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Mother's baby, father's maybe: the occurrence and frequency of multiple paternity in the European wild boar

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ABSTRACT

Background: Multiple paternity (i.e. when the litter of a pregnant female is fertilized by more than one male) is common in a variety of animal taxa, including several ungulate species. It is generally believed that dominant males of European wild boar (*Sus scrofa*) monopolize several females, suggesting that multiple paternity is a rare phenomenon in this species. However, recent studies from different populations across Europe suggest that multiple paternity occurs more often in wild boar than had previously been assumed. However, previous studies were based on small sample sizes or a background of very strong hunting pressure on males.

Aim: Clarify the number and frequency of multiple paternities in European wild boar under moderate and balanced hunting conditions.

Method: We analysed eight highly polymorphic microsatellite markers in the embryonic and uterine tissues of 35 gestating female wild boars from different but nearby hunting grounds in Lower Saxony (Germany). Then, we visually reconstructed the putative paternal genotypes. We calculated the frequency of occurrence of multiple paternity using a variety of software packages.

Results: Almost 23% (8 of 35 the uteri) of the embryonic genotypes suggested at least two different sires. A minimum of 45 different fathers had to have been involved. In fact, due to hidden incidences where both parents carried the same alleles, maximum likelihood calculations suggested an even higher rate of multiple paternity.

Keywords: alternative reproductive tactics, European wild boar, microsatellites, multiple paternity, sexual conflict, *Sus scrofa*.

INTRODUCTION

Multiple paternity (i.e. when a pregnant female carries a litter sired by more than one male) is a common phenomenon that has been observed in insects (Song *et al.*, 2007), fish (Girndt *et al.*, 2012), amphibians (Knopp and Merila, 2009), birds (Birkhead and Møller, 1992), reptiles (Meister *et al.*, 2012),

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and mammals (Dugdale *et al.*, 2007; Glen *et al.*, 2009; Vanpe *et al.*, 2009; Falcon *et al.*, 2011), and is very well documented in several ungulate species, including white-tailed deer [*Odocoileus virginianus* (DeYoung *et al.*, 2002; Sorin, 2004)], wild pronghorn antelopes [*Antilocapra americana* (Carling *et al.*, 2003)], and roe deer [*Capreolus capreolus* (Vanpe *et al.*, 2009)]. Multiple paternity has been observed not only in socially polygamous (Firman, 2014) but also in socially monogamous species (Jennions and Petrie, 2000; Arnqvist and Kirkpatrick, 2005).

Multiple mating is advantageous for males because it increases the chance to sire more offspring. However, the production of sperm can be costly for males and therefore is a limiting factor and will finally restrict the number of offspring (Dewsbury, 1982). By contrast, females cannot necessarily increase their direct fitness by mating with several males owing to a limited number of mature eggs. But a potential benefit for females actively practising multiple mating is sperm competition (Parker, 1970). Especially in mating systems where dominant males try to monopolize several females, as assumed in European wild boar, sperm competition is unavoidable if these monopolization attempts fail. This pre- and post-copulatory competition allows females to select for the most viable or suitable sperm for their offspring. Furthermore, multiple mating represents a possibility for females to enhance the genetic diversity (Bergeron *et al.*, 2011) and genetic quality (Jennions and Petrie, 2000) of their offspring, to reduce homozygosity (Charlesworth and Charlesworth, 1987), or to reduce resistance to parasites (Coltman *et al.*, 1999). However, in some cases multiple mating does not happen by choice but is the consequence of sexual harassment (Carranza and Valencia, 1999; Fox, 2002; Fitze *et al.*, 2005; Cappozzo *et al.*, 2008).

