1 Fur and faeces: an experimental assessment of non-invasive DNA sampling for the European pine marten

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- 13 Running header: Non-invasive DNA sampling for pine marten

15 Abstract

16 Non-invasive genetic sampling using materials such as faeces or hair can be used to monitor wildlife populations, 17 although DNA quality is often poor. Improving sampling efficiency and minimising factors that reduce DNA 18 quality are therefore critical. After a severe decline, European pine marten, Martes martes, has reclaimed much 19 of its former range in Scotland, UK. Recording this rapid range expansion requires developing techniques for 20 accurate monitoring, but this is hampered by the species' elusive behaviour. We tested two sampling methods, 21 hair collected from hair tubes and faeces (scat) collected along tracks, to assess the effects of key environmental 22 and sampling variables on DNA quality and sampling efficiency. For hair, we tested the influence of hair tube 23 location (distance from forest tracks) on collection rate and sex ratio of animals successfully sampled. For scats, 24 we assessed the effect of time since defecation (1 to 16 days) on genotyping error rates and success under two 25 contrasting environmental conditions (exposed to rainfall or sheltered). We found no bias in the collection rate or 26 sex ratio of animals detected by hair samples with differing proximity to forest tracks. DNA amplification failure 27 for scats exposed to rainfall increased from 28% to 65% over the 16 day experimental period. During periods of 28 low rainfall, the length of collection sessions could therefore be extended to increase sample number without risk 29 of DNA degradation. Lack of bias in hair collection rates with proximity to forest tracks provides justification for 30 tube placement close to tracks, as this reduces survey effort. These findings provide guidance for the development 31 of efficient and cost effective non-invasive sampling of Scottish pine martens.

32 Keywords: Non-invasive genetics; elusive species; DNA degradation; *Martes martes;* allelic dropout; false alleles

34 Introduction

35 Accurate baseline data on species presence, abundance and demographic rates is a key component of effective 36 wildlife management (Gibbs et al. 1999). For rare or threatened species, knowledge of population status enables 37 informed management decisions to be made and adaptive conservation relies on the ability to monitor the effects 38 of management (Nichols & Williams 2006; Head et al. 2013). In order to monitor species of conservation concern, 39 there must be a reliable method of detection. Traditional methods of detection often involve capturing animals, 40 which can be difficult when species are elusive or protected and stressful for animals vulnerable to disturbance. 41 Non-invasive genetic sampling has been suggested as an alternative survey tool, with genetic samples extracted 42 from hair, faeces or feathers potentially negating the need to physically capture or even observe the animal 43 (Taberlet et al. 1996; Taberlet & Luikart 1999). To date, non-invasive DNA methods have been used for a range of purposes including mapping distributions (e.g. the Andean cat in Peru, Orealilurus jacobita, Cossios et al. 44 45 2007; jaguar in Belize, Panthera onca, Weckel, Giuliano & Silver 2006), estimation of population densities (e.g. 46 coyote, Canis latrans, Kohn et al. 1999; the ship rat, Rattus rattus, Wilson et al. 2007) and comparisons of survival 47 estimates between the sexes (e.g. Wolverine populations Gulo gulo, Brøseth et al. 2010).

48 Genetic methods, however, are not without drawbacks. Sample processing is costly and, in the case of wideranging or low density populations, collecting sufficient samples can also be time consuming and expensive. 49 50 These issues may be exacerbated when using samples of poor quality DNA such as faeces (Lucchini et al. 2002), 51 which contain compounds that inhibit the DNA amplification process. DNA quality is measured by the rate at 52 which amplification, through polymerase chain reaction (PCR), yields a detectable quantity of DNA, quantified 53 as PCR 'success' or 'failure' rate; and the rate of occurrence of amplification errors. Two types of error are 54 prominent: allelic dropout, where one allele from a heterozygous individual fails to amplify; and false alleles, 55 where an allele differing from the consensus, or agreed, genotype is produced (Broquet, Menard & Petit 2007). 56 For practices which only require identification at species level, such as distribution mapping, researchers may be 57 concerned with maximising the rate of PCR success but, once a sample has been genotyped with a species specific 58 marker, the occurrence of error within this marker will be largely unimportant. For studies requiring individual 59 identification, such as estimates of population density, error rates must also be considered and minimised. In these 60 cases, data with an acceptable level of precision may only be achieved through larger sample sizes and repeated 61 amplifications, as well as through the use of more expensive DNA extraction techniques (Taberlet et al. 1996).

