

## MICROSATELLITE ANALYSIS REVEALS MULTIPLE PATERNITY IN A POPULATION OF WILD PRONGHORN ANTELOPES (*ANTILOCAPRA AMERICANA*)

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Although multiple paternity has been observed in various groups of mammals with litter sizes  $>1$ , it has not been documented in wild ungulate populations. With the use of DNA microsatellites, we investigated multiple paternity in pronghorn (*Antilocapra americana*) at the National Bison Range. Females in this population twin every year, and we were able to assign paternity to 25 sets of twins sired in 1999 and 2000, eleven (44%) of which were fathered by different males. Complex female mate choice is the most likely explanation for the observed pattern; however, sperm competition might contribute if a female has mated multiple times. This study represents the 1st reported example of multiple paternity in a natural ungulate population and indicates that some aspects of mammalian mating systems still are not fully understood.

Key words: *Antilocapra americana*, female mate choice, microsatellites, multiple paternity, parentage, pronghorn, sperm competition, ungulates

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Historically, mating systems have been investigated through intensive behavioral studies, often requiring long hours in the field (e.g., Byers 1997a; Clutton-Brock 1988). More recently, the increase in availability of molecular genetic techniques has allowed researchers to test hypotheses of parentage and other relationships with greater accuracy (Fleischer 1996; Marshall et al. 1998; Slate et al. 2000), often with surprising results (Hughes 1998). Accurate assessments of reproductive success, kinship, and social structure all require reliable parentage information (Neff et al. 2000).

Genetic parentage assignments have challenged so many traditional notions of mating systems that it has become common for researchers to distinguish between genetic and social mating systems (Hughes 1998). Among mammals, paternity testing

has revealed that a skew in social dominance might not correlate with a similar skew in male reproductive success (e.g., Amos et al. 1993; Coltman et al. 1999; Inoue et al. 1993). Such studies indicate that behavioral data might not serve as reliable indicators of genetic reproductive success.

In mammals with litter sizes  $>1$ , genetic techniques also have revealed the presence of multiple paternity (e.g., agile antechinus, *Antechinus agilis*—Kraaijeveld-Smit et al. 2002; black bears, *Ursus americanus*—Schenk and Kovacs 1995; common shrews, *Sorex araneus*—Tegelstrom et al. 1991; and grizzly bears, *Ursus arctos*—Craighead et al. 1995). Recently, multiple paternity has been observed for the 1st time in primates (gray mouse lemur, *Microcebus murinus*—Radespiel et al. 2002) and ungulates (white-tailed deer, *Odocoileus virginianus*—DeYoung et al. 2002).

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Multiple paternity in *O. virginianus* (DeYoung et al. 2002) is especially interesting because most ungulates have either twins or single offspring, and incomplete sampling of potential sires can make detection of such a phenomenon difficult (Xia 1999). However, it is important to note that the findings of DeYoung et al. (2002) were based on captive animals at unusually high population densities and therefore might not realistically reflect a natural population. Given the amount of research being conducted on ungulate mating systems (e.g., Byers 1997a; Coltman et al. 1999; Hogg and Forbes 1997; Pemberton et al. 1992), it remains important to investigate multiple paternity in wild ungulates.

Pronghorn (*Antilocapra americana*) could be unique among ungulates because in all well-studied populations, every female gives birth to twins each year (Byers 1997a). Behavioral evidence suggests that females have complete control over mating episodes and that females rarely mate more than once (Byers 1997a). However, because behavioral evidence cannot always be used to infer genetic paternity reliably (e.g., Amos et al. 1993; Pemberton et al. 1992), the pronghorn mating system might be more complex than current evidence suggests. The high rate of twinning makes pronghorn an ideal species for the investigation of multiple paternity among ungulates through the use of genetic paternity assessment.

#### MATERIALS AND METHODS

*Study site.*—The National Bison Range is a 7,504-ha wildlife refuge located in NW Montana. There are roughly 120 pronghorn on the National Bison Range. They are prevented from emigrating by a 2-m fence that completely surrounds the refuge boundary. A more complete description of the study site and population can be found in Byers (1997a).

*Sampling strategy.*—Tissue samples were collected in 1 of 3 ways. In the 1st method, shortly after birth, fawns were captured manually during the springs of 1999, 2000, and 2001 and restrained in a manner previously described (Byers

1997b). Briefly, solitary lactating females were observed until they nursed their fawns and led them to a new hiding location. All fawns were ear-tagged and the resultant pinna tissue was stored for molecular analysis in liquid nitrogen while in the field and transferred to  $-80^{\circ}\text{C}$  once in the laboratory. All instruments used in ear-tagging were flame-sterilized for  $\sim 30$  s before and after every use in an attempt to eliminate the possibility of cross-contamination. Because female pronghorn rarely feed fawns that are not their own (Byers 1997a), we also were able to use these nursing observations to assign maternity to all captured fawns.