Traditionally, it was assumed that multiple paternity in European wild boar litters was unlikely due to the monopolization of females by the strongest boars (Briedermann, 1986). It was first suggested that female wild boars copulate with two or more males during a reproductive cycle, resulting in multiple paternity, some years ago (Delgado *et al.*, 2008). But the authors of that study assumed this behaviour to be rare and only statistically provable. Shortly afterwards, the first physical evidence was provided in France (Poteaux *et al.*, 2009). However, it was still considered an infrequent occurrence because only two cases of multiple paternity were identified in the 21 uteri investigated. Further support for the existence of multiple paternity emerged from a transnational study conducted in Portugal, Spain, and Hungary (Costa *et al.*, 2012), in which multiple paternity was identified in 5 of 15 uteri collected, and from a multi-year study in a French population but with very strong selective hunting pressure on males (Gayet *et al.*, 2016). Over six hunting seasons, 165 pregnant females and their full litters were collected. Depending on the analytical model used, multiple paternity rates of between 33.8% and 60.6% were observed (Gayet *et al.*, 2016). However, populations with a strong overall male-selective harvest may switch to a more promiscuous and polyandrous mating system (Gayet *et al.*, 2016). As a sequel to these events, litter sizes can increase and the number of multiple paternities will be biased (Gayet *et al.*, 2016). While these examples show that multiple paternity in wild boar is more common than assumed, a study is required of its occurrence in a population where there is only a small or no impact of hunting based on selection by sex or age.

Germany has one of the highest wild boar stocks in Europe (Massei *et al.*, 2015). Only moderate and balanced hunting of wild boar is allowed under German hunting law. Most of the hunters in our sampling area follow the 'Lüneburger Model' (Teuwsen, 1980; Hennig, 1998), which mandates a significant reduction of young boars (piglets and subadults) regardless of sex, but only a sustainable cull of adults (Keuling *et al.*, 2014). Even if the numbers of harvested piglets and female subadults are below those required, the total sex ratio of the animals

killed is balanced (Keuling *et al.*, 2013). A shift to a more promiscuous and polyandrous mating system with all its consequences owing to sex-specific hunting would, therefore, be unlikely.

The first aim of this study was to confirm the occurrence of multiple paternity in a German wild boar population because to date there is no physical evidence of this. To detect multiple paternity, we used a set of eight microsatellites (Kato *et al.*, 1991; Rohrer *et al.*, 1994, 1996; Karlskov-Mortensen *et al.*, 2008) to analyse *in utero* kinship relations. If this analysis was successful, our second aim was to estimate the mean frequency of multiple paternity in the study area and to determine which females favour this behaviour. In particular, we investigated differences among maternal age classes, such as differences in body mass or mean number of embryos, and their possible influences on multiple paternity.

MATERIALS AND METHODS

Study site, sampling, and age classes

Although the European wild boar is mainly monoestrous (Briedermann, 2009) and shows a distinct seasonality of reproductive performance from March to May, pregnant piglets and subadult sows have been observed up until late summer (Gethöffer *et al.*, 2007). Gravid sows, therefore, can be observed most of the year and sampling is thus feasible throughout the year.

Sampling took place in Lower Saxony, northern Germany. Samples were taken from hunting grounds up to 100 km to the north and east of the state capital Hannover (Gethöffer *et al.*, 2007; Keuling *et al.*, 2014; Frauendorf *et al.*, 2016). The uteri of females of all age classes were collected on drive and single hunts between October 2010 and February 2011, and again between October 2011 and February 2012. The age classes were defined as follows: piglets aged up to 12 months, subadults aged 13–24 months, and adults aged 25 months and older. The body mass of each individual was weighed after dressing, i.e. after its entrails were removed. The uteri were dissected, examined, and measured in the lab (University of Veterinary Medicine Hannover, Foundation). We wished to determine whether the mean number of embryos per litter was influenced by the body mass or age class of the pregnant females. The uteri of 35 visibly pregnant females and their full litters ($n = 213$ embryos) were weighed. The tissue samples (5–15 g) that were taken from all individuals, both mothers and embryos, were stored in 5 mL tubes containing 4 mL 99.8% ethanol at 4°C for subsequent paternity analyses.

