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Effects of sampling location within feces on genotyping success in brown bears

Alexander Kopatz, Oddmund Kleven, Andrea Friebe, David Ahlqvist, Jonas Kindberg, Øystein Flagstad

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Brown bear cub © Alexander Kopatz

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Abstract

Kopatz, A., Kleven, O., Friebe, A., Ahlqvist, D., Kindberg, J. & Flagstad, Ø. 2021. Effects of sampling location within feces on genotyping success in brown bears. NINA Report 2022. Norwegian Institute for Nature Research.

Feces samples make up the largest part of the material collected for DNA analysis in brown bear (*Ursus arctos*) monitoring in Norway. However, genotyping success rates vary substantially among feces samples collected during spring, summer and autumn. We studied the influence of the sampling location or spot, i.e. outside- or inside-swabbing, of brown bear fecal samples on the genotyping success using eight microsatellite markers applied in the monitoring of the species in Norway and Sweden. We also compared the results to the success rates of collecting a piece of the sample stored on silica, as is currently applied during the non-invasive genetic sampling to monitor brown bears in Norway. Feces were collected by tracking GPS-marked individual brown bears of the Scandinavian Brown Bear Research Project. A total of 369 samples (outside swabbing, inside swabbing and piece on silica) were collected. There was large variation in the genotyping success among all samples collected and we found no statistically significant difference between outside- (50.4%) and inside-swabbing (47.2%) of the feces. Collecting a sample from feces on silica (52.8%) provided an overall similar success rate. Genotyping success rates of all three methods showed a clear seasonal pattern with very low success rates in June and July. Outside-swabbing seemed to have the tendency to be more successful on liquid and moist feces, while pieces collected on silica seemed to provide higher success on dry feces. Estimated exposure time, the period a feces was exposed to the environment (~8 days), did not affect the condition and shape of a feces, and also did not explain the variation in genotyping success. These results suggest, that visual age estimation of scats in the field should be done with caution, as the overall state of the feces may not be representative of its age. Furthermore, the results do not warrant a change of the current applied practice of scat sampling. Our study is one of the first experiments on individually tracked brown bears that has assessed sampling location within feces, exposure time and also feces characteristics in a natural environment.

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Sammendrag

Kopatz, A., Kleven, O., Friebe, A., Ahlqvist, D., Kindberg, J. & Flagstad, Ø. 2021. Effekt av prøvetakingssted på ekskrement for suksessrate for genotyping av brunbjørn. 2021. NINA Rapport 2022. Norsk institutt for naturforskning.

Ekskrementprøver utgjør den største andelen av prøver som samles inn for DNA-analyse til overvåking av brunbjørn i Norge. Suksessraten for genotyping varierer imidlertid betydelig for ekskrementprøver samlet inn i løpet av vår, sommer og høst. Vi undersøkte om prøvetakingssted, dvs. svabring på utsiden eller innsiden av brunbjørnekskrementer, påvirket suksessraten for genotyping ved å benytte åtte mikrosatelittmarkører som anvendes i overvåkingen av arten i Norge og Sverige. Vi sammenliknet også resultatene med suksessraten for prøver lagret på silica, som er den metoden som benyttes i dag for innsamling av ikke-invasive prøver for å overvåke brunbjørn i Norge. Ekskrementprøver ble samlet inn ved å spore GPS-merkede brunbjørner fra det Skandinaviske bjørneprosjektet. Totalt ble 369 prøver (svabring av utsiden, svabring av innsiden og bit på silica) samlet inn. Det var stor variasjon i suksessraten for genotyping blant alle prøvene og vi fant ingen statistisk signifikant forskjell mellom svabreprøver fra utsiden (50.4 %) og innsiden (47.2 %) av ekskrement. Prøvetaking av en bit av ekskrement lagret på silica resulterte i en tilsvarende suksessrate (52.8 %). Suksessraten for genotyping for alle innsamlingsmetodene viste en tydelig sesongvariasjon med veldig lave suksessrater i juni og juli. Svabreprøver fra utsiden av fuktige ekskrementer tenderte til å ha høyere suksessrate enn fra tørre prøver, mens biter samlet fra prøver lagret på silica tenderte til å ha høyere suksessrate fra tørre ekskrementer. Estimert eksponeringstid, perioden ekskrementene lå ute før innsamling (~8 dager), påvirket ikke tilstanden eller formen på ekskrementene og forklarte ikke variasjonen i suksessratene. Disse resultatene indikerer at visuell aldersestimering av ekskrementer i felt bør gjøres med forsiktighet ettersom tilstanden til ekskrementet ikke trenger å være representativt for dets alder. Resultatene støtter ingen endring i praksis i forhold til nåværende metode for innsamling av ekskrementer. Vår studie er et av de første på brunbjørn som har undersøkt prøvetakingssted, eksponeringstid og egenskaper ved ekskrementer i naturlige omgivelser og hvor bevegelsene til individuelle bjørner ble sporet.

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Foreword

DNA-based monitoring of brown bears in Norway has been conducted nationally and annually for more than a decade. Samples of brown bear feces collected during autumn are usually characterised by moderate genotyping success rates. Contrary, samples collected during spring and summer have provided comparably low genotyping success rates. Unambiguous identification of individual brown bears is crucial for their conservation, but especially important for an effective management and translation of counter measures to solve human-bear conflict cases. Based on our previous knowledge and in close collaboration with the Scandinavian Brown Bear Research Project, we have conducted a pilot study to identify potential differences in genotyping success rates of feces samples depending on the location and spot of the scat from where the sample was taken. Previous studies reported increased genotyping success when the outside of the scat was swabbed compared to samples taken from the inside of the scat. Here we present the results of our assessment of feces samples from individually tracked brown bears in the Scandinavian environment in order to make suggestions on how to improve DNA-based monitoring of the species, and potentially other wildlife.

Trondheim, July 2021

Alexander Kopatz

1 Introduction

The brown bear (*Ursus arctos*) is one of the five species in Norway regularly assessed under the national monitoring program for large carnivores. The assessment of the national population with DNA-based methods of regularly collected biological material and remains of the species is crucial for the implementation of the monitoring scheme. For more than a decade the population has been monitored with the help of non-invasively collected biological material, which then are subject to genetic analysis to determine the individual's genotype. Feces samples make up the largest part of the sample material collected for DNA analysis (**Figure 1**).

The average genotyping success rate for obtaining a microsatellite DNA-profile, and thus to identify a brown bear individual, was 52% in 2020 (708 of 1361 samples; Fløystad et al. 2021). The number is influenced to high degree by the notoriously low success rates of feces collected during spring and summer time. Indeed, success rates of feces collected in Norway displayed a seasonal pattern with highest genotyping success of scats collected during autumn and lower rates for spring and summer samples (Kopatz et al. 2020). Success can vary substantially between spring and fall as shown in regular monitoring data: 26 vs. 69% (Fløystad et al. 2018). Spring and summer time also represents a crucial time in brown bear biology, including the period just after leaving the den when they start searching for food resources and mating partners etc. Such high activity may also cause conflict situations with humans and, with low success rates for individual identification, this leads to challenging situations for wildlife management authorities and also the national monitoring of the species. Hence, increasing our knowledge and understanding of the interplay on the different factors influencing genotyping success rates of brown bear feces collected in spring and summer would help to improve management and conservation. Also, considerable time and effort is spend by the dedicated and professional personnel of the Norwegian Nature Inspectorate (SNO) in collecting crucial feces samples (**Figure 2**). Therefore, knowledge on how to increase genotyping success of feces overall, would potentially allow for a more effective and better monitoring and conservation management of other wildlife.

