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Assessing the current feces identification method of the European otter *Lutra lutra*

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In the past the European otter *Lutra lutra* was distributed throughout most of Europe but since the 1980s its distributional range has been reduced. Currently, the otter population is increasing. Conservation efforts have been implemented, however due to the natural elusiveness of the species it is difficult to monitor. Non-invasive sampling has proven to be the most effective method to derive population parameters such as presence/absence, genetic variability and population structure in European otters. The method to collect non-invasive samples is robust and provides reliable data. This study investigates the validity of the present state-of-the-art method of identifying otter feces, and suggests modifications and improvements of the method. Results from the comparison of field collected data and data derived from a blind test show that the method is applicable in areas abundant with otters, however the method loses its power in the periphery of the distributional range. In these areas, it would be relevant to supplement traditional sampling with DNA analysis to verify the identification of the sample.

Keywords: DNA, feces, *Lutra lutra*, monitoring, non-invasive sampling

The European otter *Lutra lutra* is widely distributed throughout Europe. It is elusive and semi-aquatic and lives along river and creek systems feeding primarily on fish, crustaceans and amphibians (Taastrøm and Jacobsen 1999, Björklund and Arrendal 2008). In the 1960s the range of the otter had been severely fragmented throughout Denmark. In 1980 there was an estimate of 200 otters left in the wild, and by the late 1980s the remaining Danish otters had withdrawn to the northern part of Jutland (Madsen et al. 2007). This loss of habitat was due to anthropogenic factors such as changes in road infrastructure, agriculture practices and the extensive use of eel traps. Furthermore, river systems were straightened and blocked off, affecting prey-availability. Thanks to recent habitat improvements and intensive conservation and monitoring efforts, the otter population is currently increasing. The range has steadily expanded southward from its core range in northwest Jutland since the mid90ties, and now includes most of Jutland. On Funen and isolated areas in western Zealand otters have probably survived in very small numbers going unnoticed for years due to the species elusiveness and currently the more frequent observations of feces suggests that the numbers might be increasing in these areas (Therkildsen et al. 2020).

Otters in other European countries such as UK, France and Sweden have experienced identical, slow recovery after having been close to extinction in greater parts of the countries owing to lower anthropogenic pressures, protection and changes in management plans (Stanton et al. 2014, Tison et al. 2015, Pigneur et al. 2019, Sainsbury et al. 2019).

Monitoring efforts were increased with the EU Habitat Directive 92/43/EEC, which committed EU countries to initiate monitoring and management of habitats of community interest to ensure favorable conservation status. In 1992 the otter *Lutra lutra* was abducted into the European Habitat

Directive (Annex II and IV). In 1996 a management plan was presented (Søgaard and Madsen 1996), which comprised a five year monitoring cycle. Due to the elusive behaviour of the otter, monitoring is conducted by non-invasive methods of collecting feces, analysing tracks and collecting road kills.

Assessment of otter spraints is known to be difficult (Hansen and Jacobsen 1999). Some samples, most commonly mink species (*Neovison vison*, *Mustela lutreola*) or European polecat *Mustela putorius* are erroneously identified as European otter feces (Hansen and Jacobsen 1999). Current practices for identifying feces rely upon skilled evaluation. Visual cues such as size and shape along with smell, texture and clues from the site are also valuable in species identification of a spraint. The method has been widely used for identifying the presence or absence of a species within an area. Unfortunately, relying on this method alone may result in false present/absence data on a given location. This became apparent in the results of the 2004 National Monitoring Survey of Otters in Denmark. DNA analysis conducted in 2006, of spraints originally collected in West Zealand in two years prior 2004 showed that samples from polecat *Mustela putorius* and mink *Neovison vison* were misidentified as otters. Newer samples taken from the same area (2006, 2017) (Andersen et al. 2016, Andersen and Søgaard 2017) revealed presence of otters. Hence, the objectives of the present study were to validate the survey method used to monitor otters in different regions of Denmark by combining field recognition, identification of spraints in the laboratory by a test-panel of experienced and inexperienced individuals, and species identification by DNA-analysis. The overriding purpose was to test whether the percentage of correct answers depended on the situation (i.e. field collection or following blind test laboratory determination) by addressing the following hypotheses:

1) The percentage of correct answers depended on the individual.
2) The percentage of correct answers depended on the geographical origin of the feces.
3) The percentage of correct answers depended on the experience level of the individual.

