Conservation Genet Resour (2013) 5:1101–1105
DOI 10.1007/s12686-013-9979-4

APPLICATION ESSAYS

Genetic sexing of stock-raiding leopards: not only males to blame

Gerald Kerth · Markus Gusset · Jari Garbely · Barbara König · Tefo Gabanapelo · Monika Schiess-Meier

Received: 27 September 2012 / Accepted: 3 June 2013 / Published online: 9 June 2013

Abstract Lethal control of stock-raiding predators is generally assumed to have fewer consequences for the species' population dynamics if it involves males only. However, very little data are available that assess whether shot "problem" animals indeed are essentially males. In this study, we used two independent genetic methods (four X-chromosomal polymorphic microsatellite loci and the sex-specific ZFXY marker) validated against known-sex samples to determine, from skin samples collected over a 6-year period, the sex of 59 leopards (*Panthera pardus*) shot by farmers in Botswana. We found that out of 53 leopards that could be sexed genetically, 21 were females (39.6 %); males were thus not significantly more often shot than females. Comparing the genetically determined sex of shot leopards to that reported by farmers showed that 58.3 % were mistaken for the opposite sex. Our genetic study revealed that more females than presumed are hunted in response to alleged livestock predation. With females frequently misidentified as males, the current practice of shooting "problem" animals is likely to negatively affect the population dynamics of leopards. These genetic data may be used to guide the development of a revised management policy for large-carnivore hunting. Importantly, models of sustainable harvest need to include female offtake as a parameter.

Keywords Genetic sexing · Hunting · Leopard · Microsatellites · *Panthera pardus* · Skin samples

Introduction

Simulation models predict that even moderate levels of hunting can lead to substantial population declines in large carnivores (Packer et al. 2009). Accordingly, harvest data suggest that in African countries and US states with the highest intensity of hunting, lions (*Panthera leo*) and cougars (*Puma concolor*) suffered the steepest population declines over the past 25 years (Packer et al. 2009). Similar effects in leopards (*Panthera pardus*) may have been masked by competitive release owing to declines in sympatric lion populations (Packer et al. 2009). Nevertheless, hunting areas in Tanzania with the highest initial harvests suffered the steepest declines in leopard populations (Packer et al. 2011).

In Tanzania, for example, hunting quotas for leopards are based on the assumption that only males are harvested. To investigate compliance with this rule, Spong et al. (2000) used molecular markers to determine the sex of 77 leopards taken by trophy hunters. They found that 29 % of the shot individuals were genetically identified as females,

G. Kerth
Applied Zoology and Conservation, Zoological Institute and Museum, University of Greifswald, 17489 Greifswald, Germany

G. Kerth · J. Garbely · B. König · M. Schiess-Meier (✉)
Institute of Evolutionary Biology and Environmental Studies, University of Zurich, 8057 Zurich, Switzerland
e-mail: monika.schiess@ieu.uzh.ch

M. Gusset · M. Schiess-Meier
Leopard Ecology and Conservation, Khutse Game Reserve, Private Bag BR7, Gaborone, Botswana

M. Gusset
Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, Abingdon OX13 5QL, UK

T. Gabanapelo
Department of Wildlife and National Parks, PO Box 93, Molepolole, Botswana

despite the fact that all skins were tagged as males. Simulation models predict a harvesting strategy that includes a considerable proportion of females to negatively affect the viability of large-carnivore populations (Packer et al. 2009), including leopards (Caro et al. 2009).

Leopards account for most livestock losses to predators (38 %) in our study area in Botswana (Schiess-Meier et al. 2007). The Department of Wildlife and National Parks implements a management policy to remove stock-raiding leopards either by translocation from communal livestock grazing to protected areas (Weilenmann et al. 2010) or by allowing farmers to shoot leopards classified as "problem" animals. However, a possible limitation of this policy, which could result in unselective and unsustainable off-take (Treves 2009), is that lethal predator control is implemented without prior knowledge of the actual sex of the leopard to be shot.

