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Microsatellite analysis of female mating behaviour in lek-breeding sage grouse

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Abstract

We used microsatellite DNA markers to genotype chicks in 10 broods of lek-breeding sage grouse, *Centrocercus urophasianus*, whose mothers' behaviour was studied by radio-tracking and observing leks. Previous behavioural studies suggested that almost all matings are performed by territorial males on leks and that multiple mating is rare. Two broods (20%) were sired by more than one male. Genetic analyses of the broods of eight females that visited an intensively studied lek were consistent with behavioural observations. Four females observed mating produced singly sired broods and males other than the individual observed copulating were excluded as sires for most or all of their chicks. Territorial males at the study lek were excluded as sires of broods of four other females that visited the lek but were not observed mating there. Radio-tracking suggested that two of these females mated at other leks. Our results confirm the reliability of mating observations at leks, but do not rule out a possible unseen component of the mating system.

Keywords: lek mating, microsatellite DNA, paternity, sexual selection, sage grouse

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Introduction

The behaviour of lek-breeding birds, in which males display on small, grouped territories visited by females only for mating, has stimulated research on the adaptive nature of mate choice (Kirkpatrick & Ryan 1991), the evolution of social courtship (Hoglund & Alatalo 1995) and the relationship between female choice and sperm competition (Gowaty 1996; Parker & Burley 1998). Current perceptions of leks are based largely on behavioural studies indicating that female choice is often relatively unconstrained and that mating distributions are highly skewed. Whether such studies reliably reveal the mating system of lekking species is, however, unclear. Strong mating skew on leks should foster alternative off-lek male mating tactics, but the focus of most previous field studies would have made detection of off-lek breeding difficult and to date only two published studies have attempted to address this question using molecular markers. Alatalo *et al*. (1996) found that paternity matched expectations from observed mating behaviour in a small sample of black grouse broods. Lanctot *et al*. (1997) documented parentage by off-lek males in the buff-breasted sandpiper, a species in which males display away from, as well as on, leks.

Behavioural studies of lekking sage grouse, *Centrocercus urophasianus* (Wiley 1973; Gibson & Bradbury 1986; Gibson *et al*. 1991) indicate that most females visit one or more leks on several mornings before mating once with a single male and that mating distributions are strongly skewed. However, other observations raise the possibility of an unobserved component of the mating system. First, males regularly court females in sagebrush around the edges of leks (Gibson 1996) and females away from leks are occasionally accompanied by displaying males (Dunn & Braun 1985; R. Gibson unpublished data), contexts in which mating would be hard to observe. Second, among radio-tagged females that visited intensely observed leks at the time when they were expected to mate, under half were observed mating (R. Gibson, unpublished data). To clarify this species' mating system we studied parentage using microsatellite DNA markers. Here we report initial results that confirm the reliability of mating observations at leks but do not rule out a possible unseen component of the mating system.

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Materials and methods

Field methods

We studied a population of sage grouse in Long Valley, California (37°40′ N, 118°50′ W) in 1997, 1998 and 2000. In each year we observed one lek (lek 4) daily during the main dawn display period from late March until territorial males stopped attending regularly 6–9 weeks later. We collected data on attendance, territorial and mating behaviour of individually recognized males as described previously (Gibson *et al*. 1991). All territorial individuals (5–11 annually) were identified using colour bands and/or individual differences in plumage (Wiley 1973). Additional non-territorial males attended the lek in all 3 years and some of these were also colour banded. Adult and yearling males were distinguished by tail length and shape in the field and by wing moult in the hand.

We trapped birds of both sexes at the study lek for marking and blood sampling by spotlighting or with cannon nets. Additional birds were trapped at other leks. A blood sample was collected into Queen's College lysis buffer (Seutin *et al*. 1991) from a clipped hallux nail. Females were fitted with radio transmitters so that we could follow their movements, detect the onset of incubation and locate nests. We attempted to locate each female daily following the morning display period.

To detect visits by females to leks between capture and the onset of incubation we monitored leks during the morning display periods for visits by radio-tagged females. This would not necessarily detect all lek visits because some may also occur in the evening and on moonlit nights and, with exception of lek 4, we did not monitor each lek daily. In previous studies (Bradbury *et al*. 1989; Gibson & Bachman 1992) females were closer to a lek on days when they visited it (1.19 ± 1.05 vs. 4.49 ± 3.12 km, $N = 14$, Wilcoxon test: $P = 0.0015$) and 95% of locations on such days fell within 2.4 km of the visited lek. Therefore, we used this distance criterion to detect possible unobserved lek visits. We detected the onset of incubation by a sustained reduction in amplitude modulation of the radio signal, indicating persistent inactivity (Gibson & Bachman 1992).