DNA extraction, amplification of microsatellite primers, and PCR conditions

DNA extraction was conducted in line with the Chelex™ 100 (Bio-Rad) protocol (Walsh *et al.*, 1991) using 25 mg tissue for each sample. The DNA concentration of each sample was measured with a NanoDrop™ 1000 spectrophotometer (Peqlab Biotechnologie GmbH) and made uniform to 70 $ng \cdot \mu L^{-1}$ by ultrapure water dilution (LiChrosolv®, Merck KGaA) to avoid DNA-amplification interference during polymerase chain reaction (PCR) caused by too much DNA. PCR was done with Peqlab's 'Taq all inclusive' kit (Peqlab Biotechnologie GmbH): amplifications used a 10 µL reaction volume containing 4.15 µL ultrapure water, 1 µL buffer solution Y, 0.2 µL dNTP (10 mM), 2 µL enhancer solution P, 2 µL DNA (70 $ng \cdot \mu L^{-1}$), 0.3 µL forward primer (10 $pmol \cdot \mu L^{-1}$), 0.3 µL reverse primer

Table 1. Primer sequences of the eight microsatellites

Microsatellite	Label	Repeat length	Forward primer	Reverse primer
KVL9495	BMN6	4	5′ CAC AGC TGG GCG AAG TTA AAC 3′	5′ CTC CTT TAA AAG CTC CTT GTG AGA G 3′
KVL9807	Cy5	4	5′ AAG TAT TAA GCA GAA CCC AGC GTG 3′	5′ CCA GTT CTT TTC AGA CCC AGA CTC 3′
TNFB	Dy-751	3	5′ CTG GTC AGC CAC CAA GAT TT 3′	5′ GGA AAT GAG AAG TGT GGA GAC C 3′
CGA	BMN6	2	5′ ATA GAC ATT ATG TAA GTT GCT GAT 3′	5′ GAA CTT TCA CAT CCC TAA GGT CGT 3′
Sw24	BMN6	2	5′ CTT TGG GTG GAG TGT GTG C 3′	5′ ATC CAA ATG CTG CAA GCG 3′
Sw632	Cy5	2	5′ TGG GTT GAA AGA TTT CCC AA 3′	5′ GGA GTC AGT ACT TTG GCT TGA 3′
SW742	Dy-751	2	5′ AAT TCT ACT TCT GGG GAG AGG G 3′	5′ CTT TTG GGA ACA TTT CTG CC 3′
Swr1941	Dy-751	2	5′ AGA AAG CAA TTT GAT TTG CAT AAT C 3′	5′ ACA AGG ACC TAC TGT ATA GCA CAG G 3′

Note: For capillary electrophoresis, a specific fluorescent label (Cy5 = blue, BMN6 = green, Dy-751 = black) was attached to the 5′ end of every forward primer of each primer pair. The repeat length of the loci varies from 2 to 4 base pairs.

Table 2. PCR conditions for primer systems

Microsatellite	Denaturation (temp./duration)	Cyclic denaturation, annealing and elongation (temp./duration)	No. of cycles	Final elongation and cooling (temp./duration)
KVL9495 KVL9807	95°C/5 minutes	95°C/60 seconds 58°C/60 seconds 72°C/60 seconds	35	72°C/15 minutes 4°C/∞
TNFB CGA	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 68°C/30 seconds	35	68°C/20 minutes 4°C/∞
Sw24 Swr1941	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 72°C/30 seconds	35	72°C/15 minutes 4°C/∞
Sw632	94°C/5 minutes	94°C/30 seconds 55°C/30 seconds 72°C/30 seconds	32	72°C/15 minutes 4°C/∞
SW742	94°C/5 minutes	94°C/30 seconds 60°C/30 seconds 68°C/30 seconds	35	68°C/20 minutes 4°C/∞

(10 pmol·μL^{-1}), and 0.05 μL peqGold Taq DNA polymerase. We used a set of eight highly polymorphic microsatellites: two tetranucleotide [KVL9495 and KVL9807 (Karlskov-Mortensen *et al.*, 2008)], one trinucleotide [TNFB (Rohrer *et al.*, 1994)], and five dinucleotide [CGA, Sw24, Sw632, SW742, and Swr1941 (Kato *et al.*, 1991; Rohrer *et al.*, 1994, 1996)] primer pairs. For genotyping, the forward sequence of each microsatellite was labelled with a fluorescent marker (biomers.net GmbH) (see Table 1). Amplification was done using the PCR conditions listed in Table 2.