Barnhurst (1986) developed a vulnerability index for the harvest of cougars. He suggested that transient males are most vulnerable to being shot, followed by resident males, transient females and resident females. Barnhurst's index corresponds to the common assumption of many wildlife management officials that most often males are killed as a consequence of human–carnivore conflict. Probably related to the more extensive ranging behaviour of males, a majority of leopards that die due to human-related causes (Balme et al. 2010a) and stock-raiding leopards that are translocated (Weilenmann et al. 2010) are in fact males. However, for a species in regular conflict with human interests, such as leopards (Inskip and Zimmermann 2009), a larger proportion of individuals indiscriminately hunted as "problem" animals than expected by wildlife management officials may be females.

The aim of the present study was to assess, in collaboration with the Department of Wildlife and National Parks, whether leopards hunted in response to alleged livestock predation in Botswana's Kweneneg District were indeed predominantly males. To this end, we used molecular markers to determine, from skin samples, the sex of leopards shot as "problem" animals. We hypothesised that, whereas males are most vulnerable to being shot, more females than presumed are killed. These genetic data may be used to guide the development of a revised management policy for the sustainable off-take of leopards.

Materials and methods

The Kweneng District in southern Botswana encompasses 38,123 km^2. There is no trophy hunting of leopards in this district and stock-raiding leopards are reported throughout the area (Schiess-Meier et al. 2007). Protected areas include the Khutse Game Reserve (2,600 km^2) and part of the Central Kalahari Game Reserve (54,000 km^2), which are bordered by communal land used mainly for livestock farming.

With permission from the Department of Wildlife and National Parks, we collected samples from untreated skins of 59 leopards shot by farmers over a 6-year period (2000–2005; there are no official records of the actual number of leopards shot). Collected samples were kept dry and dark at ambient temperatures. All skins were voluntarily brought by the farmers to one of the four stations of the Department of Wildlife and National Parks in the Kweneng District (Khutse, Dithopo, Molepolole and Dutlwe). Some farmers reported the sex of leopards as assumed prior to shooting the animal: out of 12 leopards, five were recorded as females and seven as males.

We extracted DNA from the skin samples using a salt–chloroform method (Müllenbach et al. 1989). We then, first, screened the animals at four X-chromosomal polymorphic microsatellite loci (Table 1; Menotti-Raymond et al. 1999), divided into two multiplex PCRs with two loci each (multiplex 1: FCA145, FCA018/multiplex 2: FCA674, FCA478). One primer of each pair was labelled with a fluorochrome (either FAM or HEX) and we carried out PCR amplifications in a PTC-200 Thermal Cycler (MJ Research) with the following cycling protocol: 7 min at 94 °C, 31 cycles of 30 s at 94 °C, 45 s at 50 °C and 1 min at 72 °C, and a final extension at 72 °C for 20 min. Following amplification, we added 1.5 µl of PCR product to 9 µl of formamide and analysed each probe on a Prism 310 Genetic Analyser (Applied Biosystems). Scores for alleles were compiled and analysed with GeneScan 3.1 and Genotyper 2.1 software (Applied Biosystems), using ROX 500 internal size standard. A sample was classified as originating from a male or female depending on whether either none (i.e. male) or at least one (i.e. female) of the four loci showed two alleles (results indicated that 76.2 % of samples classified as female were heterozygous at two or more loci).

Second, we ran the sex-specific zinc-finger XY (ZFXY) marker (Pecon Slattery and O'Brien 1998), which produces

Table 1 Molecular markers (four X-chromosomal microsatellites) used to determine, from skin samples, the sex of leopards shot as "problem" animals in Botswana's Kweneng District

Marker	Success rate (%)	Number of alleles	Product size (bp)
FCA018	97	3	232–238
FCA145	95	2	159–161
FCA478	93	9	178–206
FCA674	93	10	131–147

Per marker, the percentage of successfully amplified samples, the number of alleles and the size of the amplification product are given

a 500 bp X allele, a 730 bp Y allele and a weaker 900 bp band that appears in both males and females. Locus ZFXY was visualised with ethidium bromide on a 1.5 % agarose gel run for 1 h at 70 V. We used the presence of the 900 bp band to assure that the extracted DNA was sufficiently undegraded to also amplify the 730 bp Y allele, if present (in males). Consequently, to account for allele dropout, samples that showed a single X band (n = 23), but neither the Y band nor the 900 bp band, were not sexed. Similarly, one sample classified as male with the X-chromosomal microsatellites but as female with the ZFXY marker was not included.

