Molecular Ecology (1998) **7**, 1423–1428

SHORT COMMUNICATION

Empirical evaluation of preservation methods for faecal DNA

M. A. J. FRANTZEN,* J. B. SILK,† J. W. H. FERGUSON, R. K. WAYNE,‡ and M. H. KOHN‡
Department of Zoology and Entomology, University of Pretoria, Pretoria, 0001, South Africa, †Department of Anthropology, University of California Los Angeles, Los Angeles, CA 90095, USA, ‡Department of Biology, University of California Los Angeles, Los Angeles, CA 90095, USA

Abstract

We evaluate the relative effectiveness of four methods for preserving faecal samples for DNA analysis. PCR assays of fresh faecal samples collected from free-ranging baboons showed that amplification success was dependent on preservation method, PCR-product size, and whether nuclear or mitochondrial DNA was assayed. Storage in a DMSO/EDTA/Tris/salt solution (DETs) was most effective for preserving nuclear DNA, but storage in 70% ethanol, freezing at −20 °C and drying performed approximately equally well for mitochondrial DNA and short (< 200 bp) nuclear DNA fragments. Because faecal DNA is diluted and degraded, repeated extractions from faeces may be necessary and short nuclear markers should be employed for genotyping. A review of molecular scatology studies further suggests that three to six faeces per individual should be collected.

Keywords: apolipoprotein E, cytochrome *c* oxidase II, DNA preservation, molecular scatology, noninvasive sampling, *Papio cynocephalus ursinus*

Received 3 November 1997; revision received 6 April 1998; accepted 7 April 1998

Introduction

Noninvasive molecular techniques are increasingly valuable in studies of free-ranging mammals (Woodruff 1993; Morin & Woodruff 1996; Kohn & Wayne 1997). Here we evaluate the relative effectiveness of methods for preserving endogenous DNA in faecal material from free-ranging baboons (*Papio cynocephalus ursinus*) of the Okavango Delta (Botswana). The faecal samples were collected as part of a long-term study on baboon social behaviour and reproductive strategies (Silk 1987).

Although DNA has been recovered from faeces that were dried (Höss *et al*. 1992; Kohn *et al*. 1995; Foran *et al*. 1997; Taberlet *et al*. 1997a), preserved in 70% ethanol (Höss *et al*. 1992; Kohn *et al*. 1995), or absolute ethanol (Gerloff *et al*. 1995), frozen at −20 °C (Foran *et al*. 1997; Reed *et al*. 1997) or in liquid nitrogen (Constable *et al*. 1995), the relative success of these methods has not been evaluated. However, this is critical information as it affects sampling strategy, experimental design, effort and costs associated with molecular scatology projects. We compared mitochondrial DNA (mtDNA) and single-copy nuclear DNA (scnDNA) amplification success from faeces frozen (F) or dried (D), or preserved in DMSO/EDTA/Tris/salt solution (DETs), or in 70% ethanol (E70). The objective was to determine whether DNA amplification from faeces is dependent on the preservation method. Our experimental approach was based on the assumption that both amplification length and amplification success are correlated with DNA quantity and quality (Pääbo 1989; Handt *et al*. 1994a; Kohn *et al*. 1995). We did not attempt to identify factors that determined DNA degradation. However, DNA in faeces is probably affected by hydrolytic and oxidative damage, and conceivably enzymatic degradation (e.g. Linn 1981), similar to DNA in ancient tissue and bones (e.g. Lindahl 1993; Höss *et al*. 1997). Results will possibly vary according to species and ecological conditions at the study site. Therefore, our results should be applied cautiously to other populations and species.