Genotyping

Genotyping was carried out using an automated CEQ 8000 series Genetic Analysis System (Beckman Coulter). PCR products were diluted with 20 μL HPLC water, 2 μL of which was mixed with 0.15 μL of CEQ DNA Size Standard Kit 400 bp (Beckman Coulter) and 30 μL of CEQ Sample Loading Solution (Beckman Coulter). Sizing of the fragments was done following the Beckman Coulter standard protocol for the CEQ 8000 series and using GenLab software v.10.2.3 (Beckman Coulter). Allele binning was performed with R v.2.15.3 (R Development Core Team, 2013) and the package MsatAllele v.1.02 (Alberto, 2009). Calculations of allele frequency, observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), deviations from Hardy-Weinberg equilibrium (HWE), polymorphism information content (PIC) (Botstein *et al.*, 1980), probability of identity ($P_{ID/SIB}$), and presence of null alleles were performed with ARLEQUIN v.3.5.1.3 (Excoffier and Lischer, 2010), CERVUS v.3.0.3 (Marshall *et al.*, 1998; Slate *et al.*, 2000; Kalinowski *et al.*, 2007), and GIMLET v.1.3.3 (Valiere, 2002).

Repeatability, error rates, and descriptive statistics

For quality assurance, each PCR run contained an additional positive control (sample with known fragment length) and a negative control (HPLC water instead of DNA) as recommended in the literature (Budowle *et al.*, 2005; Selkoe and Toonen, 2006). Finally, to calculate the overall genotyping error rate, 10% (21 samples) of the total sample set (DeWoody *et al.*, 2006; Selkoe and Toonen, 2006) was randomly repeated (http://www.random.org/lists/) by independent DNA extraction, PCR, and genotyping steps for all eight microsatellites. Deviations in run time of more than one-half of the repetitive motif length, resulting in different numbers of alleles, were evaluated as errors. The calculated error rate is defined as the ratio of the number of deviations resulting in errors to the total number of alleles compared (Bonin *et al.*, 2004). None of the repeated samples resulted in different fragment length compared with the first run. Nevertheless, for all software packages used that require a genotyping error rate, a 5% error rate was assumed. This procedure will compensate all potentially undetected errors. Descriptive statistics and non-parametric tests were conducted with R v.2.15.3 (R Development Core Team, 2013).

Multiple paternity detection

In addition to the computational parentage analysis using the programs described in the following paragraph, microsatellite data of each sow and her piglets allowed a visual reconstruction by hand of the minimum number of paternal genotypes based on Mendelian rules of inheritance. To avoid overestimation of the number of sires, only the minimum number of paternal alleles required was used here to explain the offspring genotypes; for example, in cases where only one unambiguous paternal allele could be found, the father

was assumed to be homozygous. These fathers could also be heterozygous but owing to the absence of a second allele, the proposed assumption seems to be the most conservative way to avoid overestimating the number of alleles. In cases where two unambiguous paternal alleles were observed, only one heterozygous sire was assumed although two different homozygous fathers could also be a possible explanation. Only if the number of detected alleles allowed no other explanation was a case of multiple paternity assumed. To exclude mutations as a reason for altered allele sizes, multiple paternity was assumed only for embryos with alleles that could not be explained by a sole sire on at least two different microsatellite loci. If a case of multiple paternity was detected, the DNA of the whole family was again extracted and PCR and genotyping repeated to ensure that no technical, chemical or human factor biased the results. This was performed in addition to the above mentioned error rate calculation.