We collected clutches of 10 females after 7–9 days of incubation, sacrificed the embryos and stored tissue samples in Queen's College tissue buffer (Seutin *et al*. 1991). This procedure pre-empted sample loss due to natural nest predation (Schroeder *et al*. 1999) and was carried out under permit from the California Department of Fish and Game.

Genetic methods

Genomic DNA was extracted from blood or growing feathers using a standard Proteinase-K digestion followed by two phenol/chloroform/isoamyl alcohol extractions (Sambrook *et al*. 1989). DNA was ethanol-precipitated and pelleted in a microcentrifuge for 20 min, washed with 70% ethanol and resuspended in 1× TE buffer.

Microsatellite alleles were amplified by polymerase chain reaction (PCR) using primer sets from chickens (*Gallus gallus*) (Crooijmans *et al*. 1994; Cheng *et al*. 1995) and red grouse (*Lagopus lagopus scoticus*) (Piertney & Dallas 1997; Piertney *et al*. 1998). Approximately 30 μg of extracted DNA was added to a 25-μL PCR mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1 U *Taq* DNA polymerase (Sigma) and 5–25 pmoles of primer which had been kinased using ^{32}P dATP according to a standard protocol (Sambrook *et al*. 1989). Each amplification consisted of 28 cycles in a thermocycler (Perkin–Elmer Cetus 9600). Amplification profiles were as follows: denaturation at 94 °C for 45 s, annealing for 45 s and extension at 72 °C for 1 min. Annealing temperatures were 50 °C for *LLST1*, *LLSD5* and *LLSD8*, 55 °C for *LLSD4* and *MCW16*, and touchdown 60–50 °C for *LLSD3*. A 5-min extension step at 72 °C was carried out after all cycles were finished.

Amplification products were mixed with formamide loading dye, heated to 94 °C for 5 min and loaded onto a 6% polyacrylamide gel with a M13 size standard sequence. Gels were run at 55 W for 2–4 h, depending on expected fragment size, and visualized on autoradiographic film. Individuals were assigned genotypes based on fragment length. All birds were genotyped twice to ensure accuracy. Samples from each brood were run on the same gel as their putative mother and potential fathers.

We initially screened DNA samples for polymorphic microsatellites using 30 primer sets from chickens and 18 from red grouse. To improve amplification success, primer sequences at one locus (*LLSD4*) were redesigned (forward: 5′-TGTGAGAAAACTCACCATGG-3′, reverse: 5′-TCTTAAGGCAAAGAGGACAG-3′). We selected six polymorphic loci, five red grouse and one chicken, to analyse parentage (Table 1). We used GENEPOP 3/1d (Raymond & Rousset 1995) to test for deviations from Hardy–Weinberg and linkage equilibrium among 55 adult sage grouse sampled during the study. Two loci (*LLST1* and *LLSD4*) deviated significantly from Hardy–Weinberg equilibrium (HWE; Table 1). There was no evidence of linkage disequilibrium.

Analysis of parentage

We based parentage assignment on exclusion of individuals with genotypes incompatible with offspring. We do not report probabilistic paternity assignments (Marshall *et al*. 1998) because departures from HWE at two loci (Table 1) violate the assumptions of this approach.

Genotypes of each chick were first compared with their putative mother to identify maternal vs. paternal alleles.

Table 1 Characteristics of six loci used to infer parentage

Locus	Alleles	H_O	H_E	Exclusionary power		Null allele frequency	Deviation from Hardy–Weinberg (P)
				1st parent	2nd parent		
LLST1	2	0.685	0.484	0.115	0.182	−0.177	0.004
LLSD3	5	0.667	0.698	0.281	0.457	0.021	0.503
LLSD4	32	0.880	0.953	0.796	0.886	0.035	0.028
LLSD5	2	0.289	0.310	0.047	0.130	0.029	0.537
LLSD8	4	0.619	0.592	0.174	0.323	−0.017	0.446
MCW16	9	0.687	0.786	0.413	0.594	−0.019	0.126
Combined				0.940	0.987		

Depending on numbers of paternal alleles detected, a brood was classified as multiply sired (> two paternal alleles at ≥ one locus) or potentially singly sired (≤ two paternal alleles at each locus).

For broods whose mothers were trapped at the focal lek, we compared genotypes of each chick with its mother and each genotyped male at the focal lek; too few males were banded at other leks to warrant paternity assignment in the remaining broods. A male was excluded as the father of a chick if he could not have contributed the paternal allele at one or more loci. The slight heterozygote deficiency at locus *LLSD4* (Table 1) may indicate null alleles which can falsely indicate exclusion of an apparently homozygous parent if the offspring is also scored as homozygous (Pemberton *et al*. 1995). However, this concern does not affect our conclusions: although three males considered in the paternity analyses were homozygous at *LLSD4*, none of the offspring for which they were excluded as sires was homozygous at this locus.