Material and methods

Field collection

A total of 193 samples of spraints and jellies from presumably otter were collected from six different geographical areas in Jutland, Denmark in March and April 2006 by different collectors (Fig. 1). Collection methods were based on the technical guidelines for extensive monitoring of otter developed by Anonymous (1984) and later evaluated by Reuther et al. (2000) and Elmeros and Bussenius (2002). All samples were stored in photo canister and frozen.

Blind test

Nine collectors were invited to participate in the blind test for species-identification based on the feces. Prior to the blind test, participants were asked if they would consider themselves as experienced or inexperienced in otter feces identification (i.e. based on how long time they have been involved in monitoring). On the basis of their answers, the participants were divided into these two groups (test panel). Species identification was conducted by standard methods of subjectively evaluating the samples based on smell and visual cues. Answers were scored on an evaluation form with the following categories; otter, polecat, mink, other and 'don't know'. The category 'other' was defined as a sample that was definitely not otter. The samples were placed in petri dishes during the test and the corresponding sampling numbers were blinded. The collectors were asked to identify the species of all samples. The results from the blind test were classified until the DNA analysis was completed. Correct answers were defined as either the participants answered correctly that a sample was an otter feces or they answered one of the other species options when the sample was not otter. Thus, if for example the participant answered polecat on a mink spraint the answer was still considered correct because both polecat and mink belonged to the category 'Not otter'. Any deviation from this was scored as a wrong answer including 'don't know'. By these criteria all answers were reclassified as correct (1) or incorrect (0), in order to ease statistical analysis. Only the answers of the 141 samples that were successfully sequenced (1269 answers) were used in the statistical analysis.

DNA analysis

DNA-extractions from spraints and jelly were conducted in a laboratory dedicated to DNA-work of historical samples or fecal samples to avoid contamination from recent high quality DNA-sources as muscle tissue. The DNA-extraction from stool samples was performed with QIAGEN QIAamp DNA Stool Mini Kit following the manufacturer's protocol (QIAamp DNA Stool Handbook 2nd edn July 2007). Primers for a cyt B sequence differentiating between otter, polecat, mink and other mustelids were designed by Hansen and Jacobsen (1999). An additional primer (5′-GCCATACACTA(CT)ACATCAGACACA-3′) was designed for a nested PCR. The primer was situated downstream of the forward primer designed by Hansen and Jacobsen (1999). The expected size of the amplified segment was 189 bp (Hansen and Jacobsen 1999). DNA amplification was performed in two PCRs. First, all samples were put through a singleplex PCR with touch down cycle (PCR1). PCR1: 95°C:15 min, amplification was performed in two cycles; cycle 1: 5 cycles of 94°C:45 s, 52–48°C:45 s touch-down, 72°C:20 s. Followed by amplification cycle 2: 35 cycles of 94°C:45 s, 51°C:45 s, 72°C:30 s, followed by 72°C:10 min. Samples were checked on a 2% agarose gel. Samples with no visual band were cleaned up using the enzyme Exonuclease 1 to improve amplification of PCR products in the nested PCR (PCR2). Cycling parameters for PCR2 were: 95°C:3 min, amplification were performed in 40 cycles of 94°C:45 s, 50°C:45 s, 72°C:30 s and 72°C:10 min.