From the various available statistical programs for paternity analysis (Jones and Ardren, 2003; Jones and Wang, 2010a), we chose COLONY v.2.0 (Wang, 2004; Wang and Santure, 2009; Jones and Wang, 2010b), CERVUS v.3.0.3 (Marshall *et al.*, 1998; Slate *et al.*, 2000; Kalinowski *et al.*, 2007), and PEDIGREE v.2.2 (Smith *et al.*, 2001; Wilson *et al.*, 2003; Butler *et al.*, 2004) to identify cases of multiple paternity in combination with the visual reconstruction of paternal genotypes. While CERVUS assigns parentage based solely on a pair-wise likelihood comparison approach, COLONY infers sibship and parentage simultaneously using a full-pedigree likelihood method. PEDIGREE served as an independent means of assessing the number of sires solely based on the occurrence of half-sibs in the litters.

To estimate critical values for CERVUS log-likelihood statistics with a 95% level of confidence, we applied a simulation with the following parameters: 100,000 cycles, 266 candidate parents (all maternal and all possible combinations of paternal genotypes), 0.8 for the proportion of candidate parents sampled, 0.99 for the proportion of loci typed, 0.05 for the proportion of loci mistyped, and a 0.05 error rate in likelihood calculations. COLONY was either provided with the same dataset (all parents known) or with the option 'all males unknown' in a polygynous-polyandric mating system with inbreeding and a full-likelihood model with no sibship prior. PEDIGREE was provided with the same dataset and the following parameters: 10 runs, one million iterations, full-sib constraint, temperature of 5 to 50°C in steps of 5°C, weight 1, and random seed.

RESULTS

The 35 analysed gestating sows represented all age classes: piglets ($n = 11$), subadults ($n = 17$), and adults ($n = 7$). Litter sizes ranged from 2 to 10 with a mean of 6.057 ± 2.222 (±SD) embryos per litter (median = 6; see Fig. 1).

We found significant differences between maternal age groups in the number of embryos (Kruskal-Wallis rank sum test: $\chi^2 = 11.215$, $df = 2$, $P < 0.004$; see Fig. 1). Subadult females had significantly more embryos than piglet females (Mann-Whitney *U*-test: $W = 21.5$, $P < 0.001$) but did not differ from adult females ($W = 48.5$, $P = 0.499$), while adults tended to have more embryos than piglets ($W = 57$, $P = 0.098$; see Fig. 1). The number of embryos increased with female body mass regardless of their age (Spearman rank correlation: $r = 0.556$, $P = 0.0005$). However, this held true only because of the highly significant correlation between body mass and number of embryos for females with a body mass below 60 kg ($r = 0.796$, $P < 0.001$); no such correlation was observed for females with a body mass of 60 kg and above ($r = 0.019$, $P = 0.942$) (see Fig. 2).

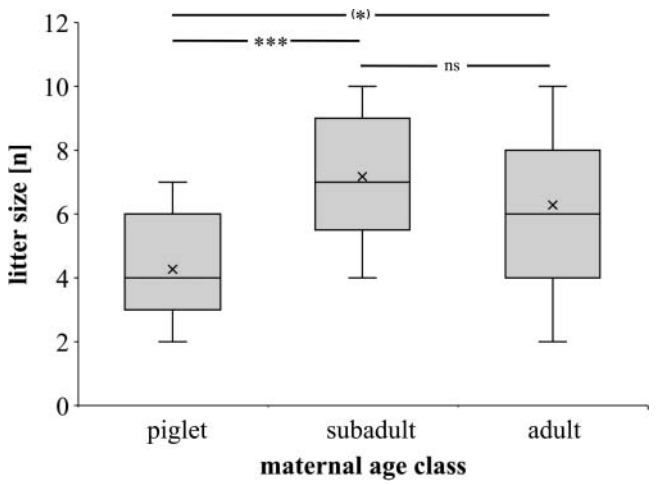

Fig. 1. Mean litter size of piglets, subadults, and adults. Shown are mean (×), median, first and third quartiles, and range span. ***$P < 0.001$, (*) = non-significant but a tendency $0.1 > P < 0.05$, ns = $P > 0.05$.