Correspondence: M. H. Kohn. Fax: +01-310-206-3987; E-mail: michaelk@biology.ucla.edu

Materials and methods

Sampling

Faecal samples from 22 members of a free-ranging group of baboons were collected immediatly after defaecation

between June and August 1995. Disposable gloves were used to collect samples from both the inside and outside of faeces, which were subsequently mixed. Each faecal sample was divided into four parts and preserved using four methods: (1) dissolved in DMSO salt solution (DETs; 20% DMSO, 0.25 M sodium-EDTA, 100 mM Tris, pH 7.5, and NaCl to saturation; Seutin *et al*. (1991)); (2) dissolved in 70% ethanol (E70); (3) frozen (F) in a standard commercial freezer (– 20 °C); and (4) air dried (D) on a shelf in a cool, dry room of a thatched shed (complete dessication took between 1 and 2 weeks). About 2 g (wet weight) of sample was added directly to 1 mL of preservant (DETs, E70) kept in 2 mL screw-top vials. Dried and frozen samples were kept in paper bags and ziploc bags, respectively. Faeces were shipped to UCLA where DNA analysis started in November 1995. In addition, control baboon blood samples were obtained from J. Dubach (Brookfield Zoo).

DNA extraction

Extractions were carried out in a separate room exclusively dedicated to DNA analysis of faeces. Two blanks (reagents only) were included in each extraction to monitor for contamination (Handt *et al*. 1994). To insure that differences in the amount of starting faecal material included in the extraction would not affect our subsequent analysis, we desiccated aqueous and frozen samples in a speed vacuum and compared their dry weight; 100 μL of aqueous sample yielded about 30 mg of dried solid sample. Thus, either 100 μL of liquid sample (DETs, E70) or 30 mg solid sample (D, F) was suspended in 1 mL of extraction buffer (5 M guanidium thiocyanate [GuSCN]; 0.1 M Tris-HCl pH 7.0; 0.2 M EDTA pH 7.0; and 1.3% Triton X-100). We employed the silica-based extraction procedure (Boom *et al*. 1990; Höss & Pääbo 1993) following Kohn *et al*. (1995).

PCR amplification and DNA sequencing

Each faecal sample was divided into four parts and stored in DETs, E70, frozen, and dried. DNA extracted from these 88 samples was used as template in PCR amplification of 'short' (100–200 bp), 'medium' (300–400 bp), and 'long' (600–700 bp) mtDNA and scnDNA fragments. Consequently, 528 PCR reactions were scored for presence or absence of product.

Primers for the amplification of fragments of 190 bp, 393 bp and 666 bp from the mtDNA cytochrome *c* oxidase subunit II gene were designed based on Disotell *et al*. (1992). Similarly, primers for intron 2 of the nuclear apolipoprotein E gene were designed based on Hixon *et al*. (1988) to amplify segments of increasing length of 149 bp, 365 bp and 614 bp. To reduce the risk of accidental amplification of mtDNA or scnDNA from human template, the heavy-strand primer employed for all mtDNA amplifications (H Pcox; Table 1) was designed such that six mismatches could be identified with the homologue human sequence (Disotell *et al*. 1992) and the forward primer for scnDNA amplification (F apoE; Table 1) was located at a position deleted in the human homologue (Hixon *et al*. 1988). These markers were chosen because the sequences were already published allowing for the design of species-specific primers.

Wax-mediated hot start PCR reactions were set up as described previously (Kohn *et al*. 1995). PCR blanks (reagents only) were included with each experiment. After amplification in a programmable thermal cycler (Perkin Elmer Cetus 480) for 40 cycles with denaturation for 60 s at 94 °C, annealing for 60 s at the suggested temperature (Table 1), and extension for 60 s at 72 °C, products were separated on 6% polyacrylamide mini-gels, visualized under UV light after ethidium bromide staining, and were compared to a 100 bp size ladder (Promega).

Prior to sequencing, PCR products were purified directly from the PCR reaction mixture (Ultrafree-MC 30000 filter units, Millipore Co.) following the supplier's instructions, sequenced using L Pcox (Table 1) following Bachman *et al*. (1990) with a Sanger DNA sequencing kit (USB), and aligned to the homologue sequences of human (Anderson *et al*. 1981), *Papio cynocephalus hymadryas*, and *P.c. anubis* (Disotell *et al*. 1992).