The DNA was sequenced at The Department of Genetics and Biotechnology, Aarhus University Research Center, Foulum and by MACROGEN (South Korea). Species identification of each sequence was done in combination of Finch TV ver. 1.4.0 (Geospiza, Inc.) (<www.geospiza.com/

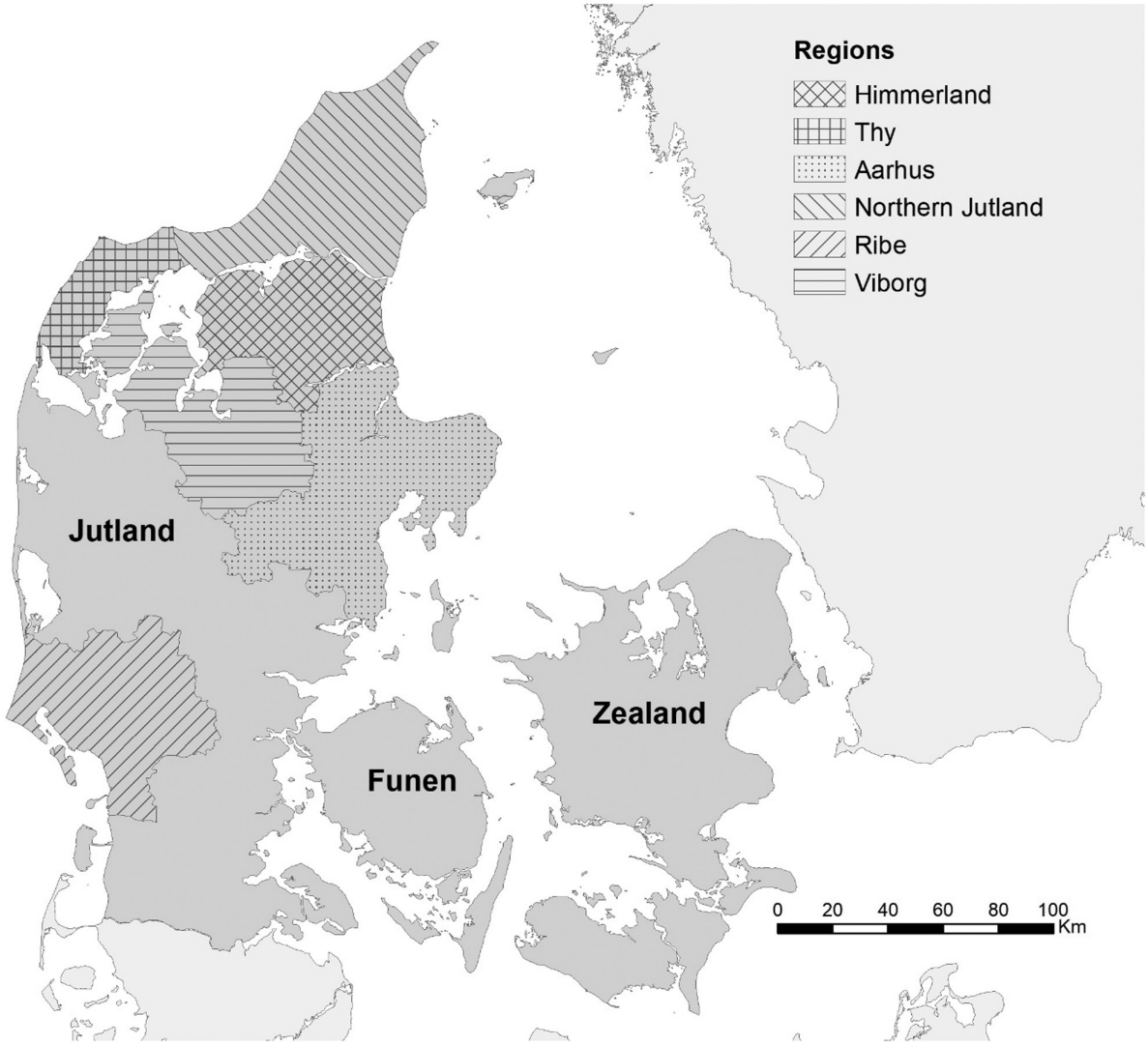

Figure 1. Location of collection sites (areas) in Denmark. Sites are situated on the peninsula of Jutland.

finchtv>), Sequencher 4.2 (GeneCode) and the website National Center for Biotechnology Information (NCBI), and Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The DNA-species identifications were performed before the results from the blind test were disclosed.

