95, Gaillard *et al.*, 1992; Hewison, 1996). Hence, inbreeding avoidance is likely not the only explanation for the evolution of female multiple mating in roe deer, and multiple benefits, including notably fertilization insurance, probably occur. Testing the fertility insurance hypothesis in roe deer, however, would require a comparison of the conception success or size of litters sired by one versus

several males, which is not possible with the current available data.

ROE DEER MATING SYSTEM REVISITED

The roe deer was considered up to now as weakly polygynous (Liberg *et al.*, 1998; Vanpé *et al.*, 2008a). In the present study, we provide evidence that both males and females may actually mate with several partners during a given breeding season in this species so that the roe deer mating system might actually be somewhat promiscuous, with important consequences in terms of the operational sex ratio, the opportunity for sexual selection, and the maintenance of genetic diversity in this territorial species. Our results also confirm that territoriality in roe deer is definitively not a male mating tactic that ensures paternity through monopolizing oestrous females present within a defined area of dominance (for similar arguments, see also Vanpé *et al.*, 2008b)

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