To validate our two genetic sexing methods, we sampled blood and hair of 10 leopards of known sex (six females and four males) that were captured using baited cage traps with expanded mesh walls and were immobilised by a licensed veterinarian using a tiletamine–zolazepam (Zoletil) or a ketamine–xylazine combination. Drugs were remotely administered with a CO_2-injection rifle (Telinject). Applying our molecular analysis to these samples correctly identified the sex of each individual with both methods (the six females were heterozygous at one to three X-chromosomal loci; the ZFXY marker for one female was not conclusive, as the 900 bp band did not amplify). The use of two independent genetic sexing methods thus minimised the probability of samples being misclassified (e.g. due to allele dropout).

A possible deviation from an even sex ratio of shot leopards was statistically evaluated in Systat 12 (Systat Software) using a two-sided single proportion test, with the significance level set at $P \leq 0.05$. We also compared the genetically determined sex of shot leopards to that reported by farmers.

Results and discussion

From skin samples, 32 leopards were identified as males (24 with both methods, five with the X-chromosomal microsatellites only and three with the ZFXY marker only), while 21 leopards were identified as females (six with both methods and 15 with the X-chromosomal microsatellites only) (Fig. 1). The sex of six leopards could not be determined with either method, because no DNA of sufficient quality could be extracted from a sample (n = 2) or the molecular analysis for both the X-chromosomal microsatellites and the ZFXY marker of a sample was not conclusive (n = 4). Although many wildlife management officials assume that predominantly males are hunted in response to alleged livestock predation, we found that out of 53 genetically sexed leopards shot by farmers, 39.6 % were females. In accordance with our hypothesis, males were not significantly more often shot than females ($z = 1.52$, $P = 0.13$). Although confirmed by genetic evidence for the first time, this does not come completely unexpected: out of 12 leopards shot by farmers with the sex reported to be known prior to shooting the animal, five individuals (41.7 %) were recorded as females.

Leopards show sexual size dimorphism (Balme et al. 2012), but to discriminate between the sexes on the basis of body size alone can be difficult (e.g. large females may be misidentified as small males). Indeed, we found that out of 12 leopards shot by farmers with the sex reported to be known prior to shooting the animal, seven individuals (four of the five alleged females and three of the seven alleged males) were mistaken for the opposite sex (58.3 %). Our molecular analysis of skin samples was thus essential, showing that in the current practice of shooting leopards, identifying the sex in the field and/or reporting it to the Department of Wildlife and National Parks by the farmers was not entirely reliable. Therefore, rather than shooting "problem" animals, efforts should focus on reducing the potential for problems to develop (Loveridge et al. 2010), most importantly on improving livestock husbandry practices. Non-selective removal was found to be unsuccessful as a management strategy to mitigate human–felid conflict (Inskip and Zimmermann 2009). If lethal control is deemed inevitable, it should be implemented by the Department of Wildlife and National Parks rather than the farmers (see e.g. Weilenmann et al. 2010).

The effect of shooting stock-raiding predators on population dynamics will depend not only on numbers taken but also on the sex and age of animals that are shot (Packer et al. 2009). Selective male-biased harvesting may have negative demographic effects (Milner et al. 2007), for example if it results in a disruption of the social structure, as shown for lions (Loveridge et al. 2009). However, in most other polygynous species like leopards, hunting of

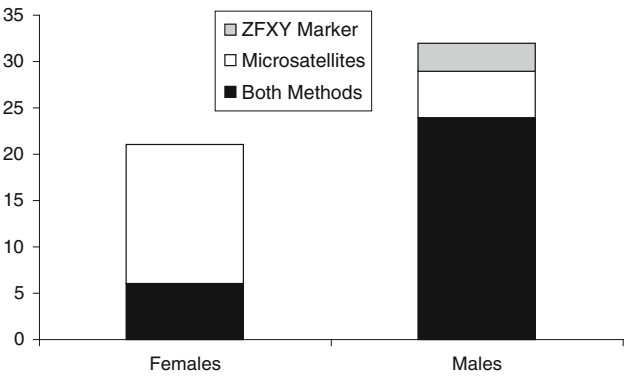

Fig. 1 Sex of leopards shot as "problem" animals in Botswana's Kweneng District, genetically determined from skin samples using four X-chromosomal polymorphic microsatellite loci and the sex-specific ZFXY marker