Figure 1*. Sampling a piece from brown bear feces into a tube filled with silica for DNA-analysis in June 2020. Photo: Jonas Kindberg.*

Seasonality of genotyping success of especially feces samples is a wide-standing challenge in wildlife monitoring, across multiple species and geographical regions (see e.g. Lucchini et al. 2002, Maudet et al. 2004, Piggott 2004). Variation in genotyping success does not occur equally throughout the year. Lower success rates of genetic analyses may occur due to a combined effect of multiple reasons, including the time a biological sample, i.e. feces, is exposed to the environmental and different weather conditions such as e.g. temperature, ultraviolet light, humidity and precipitation, as well as other microclimatic conditions. All these factors have been described as possible limiting factors, which can have significant impact on the DNA-molecule (e.g. its integrity and degradation) and on the success of the DNA-analysis. Further, the content of the feces itself, i.e. the diet of the brown bear, showed strong seasonality in the availability of different food resources and can therefore have significant effects on genotyping success of feces samples of the target species (**Figure 2**; see e.g. Piggott 2004, Hajkova et al. 2006, Murphy et al. 2007, Stenglein et al. 2010, Woodruff et al. 2015, Gulsby et al. 2016).

***Figure 2**. Brown bear feces in Scandinavia show variation in shapes and conditions across seasons and month of the year (selection): A: May, B: June, C: August, D: September. Photos: Jonas Kindberg (A, C) and Tore Solstad (B, D).*

In a previous study, we assessed the genotyping success of different DNA-extraction techniques, which have been successfully applied in the analysis of feces of other large carnivore species, in order to evaluate a potential increase of success rates also for brown bears (Kopatz et al. 2020). However, all DNA-extraction methods tested displayed similar results, including the same seasonal effect, and without one technique appearing to be superior. Overall, genotyping success for feces collected in spring and summer did not increase according to the method. We concluded, that an increase in genotyping success would likely require substantially more effort

and thus costs in technical development, but also with presumably small increases in the rate of success. Based on the results of our previous study, we aimed for a more comprehensive investigation of the sample-taking process itself. We therefore proposed a pilot study to gather more knowledge on the sampling location on a single feces in the field to test and to potentially improve genotyping success.

Surprisingly, only a few studies reported or evaluated the location on or in the feces, where the sample material for DNA-analysis had been taken from. Hence, circumstances and potential effects of the sample location remain understudied, although previous studies made such recommendations (see e.g. Rutledge et al. 2009, Stenglein et al. 2010, Bourgeois et al. 2019). A previous study on brown bear and grey wolf, reported that outside or surface swabbing to sample feces led to higher genotyping success than samples which were taken from the inside of the scat (Stenglein et al. 2010). Also, other studies on different wildlife species, e.g. on coyote *Canis latrans* (Gulsby et al. 2016), jaguars *Panthera onca* (Wultsch et al. 2015) or on the forest elephant *Loxodonta cyclotis* (Bourgeois et al. 2019) showed that surface swabbing led to a higher DNA extraction and genotyping success. The reasons for a higher success are caused by a higher number of epithelial cells of the intestinal lining, which can be found mainly on the outside or surface of a scat (Albaugh et al. 1992, Flagstad et al. 1999). Further, the amount of possible inhibitors, interfering with the DNA-analysis, are less on the feces' surface but are usually frequently abundant inside the feces (Deuter et al. 1995, Kohn et al. 1995, Reed et al. 1997, Vynne et al. 2012, Wasser et al. 1997). Therefore, we tested the effect and success of the surface or outside sampling versus inside sampling of brown bear feces in the monitored study environment of the Scandinavian Brown Bear Research Project in Sweden.

Our objectives were to assess the DNA-success rate of the outside and inside sampling location of each feces collected from GPS-collard individuals. Knowledge of the location and time of presence of each individual allowed us to estimate the feces' approximately maximum exposure time to the environment. Further, we also collected additional samples of the same feces and processed them in the same way as currently done for the Norwegian national monitoring program for large carnivores, to evaluate the genotyping success of all three methods.

2 Material and Methods

2.1 Study area and sample collection

The sample collection was carried out over a period of six months from May to October 2020 in the research area of the Scandinavian Brown Bear Research Project in central Sweden (**Figure 3**). The research area of approximately 5,802 km^2 is located in the southernmost reproductive area of the Scandinavian brown bear population at about 61°N, 15°E. Coniferous forest covers most (80%) of the area, along with lakes and bogs (Martin et al. 2010).

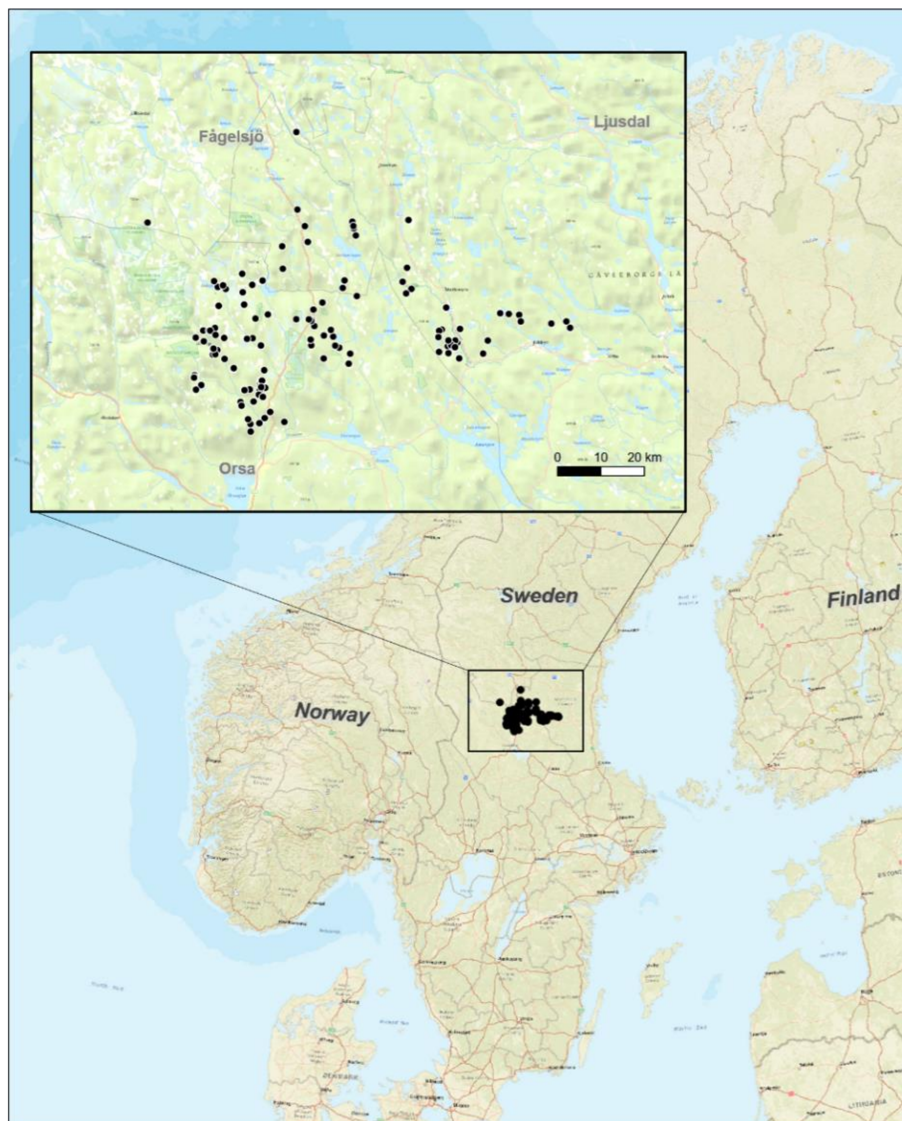

***Figure 3**: Sampling locations of the 123 brown bear feces in central Sweden.*

Feces samples were collected by two staff members of the Scandinavian Brown Bear Research Project, both trained wildlife professionals, from locations visited by individual GPS-collared brown bears prior to sampling. The sampling took place sometime after the marked individuals had left the area. This way of sampling allowed us to record the time and date when a marked individual was in that area. Prior to sampling, the staff recorded information on the feces' shape