Results

Single vs. multiple parentage of broods

We genotyped 72 offspring from broods of 10 females, including eight trapped at the focal lek and two at another lek. An allele consistent with the putative mother's genotype was present at each typed locus in every offspring. In eight broods there were ≤ 2 paternal alleles at each locus, consistent with a single father. Two other broods contained three paternal alleles at each of three loci and were therefore sired by at least two different males (Table 2). Because mothers of both multiply sired broods visited leks that we did not monitor closely, we did not observe their mating behaviour. However, radio-tracking indicated that one (423) shifted her nesting range and possibly visited another lek before laying the eggs we collected. In this case, multiple paternity might imply sperm storage between breeding attempts.

Table 2 Genotypes of two multiply sired sage grouse broods at three microsatellite loci. Alleles are identified by size (base pairs)

Individual	Locus		
	LLSD3	*LLSD4*	*MCW16*
Female 423	137/141	460/460	183/183
chick 1	137/141	336/460	183/183
chick 2	137/157	262/460	183/177
chick 3	137/157	336/460	183/185
chick 4	137/137	336/460	183/183
chick 5	137/145	258/460	183/183
Paternal alleles	137, 145, 157	262, 258, 336	177, 185, 183
Female 425	139/157	462/500	149/179
chick 1	137/157	288/500	179/179
chick 2	139/145	414/462	149/179
chick 3	139/145	—	149/179
chick 4	141/157	336/500	149/149
chick 5	137/157	414/500	149/179
chick 6	141/157	414/500	179/179
chick 7	137/139	—	179/179
chick 8	—	—	149/177
chick 9	137/139	288/462	149/177
Paternal alleles	137, 141, 145	288, 336, 414	149, 177, 179

Mating behaviour and paternity

All matings observed at lek 4 were by territorial adult males and mating distributions were typically skewed: in descending rank order, copulations per territorial adult male were 16, 16, 4, 2 and 0 in 1997, 24, 6, 4, 1, 0 and 0 in 1998 and 12, 1, 1, 1, 1, 0, 0, 0, 0, 0 and 0 in 2000. In each year the lek was also attended by additional non-territorial adult and yearling males but none was observed mating.

We tested paternity assignments for the broods of eight females captured at the focal study lek. Four subsequently returned to the lek and were observed copulating there, each once with a single territorial adult male. Each of their

Table 3 Paternity exclusions for families of seven females trapped at lek 4. One female (422) that mated with an un-typed male is omitted. Listed males include all territorial adults at the lek on the day(s) the female visited (or all but two males for females 441 & 442). Exclusions are based on 1.9 ± 0.8 loci per chick (mean ± SD, range 1–5)

Female	Male	Chicks excluded	Female	Male	Chicks excluded	Female	Male	Chicks excluded	Female	Male	Chicks excluded
(a) Females observed mating at lek 4 (mating male underlined)											
411	403	5/6	441	407	0/7	442	407	0/7			
	404	5/6		433	7/7		433	7/7			
	405	6/6		436	7/7		436	6/7			
	407	5/6		438	7/7		438	7/7			
	413	0/6		439	6/7		439	7/7			
	446	5/7		446	6/7						
	447	4/7		447	6/7						
	448	7/7		448	7/7						
	449	7/7		449	7/7						
(b) Females not observed mating at lek 4											
409	403	5/5	421	403	8/9	423	403	5/5	424	403	8/8
	404	4/5		404	3/9		404	4/5		404	8/8
	405	5/5		405	9/9		405	5/5		405	8/8
	407	5/5		407	9/9		407	5/5		407	8/8
	413	5/5		408	9/9		408	5/5		408	8/8
				Bubba*	9/9		Bubba*	3/5		Bubba*	8/8

*Bubba is an unbanded male whose genotype was deduced from the singly sired brood of female 422 who mated with him.

broods was singly sired. Three of these females (411 in 1997, 441 and 442 in 2000) mated with genotyped males. In each case, the male seen mating was the only male not excluded as the sire of any offspring and other sampled territorial adults were excluded for all or most of the chicks (Table 3a). The fourth hen (422), not shown in Table 3, mated with the only unbanded territorial adult at the lek in 1998 (he disappeared soon afterwards, before we could obtain a blood sample) and all other territorial adults at the lek were excluded for all eight chicks. Thus, in all four cases the genetic data are consistent with observed mating behaviour.