62 Improving the efficiency of sampling and minimising the factors that reduce DNA quality are therefore critical63 when designing a cost effective surveying strategy.

64 Despite previous findings that suggest a decrease in faecal DNA quality over time (Brinkman et al. 2010; Panasci 65 et al. 2011), and with increased rainfall (Nsubuga et al. 2004; Murphy et al. 2007; Brinkman et al. 2010), there is 66 considerable variation in the effect of these factors between taxa. For example, rainfall significantly degrades 67 DNA in Sitka black-tailed deer pellets (Odocoileus hemionus sitkensis), but does not affect DNA sample quality 68 from mountain gorilla faeces (Beringei beringei; Nsubuga et al. 2004). Similarly, amplification success as faecal 69 samples aged (up to one month) decreased by 65% for the brush-tailed rock-wallaby (Petrogale penicillata; 70 Piggott & Taylor 2003), but only 5% for coyote (Canis latrans; Panasci et al. 2011). Genotyping success has been 71 higher for hair samples than scats for pine martens in previous studies (Mullins et al. 2007), but success rates for 72 hair can still vary, with factors such as the number of hairs that are used in the extraction process having a 73 significant effect, as seen for the Asiatic black bear (Ursus tibetanus, Uno et al. 2012), although it remains unclear 74 if differences exist between species.

75 Pine marten populations in Scotland have shown a recent range expansion after near-extinction in the early 20th 76 century (Lockie 1964; Croose et al. 2013). As a protected native species, there is strong stakeholder interest in the 77 conservation of pine martens, particularly since the suggestion that they may play a role in controlling the invasive 78 American grey squirrel (Sciurus carolinensis; Sheehy et al; 2014). There is concern, however, about the effect of 79 pine martens on vulnerable prey species through, for example, nest predation of capercaillie (Tetrao urogallus) 80 populations (Summers, Willi & Selvidge 2009). Their elusive behaviour makes non-invasive sampling such as 81 DNA extraction from hair or faeces potentially useful. Genetic analyses of scat have been successfully used for 82 species identification and for determining the distribution of martens in Scotland (Caryl et al. 2012a; Croose et al. 83 2013) but have thus far been unsuccessful in individual-level analyses due to poor quality DNA. This has 84 prompted the need for an assessment of the factors affecting DNA quality in order for these factors to be minimised 85 in future studies.

Sampling regimes used to estimate population abundance and density should account for differences in detectability, either through sampling design or through statistical methods. For studies using non-invasive hair sampling, time constraints usually make it unfeasible to relocate hair tubes between sampling sessions, which may introduce a temporal bias and violate assumptions of sampling independence (Boulanger et al. 2006). For example, heterogeneity in the probability of capture between individual pine marten has been observed in an Irish study, 91 with hair tubes placed in lowland forests collecting more samples than those in upland forests, despite similar 92 population densities in both habitats (Lynch et al. 2006). Spatial biases can also occur; hair tubes are most 93 accessible if placed close to forest tracks; pine marten scats are also collected from forest tracks due to the relative 94 ease of collection compared to searching the densely vegetated, forest floor. If some individuals use forest tracks 95 less frequently than others, the samples collected may only represent a sub-set of the population. Female pine 96 martens, for instance, are thought to be more risk averse than males due to the reporting of a higher proportion of 97 male road casualties (Rob Coope, pers. comm.); females also maintain smaller home ranges than males (Caryl et 98 al. 2012b), which therefore could be less likely to contain forest tracks. As a consequence, the effect of different 99 sampling techniques and designs on the outcome of non-invasive hair sampling is currently unclear.

In this paper we assess the effects of key environmental and sampling variables on the quality of pine marten
DNA sampled non-invasively through hair and scats (with the latter divided into experimental treatments to test
for the effect of exposure to rainfall), and examine the implications for developing efficient sampling protocols.
Specifically, we address the following questions:

104 1. How does time (measured as consecutive sampling sessions) influence hair tube sample independence (hairsamples only)?

