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

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Running header: Non-invasive DNA sampling for pine marten

Abstract

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

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

Introduction

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

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

techniques (Taberlet et al. 1996). Improving the efficiency of sampling and minimising the factors that reduce DNA quality are therefore critical when designing a cost effective surveying strategy.

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

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

94 Irish study, with hair tubes placed in lowland forests collecting more samples than those in upland forests,
95 despite similar population densities in both habitats (Lynch et al. 2006). Spatial biases can also occur; hair tubes
96 are most accessible if placed close to forest tracks; pine marten scats are also collected from forest tracks due to
97 the relative ease of collection compared to searching the densely vegetated, forest floor. If some individuals use
98 forest tracks less frequently than others, the samples collected may only represent a sub-set of the population.
99 Female pine martens, for instance, are thought to be more risk averse than males due to the reporting of a higher
100 proportion of male road casualties (Rob Coope, pers. comm.); females also maintain smaller home ranges than
101 males (Caryl et al. 2012b), which therefore could be less likely to contain forest tracks. As a consequence, the
102 effect of different sampling techniques and designs on the outcome of non-invasive hair sampling is currently
103 unclear.

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

108 1. How does time (measured as consecutive sampling sessions) influence hair tube sample independence (hair
109 samples only)?
110 2. Does distance from forest track affect the visitation rates of pine marten, and does this vary between the sexes
111 (hair samples only)?
112 3. How is PCR success affected by the number of hair follicles included in the reaction (hair samples only)?
113 4. What are the effects of time since defecation and exposure to rainfall on DNA genotyping success and error
114 rates (scats only)?

115 **Materials and methods**

116 *Study areas*

117 Four forests in the Scottish Highlands known to have pine martens present were surveyed. Abernethy Forest
118 National Nature Reserve (57°15′N, 3°40′W; hereafter Abernethy) is a Royal Society for the Protection of Birds
119 (RSPB) reserve in the northern Cairngorms covering 36 km^2 of both ancient native pinewood (approx. 24 km^2)
120 and Scots pine (*Pinus sylvestris*) plantation (Summers, Dugan & Proctor 2010). Mar Lodge Estate (57°00'N,
121 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.

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.

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

150 enabling the time since defecation to be established, where the day of collection was 'day zero'. Twenty two
151 scats were collected in individual pots and labelled with a unique identifier, then frozen at -20 ˚C within 8hrs
152 before transfer to a -80 ˚C freezer. In order to test the effect of exposure to rainfall and time since defecation on
153 DNA quality, scats were thawed and a small section taken for DNA extraction (day zero samples). The
154 remainder of the scat was split into two equal sections and allocated to one of two treatment groups. Samples in
155 treatment one (exposed) were placed directly on a woodland floor in the University of Stirling grounds to
156 replicate the conditions in which they were found. Samples in treatment two (sheltered) were placed in the same
157 location, but raised off the ground and covered by a waterproof canopy. To test the effect of time since
158 defecation (hereafter 'time'), a small section of each scat was taken from both treatments at intervals of 2, 5, 9,
159 12 and 16 days.

160 *Genetic analysis*

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

175 For scat samples, genomic DNA was immediately extracted from day zero samples using the QIAamp DNA
176 stool mini kit (Qiagen, #51504) with a negative control. To avoid contamination, extractions were performed in
177 an area of the laboratory reserved for DNA extraction. To test DNA amplification failure and error rates, two
178 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 used followed by a final extension step of 60 °C for 30 mins. Fragment analysis was performed at DNA Sequencing and Services (University of Dundee, Scotland, DD1 5EH) with negative and positive controls. Samples were scored using GeneMarker (Version 2.4.0) and verified by eye. Consensus genotypes were obtained for day zero samples following the comparative multi-tubes approach (Frantz et al. 2003); each sample was initially amplified twice, then further replications were performed until a consensus was reached. Samples without a consensus after seven amplifications were discarded. Samples from each treatment and time period were extracted and amplified twice then compared to the consensus to quantify error rates, with a negative and positive control in each plate.

Statistical analysis

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

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

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

$$p = \bar{p}_w = \frac{\sum_{j=1}^{L} D_j}{\sum_{j=1}^{L} A_{het_j}} \qquad f = \bar{f}_w = \frac{\sum_{j=1}^{L} F_j}{\sum_{j=1}^{L} A_j}$$

211

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

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

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

231 **Results**

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

234 *Hair tube placement*

235 Visitation rate varied over time, with a higher predicted visitation rate as sessions progressed from one (0.07;
236 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
237 distance of the tube from the nearest track nor the identity of the forest significantly improved model fit for hair
238 tube visitation rate or the sex ratio of visitors (Table 1).