Data analysis

Data analysis was conducted using two statistical programs; statistical analysis software (SAS) and JMP ver. 7.0.

We used a Type 3 GEE-model Binary Data with Logit model to compare categorical explanatory variables with two levels of answers (correct and incorrect answer 0;1). This model incorporated repeated measurements as each individual assessed several spraints. Three variables were included in the model; 'respondent (individual)', 'geographical origin of the feces' and 'experience level'. Two different analyses were carried out, one overall model for respondents and one for experience level and geographical origin, in which the effect of variable 'respondent' was incorporated. The χ^2 test was used to analyze differences in frequencies between groups of data. Wald χ^2 test and likelihood χ^2 tests were presented to show if there was a significant effect of a given variable, defined as $p < 0.05$.

Results

DNA verification of field sampling

Data comprised 193 collected samples. Sequence success from the total set of samples was 73.06% (141 samples). The number of sequences identified as otter was 127 (90.1%). Aligning the sequences revealed two different haplotypes, OD1 (GenBank acc. no. MW303425) and OD2 (GenBank acc. no. MW303426). OD1 was almost identical to part of the CytB sequence in the unpublished mitogenome with GenBank acc. no. MN122838 differing with a single mutation (transition C to T in the mitogenome) at site 14 336 and 14 501. OD2 differed additionally from the mitogenome CytB sequence with a transition (T to C in the mitogenome) at site 14 414. Of the 127 sequences it was possible to assign haplotypes to 101 sequences (85 OD1 and

16 OD2) while 26 were only identified to the species level. The other 14 samples identified as not otter were: American mink *Neovison vision* (8, 5.7%), pine marten *Martes martes* (2, 1.4%), European polecat *Mustela putorius* (2, 1.4%), stoat *Mustela erminea* (1, 0.7%) and dog *Canis familiaris* (1, 0.7%). First, we tested if there was a difference in the frequencies among the five other species detected, despite the low sample size. No significant difference was observed (χ^2 (likelihood) = 4.819; df = 4; p = 0.055).

Blind test in laboratory

In total, performing the blind test on the 141 samples with available DNA sequences, the nine participants provided 1269 answers. In 67.1% of the answers, the participants gave a correct answer, in 10.6% the participants were uncertain and in 22.4% of the answers the participants provided a wrong answer.

Testing how skilled the participants were to identify otter spraint in the laboratory we tested the frequencies of the wrong answers when the sample DNA sequence was identified as otter and when the sample sequences were not otter (Table 1). When the sample was identified as 'otter' 31% of the answers were wrong, opting for 'not otter' or 'uncertain', and vice versa when the sample was not identified as 'otter' 50.8% of the answers opted for 'otter' or they were uncertain. Using a χ^2 test with correction for continuity, there were significantly more wrong answers when the feces were NOT from an otter.

Blind test versus field identification

Comparing the frequencies of correct identification of otter spraint in the blind test and the field (Fig. 2), there was a significant higher success of identifying otter feces correctly when collected in the field ($\chi^2 = 30.54$, df = 1, $p < 0.0001$).

Hypothesis testing

We addressed the hypotheses 1) and 2) by incorporating 'geographical origin of the feces' as a variable in the statistical model, which represented the collector of the area or the 'respondent', representing the person in the blind test in the laboratory. The results of the influence of the 'geographical origin of the feces' indicated a significant effect ($\chi^2 = 286.7$, df = 5, $p < 0.0001$). The area 'Ribe' contributed with a low frequency of correct answers (Fig. 2). The results of the analysis of the effect of 'respondent' in the blind test disclosed a significant difference between the correct answers amongst the participants (Wald χ^2 test = 127.28, df = 8, $p > 0.0001$) (Fig. 3).