Forward primer	Reverse primer	5' to 3' primer sequence	Annealing temp. (°C)	Expected product (bp)	Length attribute
L Pcox1		TCACATCTCAAGACGTACTAC	53	190	Short
L Pcox3		GACAGACGAAATCAATAACC	52	393	Medium
L Pcox6		CTCATCCAGTGCAACTAGGC	55	666	Long
	H Pcox	TTTAGTGGAATCAGCTCTGC			
F apoE		TGTACTTTGAGTAGGGAAGGG			
	R apoE1	ACTCCTATATTAAGGATGGG	57	149	Short
	R apoE3	AGTTAGTCGGGTGTGGTGG	57	365	Medium
	R apoE6	TATTGAGTGTCCTTGTGTGC	57	614	Long

Table 1 Primer sequences, annealing temperatures, and expected PCR-product sizes. Pcox primers amplify portions of the mitochondrial cytochrome *c* oxidase subunit II gene. ApoE primers amplify portions of the baboon apolipoprotein gene E. Fragments between 100 and 200 bp were called 'short', 300–400 bp 'medium' and 600–700 'long'

Data analysis

Successful amplification was measured by the presence of PCR products of the expected size. We ignored intensity of amplification products and unspecific amplification products as long as they were of different size than the expected product. Data were analysed in a three-step procedure: (i) results were arranged as a three-way contingency table (Table 2) which was analysed using log-linear models (SYSTAT for Macintosh: *Statistics, Version 5.2*. Evanson, IL: SYSTAT, Inc. 1992). As factors, we tested the significance of preservation method (DETs, E70, F, and D), fragment length ('short' [100–200 bp], 'medium' [300–400 bp], and 'long' [600–700 bp]), and molecule (mtDNA and scnDNA) including their interaction terms. Pearsons chi-square and likelihood ratio chi-square were computed to test for significance of terms; (ii) results of attempted amplifications of scnDNA (which should be more sensitive to degradation than mtDNA) from faecal extracts were arranged as randomized blocks and were tested by computing Cochran's Q value and comparison to the chi-square distribution (Sokal & Rohlf 1995); (iii) finally, preservation methods were ranked based on their performance (Table 2). Methods ranked as 1 and 2, respectively, were compared with a McNemar test and the *G*-value was compared to the chi-square distribution after application of William's correction (Sokal & Rohlf 1995).

Results and Discussion

Sequence authenticity

A portion of the cytochrome *c* oxidase II gene was sequenced from a baboon faecal sample to verify that amplification products obtained from faeces were endogenous. The obtained sequence was distinguished from the human sequence at 27 sites but differed at three positions only from the *Papio cynocephalus hymadryas* and *P.c. anubis* sequences. Prior to this study, DNA had never been isolated from any other primate in our laboratory. If DNA from our positive controls was amplified accidentally, we would have observed high levels of contamination in our PCR controls, extraction blanks, and other scnDNA amplifications throughout our experiments.

Amplification efficacy

We were successful in amplifying baboon DNA in 340 of 528 (64%) PCR attempts. Positive PCR controls consisting of DNA isolated from blood always amplified indicating that the PCR mixture was viable. Preservation method had little effect on success or failure of PCR of mtDNA, regardless of target length. In contrast, amplifications of scnDNA from faeces preserved in DETs were more often successful (42%) than amplifications from dried (35%) and frozen (27%) samples, and samples stored in E70 (27%) (Table 2). DETs-preserved extracts were the only ones that proved successful in amplifications of medium-sized (7/22) scnDNA fragments. Only dried (2/22) and DETs samples (2/22) produced long PCR fragments.

In general, mtDNA was more easily amplified than scnDNA (96% vs. 33%), and when scnDNA was examined separately it was obvious that short fragments were much more frequently amplified than medium and long fragments, respectively (86% vs. 8% and 5%). In fact, PCR success dropped by about 91% between short- and medium-length amplifications. In contrast, short, medium and long mtDNA fragments were easily retrieved (92–100%, Table 2). Amplifications of long template generally seemed weaker and, in addition to the product of expected length, unspecific product of different length was occasionally observed (data not shown).