2. Does distance from forest track affect the visitation rates of pine marten, and does this vary between the sexes(hair samples only)?

108 3. How is PCR success affected by the number of hair follicles included in the reaction (hair samples only)?

4. What are the effects of time since defecation and exposure to rainfall on DNA genotyping success and errorrates (scats only)?

111 Materials and methods

112 Study areas

Four forests in the Scottish Highlands known to have pine martens present were surveyed. Abernethy Forest National Nature Reserve (57°15′N, 3°40′W; hereafter Abernethy) is a Royal Society for the Protection of Birds (RSPB) reserve in the northern Cairngorms covering 36 km² of both ancient native pinewood (approx. 24 km²) and Scots pine (*Pinus sylvestris*) plantation (Summers, Dugan & Proctor 2010). Mar Lodge Estate (57°00'N, 3°37'W; hereafter Mar), owned by the National Trust for Scotland, comprises Caledonian pinewood concentrated mainly along Glen Lui and Glen Quioch, north west of Braemar (Davies & Legg 2008). Inshriach Forest (57°06'N, 3°56'W, hereafter Inshriach) is a Forestry Commission owned site in the Northern Cairngorms consisting mainly
of managed Scots pine plantation with some remnants of Caledonian pinewood (Twiddle & Quine 2011).
Darnaway Forest (57°33'N, 3°45'W; hereafter Darnaway), which is managed by Moray Development Company
Ltd, consists of commercial Scots pine, Sitka spruce (*Picea sitchensis*) and Douglas fir (*Pseudotsuga* sp.)
plantation, with some areas of deciduous woodland.

124 Sample collection

Hair was sampled during September to November at two forests in 2011 (Abernethy, Mar) and two forests in 2012
(Darnaway, Inshriach) using hair tubes fitted with sticky pads (Mullins et al. 2009) and labelled with a unique
identifier (Hairtube ID). Four sampling sessions were held in Abernethy and Darnaway, and five each at Inshriach
and Mar (Online resource 1), with each session taking five (Mar, Inshriach) or six consecutive days (Darnaway,
Abernethy). Hair samples from each tube were collected in individual polythene bags and labelled with a unique
identifier. All samples were frozen at -20 °C within 8 hrs and transferred to -80 °C within three weeks to await
DNA analysis.

132 Hair tube placement within each forest was planned using 1:25,000 Ordnance Survey maps. To ensure that at least 133 one hair tube was placed in each potential home range (Caryl et al. 2012b), one (Abernethy, Mar) or two 134 (Inshriach, Darnaway) hair tubes were placed in each 1 km² grid cell within the study area (Fig 1), giving a total 135 of 33 hair tubes at Abernethy, 26 at Mar, 64 at Inshriach and 47 in Darnaway. For ease of access, only cells 136 containing forest tracks were used. In the field, fine scale placement was chosen based on the presence of 137 woodland. Cells that did not contain trees were excluded. Hair tubes were placed at distances of between 0 m and 138 200 m from the nearest forest track (in increments of 50 m) with approximately the same number of tubes at each 139 distance within a forest. A combination of Hawbakers marten lure (F&T Fur Harvester's Trading Post, 10681 140 Bushey Road, Alpena, MI 49707), peanut butter and bread were used as attractants as these have previously 141 proven effective (Chandrasekhar 2005; Roche 2008; Burki et al. 2009). Details of hair tube construction can be 142 found in Online Resource 2).