All microsatellite loci were highly polymorphic and suitable for individual discrimination purposes (see Table 3) (Botstein *et al.*, 1980; Waits *et al.*, 2001). The mean gene diversity over all loci was estimated at 0.765 ± 0.403 ($\pm$SD) and the polymorphism information content (PIC) ranged from a minimum of 0.572 for locus Swr1941 to a maximum of 0.929 for locus KVL9495 with a mean of 0.769 ± 0.129. The combined non-exclusion probability of identity (P_{ID}), the probability that the genotypes do not differ between two randomly chosen individuals, was estimated at 3.78×10^{-11}, and between full-sibs (P_{SIB}) at 3.16×10^{-4}. There was no significant deviation from HWE except for locus TNFB and no evidence for null alleles F(Null).

As mentioned previously, by visually reconstructing by hand the minimum number of paternal genotypes based on Mendelian rules of inheritance, at least 45 different sires would be required to explain the genotypes of all 213 embryos. In 6 of the 35 investigated uteri, one or more embryos could only be explained by two or more different sires. For another two uteri, the allelic distribution of the embryos could only be explained by three or more different sires. In summary, 8 of 35 females were fertilized by multiple males.

With these 45 reconstructed putative paternal genotypes set as priors in the genetic analyses, all eight cases of multiple paternity could be validated by adjacent CERVUS and COLONY runs. Without prior male genotype information, the CERVUS, COLONY, and PEDIGREE analyses provided slightly different results. In addition to the 8 cases of multiple paternity confirmed by visual reconstruction (8 of 35), CERVUS suggested an additional two cases of cryptic multiple paternity (10 of 35) where the combination of alleles was likely due to multiple sires. The analysis by COLONY suggested an additional three cases of cryptic multiple paternity (11 of 35). Using PEDIGREE for clustering the offspring in full- and half-sib groups resulted in 10 cases of multiple paternity. All confirmed and suggested cases of multiple paternity were for the same individuals, except for the eleventh case proposed by COLONY (see Fig. 2).

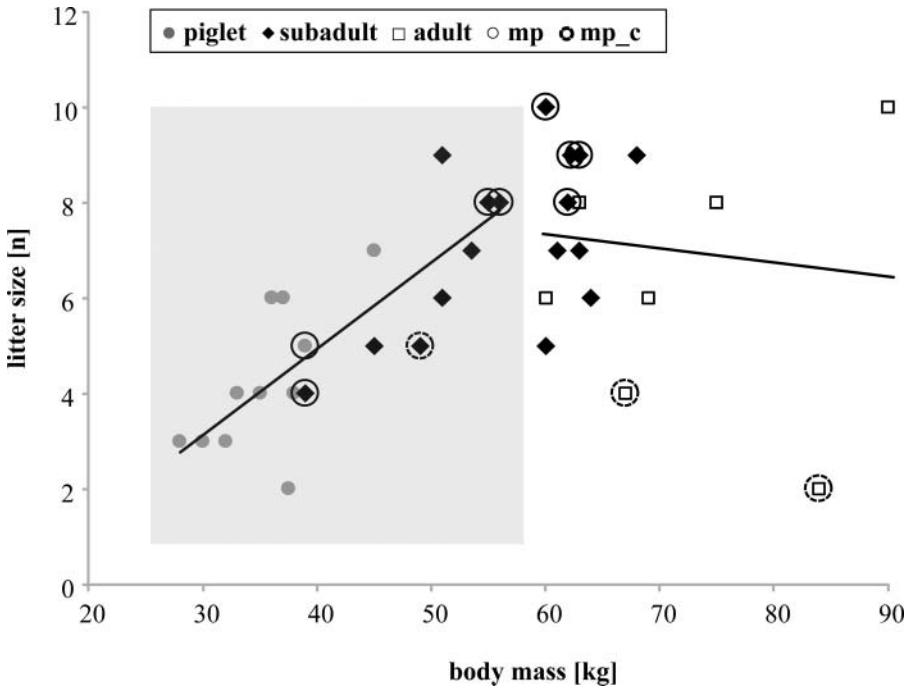

Fig. 2. Correlation between female body mass and litter size. The grey dots (●) show piglets, the solid rhombi (◆) subadults, and the open squares (□) adults. There was a highly significant correlation between body mass and number of fetuses (highlighted by the grey background) for females below 60 kg, but not for females of 60 kg or above. The eight unbroken black circles indicate females with multi-paternal offspring (mp) from a visual reconstruction by hand of the minimum number of paternal genotypes based on Mendelian rules of inheritance. The three broken circles indicate females with multi-paternal offspring (mp_c) using the program COLONY.