(solid or liquid) and condition (dry, half-dry or moist). Then three different samples were taken from each unique feces separately. Two samples were collected with sterile swabs separately and stored separately in Eppendorf tubes containing a high-salt solution, Queen's lysis buffer, for the preservation of the sample material (Seutin et al. 1991): 1) a swab from the outside or surface layer of the specimen, 2) a swab from the inside of the feces (**Figure 4**). In addition, although a statistical comparison would not be feasible as the laboratory pipeline differed, but in order to be able to evaluate and compare overall performance, we collected a third sample by 3) taking a piece of the feces in tubes containing silica, as currently applied to monitor brown bears in Norway (see **Figure 4**; Kopatz et al. 2020). Swab-samples were kept in the fridge and silica-samples at room temperature until laboratory analysis (see **Appendix A1**).

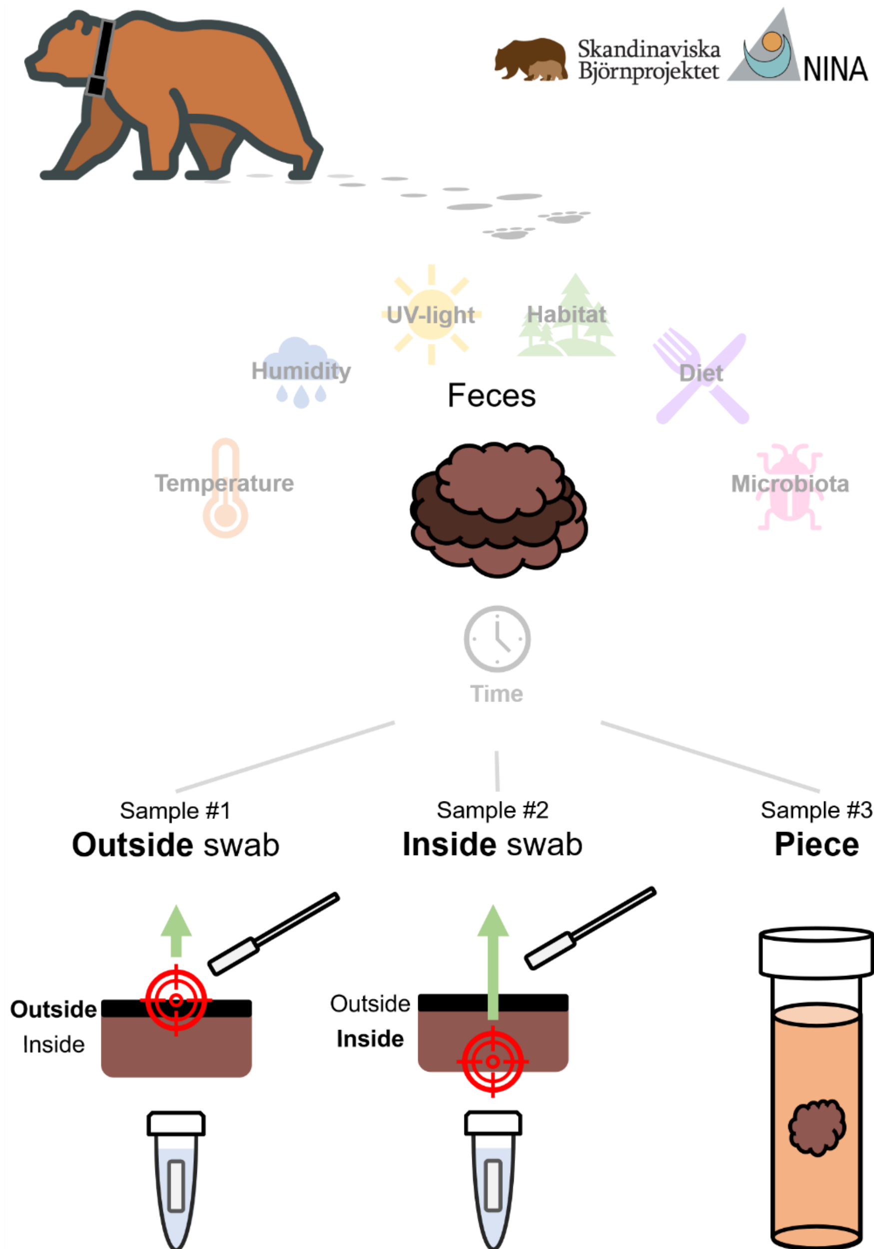

***Figure 4**. Sampling in the field: schematic flow of the sample collection of brown bear feces from locations visited by the GPS-marked individuals: with a swab from the outside surface (sample no. 1), a swab from the inside (sample no. 2), and a small piece of the feces (sample no. 3). Bear icon by Freepik and has been designed using resources from Flaticon.com.*