Scats were collected from Abernethy during May 2011 (Fig 1). Scats were cleared 24 hrs prior to the first survey, and then two surveys were conducted on consecutive days so that all scats were ≤ 24 hrs old. All of the encountered scats were collected, essentially re-clearing transects of scats for subsequent collection rounds and enabling the time since defecation to be established, where the day of collection was 'day zero'. Twenty two scats were 147 collected in individual pots and labelled with a unique identifier, then frozen at -20 °C within 8hrs before transfer 148 to a -80 °C freezer. In order to test the effect of exposure to rainfall and time since defecation on DNA quality, 149 scats were thawed and a small section taken for DNA extraction (day zero samples). The remainder of the scat 150 was split into two equal sections and allocated to one of two treatment groups. Samples in treatment one (exposed) 151 were placed directly on a woodland floor in the University of Stirling grounds to replicate the conditions in which 152 they were found. Samples in treatment two (sheltered) were placed in the same location, but raised off the ground 153 and covered by a waterproof canopy. To test the effect of time since defecation (hereafter 'time'), a small section 154 of each scat was taken from both treatments at intervals of 2, 5, 9, 12 and 16 days.

155 Genetic analysis

156 Hair samples were removed from sticky pads with xylene. Extractions were performed using an adapted chelex-157 100 method (Walsh, Metzger & Higuchi 1991); a 1 cm root-section of hair was placed in 200 µl chelex (5%) 7 µl 158 dithiothreitol (DDT) and 1 µl proteinase K and agitated at 56 °C for approx. 5 hrs, centrifuged for 3 minutes and 159 the supernatant incubated at 95 °C for 10 minutes. DNA was stored at -20 °C until required. The number of hair 160 follicles in each extraction was recorded. Sex typing was performed using a 5' nuclease TaqMan assay developed by Mullins (2009) and Real-time PCR using 5 µl Precision Master Mix (Primer Designs), 0.2 mM of either MMX 161 or MMY forward and reverse primers and probes (MMX and MMY probe sequences are reversed from the text 162 provided in Mullins et al. 2009 and are as follows: MMX, 5'-VIC-CCTGGTCTGAAAACT-MGB-3' and MMY 163 164 5'-6FAM-TGTGTCTCTCTCTGTCAAMGB-3'.) and 3 µl DNA template in a total volume of 10 µl. Amplification of ZFX (MMX) only signifies female DNA, whereas amplification of both ZFX and ZFY (MMY) 165 signifies male DNA (Mullins et al. 2009). The PCR conditions were 2 min at 50 °C, 10 min at 95 °C, then 50 166 cycles of 15 s at 95 °C and 1 min at 60 °C. Two replicate amplifications were performed for each primer/probe. 167 168 For real-time product detection, Ct value (i.e. the number of PCR cycles needed to obtain the required quantity of 169 DNA) was recorded at a Δ Rn threshold of 0.2.

For scat samples, genomic DNA was immediately extracted from day zero samples using the QIAamp DNA stool
mini kit (Qiagen, #51504) with a negative control. To avoid contamination, extractions were performed in an area
of the laboratory reserved for DNA extraction. To test DNA amplification failure and error rates, two
microsatellite loci were amplified (Mar08, Mar43; Natali et al. 2010) in one multiplex reaction of 10 µl containing
0.4 µM forward and reverse primers, 5 µl Qiagen Type-it PCR mastermix, 1 µl Q solution and 2 µl DNA template.
After initial denaturation at 95 °C for 5 mins, 40 cycles of 95 °C for 30 s, 63 °C for 90 s and 72 °C for 30 s were

176 used followed by a final extension step of 60 °C for 30 mins. Fragment analysis was performed at DNA 177 Sequencing and Services (University of Dundee, Scotland, DD1 5EH) with negative and positive controls. 178 Samples were scored using GeneMarker (Version 2.4.0) and verified by eye. Consensus genotypes were obtained 179 for day zero samples following the comparative multi-tubes approach (Frantz et al. 2003); each sample was 180 initially amplified twice, then further replications were performed until a consensus was reached. Samples without 181 a consensus after seven amplifications were discarded. Samples from each treatment and time period were 182 extracted and amplified twice then compared to the consensus to quantify error rates, with a negative and positive 183 control in each plate.