Statistical analysis showed that all factors (preservation method, molecule, and fragment length) have significantly

		Preservation method				
Molecule	Product	DETs	E70	F	D	Total
MtDNA	short	22	22	22	22	88 (100)
	medium	22	19	22	22	85 (97)
	long	20	20	19	21	80 (92)
Subtotal		64 (97)	61 (92)	63 (95)	65 (99)	253 (96)
ScnDNA	short	19	18	18	21	76 (86)
	medium	7	0	0	0	7 (8)
	long	2	0	0	2	4 (5)
Subtotal		28 (42)	18 (27)	18 (27)	23 (35)	87 (33)
Total		92 (70)	79 (60)	81 (61)	88 (67)	340 (64)
Rank		1	4	3	2	

Table 2 Three-way table summarizing results of 528 PCR attempts from 'short', 'medium', and 'long' mtDNA and single-copy nuclear (scn) DNA isolated from faeces. Faecal samples were stored in DMSO/EDTA/Tris/salt solution, (DETs); 70% ethanol, (E70); frozen, (F); and dried, (D). Percentages were rounded to whole numbers and are given in parenthesis

influenced amplification success and that they were significantly associated (Pearsons chi-square and likelihood ratio chi-square; $P < 0.05$). Thus, PCR-amplification success depended on the choice of molecule and size of PCR product. Computation of Cochran's Q supported the fact that PCR amplification success of scnDNA varied between preservation methods ($P < 0.05$). As suggested by ranks assigned to preservation methods in Table 2, we found significant support ($P < 0.05$) for DETs buffer as the most effective preservation method for scnDNA (chi-square 4.05 compared to the critical value 3.84; Sokal & Rohlf 1995).

Nuclear loci such as microsatellites are often analysed when studying paternity and kinship (e.g. Queller *et al*. 1993). On average, in 86% of attempted amplifications of short scnDNA PCR product was obtained. In general, repeated PCR of extracts which had not yielded product in a first attempt did not yield PCR product (data not shown). However, for all 22 faeces (i.e. all members of the troop) further extractions eventually yielded short scnDNA suitable for PCR amplification. On average, we needed 1.16 extractions (range 1–4) per faecal sample (i.e. baboon) to achieve at least one successful amplification of short scnDNA.

Implications for sampling and experimental design

We recommend that DETs buffer should be used to preserve faeces collected in the field. Our results are consistent with those of Seutin *et al*. (1991) who found that DETs buffer was as efficient as cryopreservation (– 70 °C) at slowing DNA degradation in tissue samples and greatly outperformed 70% ethanol. However, as evident from our results and previous studies, other preservation methods are also useful for faecal DNA storage and perform almost equally well for mtDNA or short nuclear DNA analysis (Table 2). For example, PCR amplification of mtDNA and of short scnDNA from dried samples was more often successful (although not significantly) than amplification from DETs-preserved samples (Table 2). Note that our drying procedure under field conditions has not been optimal as samples required up to 2 weeks to dessicate completely. Addition of silica particles to faeces causes drying and storage to be more effective (Wasser *et al*. 1997). Moreover, preservation in 70% ethanol might have been more effective when either less than 2 g of sample was mixed with 1 mL of fluid or a higher percentage of ethanol was used as the ethanol might have been too dilute. However, Wasser *et al*. (1997) found that 70% ethanol was less effective than drying, freezing and DMSO-salt buffer even when mixed at a ratio of 1 g per 5 mL of fluid. The fact that all methods tested in our study perform well is pertinent information when logistic or other practical obstacles prevent the use of the optimal storage method.

An average of 1.16 extractions (range 1–4) was necessary to achieve at least one successful PCR of short scnDNA. Repeated PCR on the same extraction usually did not result in product when the initial PCR failed. However, replication of PCR may be necessary for verification of results. Elsewhere it has been shown that simply obtaining PCR product is easier than obtaining a correct sequence or genotype (e.g. at a microsatellite locus), especially when amplifying from very dilute or degraded template (Foulcault *et al*. 1996; Taberlet *et al*. 1996b; Gagneux *et al*. 1997).