breeding-age females generally is often more detrimental to the population than the removal of adult males (Caro et al. 2009). Fewer remaining males will presumably mate with more females, contrasting to the effect of shooting females where a population's reproductive potential will be reduced and any dependent cubs will die (Milner et al. 2007). A population viability analysis conducted for the South African leopard population showed that the risk of extinction almost doubled in one province when females were included in harvest quotas (Daly et al. 2005). While this mainly refers to trophy hunting (also see Spong et al. 2000), our study revealed that "problem" animal off-take too can include a considerable proportion of females.

Our molecular analysis of skin samples differed from previous studies using similar markers to determine the sex of leopards from non-invasive samples (Spong et al. 2000; Perez et al. 2006; Dutta et al. 2012) in that we used two independent marker systems, both validated against known-sex samples. Even though nuclear DNA fragments of 500–900 bp may often not be expected in skin samples, in our study the ZFXY method worked rather well (also see Spong et al. 2000). This is probably because the skins were untreated and thus resulted in sufficient amounts of un-degraded DNA. Nevertheless, the ZFXZ maker could be successfully amplified in only 57.6 % of samples and low amount of un-degraded DNA is a potential limitation for the genetic sexing of other non-invasive samples (for a review, see Rodgers and Janečka 2013).

In line with this, we could determine the sex from more samples with the X-chromosomal microsatellites, which were based on much smaller fragment sizes than the ZFXY method (Table 1), especially in females (71.4 % of samples identified; Fig. 1). Despite the fact that the X-chromosomal microsatellites had a high amplification success rate (Table 1), there is a small probability that a female is homozygous for all four loci (we only had one such sample according to the ZFXY method). An alternative method could be to use just one X-linked microsatellite and a Y-linked microsatellite (Pilgrim et al. 2005). As long as their allele ranges do not overlap, a male will show two alleles (one Y-specific and one X-specific), a heterozygous female will show two X-specific alleles, and a homozygous female will show a single X-specific allele.

Policy application

Concerns have been raised over the allocation of leopard harvest quotas, implementation of hunting practices and level of "problem" animal off-take (Balme et al. 2010b). Our genetic study revealed that more females than presumed are hunted in response to alleged livestock predation (as previously also found in trophy hunting, see Spong et al. 2000), with females frequently misidentified as males, which is likely to negatively affect the population dynamics of leopards (Packer et al. 2009). Therefore, these genetic data can be incorporated into a revised management policy for hunting large carnivores (Treves 2009): (1) In Botswana and elsewhere, keeping records of leopards shot would allow for better assessing the sustainability of hunting and its impact on population viability (Daly et al. 2005). (2) Lethal predator control implemented without prior knowledge of the actual sex of the leopard to be shot results in indiscriminate off-take, potentially perturbing the population's sex ratio and age structure (Milner et al. 2007). (3) It is evidently difficult to restrict the removal of "problem" animals to males only, thus models of sustainable harvest need to include female off-take as a parameter.

Acknowledgments We thank Botswana's Department of Wildlife and National Parks for permission to conduct the study and for continuing support. David Mills kindly helped with sample collection. Samples were exported and imported under CITES permits issued to the University of Zurich by Botswana's Department of Wildlife and National Parks and the Swiss Federal Veterinary Office, respectively, as well as a veterinary permit issued by Botswana's National Veterinary Laboratory. The study was funded by the PSP Publishing Foundation, the Messerli Foundation, the African Cats & Conservation Foundation and BGS Architekten. The manuscript benefitted from comments provided by Rus Hoelzel and two anonymous referees.