184 *Statistical analysis*

185 Darnaway was excluded from all analyses due to lack of hair samples. Visitation rate to hair tubes was analysed 186 using a Generalised Linear Mixed effects Model (GLMM) with a binomial error distribution. The response 187 variable was recorded as 'visit' or 'no visit' for each hair tube, replicated per session. To allow us to specifically 188 test the effect of time on the rate of visitation, we included session as a proxy for time elapsed as a fixed covariate, 189 as well as distance (question 1). Forest was included as a fixed factor and two way interaction terms between 190 distance and forests, and distance and session were included (question 2). Hair tube ID was included as a random 191 factor. To test the effect of these variables on the sex-ratio of visitors, the same analysis was used, but with the 192 proportion of males as the response variable restricting analyses to samples with a positive sex ID only (question 193 2).

194 To determine whether PCR success for pine marten sex-typing is affected by the number of hair follicles used in 195 the extraction process, we calculated the mean Ct value per sample over positive rtPCR replicates. As the ZFX 196 region is present in male and female pine martens and a Ct value is only obtained for positive samples, we included 197 positive amplifications using the MMX locus only. There are, however, two copies of the ZFX region in female 198 DNA for every one copy in male DNA, so it may take fewer cycles to obtain the threshold level of DNA template 199 for female samples than for males; the effect of this bias should, however, be negligible as Ct value is unlikely to 200 be reduced by more than one for females as compared to males. A Generalised Linear Model (GLM) with poisson 201 error distribution was used with Ct value as the response variable and number of hairs as the explanatory variable 202 (question 3).

Genotyping errors per amplification were categorised as allelic drop out (p), false alleles (f) and failure as
 described in Murphy et al. (2007), relative to the consensus genotype for each sample. Overall error rates were
 calculated using equations from Broquet (2004):

$$p = \bar{p}_{w} = \frac{\sum_{j=1}^{L} D_{j}}{\sum_{\substack{j=1\\j=1\\200}}^{L} A_{het_{j}}} \qquad f = \bar{f}_{w} = \frac{\sum_{j=1}^{L} F_{j}}{\sum_{j=1}^{L} A_{j}}$$

Where p and f are the probability of allelic drop out and false alleles, respectively, at locus j. L refers to each scat within the treatment block, A_j and A_{hetj} are the number of positive amplifications, and the number of positive heterozygous amplifications, respectively, for the scat at locus j. Dj and Fj are the number of amplifications at locus j containing an allelic drop out and a false allele respectively (Broquet et al. 2004).

211 The effects of time and exposure to rainfall on error rates (allelic drop out, false alleles and failure) were analysed 212 using three GLMMs with a binomial (logit) distribution using proportional data from two repeated amplifications 213 per sample for each combination of treatment, time and locus (question 4). As treatment commenced on day two, 214 samples from day zero were not subject to the treatment conditions and so were not included in the models. Only 215 successful samples (i.e. those that produced DNA) were included in the models for false alleles and allelic drop 216 out. Treatment (exposed, sheltered) and locus were included as fixed factors, time (days) as a fixed covariate and 217 an interaction between time and treatment included in all models. To account for pseudo-replication of scat 218 samples, scat and 'scat-half' (i.e. the division of each scat between the two treatments), were included as random 219 effects, with scat-half nested within scat.

For all analyses, we present estimates of the full model to avoid bias associated with stepwise deletion of nonsignificant terms (Whittingham et al. 2006). We present likelihood ratio test results for the deletion of each interaction term from the full model, or each main effect from a model with main effects only (Faraway 2005; Zuur et al. 2009). Prediction uncertainty of the full models is calculated using N = 1,000 random draws from the estimated parameter distributions and presented as the 95 % quantiles of the resulting distributions (Gelman & HIII 2007; Zuur et al. 2009). Analyses were performed in R version 3.1.0 (R core team 2014).

226 Results

Overall, hair samples were obtained on 20 % of occasions (115 samples, 572 tube nights; Online resource 1). Of
the 115 samples, 69 (60%) provided a positive sex-type, with 23 samples from males and 46 from females.

229 *Hair tube placement*

Visitation rate varied over time, with a higher predicted visitation rate as sessions progressed from one (0.07; 0.01
- 0.10) to four (0.18; 0.13 – 0.25; Fig 2), but did not significantly affect the sex-ratio of visitors. Neither the
distance of the tube from the nearest track nor the identity of the forest significantly improved model fit for hair
tube visitation rate or the sex ratio of visitors (Table 1).