A review of molecular scatology studies shows that on average 31% of faecal samples did not yield scnDNA or mtDNA, even after repeated extractions (Table 3). Consequently, given a frequency of failure of 0.31, based on the binomial theorem the resulting probability of obtaining no PCR product from three or six samples is < 0.05 or < 0.01, respectively. Therefore, we recommend that at least three to six faeces per individual animal should be collected to ensure recovery of both short scnDNA and mtDNA from at least one of these. However, it was not clear if all previous studies systematically attempted to recover DNA from each sample by repeated extractions, such that our recommendation may be somewhat overcautious. For example, human faeces consists of large amounts of intestinal mucosal cells sloughed off during digestion (Despopoulos 1986; Albaugh *et al*. 1992); thus, if properly preserved, one might expect that every faecal samples should yield sufficient DNA for analysis. Nonetheless, more samples may be preferable, as they provide some margin of error given the variation in preservation conditions, ecological- and species-specific factors influencing DNA preservation and concentration. Based on our findings and

Table 3 Review of recovery-success of mtDNA and scnDNA in molecular scatology studies. The number of faecal samples which yielded DNA (numerator) is compared to the number of faecal samples examined (denominator). Percentages were rounded to whole numbers and are given in parenthesis

	mtDNA	scnDNA	Successful DNA recovery from faecal samples
This study	×	×	22/22 (100)
Kohn *et al*. 1995	×	×	8/12 (67)
Gerloff *et al*. 1995		×	31/33 (94)
Reed *et al*. 1997		×	156/173 (90)
Taberlet *et al*. 1997		×	21/105 (20)
Mean			238/345 (69)

data available to date it is yet unclear how to ensure long-term curation of faecal samples. It has been noted that DETs solution is effective for at least 24 weeks (Seutin *et al*. 1991) and that all storage methods are more effective at −20 °C than at room temperature (Wasser *et al*. 1997).

Molecular scatology may be augmented with faecal steroid hormone analysis (Kohn & Wayne 1997). Either an additional sample can be stored in the appropriate buffer for hormone analysis (Wasser *et al*. 1991), or a pilot study may determine if a single preservation method is suitable for both DNA and hormone analysis.

In conclusion, we have confirmed that it is possible to extract both mtDNA and scnDNA from faecal samples preserved in four different ways. Fragments less than 200 bp are more reliably amplified than fragments longer than 300 bp. Our results suggest that the DMSO salt solution (DETs) is the most appropriate method to preserve nuclear faecal DNA if fragments longer than 300 bp are desired products. Investigators should aim to collect three to six faeces per individual, anticipate multiple extractions per sample, and employ short scnDNA markers only to ensure effective and accurate genotyping.

Acknowledgements

We thank L. van Rooyen (Du Buisson and partners, Pretoria, South Africa) and the University of Pretoria (South Africa) for generous financial support of M. Frantzen. Further, we thank M. Bruford for DNA samples and advice, J. Dubach for blood and faecal samples and J. Altmann for cooperation. Colleagues who collected with J. Silk were M. Mokupi, R. Seyfarth, and D. Cheney. M. Kohn was funded by a nonresident tuition fellowship (UCLA).

References

Albaugh GP, Iyengar V, Lohani A *et al.* (1992) Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers. *International Journal of Cancer*, **52**, 347–350.

Anderson S, Bankier AT, Barrell BG *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.

Bachmann B, Luke W, Hunsmann G (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Research*, **18**, 1309.

Boom R, Sol CJA, Salimans MMM (1990) Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology*, **28**, 495–503.

Constable JJ, Packer C, Collins DA, Pusey AE (1995) Nuclear DNA from primate dung. *Nature*, **373**, 393.

Despopoulos A (1986) *Color Atlas of Physiology*. Thieme Verlag, New York.

Disotell TR, Honeycutt RL, Ruvolo M (1992) Mitochondrial DNA phylogeny of the old-world monkey tribe Papionini. *Molecular Biology and Evolution*, **9**, 1–13.