For the 2nd sampling method, we captured almost all individual pronghorn in the population with a helicopter and wing-trap in autumn 1999. All captured individuals were ear-tagged, and tissue samples were taken. Tissue collection and storage was the same as described for the fawn captures. In the 3rd method, the remaining unsampled individuals, either surviving untagged fawns or adults that evaded the roundup, were sampled opportunistically with a remote biopsy dart rifle (Paxarms NZ Ltd., Timaru, New Zealand—Karesh et al. 1987).

*Molecular analysis.*—Total genomic DNA was extracted from all sampled individuals with Qiagen QIAamp DNA minikits per the manufacturer's protocol (Qiagen Inc., Valencia, California). Individuals were genotyped by a suite of pronghorn microsatellite loci (Table 1). Amplifications for polymerase chain reactions (PCRs) were performed as previously described (Carling et al. 2003). PCR products were multiplexed and visualized with an ABI 377 automated DNA sequencer with GENESCAN and GENOTYPER software (PE Applied Biosystems, Foster City, California).

*Paternity analysis.*—Paternity was assigned with CERVUS software (Marshall et al. 1998). CERVUS estimates the difference in log-likelihood scores between the 1st and 2nd most likely fathers by a maximum likelihood approach. We chose 0.95 as the strict confidence interval and 0.80 as the relaxed level. CERVUS also requires the user to input the number of candidate males (41 in our study), the percentage of males sampled (95%), the percentage of loci typed (99%), and the estimated genotyping error rate (1%). The number of candidate males and percentage of those males sampled were estimated from census data collected during the roundup in

TABLE 1.—Microsatellite loci used in study of *Antilocapra americana*, including polymorphism information, observed ( $H_O$ ), and expected ( $H_E$ ) heterozygosities, and estimates of probabilities for paternity exclusion.

Locus	No. of alleles	Size range	Heterozygosity		Exclusion probability
			$H_O$	$H_E$	
<i>Aam1</i>	3	218–222	0.14	0.139	0.066
<i>Aam2</i>	6	141–159	0.753	0.718	0.483
<i>Aam3</i>	7	163–189	0.661	0.736	0.488
<i>Aam4</i>	11	224–262	0.808	0.811	0.626
<i>Aam5</i>	4	163–169	0.536	0.528	0.242
<i>Aam6</i>	4	124–130	0.534	0.494	0.194
<i>Aam7</i>	5	209–217	0.585	0.54	0.325
<i>Aam8</i>	7	194–224	0.743	0.743	0.499
<i>Overall</i>	5.88		0.589	0.588	0.981

TABLE 2.—First and 2nd most likely candidate males for 11 sets of *Antilocapra americana* twins assigned different fathers. Confidence level indicates the level (95% or 80%) at which CERVUS assigned paternity to the most likely father.

Mother	Offspring	Most likely father	Confidence level (%)	Second most likely father
F1	F1-A	M1	80	M18
	F1-B	M2	80	M19
F2	F2-A	M3	95	M1
	F2-B	M4	95	M20
F3	F3-A	M5	95	M7
	F3-B	M6	80	M19
F4	F4-A	M7	80	M21
	F4-B	M8	80	M22
F5	F5-A	M9	80	M3
	F5-B	M10	80	M8
F6	F6-A	M11	95	M23
	F6-B	M12	80	M11
F7	F7-A	M3	80	M17
	F7-B	M13	80	M9
F8	F8-A	M14	95	M24
	F8-B	M15	80	M24
F9	F9-A	M3	80	M15
	F9-B	M2	80	M19
F10	F10-A	M7	80	M10
	F10-B	M16	95	M24
F11	F11-A	M17	95	M18
	F11-B	M15	80	M25

1999 and each winter. The genotyping error rate was estimated from known mother–offspring mismatches, as well as by reamplifying ~15% of loci and comparing the resultant genotypes with the originals.