234 Hair sex-typing success

The number of hair follicles used for DNA extraction had a significant effect on the number of PCR cycles needed to obtain the required quantity of DNA, as measured by Ct value (χ^2 = -2.08, df = 61, p =0.036). As the number of hair follicles increased from one to >13, the Ct value decreased by 13% (Fig 3).

238 Scat genotyping success

- For the experimental study, a consensus genotype was established for 28 of 44 sample loci (22 samples, two loci).
 DNA amplification was successful in 63 % (421/666) of attempts over all loci, treatments and time periods. The
 average temperature for the duration of the study was 15 °C (7.7 23.7 °C), with 21.6 mm rainfall overall
 (University of Stirlng weather station).
- 243 *PCR failure:*

Time, treatment and locus all significantly affected failure rate (Table 2). Failure rate increased from 0.28 (0.18 0.43) at day two to 0.65 (0.48 - 0.79) at day 16 for exposed samples, but did not change significantly for sheltered
samples: 0.22 (0.13 - 0.35) at day two to 0.29 (0.15 - 0.42) at day 16 (Fig 4). Locus also improved model fit with
the average failure rate over all treatments and time periods being higher for locus m08 than locus m43, with
proportions of 0.58 (0.51 - 0.61) and 0.44 (0.37 - 0.51) respectively.

249 Allelic drop out and false alleles:

For successful amplifications, overall rates of allelic drop out and false alleles were 0.25 and 0.33 respectively. Neither treatment, time, nor genetic locus significantly improved model fit for allelic drop out (Table 2). The rate of false alelles increased with time for exposed samples only, from 0.19 (0.10 - 0.38) to 0.52 (0.28 - 0.78; Table 2; Fig 5). Samples amplified using locus m08 contained false alleles in 0.47 (0.37 - 0.58) of cases, compared to 0.30 (0.21 - 0.41) of cases for samples amplified with locus m43.

255 Discussion

256 We tested temporal and spatial hair tube use by pine marten populations in Scotland and assessed the impacts of 257 time and exposure to rainfall on scat DNA quality. Hair tube visitation rates increased over time with, on average, 258 2.6x as many samples collected in the fourth session compared to the first. This increase supports previous findings 259 in Ireland, where sampling success increased with time when hair tubes were checked every four to six weeks for 260 six months (O'Mahony et al. 2012). The shorter time period of the current study means that this effect is not due 261 to increased population density, but suggests the influence of two factors; an increase in the likelihood of different 262 animals locating hair tubes over time; and the habituation of individual pine martens to particular hair tubes. The 263 latter of these factors is less apparent as, of 15 identified individuals that made multiple visits during the study 264 period, only one individual used a single hair tube for all of their visits (Kubasiewicz et al. Unpublished data).

The proximity of hair tubes to forest tracks did not affect the overall visitation rate, or the sex-ratio of visitors, suggesting that tubes along tracks are not avoided by either sex and that surveying along tracks does not bias the sample towards more males than females. There is no evidence to suggest that placing hair tubes on the edge of forest tracks favours certain individuals; of the 15 pine martens that visited more than one hair tube, 14 (93%) visited tubes at multiple distances (Kubasiewicz *et al.* Unpublished data). Placing hair tubes directly next to forest tracks reduces sampling effort, potentially allowing more samples to be collected per session or more sessions to be conducted.

The amount of amplifiable DNA obtained from hair samples is significantly increased by including more hair follicles in each reaction. Previous studies suggest that one hair is sufficient for accurate genotyping (Higuchi et al. 1988). Our analysis, however, suggests that including more follicles (up to 13) reduces PCR failure rates. Where funding, or time, prevent processing of all samples, researchers should favour samples with the most follicles to increase PCR success. However, as hair tubes do not prevent visitation by more than one animal per session, the risk that including more than one hair per reaction may produce erroneous genotypes (i.e. via contamination from the second visitor) must be considered. During a larger scale study of pine marten population density in Scotland, including 136 – 320 hair-tube nights per forest, no erroneous genotypes were detected (Kubasiewicz et al, Unpublished data). We cannot rule out the possibility that more than one individual was present in a sample, with different homozygous genotypes at one or more loci (i.e. this would present as a heterozygous genotypes which we would not recognise as erroneous). However, as pine martens are attracted to hair tubes with bait, which is removed once a visit has occurred, the chance of multiple visits is low.