None of four remaining females was observed mating at the focal lek even though three returned after capture. All territorial adult males at the lek were excluded as sires for most or all of these females' broods (Table 3b). The only possible exception is male 404 who was excluded for only three of female 421's eight offspring. However, male 404 is also an unlikely father for the non-excluded chicks because this brood was singly sired. Thus the genetic data are inconsistent with the idea that these females copulated unseen with the same males that we observed mating with other females.

The behavioural data clarify some alternative scenarios. It is unlikely that any of these four females had mated at another lek prior to capture. All visited leks after capture, whereas closely monitored radio-tagged hens seen mating in this and prior studies (Bradbury *et al*. 1989; Gibson & Bachman 1992; $N = 13$) stopped attending leks immediately after mating and did not return unless an initial clutch was destroyed. Also all four were trapped 1 day after the onset of mating in the year in which they were studied, insufficient time for a failed prior nesting attempt.

Two of the four females (409 and 411) were subsequently radio-tracked to at least one other lek where they could have mated. One (411) visited three leks over a 5-day period. The other two hens (421 and 424) both returned to lek 4 after capture in 1998. Subsequently, female 421 was located > 2.4 km from any lek (see Materials and methods) except on one occasion 6 days before starting to incubate. Female 424 nested < 1 km from lek 4 and was > 2.4 km from all other leks except once, 2 days before beginning to incubate. Because sage grouse lay no more than two eggs every 3 days (Schroeder *et al*. 1999), neither female could have mated on the days they were < 2.4 km from another lek and laid a singly sired clutch of the observed size before incubation started. Thus the radio-tracking data do not indicate that either mated at another lek. However, gaps in the tracking records of both females leave open the possibility of an undetected lek visit.

Thirteen non-territorial males (three adults and 11 yearlings) attended lek 4 on the morning female 421 visited and 10 (all yearlings) when female 424 visited. We genotyped only four non-territorial males in that year, all yearlings, and thus were unable to test whether most non-territorial males could have mated unobserved with either these two females. The four genotyped yearlings were excluded as sires for all of female 421's chicks (three

males) or all but one chick (one male). All four were excluded as sires for all of female 424's brood.

Discussion

The genetic data broadly confirm our behavioural observations. Among females trapped at the focal lek, the genotypes of offspring whose mother was seen copulating excluded all territorial males except the putative father. Conversely, when we did not see a female mate at the lek, all territorial males, a subset of whom performed all observed matings with other females, were excluded as sires of her brood indicating that she mated in some other context.

Although genetic confirmation of behaviourally inferred paternity supports the earlier behavioural studies, interpretation of cases in which we failed to observe a mating is more difficult. Contextual evidence precludes prior mating elsewhere (see Results), whereas the genetic mismatch to males seen mating with other females on the lek rules out observer error or mating with territorial males at the study lek during unmonitored evening or nocturnal display. Alternative possibilities include later mating at another lek, unseen mating with non-territorial males at the study lek or, more speculatively, mating with males away from leks (see Introduction). The first possibility would be the least surprising and is suggested in two cases by our radio-tracking data. In the remaining cases none of the three options can be ruled out. Thus, our data do not rule out the last two novel scenarios. Their confirmation will require molecular identification of sires and would have the potential to substantially revise current interpretations of this mating system.

Although earlier behavioural studies suggested that multiple mating is rare in sage grouse, we also identified cases of multiple paternity. Multiple paternity has now been detected in one of two species of lekking grouse (Alatalo *et al*. 1996; this study) and two species of lekking sandpiper (Lanctot *et al*. 1997; Lank *et al*. unpublished, cited by Lanctot *et al*. 1997). Sperm competition may therefore be more widespread in lek breeding birds than was inferred from earlier comparative studies of testis size (Birkhead & Moller 1992). Multiple mating in sage grouse has been linked to social interference in mating and subsequent re-mating (Gibson & Bradbury 1986), though in black grouse similar behaviour does not necessarily generate multiply sired broods (Alatalo *et al*. 1996). A further scenario, possibly relevant to one case in our study, is that a second brood may be multiply sired if a female mates with different males in her first and second breeding attempts (Oring *et al*. 1992). In view of its relevance to the idea that unconstrained mate choice promotes monogamous mating by females (Gowaty 1996), a more detailed analysis of the contexts associated with multiple paternity would be of interest.

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This study is part of a collaborative project in which we are using molecular markers to characterize the mating and social systems of lekking birds. Kathleen Semple is a PhD candidate in Robert Wayne's laboratory at UCLA, currently studying the behavioural ecology and evolution of island birds.