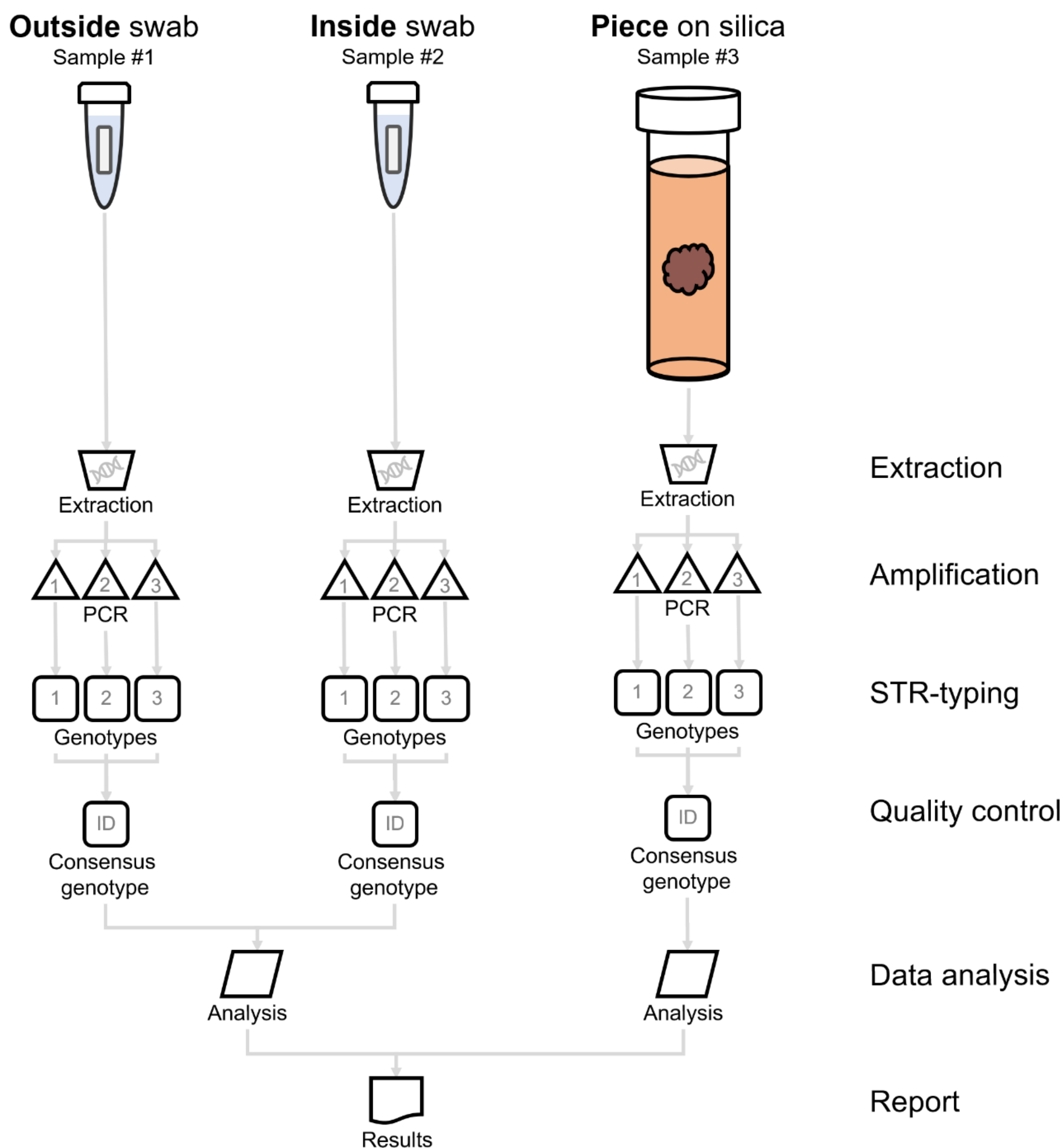

***Figure 5**. Analysis of the samples: schematic work-flow of the analysis pipeline in the genetic laboratory with two different DNA-extraction methods for swabs and pieces collected on silica.*

2.2 Genetic and data analysis

Every sample was subject to DNA-extraction and amplification of three replicates for eight STRs (STR=short tandem repeat or microsatellite), used to monitor brown bears in Norway and Sweden, and as applied in our previous study (Kopatz et al. 2020). DNA from the swab samples was extracted using DNeasy Blood and Tissue Kit (Qiagen), while the silica samples were extracted using Maxwell 16 Tissue DNA Purification Kit in combination with the Maxwell 16 Instrument (Promega) for automated purification of genomic DNA. DNA-extractions followed the manufacturer's instructions. A schematic overview over the analysis-pipeline can be seen in **Figure 5**. After extraction, samples were genotyped with eight STR markers and a sex-typing marker (see Andreassen et al. 2012, Kopatz et al. 2012). Specificity, sensitivity and forensic evaluation of the STR markers are reported in Andreassen et al. (2012). All samples were analysed in three independent PCR replicates including negative and positive controls. Followed by quality assurance,

a consensus genotype for each sample was constructed based on the following criteria: loci with a heterozygote result had to be consistent in two independent PCRs and loci with a homozygote result had to be consistent in three independent PCRs. Samples with a consensus genotype containing at least six markers were defined as successfully genotyped, as currently also applied in the DNA-based monitoring of brown bears in Scandinavia.

We calculated the maximum exposure time or age of a feces sample by taking the time between the first transmission of the location of the GPS-collared brown bear at arrival in the area (date and time) and the collection of the sample (date and time) from that location. Potential temporal trajectory of the results was analysed. We tested normality of the data from outside and inside sampling (Massey Jr. 1951) and difference in success using the Wilcoxon signed rank test (Wilcoxon 1945) as well as correlation of each method's success rate against time with the recorded information on exposure time, feces shape and feces condition (R Development Core Team 2021).

3 Results

From May to October 2020 we collected a total 369 samples from 123 different brown bear feces (**Figure 3**) by taking samples with swabs on the outside and the inside as well as by collecting a random piece from the same feces (see **Figure 4**). The number of feces collected varied throughout the study period with most samples collected in June, August and September (**Table 1**). Genotyping success, i.e. the number of amplified STR markers and with that the number of accepted genotypes, were comparably high in May, August, September and October, lower in June and lowest, with no genotypes accepted, in July (**Table 1**, **Figure 6**). Overall, standard deviations were comparably large (31.8-40.8%). Across the whole study period, swabbing the outside (50.4%, 62 genotypes) and collecting a piece (52.8%, 65 genotypes) of the feces showed overall similar results in the proportion of accepted genotypes. Inside swabbing resulted in the lowest success rate (47.2%, 58 genotypes). All methods combined resulted in 82 (66.7%) of the 123 samples successfully genotyped for accepting an individual genotype (**Table 1**).

***Table 1**. Genotyping success: results of the genotyping of 123 brown bear fecal samples with samples collected by swabbing the outside, the inside or by taking a piece of the scat, as well as all methods combined, showing the number of accepted genotypes (top) and proportion of accepted genotypes (bottom).*

		Accepted genotypes (count)			
Month	***N***	**Outside (swab)**	**Inside (swab)**	**Piece (silica)**	**All combined**
May	5	5	2	4	5
June	29	2	2	11	11
July	12	0	0	0	0
August	25	18	16	20	21
September	39	27	28	20	32
October	13	10	10	10	13
Sum	123	62	58	65	82

		Proportion of accepted genotypes (%)			
Month	***N***	**Outside (swab)**	**Inside (swab)**	**Piece (silica)**	**All combined**
May	5	100.0	40.0	80.0	100.0
June	29	6.9	6.9	37.9	37.9
July	12	0.0	0.0	0.0	0.0
August	25	72.0	64.0	80.0	84.0
September	39	69.2	71.8	51.3	82.1
October	13	76.9	76.9	76.9	100.0
Mean	20.5	50.4	47.2	52.8	66.7

The number of successfully amplified STR markers and with that the overall genotyping success rates indicated a temporal, seasonal pattern. Numbers were notoriously low in June and July, but the trajectories across months were not statistically significant (**Figure 6** and **7a**). However, with the success rate of each sample (**Table A1**) plotted according to the day it was collected, a significant seasonal pattern throughout the study period and year was visible (**Figure A1**). Trends for outside and inside sampling were quite similar and slope for the pieces-samples taken on silica was slightly flatter across the sampling period. Also, the average number and trajectory of accepted genotypes overall and if all methods combined, increased significantly from May to

September (**Figure A2**). Success rates of all samples and per method (**Table A1**) were not normally distributed (*p*<.001; Kolmogorov-Smirnov-test). Comparison of these results, outside sampling location and inside sampling location, did not differ statistically (*p*=.17; Wilcoxon-test; see also **Figure 6**). When testing success rates per month for outside and inside sampling location, both did not differ from normal distribution (outside: *p*=.46, inside: *p*=.76; Kolmogorov-Smirnov-test). The comparison of the trajectory of the success rates of both techniques throughout the study period and per month, did not differ statistically (*p*=.34; t-test; see **Figure 7a**). Although not statistically comparable, the trajectory of the samples collected as piece and put on silica, displayed a similar trend throughout the period, with the exception of more genotypes identified in June but less in September, compared to the outside and inside sampling (**Figure 7b**). Overall, all three methods combined showed a similar pattern of successful genotyping, with all samples successfully genotyped in May and October; and over 80% in August and September (**Figure 7c**). Samples collected in June and July showed the lowest number of accepted genotypes (**Figure 7c** and **7d**).