Foran DR, Crooks KR, Minta SL (1997) Species identification from scat: an unambiguous genetic method. *Wildlife Society Bulletin*, **25**, 835–839.

Foulcault F, Praz F, Jaulin C, Amor-Gueret M (1996) Experimental limits of PCR analysis of $(CA)_n$ repeat alterations. *Trends in Genetics*, **12**, 450–451.

Gagneux P, Boesch C, Woodruff DS (1997) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology*, **6**, 861–868.

Gerloff U, Schlötterer C, Rassmann K *et al.* (1995) Amplification of hypervariable simple sequence repeats (Microsatellites) from excremental DNA of wild living Bonobos (*Pan paniscus*). *Molecular Ecology*, **4**, 515–518.

Handt O, Höss M, Krings M, Pääbo S (1994) Ancient DNA – methodological challenges. *Experientia*, **50**, 524–529.

Hixon JE, Cox LA, Borenstein S (1988) The Baboon Apolipoprotein E gene: structure, expression, and linkage with the gene for Apolipoprotein C-1. *Genomics*, **2**, 315–323.

Höss M, Pääbo S (1993) DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Research*, **21**, 3913–3914.

Höss M, Kohn M, Knauer F, Schröder W, Pääbo S (1992) Excrement analysis by PCR, *Nature*, **359**, 199.

Höss M, Jaruga P, Zastawny TH, Dizdaroglu M, Pääbo S (1997) DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Research*, **24**, 1304–1307.

Kohn MH, Wayne RK (1997) Facts from feces revisited. *Trends in Ecology and Evolution*, **12**, 223–227.

Kohn M, Knauer F, Stoffella A, Schröder W, Pääbo S (1995) Conservation genetics of the European brown bear – a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology*, **4**, 95–103.

Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709–715.

Linn S (1981) Deoxyribonucleases: a survey and perspectives. In: *Enzymes*, Vol. 14. (ed. Boyer PD), pp. 121–135. Academic Press, New York.

Morin PA, Woodruff DS (1996) Noninvasive genotyping for vertebrate conservation. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB and Wayne RK), pp. 298–313. Oxford University Press, Oxford.

Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proceedings of the National Academy of Sciences of the USA*, **6**, 1939–1943.

Queller DC, Strassmann JE, Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology and Evolution*, **8**, 285–288.

Reed JZ, Tollit DJ, Thompson PM, Amos W (1997) Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology*, **6**, 225–234.

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analysis. *Canadian Journal of Zoology*, **69**, 82–90.

Silk JB (1987) Social behavior in evolutionary perspective. In: *Primate Societies* (eds Smuts BB, Cheney DL, Seyfarth RM *et al.*), pp. 318–329. University of Chicago Press, Chicago.

Sokal RR, Rohlf FJ (1995) *Biometry*, 3rd edn, W.H. Freeman and Co., New York.

Taberlet P, Camarra JJ, Griffin S *et al.* (1997a) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **9**, 869–876.

Taberlet P, Griffin S, Goossens B *et al.* (1996b) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Wasser SK, Monfort SL, Wildt DE (1991) Rapid extraction of faecal steroids for measuring reproductive cyclicity and early pregnancy in free-ranging yellow baboons (*Papio cynocephalus cynocephalus*). *Journal of Reproduction and Fertility*, **92**, 415–423.

Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997) Techniques for application of faecal DNA methods to field studies of Ursids. *Molecular Ecology*, **6**, 1091–1097.

Woodruff DS (1993) Non-invasive genotyping of primates. *Primates*, **34**, 333–346.

M. Frantzen is a graduate student of J. Ferguson of the University of Pretoria (South Africa). This pilot study was initiated by J. Silk to investigate which preservation method would be most useful and practical for planned large-scale sampling of baboon faeces in the Okavango Delta. The eventual goal is a genetic analysis of the social behaviour and reproductive strategies of baboons. The work and analysis were conducted in the laboratory of R. Wayne by M. Frantzen and M. Kohn. All of the authors are captivated by the possibility of using noninvasive sampling to study evolution, behaviour and conservation of free-ranging animal populations.