## RESULTS

Every fawn genotyped at  $\geq 5$  loci was included in the analysis, regardless of whether its mother was sampled, and all offspring were compared with all sampled males. Two hundred nine individuals were genotyped at  $\geq 5$  loci—107 fawns, 41 adult males, and 61 adult females. The combined probability of exclusion across all 8 loci was 0.981 (Table 1). Overall, paternity was assigned to 78 fawns (out of 107 genotyped) sired during the ruts of 1999 and 2000. Included in the 78 fawns that were assigned paternity at  $\geq 80\%$  confidence level were 25 sets of twins. In 14 of the 25 sets (56%), both fawns were assigned the same father at a confidence level of  $\geq 80\%$ . In the other 11 sets of twins (44%), different fathers were assigned to each offspring. Of the 22 paternity assignments made for the twins assigned different sires (11 sets of twins), 7 were assigned at the strict 95% confidence level and the remaining 15 assignments were made at the relaxed, 80% confidence level (Table 2). For the 14 sets of twins each assigned the same sire, 15 assignments (out of 28) were made at the strict 95% level and the other 13 assign-

ments were made at the relaxed 80% confidence level.

In 1 set of twins assigned different fathers, closer examination revealed that the most likely candidate male (i.e., the assigned father) for 1 fawn was the 2nd most likely candidate male for the other fawn (Table 2). Because both of these paternities were assigned at the 80% confidence level, it could be that 1 male did indeed father both fawns but was erroneously deemed the 2nd most likely candidate male for one of the fawns. Although this or another similar situation could occur 20% of the time when conclusions are drawn based on the 80% confidence levels, this particular scenario is unlikely to bias the overall pattern of multiple paternities in pronghorn.

#### DISCUSSION

To our knowledge, the only previous study to show multiple paternities in an ungulate was conducted on a captive population of unrealistically high population densities (DeYoung et al. 2002). Our data demonstrate that multiple paternities can occur in natural ungulate populations, at least among pronghorn. In our study, different males sired each offspring in a set of twins in nearly half (44%) of the litters in which we were able to assign paternity to both offspring. These findings contradict behavioral observations, which indicate that very few females mate more than once during a given estrus cycle (Byers 1997a). There are a number of possible explanations for the observed pattern of multiple paternities in pronghorn.

First, many fawns were assigned fathers at the 80% confidence level, meaning 1 in 5 paternity assignments at that level might be incorrect. An incorrect paternity assignment could result in fawns from a single female being assigned different sires when they were actually fathered by the same male. However, many twins were assigned the same father at the 80% confidence level, and one must realize that 20% of those assignments also might be incorrect. There-

fore, although it seems plausible that some of the twins assigned different fathers were actually fathered by the same male but assigned different sires incorrectly, the opposite situation is equally likely. A portion of the fawns assigned the same sire might have been fathered by different males, meaning it is also possible for the incidence of multiple paternity to be higher than we report. In either case, assignment of some paternities at the 80%, rather than the 95%, confidence level cannot explain completely the observed pattern of multiple paternities.

Second, some forced matings could have occurred, and some males might be able to gain paternities through alternative mating strategies similar to those reported in other ungulates (Coltman et al. 1999; Hogg and Forbes 1997). In pronghorns, available behavioral data suggest it is unlikely that surreptitious copulations account for the high rate of multiple paternities, due to the extreme rarity of observing a female mating with multiple males (Byers 1997a). Also, unlike other ungulates where females often copulate with many males over a short period of time (e.g., Coltman et al. 1999), pronghorn females are rarely observed to mate more than once per estrus (Byers 1997a). In order for a male pronghorn to gain intromission, he must have complete cooperation from the female (Byers 1997a); a single forward step by the female precludes intromission. Copulation control, combined with the observation that females are always successful in their attempts to evade harassing males (Byers 1997a), effectively eliminates forced copulations as an explanation for multiple paternity. However, once a female decided to mate with another male, sperm competition between males would surely become important. The most likely explanation for the observed pattern is one dependent on female choice, which might or might not be influenced by male mating strategies.

A variety of benefits to multiple paternity have been reported in the literature, including fertility insurance, material benefits, ge-

netic diversity, genetic quality, and avoidance of infanticide (Birkhead and Møller 1992; Birkhead and Pizzari 2002; Jennions and Petrie 2000). Because all females in this population become pregnant every year (Byers 1997a), it seems unlikely that they would need to mate with multiple males solely to ensure fertilization. The material benefits explanation also is unsatisfactory in this population because the mating system is not resource based (Byers 1997a). Similarly, infanticide has never been reported in pronghorn, so it is highly unlikely that females mate multiply to reduce this risk. Therefore, the hypotheses that multiple mating can increase genetic quality or diversity among littermates are the most interesting, particularly because fawn survival is very low (<1%) in some years at the National Bison Range (Byers 1997a, 1997b).