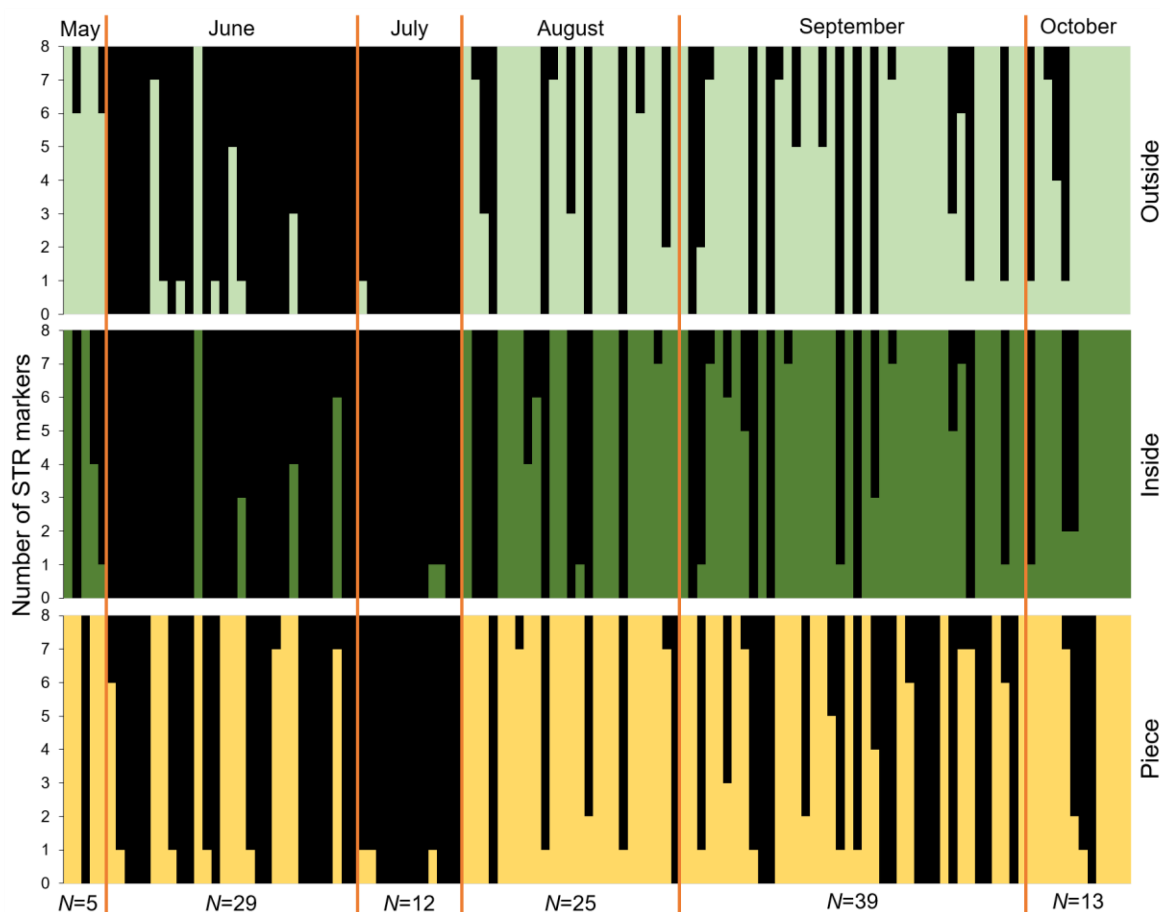

***Figure 6**. Genotyping success: number of amplified STR-markers from each feces sample (each coloured bar) throughout the study period and the three applied sampling methods: outside swabbing (top), inside swabbing (middle) and collecting a piece (bottom). Number of not amplified markers per sample (bar) are in black.*

A clear seasonal pattern was also the occurrence of feces shapes: while samples originated mainly from solid feces in May, June and July, the proportion shifted in August and the majority of feces were mainly liquid in September and October ($b=17.68$ *(CI 3.69, 31.68)*, $R^2=0.69$, $p<.05$; **Figure 8a**). A similar pattern emerged when looking at the trajectory of the feces condition: while

the proportion of dry samples decreased from 100% in May to zero in September and October (*p=.056*), the proportion of moist feces increased from zero in May to 100% in October (*b=19.95 (CI 9.18, 30.71),* R^2*=0.84, p<.01*). Half dry samples occurred mainly in June, peaked in July at 67%, and August (*p*=.62; **Figure 8b**). Results on the number of successfully amplified STR markers as well as the overall success rate of liquid and solid samples did not differ between outside and inside sampling of the feces; 5.4 to 5.5 STR-markers and 70-72% success for liquid samples, and 3.0 to 2.3 STR markers, 41 to 30% for solid samples (**Figure 8c** and **8d**). Also, results from samples taking a piece on silica appeared slightly lower for liquid samples while slightly higher for solid samples. But differences of the results were not significant (**Figures 8c** and **8d**). A similar picture emerged when comparing the results based on feces condition, with outside vs. inside sampling: 5.4 vs. 5.6 STR markers; 70 vs. 72% for moist samples; 2.8 to 2.2; 37 to 30% for half-dry samples and, 3.5 vs. 2.1 STR markers; 48 vs. 28% for dry samples. Also here, DNA-analysis of a piece on silica showed higher success for the dry samples with 4.5 STR markers (58%) amplified (**Figure 8e** and **8d**).

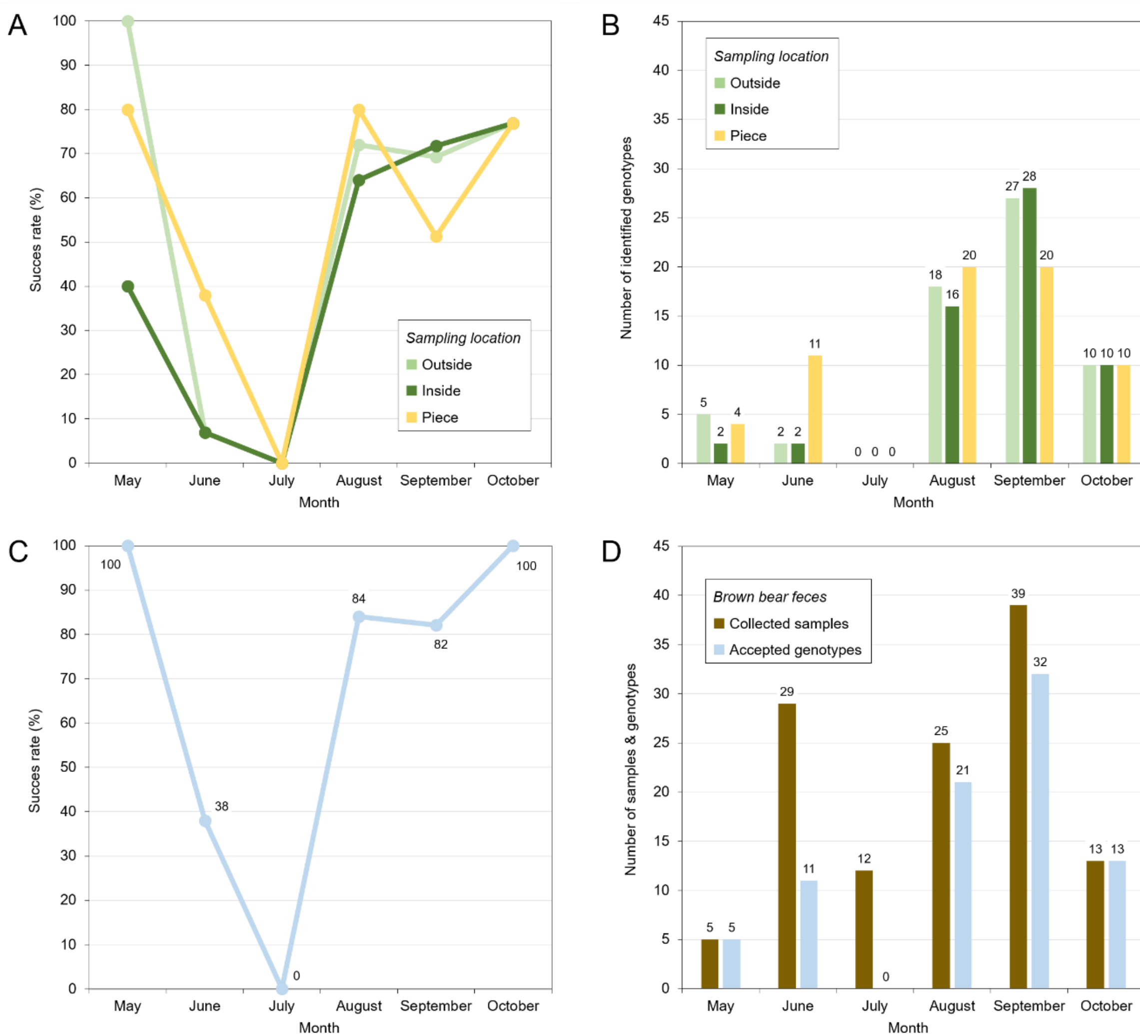

Figure 7. *(a) Relationship between sampling month and the genotyping success rate for outside (p=.72) and inside swabbing (p=.12), as well as for collection of a piece on silica (p=.74) from 123 brown bear feces samples in 2020 and (b) the number of accepted genotypes. Further, (c) the overall genotyping success (p=.58) and (d) number of accepted genotypes from all methods combined.*