239 *Hair sex-typing success*

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

243 *Scat genotyping success*

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

248 *PCR failure:*

249 Time, treatment and locus all significantly affected failure rate (Table 2). Failure rate increased from 0.28 (0.18
250 - 0.43) at day two to 0.65 (0.48 - 0.79) at day 16 for exposed samples, but did not change significantly for
251 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
252 model fit with the average failure rate over all treatments and time periods being higher for locus m08 than locus
253 m43, with proportions of 0.58 (0.51 - 0.61) and 0.44 (0.37 - 0.51) respectively.

254 *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.

Discussion

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

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

For scat collection, researchers must strike a balance between leaving sufficient time for samples to accumulate and collecting samples before DNA degrades, particularly during periods of rainfall. If longer sampling sessions are required where populations are thought to be at low density, genotyping success may be improved by sampling during drier periods. For hair, samples are usually collected from stationary sources such as hair tubes. As such, the time between sampling sessions must also take into account sample independence. For pine martens in Scotland, our data suggest that sessions of longer than four days are required to achieve this independence. Compared to hair samples, scats are relatively easy to collect in large numbers, making this a

preferable method of data collection for large scale studies. Scat samples, however, are difficult to genotype due to high levels of genotyping error associated with the poor quality DNA recovered (Lucchini et al. 2002). It may be beneficial for future studies to evaluate the use of SNPs (single nucleotide polymorphisms), which are more successful for degraded samples (Fabbri et al. 2012). Sample quality, however, can be maximised by using as many hair follicles as possible per sample in the DNA extraction process. Sampling efficiency can also be improved by placing hair tubes on the edge of forest tracks to improve access by surveyors. Given the high rate of error associated with non-invasive genetic sampling, refinement of the process and consideration of environmental conditions associated with each species is paramount to making the process efficient and cost effective. This study provides guidance for improvements to non-invasive surveys of pine martens in Scotland, and also highlights key areas for assessment prior to surveys of other mammalian species.

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

421 **Figure 2.** Visitation rate to hair tubes by pine marten in Scotland. Data points represent predicted visitation
422 rate from the GLMM (Table 1) and error bars represent the 95% confidence intervals for the model from
423 repeated model simluations using random draws from the estimated parameter distributions (Gelman & Hill
424 2007). The 'forest' parameter was set for Inshriach. The 'distance' parameter was set to its median value.

425 **Figure 3**. Ct value obtained from rtPCR of the ZFX region of each pine marten hair sample plotted against the
426 amount of hair used in the extraction process. Data points are for observed data, solid lines represent
427 predicted Ct value from the GLM and dashed lines represent the 95% confidence intervals for the model
428 prediction from repeated model simluations using random draws from the estimated parameter distributions
429 (Gelman & Hill 2007).

430 **Figure 4**. Failure rate of PCR amplifications with increasing sample age, for samples exposed to rainfall (black
431 line) and those under shelter (grey line). Data points are for observed data, solid lines represent predicted
432 failure rates from the GLMM (Table 2) and dashed lines represent the 95% confidence intervals for the model
433 prediction calculated from repeated model simulations using random draws from the estimated parameter
434 distributions (Gelman & Hill 2007). The 'locus' parameter was set to locus m43.

435 **Figure 5**. Rate of occurrence of false alleles with increasing sample age, for exposed (black line) and sheltered
436 (grey line) samples. Data points are for observed data, solid lines represent predicted failure rates from the
437 GLMM (Table 2) and dashed lines represent the 95% confidence intervals for the model from repeated model
438 simluations using random draws from the estimated parameter distributions (Gelman & Hill 2007). The 'locus'
439 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 terms). Darnaway was excluded from the analysis due to lack of data.

	Visitation rate			**Visitation rate (proportion males)**		
Predictor	**Estimate ± SE**	χ^2_{df}	**P**	**Estimate ± SE**	χ^2_{df}	**P**
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	3.54_2	0.170	-0.004 ± 1.439	1.26_2	0.532
Forest (Mar)	-0.645 ± 0.923			1.084 ± 1.559		
Distance*Forest (Inshriach)	0.007 ± 0.004	3.68_2	0.159	0.001 ± 0.006	0.35_2	0.838
Distance*Forest (Mar)	0.008 ± 0.005			-0.001 ± 0.007		
Session*Forest (Inshriach)	-0.229 ± 0.221	5.19_2	0.075	-0.012 ± 0.412	0.42_2	0.812
Session*Forest (Mar)	-0.479 ± 0.234			-0.174 ± 0.433		

Table 2. Coefficient estimates for the GLMM's for failure, allelic dropout and false alleles associated with PCR 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 main effect terms).

	Failure			Allelic drop out			False alleles		
Predictor	**Estimate ± SE**	χ^2_{df}	**P**	**Estimate ± SE**	χ^2_{df}	**P**	**Estimate ± SE**	χ^2_{df}	**P**
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