Trivers (1972) suggested that when a female's investment in offspring production is far greater than a male's, females should select mates on the basis of expressions of health and vigor, which are likely indicators of "good genes." Multiple mating in females should be favored if there are initial constraints that prevent a female from mating with the most genetically preferred male (Jennions and Petrie 2000). Females could "trade up" if they encounter genetically superior males after they have already mated. Considerable evidence among bird species in which females engage in extra-pair copulations suggests that they typically mate with older, more dominant males—males who are more likely than their social partners to have good genes (Møller 1992). Female birds might select their initial mate based on his access to a nest site or parental care abilities but then mate outside this social bond in hopes of increasing the genetic fitness of their offspring. Polyandry also might be favored if there are no reliable precopulatory cues to distinguish ideal mates from other less desirable ones (Jennions and Petrie 2000). In these situations, females could choose between mates using

in copula or postcopulatory mechanisms. Female pronghorn might be choosing mates either by the trade-up mechanism or by exercising postcopulatory choice or "cryptic" choice.

Evidence from pronghorn suggests that females choose vigorous mates, which could be males with superior genes (Byers et al. 1994). In each rut, some females "incite" fights between males by fleeing a harem male and attracting the attention of other, nearby males. Often a female will then watch the ensuing competition between the males and mate with the male that successfully defends her (Byers et al. 1994). In these cases, it appears obvious that female pronghorn are actively practicing mate choice and that they are attempting to mate with those males who best display traits that could be associated with good genes. Other times, it appears that a female chooses a harem based on its remote location and remains with that male until she is ready to mate (Byers et al. 1994). These "quiet" females might be choosing mates on the basis of the secluded nature of the harems, perhaps to avoid the potential costs associated with inciting displays among males, instead of choosing mates on the basis of their vigor (Byers et al. 1994).

Some females might continue to exercise mate choice after they have mated once. A female could mate with 1 male because he successfully protects her from other males, but if that male is later displaced from his harem, she could become an inciter and mate a 2nd time. Some evidence suggests that females switch mating strategies between seasons (Byers 1997a), and it could be that some females switch strategies within a single breeding season. Quiet females might remate if they subsequently encounter a male who prominently displays traits associated with superior genes. Because female mammals are typically unable to store sperm for very long (Ginsberg and Huck 1989), female pronghorn likely remate quickly. However, given the data, we are unable to eliminate the trade-up hypothesis

from the repertoire female pronghorn have available to use when choosing mates.

Mating with multiple males could also serve to increase the genetic diversity of a female pronghorn's offspring. Bumblebee (*Bombus terrestris*) colonies with high genetic diversity exhibited higher reproductive output and lower parasite loads than less diverse colonies (Baer and Schmid-Hempel 1999). In a study of multiple paternity in loggerhead turtles (*Caretta caretta*), Moore and Ball (2002) suggest that because new beaches are often colonized by a lone gravid female, greater genetic diversity among the hatchlings might increase the chance of colony persistence. If precopulatory clues of genetic quality are insufficient, pronghorn females might mate with multiple males in an attempt to raise the probability that 1 offspring might survive due to an inherited trait. Because fawn mortality at the National Bison Range is so high (56.0%–99.2% from 1981 to 1996—Byers 1997b), increased genetic diversity between littermates could increase the probability that at least 1 fawn will survive.

The idea that female pronghorn are able to control fertilization by some method of postcopulatory, or cryptic, choice is particularly interesting. Female pronghorn are polyovulators; yearlings typically ovulate between 2 and 7 eggs, and older females usually ovulate between 3 and 9 eggs per estrus (Mitchell 1980). Intrauterine mortality reduces litter size to 2, one embryo in each uterine horn (O'Gara 1969). Polyovulation and the subsequent reduction in litter size might be an adaptation to obtain a balanced uterus (Birney and Baird 1985), and this winnowing process could provide a mechanism for cryptic female choice between different males.

If increasing the genetic variation in their offspring is important to females, they might use multiple ovulation as a way to increase the chance that each twin has a different father. Conversely, if females are just trying to provide their offspring with the best genes possible, the interplay between

female choice and sperm competition could explain why so many females give birth to offspring fathered by different males. Perhaps sperm from the 1st male a female mates with has a distinct advantage, which can only be partially overcome by subsequent matings.

There are a number of possible explanations for multiple copulations per estrus in pronghorn. At present, our data permit only weak discrimination among the possibilities. Elucidation of the relationships between sperm competition and female mate choice will require further analysis of genetic paternity combined with focused field studies. Furthermore, an accurate estimate of the frequency of multiple paternities among ungulates is needed before researchers can accurately address questions for which a thorough understanding of the mating system is essential.

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