284 Both time since defecation and the level of exposure affected DNA amplification, reinforcing previous findings 285 of the importance of these factors. An increase in PCR failure occurred with time up to 16 days after deposition, 286 but only for scats that were exposed to rainfall. Although this effect was also seen for false alleles, allelic drop out 287 did not increase significantly with time or treatment. As only a small number of repeat amplifications were 288 performed, the increased failure rate over time could have masked any decrease in quality i.e. fewer successful 289 amplifications were available for errors to occur in. Nevertheless, our results highlight the interacting effects of 290 time and rainfall on pine marten scats and we would encourage other studies to assess the drivers of DNA 291 degradation in faecal samples from other mammals. The finding that PCR failure increases with time for scats 292 exposed to rainfall, as opposed to error rates alone, indicates that studies which require identification only at the 293 species level, as well as those requiring accurate individual identification, need to minimise the effect of these 294 factors for ensure a cost and time effective strategy.

There was a significant difference in DNA quality and amplification success between the two loci tested. During initial planning of a project, we would recommend testing a range of potential microsatellite loci for relative success and error rates so that the most effective panel can be chosen. This should be considered as essential as optimising sample collection and storage conditions in developing an efficient and cost effective process.

299 For scat collection, researchers must strike a balance between leaving sufficient time for samples to accumulate 300 and collecting samples before DNA degrades, particularly during periods of rainfall. If longer sampling sessions 301 are required where populations are thought to be at low density, genotyping success may be improved by sampling 302 during drier periods. For hair, samples are usually collected from stationary sources such as hair tubes. As such, 303 the time between sampling sessions must also take into account sample independence. For pine martens in 304 Scotland, our data suggest that sessions of longer than four days are required to achieve this independence. 305 Compared to hair samples, scats are relatively easy to collect in large numbers, making this a preferable method 306 of data collection for large scale studies. Scat samples, however, are difficult to genotype due to high levels of 307 genotyping error associated with the poor quality DNA recovered (Lucchini et al. 2002). It may be beneficial for 308 future studies to evaluate the use of SNPs (single nucleotide polymorphisms), which are more successful for 309 degraded samples (Fabbri et al. 2012). Sample quality, however, can be maximised by using as many hair follicles 310 as possible per sample in the DNA extraction process. Sampling efficiency can also be improved by placing hair 311 tubes on the edge of forest tracks to improve access by surveyors. Given the high rate of error associated with 312 non-invasive genetic sampling, refinement of the process and consideration of environmental conditions 313 associated with each species is paramount to making the process efficient and cost effective. This study provides 314 guidance for improvements to non-invasive surveys of pine martens in Scotland, and also highlights key areas for 315 assessment prior to surveys of other mammalian species.

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- 409 Figure 1. Hairtube placement (a) and scat transects (b) in Abernethy NNR, with a grid of 1 km⁻². The grey dots 410 are hairtubes, placed at approximate density of 1 km⁻¹. Dashed lines are transects used for scat collection and 411 are placed along vehicle tracks. Transects were surveyed by walking up one side of the track and down the other, 412
- hence checking each track twice per survey.

413 Figure 2. Visitation rate to hair tubes by pine marten in Scotland. Data points represent predicted visitation rate 414 from the GLMM (Table 1) and error bars represent the 95% confidence intervals for the model from repeated 415 model simluations using random draws from the estimated parameter distributions (Gelman & Hill 2007). The 416 'forest' parameter was set for Inshriach. The 'distance' parameter was set to its median value.