Table 3. Diversity indices of the eight microsatellites for mothers and fetuses

MS-system	A	N	H_{obs}	H_{exp}	PIC	P_{HWE}	F(Null)
TNFB	7	239	0.619	0.727	0.687	<0.001	0.0913
CGA	20	242	0.831	0.902	0.891	0.0144	0.0409
KVL 9495	24	246	0.886	0.934	0.928	0.0241	0.0251
KVL 9807	13	245	0.869	0.883	0.869	0.0914	0.0054
SW 742	16	242	0.736	0.843	0.829	0.0185	0.0697
Swr1941	5	248	0.605	0.619	0.572	0.1699	0.0037
Sw24	5	247	0.680	0.701	0.646	0.0444	0.0080
Sw632	8	248	0.617	0.685	0.633	0.1444	0.0515

Note: Microsatellite system (MS-system), number of alleles per locus (A), number of genotyped individuals (N), observed (H_{obs}) and expected rate of heterozygosity (H_{exp}), polymorphism information content (PIC), results of Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HWE}), and calculated frequency of null alleles F(Null).

Table 4. Different computations revealed different numbers of sires and multiple paternities

	Visual	CERVUS	COLONY	PEDIGREE
Sires	45	45	39	45
Multiple paternities	8	10	11	10

While all 45 visually reconstructed putative paternal genotypes were confirmed with and without prior male genotype information by CERVUS, only 39 sires were suggested by COLONY without prior male genotype information, assuming some of the females to be inseminated by the same males (see Table 4).

Although none of the litters of adult females resulted in an unambiguous case of multiple paternity, the effect of female age on the occurrence of multiple paternity was not significant (Pearson's chi-squared test: $\chi^2 = 4.2805$, $df = 2$, $P = 0.1176$). However, litters that are the result of multiple paternity tend to be larger than those that are not (Mann-Whitney U-test: $W = 163$, $P < 0.031$).

DISCUSSION

The present study provides conclusive evidence for the regular occurrence of multiple paternity in wild boars in Germany. In 22.8% of the investigated uteri, clearly recognizable cases of multiple paternity were highlighted by reconstruction of paternal genotypes. According to this, two or more different sires would need to be involved to explain all fetal genotypes. When including those cases where the allelic distribution makes a single sire statistically less likely than a case of multiple paternity, this should be considered as the minimum frequency of occurrence in the study area. The software tools used to calculate the occurrence of these cryptic events – CERVUS, COLONY, and PEDIGREE – proposed two to three additional cases of multiple paternity. This results in a total of 11 of 35 cases, which means about 30% of all examined gestating females successfully engaged in multiple copulations with different sires. Repetitive DNA extraction, amplification, and genotyping of all potential cases of multiple paternity should have minimized possible errors to a rate below that expected to influence the study results significantly.

In contrast to previous studies (Delgado *et al.*, 2008; Poteaux *et al.*, 2009), multiple paternity appears not to be a rare phenomenon but a regular trait. The high proportion of females mating several times in this study is in line with research in other European countries, which showed rates of multiple paternity of 33% (Costa *et al.*, 2012), 50% (Say *et al.*, 2012), and even 60% (Gayet *et al.*, 2016). With half-sibs in every second to fourth litter, this behaviour is far more common than originally thought (Briedermann, 2009) and clearly not a local phenomenon – it has been shown from Portugal and Spain to Hungary (Costa *et al.*, 2012), France (Say *et al.*, 2012; Gayet *et al.*, 2016), and now Germany. While the occurrence of this behaviour is clear, its causes remain unknown. It might be beneficial for females to engage in multiple mating as a protection against functional infertility of their mates (Sheldon, 1994). Cryptic female choice is also a possibility (Eberhard and Cordero, 1995), in which case females would release competent spermatozoa from a sperm reservoir accumulated through mating with competing sires (Parker, 1970; Birkhead and Møller, 1998; Simmons, 2001). To date, no proof has been provided for cryptic female choice in wild boar. All necessary mechanisms seem to be available though: females are able to