We tested whether these effects could be attributed to the experience level of the participants or to the geographical origin of the feces (hypothesis 2 and 3). Hence, these effects were incorporated as variables in a GEE-model. It was not possible to incorporate experience of the field-collectors, as their experience was not monitored consistently. The GEE-model showed a significant effect of the geographical sampling area (Wald $\chi^2 = 41.7$, df = 5, $p < 0.0001$) whereas the experience level of the respondents had no effect (Wald $\chi^2 = 2.51$, df = 1, p = 0.11).

Discussion

Counting/collecting spraints is the most used method to monitor otter distribution to estimate relative population density and habitat selection (Reuther et al. 2000, Romanowski et al. 2013). It is however, questionable if the method is valid and accurate for estimating population size and habitat selection in connection with evaluation of conservation status (Reid et al. 2013). The issues raised are problems regarding variation in the frequency of seasonal as well as individual marking (Kruuk 2006) but also the traceability could vary across seasons (Kruuk et al. 1986, Reuther et al. 2000, Lampa et al. 2008). Consequently, Sittenthaler et al. (2020) investigated the relationship between spraint-abundance (old and fresh) monitored along a stream and population density together with marking behavior connected to individuals and/or sex-specificity using genetic monitoring. They observed that age of spraints and the spatial scale of the monitoring were the critical factors for indexing the otter density using spraint counts. This was caused by the fact that it was easier to find fresh spraints with increased population densities when a large part of the territories was covered, while this relationship was not found in single marking sites. Thus, Sittenthaler et al. (2020) further concluded that only fresh spraints should be used for assessment of population density and trends, and old spraints can be used for surveillance of distribution and expansion.

However, spraints/feces are also used for diet analysis (Marcolin et al. 2020) where species identification of the spraint is just as important as for surveillance studies emphasizing the importance of using DNA for species verification. A prerequisite for the applicability of the non-invasive DNA-method is the freshness and following preservation of spraint after collection. In the present study, we sampled whole spraint/feces in ethanol for further procession but using swabs is an alternative method (Velli et al. 2019) for species identification. The method of choice depends on the question addressed in the study, i.e. collecting whole spraints permit following diet-analysis, which is not possible with the swab method.

No study has so far investigated how dependent the monitoring method was on the geographical origin of the feces

Table 1. Comparison of the two types of answers, either the correct answer was 'otter' but the respondent opted for other ('unsure', 'not otter') or the correct answer was another species but the respondent opted for 'otter'. N = total number of answers, NW = number of wrong answers.

	N	NW	%
Species is otter (DNA) and respondent opted for 'not otter' or 'unsure'	1143	354	31
Species not otter (DNA) and respondent opted for 'otter' or 'unsure'	126	64	50.8
Significantly more wrong answers when feces are not from otter	$\chi^2 = 19.30$, df = 1, $p < 0.0001$*		

* χ^2 test, with correction for continuity.

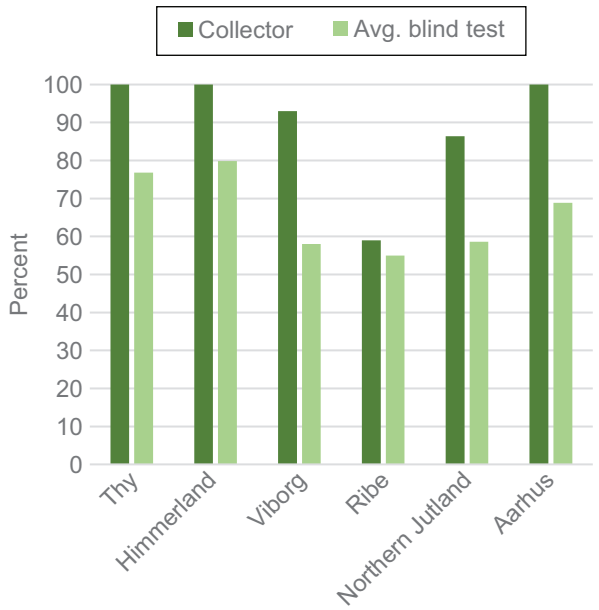

Figure 2. Results of the field identification of otter spraints conducted by the collectors (dark green) and the identification performed in the laboratory in the blind test (light green) according to the geographical sampling area.