References

Balme GA, Slotow R, Hunter LTB (2010a) Edge effects and the impact of non-protected areas in carnivore conservation: leopards in the Phinda–Mkhuze Complex, South Africa. Anim Conserv 13:315–323

Balme GA, Hunter LTB, Goodman P, Ferguson H, Craigie J, Slotow R (2010b) An adaptive management approach to trophy hunting of leopards (*Panthera pardus*): a case study from KwaZulu-Natal, South Africa. In: Macdonald DW, Loveridge AJ (eds) Biology and conservation of wild felids. Oxford University Press, Oxford, pp 341–352

Balme GA, Hunter L, Braczkowski AR (2012) Applicability of age-based hunting regulations for African leopards. PLoS ONE 7:e35209

Barnhurst D (1986) Vulnerability of cougars to hunting. Dissertation, Utah State University, Logan

Caro TM, Young CR, Cauldwell AE, Brown DDE (2009) Animal breeding systems and big game hunting: models and application. Biol Conserv 142:909–929

Daly B, Power J, Camacho G, Traylor-Holzer K, Barber S, Catterall S, Fletcher P, Martins Q, Martins N, Owen C, Thal T, Friedmann Y (2005) Leopard (*Panthera pardus*) population and habitat viability assessment. Endangered Wildlife Trust, Johannesburg

Dutta T, Sharma S, Maldonado JE, Wood TC, Seidensticker J (2012) A reliable method for individual identification and gender determination of wild leopards (*Panthera pardus fusca*) using non-invasive samples. Conserv Genet Resour 4:665–667

Inskip C, Zimmermann A (2009) Human–felid conflict: a review of patterns and priorities worldwide. Oryx 43:18–34

Loveridge AJ, Packer C, Dutton A (2009) Science and the recreational hunting of lions. In: Dickson B, Hutton J, Adams WM (eds) Recreational hunting, conservation and rural livelihoods: science and practice. Blackwell Publishing, Oxford, pp 108–124

Loveridge AJ, Wang SW, Frank LG, Seidensticker J (2010) People and wild felids: conservation of cats and management of conflicts. In: Macdonald DW, Loveridge AJ (eds) Biology and conservation of wild felids. Oxford University Press, Oxford, pp 161–195

Menotti-Raymond M, David VA, Lyons LA, Schäffer AA, Tomlin JF, Hutton MK, O'Brien SJ (1999) A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics 57:9–23

Milner JM, Nilsen EB, Andreassen HP (2007) Demographic side effects of selective hunting in ungulates and carnivores. Conserv Biol 21:36–47

Müllenbach R, Lagoda PJ, Welter C (1989) An efficient salt-chloroform extraction of DNA from blood and tissues. Trends Genet 5:391

Packer C, Kosmala M, Cooley H, Brink H, Pintea L, Garshelis D, Purchase G, Strauss M, Swanson A, Balme G, Hunter L, Nowell K (2009) Sport hunting, predator control and conservation of large carnivores. PLoS ONE 4:e5941

Packer C, Brink H, Kissui BM, Maliti H, Kushnir H, Caro T (2011) Effects of trophy hunting on lion and leopard populations in Tanzania. Conserv Biol 25:142–153

Pecon Slattery J, O'Brien SJ (1998) Patterns of Y and X chromosome DNA sequence divergence during the Felidae radiation. Genetics 148:1245–1255

Perez I, Geffen E, Mokady O (2006) Critically Endangered Arabian leopards *Panthera pardus nimr* in Israel: estimating population parameters using molecular scatology. Oryx 40:295–301

Pilgrim KL, McKelvey KS, Riddle AE, Schwartz MK (2005) Felid sex identification based on noninvasive genetic samples. Mol Ecol Notes 5:60–61

Rodgers TW, Janečka JE (2013) Applications and techniques for non-invasive faecal genetics research in felid conservation. Eur J Wildl Res 59:1–16

Schiess-Meier M, Ramsauer S, Gabanapelo T, König B (2007) Livestock predation—insights from problem animal control registers in Botswana. J Wildl Manag 71:1267–1274

Spong G, Hellborg L, Creel S (2000) Sex ratio of leopards taken in trophy hunting: genetic data from Tanzania. Conserv Genet 1:169–171

Treves A (2009) Hunting for large carnivore conservation. J Appl Ecol 46:1350–1356

Weilenmann M, Gusset M, Mills DR, Gabanapelo T, Schiess-Meier M (2010) Is translocation of stock-raiding leopards into a protected area with resident conspecifics an effective management tool? Wildl Res 37:702–707