- 417 Figure 3. Ct value obtained from rtPCR of the ZFX region of each pine marten hair sample plotted against the 418 amount of hair used in the extraction process. Data points are for observed data, solid lines represent predicted 419 Ct value from the GLM and dashed lines represent the 95% confidence intervals for the model prediction from 420 repeated model simluations using random draws from the estimated parameter distributions (Gelman & Hill 421 2007).
- 422 Figure 4. Failure rate of PCR amplifications with increasing sample age, for samples exposed to rainfall (black 423 line) and those under shelter (grey line). Data points are for observed data, solid lines represent predicted failure 424 rates from the GLMM (Table 2) and dashed lines represent the 95% confidence intervals for the model prediction 425 calculated from repeated model simulations using random draws from the estimated parameter distributions 426 (Gelman & Hill 2007). The 'locus' parameter was set to locus m43.
- 427 Figure 5. Rate of occurrence of false alleles with increasing sample age, for exposed (black line) and sheltered 428 (grey line) samples. Data points are for observed data, solid lines represent predicted failure rates from the 429 GLMM (Table 2) and dashed lines represent the 95% confidence intervals for the model from repeated model 430 simluations using random draws from the estimated parameter distributions (Gelman & Hill 2007). The 'locus' 431 parameter was set to locus m43.

- Table 1 Coefficient estimates for the GLMM for visitation rate of hair tubes. Results are shown for visitation rate of all pine marten (overall) and proportions of visits to hair tubes attributed to male pine marten (proportion males). Estimates are for the full model. Log-likelihood χ^2 statistic and associated p-values are for the deletion of each term from the full model (for interaction terms); or the model with main effects only (for main effect
- 436 terms). Darnaway was excluded from the analysis due to lack of data.

	Visitation rate			Visitation rate (proportion males)			
Predictor	Estimate ± SE	imate ± SE χ^2_{df}		Estimate ± SE	$\chi^2 df$	Ρ	
Intercept	-2.863 ± 0.695			-0.726 ± 1.28			
Distance	-0.005 ± 0.003	0.01 1	0.906	-0.001 ± 0.005	0.15 1	0.696	
Session	0.544 ± 0.191	15.12 ₁	<0.001	0.149 ± 0.379	0.70 1	0.404	
Forest (Inshriach)	-0.509 ± 0.866	2 5 4	0.170	-0.004 ± 1.439	1.26 ²	0.532	
Forest (Mar)	-0.645 ± 0.923	3.54 2		1.084 ± 1.559			
Distance*Forest (Inshriach)	0.007 ± 0.004	2 69	0.159	0.001 ± 0.006	0.25	0.838	
Distance*Forest (Mar)	0.008 ± 0.005	5.00 2		-0.001 ± 0.007	0.55 2		
Session*Forest (Inshriach)	-0.229 ± 0.221	F 10	0.075	-0.012 ± 0.412	0.42 2	0.812	
Session*Forest (Mar)	-0.479 ± 0.234	5.19 2		-0.174 ± 0.433			

- 439 Table 2. Coefficient estimates for the GLMM's for failure, allelic dropout and false alleles associated with PCR
- 440 **amplification results.** Estimates are for the full model. Log-likelihood χ^2 statistic and associated p-values are for
- the deletion of each term from the full model (for interaction terms); or the model with main effects only (for
- 442 main effect terms).

	Failure			Allelic drop out			False alleles		
Predictor	Estimate ± SE	$\chi^2 {}_{df}$	Ρ	Estimate ± SE	$\chi^2 _{df}$	Ρ	Estimate ± SE	$\chi^2 {}_{df}$	Ρ
Intercept	0.699 ± 0.358			1.659 ± 0.478			0.382 ± 0.435		
Treatment (exposed)	-0.150 ± 0.394	8.85 ₁	0.003	0.115 ± 0.590	0.05 1	0.817	0.366 ± 0.590	1.31 ₁	0.251
Time	-0.023 ± 0.028	10.80 ₁	0.001	-0.040 ± 0.042	1.95 ₁	0.162	0.004 ± 0.036	2.08 ₁	0.149
Locus (43)	0.594 ± 0.197	9.07 ₁	0.003	-0.534 ± 0.339	2.36 ₁	0.125	0.930 ± 0.279	10.80 ₁	0.001
Treatment* Time	-0.086 ± 0.040	4.55 ₁	0.033	-0.027 ± 0.071	0.14 ₁	0.705	-0.118 ± 0.058	4.02 ₁	0.045



- Figure 1









460 Figure 4