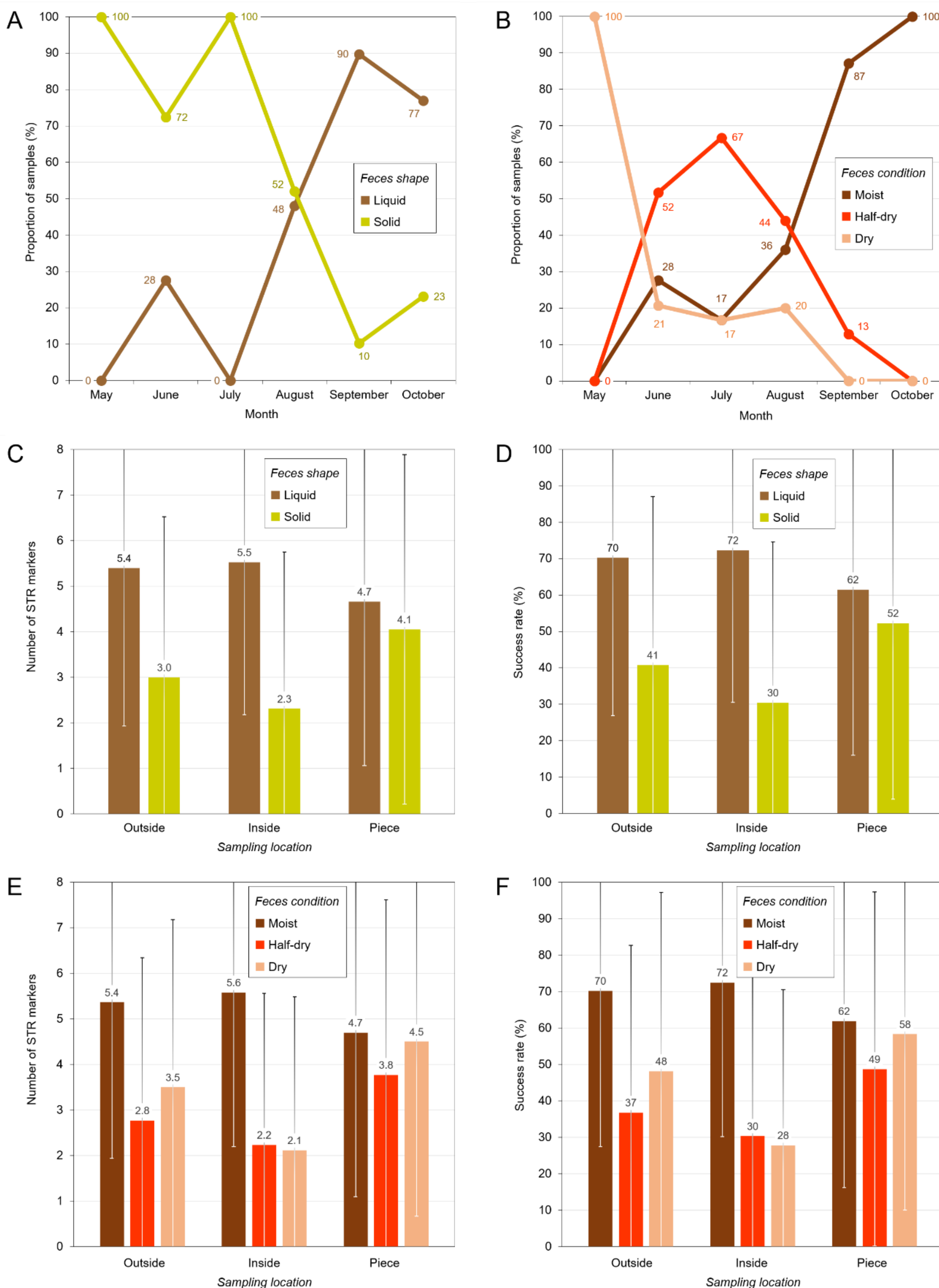

***Figure 8**. Trajectories of shape, condition and genotyping success of 123 brown bear feces samples over the course of the study period: (a) Proportion of liquid shape solid shape, (b) proportion of the samples with the condition being dry and moist, half-dry; (C) number of successfully amplified STR markers per sampling location and shape and (D) their respective success rates; number of successfully amplified STR markers per sampling location and condition (E) and their respective success rates (F). Error bars show the standard deviation.*