(a potential proxy for population density) and experience of the personnel conducting the monitoring directly as in the present study.

The results of our study showed that there was a difference among participants in the laboratory blind test, the collectors and geographical origin of the feces with respect to how they correctly determined a sample that was indeed an otter. Contrary to expectations, geographical origin was found to be the most important factor explaining the correct answer compared to the experience level of the participants in the blind test.

Species identification based on smell and vision

The varied diet consumed by the different mustelid species can be used as an indicator when evaluating feces. The characteristic item in otter spraints are the predominant presence of fish bones, while mink and polecat feces may contain more feather, mammalian (rodent) bones and fur, reflecting the diet of a generalist. Mink feces are also characterized by a tapered end of the spraint and a strong scent (Taastrøm and Jacobsen 1999, Hammershøj et al. 2004). Otter feces have a more sweet fishy odor (Hansen and Jacobsen 1999). These characteristic differences between the three mustelid species' diet support that examination of prey remains found in feces provides a good indicator of feces identity. However, similar morphological characteristics of mink and polecat feces can be mistaken for otter feces. These species use identical territorial marking strategies leaving spraints and jellies along riversides, tree stumps, mounds, etc. (Hansen and Jacobsen 1999). This was illustrated in the present study as the species that was most frequently mistaken for otter was mink. Mink are commonly found across Denmark and share some of the same habitats as otters. Especially in areas near commercial mink farms, there is a higher frequency of mink in the surrounding area due to escaped animals (Hammershøj 2004).

Generally, the field sampling method provided a rather high percentage (~90%) of correct identification of otter spraints suggesting that the morphological method used is quite reliable. In the blind test performed in the laboratory, the morphological and scent identification of otter spraints were considerably lower, only ~67%. This does not imply that the participants conducting the blind test were unable to identify otter spraints, as they were significantly better at identifying an otter spraint compared to spraint from the other species. This suggests that transporting the spraints to the laboratory had a negative effect on the identification ability. The spraints were not fresh, had lost scent and probably the characteristic morphological appearances, and finally, they were not considered in an ecological context. These factors might have complicated the correct identification. This was supported by the comparison of the results of the correct answers from the two approaches, showing a significantly better spraint identification in the field.

Geographical origin of the feces

Due to the uneven distribution of otters in Denmark (Elmeros et al. 2006), the chances of finding otter feces is site-dependent. Locations in the northern part of Jutland have a relatively long monitoring history (since 1984) compared to other areas in Denmark because of a relatively high abundance and range dynamics of the otter. This is reflected by the fact that areas having long-time established otter

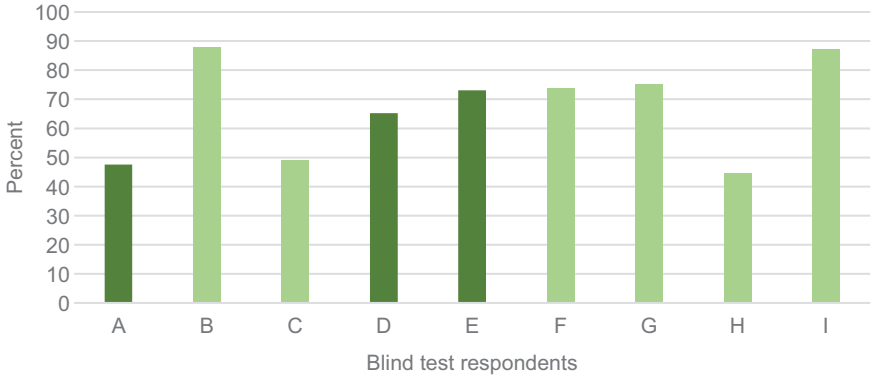

Figure 3. The percent distribution of correct answers given by participant A–I. Experience levels of the participants are categorized by inexperienced A = dark green, and experienced B = light green.