store and release male sperm for up to 30 hours (Gualtieri and Talevi, 2003; Talevi and Gualtieri, 2004; Satake *et al.*, 2006; Bonet *et al.*, 2013), they usually engage in multiple copulations during their fertile period (Meynhardt, 1978; Briedermann, 1986), and boar semen is potentially highly variable (Kozdrowski and Dubiel, 2004; Smital, 2009; Klimas *et al.*, 2012). However, alterations in the population structure might be an additional factor in female selection behaviour. Such a social change might be created by selective hunting pressure, resulting in a sounder of adult females losing the protection of their dominant male during the oestrous cycle. It is known that populations with a strong overall male-selective harvest may switch to a more promiscuous and polyandrous mating system owing to the lack of dominant and monopolizing males (Say *et al.*, 2012; Gayet *et al.*, 2016). Thus, frequencies of multiple paternities of up to 60% have been documented (Gayet *et al.*, 2016). Also, a surplus of sexually mature young males (i.e. sexually mature male piglets and subadults), owing to the selective hunting of older males, influences the population structure and hence the reproductive behaviour of piglets and subadults (Meynhardt, 1978; Briedermann, 1986). Both may induce a shift from pre- to more post-copulatory selection mechanisms to counteract the increasing risk of genetic incompatibility (Zeh and Zeh, 1997) in reduced and oversupplied male contingents.

Interestingly in the present study, although litters that had multiple paternity tended to be larger than those that did not, which is supported by other findings (Gayet *et al.*, 2016), all cases of multiple paternity were observed in the two younger age classes. We could not detect any unambiguous case of multiple paternity in adult female litters. Even though all cases of multiple paternity were assigned to younger females (piglets and subadults), no age-dependent influence on the occurrence of multiple paternity was observed. The findings of the present study rather suggest that the body mass of females is a better predictor of reproductive performance than their age. Accordingly, recent studies have revealed that nutritional status is an important factor in the onset of sexual maturation and consequently litter size (Gethöffer *et al.*, 2007; Frauendorf *et al.*, 2016). Female age as well as female body mass and number of embryos showed the expected associations (Briedermann, 2009). Older and heavier females had more embryos than younger and lighter ones but with one proviso: a highly significant correlation was observed between body mass and number of embryos for females with a body mass below 60 kg, whereas no such correlation was observed for females with a body mass of 60 kg and above. This result is predicted by the 'coin-flipping' hypothesis proposed for wild boar (Gamelon *et al.*, 2013). Coin-flipping is a form of phenotypic plasticity. Fertile heavy female wild boars seem to be able to diversify their offspring's phenotype by differential investment in the embryos within a litter. In contrast, lighter females tend to produce similar-sized embryos. In this context, it would be reasonable to assume that lighter females therefore increase the genetic diversity of their offspring instead of phenotypic diversity as a result of multiple mating.

It is conceivable that female boars with a lower body mass might be more vulnerable to sexual harassment by stronger males. Sexual harassment could also be induced by a surplus of sexually mature non-adult males, as mentioned above. Especially in the case of polygynous species, sexual coercion and sexual harassment arise owing to intense male–male competition for mates (Smuts and Smuts, 1993). Also, the females of such species may voluntarily engage in multiple copulations to dilute the probability of inbreeding when close relatives or male littermates are involved (Meynhardt, 1978).

In conclusion, we have shown that multiple paternity is a relatively common phenomenon among wild boar litters in Germany. Questions to be addressed in future research include whether this behaviour is related to age (i.e. are adult females less affected by this than

subadults and piglets) and whether there is support for the 'coin-flipping' hypothesis. The underlying mechanisms why females engage in multiple mating with different sires is a more complicated question to answer and will require more research on larger data sets.

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