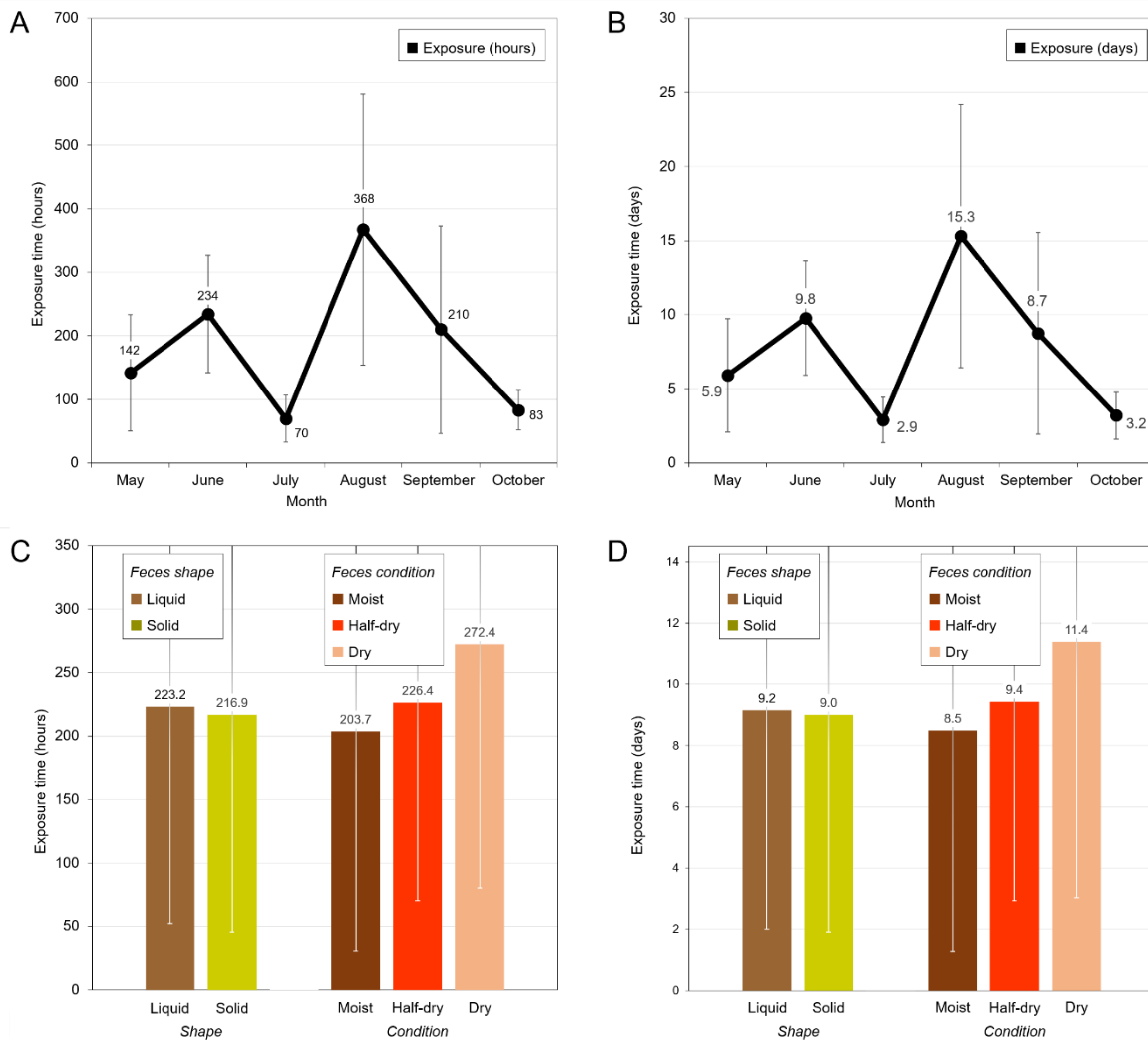

***Figure 9**. Exposure time of the collected feces across the study period in hours (A) and days (B), as well as average exposure time of feces according to their shape and condition in hours (C) and days (D). Error bars show the standard deviation.*

Maximum exposure time could be calculated for 120 collected feces. For three feces, the exact date and time when the bear was entering the area were not available. Overall, the maximum exposure time or age of feces which were exposed in the field were 7.6 (±4.7) days or 184.4 (±111.3) hours (**Table A2**). Exposure time of the feces was longest in August (15.3 (±8.9) days or 367.6 (±213.5) hours) and shortest in July (2.9 (±1.5) days or 69.7 (±36.8) hours (**Figure 9a** and **9b**). Feces of both different shape and condition had similar average exposure time of liquid (9.2 (±7.2) days), solid (9 (±7.1) days), moist (8.5 (±7.2) days) and half-dry (9.4 (±6.5) days), but slightly longer for dry samples (11.4 (±8.4) days); see results, including exposure times for hours in **Figure 9a** and **9b**. Genotyping success rates per sample did not correlate with the age of the sample, i.e. exposure time. Also, the exposure time did not correlate with the day (of the year) of sampling. We did not find relationships between results and the number of samples collected.

4 Discussion

The DNA-success rates from the outside-swabbing of the 123 different feces were slightly higher (avg. 50.4%) than from the inside-swabbing (avg. 47.2%). Overall, genotyping success showed large variation for all methods applied and thus led to large values of standard deviation. The success rates for both methods were not statistically different (**Table 1**, **Figure 6**). A comparable study, reported statistically significant difference between outside and inside swabbing and an average genotyping success of 59% outside and 48% from the inside of 25 brown bear feces (Stenglein et al. 2010). The difference in our study was much smaller. We consider our larger sample size more representative, at least for Scandinavia. It is likely that the margin between the success rates of both sampling methods decreases with increasing number of samples and when more data is collected. Wultsch et al. (2015) also reported higher success rates of outside versus inside sampling of jaguars and other Neotropical felids feces as well as Bourgeois et al. (2015) on forest elephants. However, these studies were conducted in different areas of the world, northern Italy (Stenglein et al. 2010), Belize (Wultsch et al. 2015) and Gabon (Bourgeois et al. 2019) and therefore may not be directly comparable to brown bears in Scandinavia, especially the results from regions of Central America or Central Africa and with the conditions described in Wultsch et al. (2015) and Bourgeois et al. (2019). Rutledge et al. (2009) reported that fresh swabbing of *Canis lupus* and *Canis lycaon* scats in Ontario, Canada, improved success and efficiency. However, also here, results cannot be directly compared as there are substantial differences in diet and thus feces content between *Canidae* and brown bear, and, also environmental conditions are also not directly comparable. Variations in genotyping success of the same target species but from different regions can deliver different genotyping success rates (Murphy et al. 2007). Although our results were not significant, there appears to be a tendency that the outside swabbing of fresh scats can increase success rates, especially on liquid and moist samples in Scandinavia (**Figure 7**). Although a direct, statistical comparison among the swab sampling and the sampling of a piece on silica was not feasible due to different DNA-extraction techniques, the overall genotyping success was similar (**Table 1**, **Figure 7**), but varied throughout the year.

A distinct seasonal pattern (**Figure 6**) in genotyping success was visible when plotting each sample in accordance to the day of the year it was sampled (**Figure A1**), while the display of seasonality appeared less pronounced when averaged over the months of the study period (**Figure 7a**). Across the study period, the success rates of especially the outside and inside sampling location increased significantly until autumn. Success for pieces sampled on silica also showed a similar trajectory, however, the overall trend was less distinct and showed a tendency of a more evenly distributed success rate across the year. All methods provided much lower success rates in June and July compared to May, August, September and October. The results may underline challenges to DNA-preservation in feces during these two months. The only factor considered in this study, possibly related to the challenge in our study, is the sudden increase of half-dry samples in June and July. However, a high proportion of feces were half-dry in August as well, but DNA-analyses still resulted in higher success rates compared to June and July. These results, however, may also be influenced by the sudden shift from solid to liquid samples during August (**Figure 6**). Genotyping success of pieces collected in May and June showed slightly higher success in spring (May, June) and lower in autumn (August, September, October; **Figure A1**). The results indicated that for May, June and July, where samples appeared to be rather solid and dry to half-dry, a piece collected on silica may lead to higher genotyping success. On the other hand, the highest genotyping success for liquid and moist samples, collected mainly in August, September and October, were provided by the swabbing methods. All results considered, there was a tendency of outside sampling being overall more successful than sampling the inside or a piece on silica (**Figure A1**, **7a** and **8c-f**). The differences among genotyping success of the methods applied seemed nuanced, but may also be dependent on overall annual conditions and especially other factors such as weather conditions, which were not considered in the frame of this pilot study. Likely more data over consecutive years would be required for comprehensive testing and evaluation of the results as well as possible interannual variations.

Liquid or moist feces samples usually pose a challenge for studies conducted in more temperate regions and more humid environmental conditions. Therefore, these studies recorded higher rate of success using dry samples or samples from dry environments and thus recommended to conduct sampling during dry and possible cold seasons or periods (Farrell et al. 2000, Maudet et al. 2004, Murphy et al. 2007, Brinkman et al. 2009). In contrast to these studies, genotyping liquid samples appeared not to be the major challenge in our pilot assessment. The success we measured on liquid and moist samples was evident, especially when applying outside-swabbing during autumn, when temperatures drop and sun light diminishes.