populations in 2007, when the samples were collected, were also the areas where the collectors have scored 100% (Thy, Himmerland, Aarhus) or high percent scores of 93% and 86% both from northern Jutland. Further, the area with the lowest percentage of sampled otter feces was found at the edge of the otter distributional range in 2006–2007 in the southwestern part of Jutland (Ribe), where only recent observations of established otter territories existed. Thus, geographical origin of the feces had an effect on the difficulty level and probability of finding feces within an area, giving the collectors in otter-rich areas the luxury of choosing samples that were most likely from otters. In southern Jutland samples were few and far apart, adding to identification difficulty, and hence, the collectors had to settle with samples that were degraded or samples in which the identification was obscured. Furthermore, as the collectors were knowledgeable about areas with otter presence, the combination of habitat familiarity and abundance of otters especially in the north of Jutland facilitates the finding of otter feces. This also explains the higher percentage of correct identification in the field as opposed to the scores in the blind test (Fig. 2). However, the data also suggested a variation in the percentage of correct answers that could be attributed to the participants as such. Whether this could be explained by the experience in identifying otter spraints was further analysed.

Experience level of participants

Examining the influence of geographical origin of the feces together with experience level of the participants suggested that only the collection area was important. This was surprising as it was expected that experience level was important. For example, researchers working in areas with high otter density will encounter otter feces more frequently, acquiring a routine and expertise in identifying otter feces. Furthermore, the higher otter density will inevitably give more feces to choose amongst, increasing the probability of choosing an otter spraint.

The separation of the participants into the two categories, experienced and inexperienced, was based on how long time they have been involved in monitoring and their perception of their own skills. This might introduce a bias to the results as this kind of self-assessment is subjective and many psychological aspects interfere, not necessary presenting the true expertise/skills of the respondent. Another factor is how conservative a participant answered. For example, participant H answered, 'don't know' more often compared to the other participants in the panel. This may be due to the person being cautious in answering, rather opting for 'don't know' than to give an incorrect answer. Unfortunately, all answers given by H in this category were scored as incorrect. These flaws could explain the discrepancies in the analysis of experience level (Fig. 3). It was expected that participants C and H had performed better according to their experience level. Conversely, inexperienced participants D and E scored high, and just as well as the experienced F and G.

This hypothetical exercise emphasizes the challenges with misidentification of feces in the field. The most frequent misidentification was when otter was the correct answer but the test person opted for 'not otter' or another species. This suggests that if field monitoring carried the same 'answer-bias', it could lead to an under-estimate of otter spraint identified in an area. This would be critical at the edges of known otter ranges or in newly occupied areas.

Management applications and implications

This study showed that the commonly applied identification method was reliable in areas with high population density combined with a prior knowledge of population movements. However, the method loses strength in peripheral areas of the distributional range. In these areas and in recently newly discovered areas of otter presence, the method would benefit from a supplementing DNA analysis in order to validate the species identification. Therefore, samples found in zones around the edge of a distributional range or feces found in newly occupied areas should be subjected to DNA-analysis in order to verify species identity. This will increase the effectiveness of monitoring and management programs saving time and resources. Launching actual genetic monitoring of otters, including species- as well as individual identification, performed repeatedly on a temporary basis, will uncover population trends making management programs not only more informative, but also allow researchers to adjust management efforts accordingly.

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Author contributions – The project was based on HSS master thesis. LWA, ABM and BH designed and planned the project, LWA and HSS were responsible for the laboratory work, AL, OHJ, EAT, FS, HJB, JF, BH and HJ were responsible for sampling and blind testing, LWA, HSS and JK performed the data analysis. ABM, VL, JK, HSS and LWA contributed to writing the manuscript.
Conflicts of interest – The authors declare no conflicts of interests.

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