Exposure time can have substantial, negative effect on the genotyping success of feces samples (see e.g. Farrell et al. 2000, Friedberg 2003, Goossens et al. 2000, Lucchini et al. 2002, Nsubuga et al. 2004, Murphy et al. 2007, Vynne et al. 2012, DeMay et al. 2013, Skrbinšek 2020). However, according to our results, estimated sample age did not have any significant, direct effect on a sample's genotyping success and neither on the shape and condition of the feces sample. Other studies also reported large variances of success among samples, independently of exposure time (Piggott 2004, Wehausen et al. 2004, Santini et al. 2007). Apparently, sampling periods and age of the samples vary over different studies and it may be likely that prompt sampling of fresh feces, as conducted in our study, was in time and quick enough to avoid possible, major negative effects on the genotyping effort. Also, this result is in line with the findings of our previous project, in which the time of feces samples - even when collected in different storage systems - being on transit 2 to 21 days to the laboratory, had no detectable or negative effect on their genotyping success (Kopatz et al. 2020). However, one has to interpret these findings with caution. Even though we could not identify a direct influence, exposure time potentially confounds with other factors and affects the integrity of the feces and DNA. Also, the fact that we did not find any direct effect of the exposure time on the feces shape and condition, indeed shows that other factors may influence such characteristics, such as the local weather, habitat and landscape conditions. For instance, constant shade provided by e.g. vegetation, protecting a feces from direct sun light will help to preserve e.g. moisture and liquidness for longer, and in combination with cooler temperatures, can also avoid the feces to desiccate. The opposite seems to occur for feces that were e.g. dropped on open grass or hillsides (Murphy et al. 2007). Here, direct ultraviolet light and rainfall can have harmful effect on scat's integrity and the DNA-molecule (Brinkman et al. 2009).

Based on earlier studies and our results, it seems likely that the time of exposure combined with the diet content and actual temperature during the actual sampling followed by the characteristics of the storage medium have substantial influence on the long-term genotyping success of feces samples (Murphy et al. 2002, Nsubuga et al. 2004, Murphy et al. 2007). This is supported by the findings that genotyping success rates can drop drastically after a specific period of time of the specimen in the field, e.g. one month (Murphy et al. 2002). Feces samples may contain sufficient amount of DNA for successful genotyping even after being exposed for 60 days to the environment, however, the time of sampling (exposure time) can affect their genotyping success drastically: amplification success can decrease significantly after the first two days in the open (Murphy et al. 2007, Skrbinšek 2020). Our results and the results from other studies on sample age and storage time of feces appeared so far ambivalent and warrant further investigation. Based on our current knowledge, one should apply caution when using feces shape and condition as a proxy to estimate the age of a sample. The application is, however, also dependent on the landscape. A more homogenous landscape may allow for visual identification of the age of a scat (Piggott 2004, Skrbinšek 2020).

The clear seasonal pattern on genotyping success rate identified in our study might be explained by the diet (Panasci et al. 2011, Lonsinger et al. 2015a, Gulsby et al. 2016). Although all major food sources are utilized by brown bears throughout the year, the volumes consumed can differ substantially depending on the season and availability. Brown bears in central Sweden were reported to mainly consume moose (*Alces alces*), ants (*Formica* spp. and *Camponotus herculeanus)* and graminoids during spring and summer, and berries, mainly bilberry (*Vaccinium myrtillus)*, crowberry (*Empetrum hermaphoditum)* and lingonberry (*Vaccinium vitis-idaea)*, were the

major food items in autumn. Berries from the previous year were also consumed during spring (Stenset et al. 2016). Differences in food items can cause variation in DNA amplification and genotyping success of feces as different food types vary in digestion speed and hence alter contact between fecal matter and the intestinal membranes, but likely also influence the amount of inhibitors (see e.g. Murphy et al. 2003, Maudet et al. 2004, Elfström et al. 2013, Lonsinger et al. 2015b). However, even with information on the diet, reasons for variation in the results on genotyping success can remain ambiguous (Broquet et al. 2006).

The likelihood that we collected samples from feces of the wrong target species can be considered as highly unlikely as our staff was trained personnel with many years of experience in wildlife research and collecting brown bear feces (Prugh & Ritland 2005). Our sampling was further closely connected to the locations of GPS-marked animals. We used samples with an average age of roughly eight days (**Table A1**), which is well within the recommended period for feces sample collection made by other studies (see e.g. Murphy et al. 2007). Further, the amplification of non-target DNA, e.g. from other species seems also unlikely as most of the genetic markers are species specific and, especially for the outside-swabbing, mainly sloughed intestinal cells from our target species, brown bear, were collected (Rutledge et al. 2009). We used a high-salted sample preservation solution which has demonstrated in earlier studies to perform equally well to high percentage ethanol, and has therefore been recommended for DNA-based assessments on wildlife feces (see e.g. Seutin et al. 1991, Kohn & Wayne 1997, Reed et al. 1997, Murphy et al. 2002, Murphy et al. 2003, Panasci et al. 2011, Tende et al. 2014).

We can summarize that the genotyping success of feces appears to be influenced by a convoluted interplay of multiple factors which are challenging to disentangle in order to be able to understand how these affect each other and the genotyping success. Although multiple studies suggested similar effects of the same factors, other studies on the other hand, remained inconclusive. It appears as generalization should be done with caution as the effects appear to alter the sample material in multiple, confounding ways. To our knowledge, this is one of the first studies on brown bear assessing sampling location, feces sample age and characteristics in a natural environment and where the movement of individual brown bears was tracked. We encourage further studies looking into the interactive system of feces, DNA and tracking of environmental conditions.

Implications for the management

The majority of feces samples in Scandinavia are collected and stored using silica filled (Norway) or empty tubes (Sweden) during the period August to October, supported by substantial contribution of volunteers. This seems also to be the most efficient time period applying the current method and our results do not warrant a change of the current applied practice. However, based on the findings, the application of outside swabbing could be discussed and evaluated further. While sampling pieces of the feces on silica seems to deliver slightly better results on dry samples, moist and liquid feces could be sampled by outside-swabbing to increase DNA-yield; at least by professional personnel. Sampling should nevertheless be done as soon as possible to minimize exposure time of the specimen, and with that, to increase likelihood for positive genotyping success.

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7 Appendices

Table A1*. Results of the different sampling location within 123 different brown bear feces from Sweden, collected from May to October 2020 on the number of successfully amplified microsatellite markers and success rate.*

		Number of STR markers			Success rate (%)		
Sample	**Month**	**Outside**	**Inside**	**Piece**	**Outside**	**Inside**	**Piece**
1	May	8	8	8	100.0	100.0	100.0
2	May	6	0	8	100.0	0.0	100.0
3	May	8	8	0	100.0	100.0	0.0
4	May	8	4	8	100.0	66.7	100.0
5	May	6	1	8	100.0	16.7	100.0
6	June	0	0	6	0.0	0.0	100.0
7	June	0	0	1	0.0	0.0	16.7
8	June	0	0	0	0.0	0.0	0.0
9	June	0	0	0	0.0	0.0	0.0
10	June	0	0	0	0.0	0.0	0.0
11	June	7	0	8	100.0	0.0	100.0
12	June	1	0	8	16.7	0.0	100.0
13	June	0	0	1	0.0	0.0	16.7
14	June	1	0	0	16.7	0.0	0.0
15	June	0	0	0	0.0	0.0	0.0
16	June	8	8	8	100.0	100.0	100.0
17	June	0	0	1	0.0	0.0	16.7
18	June	1	0	0	16.7	0.0	0.0
19	June	0	0	8	0.0	0.0	100.0
20	June	5	0	8	83.3	0.0	100.0
21	June	1	3	8	16.7	50.0	100.0
22	June	0	0	1	0.0	0.0	16.7
23	June	0	0	0	0.0	0.0	0.0
24	June	0	0	0	0.0	0.0	0.0
25	June	0	0	7	0.0	0.0	100.0
26	June	0	0	8	0.0	0.0	100.0
27	June	3	4	8	50.0	66.7	100.0
28	June	0	0	0	0.0	0.0	0.0
29	June	0	0	0	0.0	0.0	0.0
30	June	0	0	0	0.0	0.0	0.0
31	June	0	0	0	0.0	0.0	0.0
32	June	0	6	7	0.0	100.0	100.0
33	June	0	0	0	0.0	0.0	0.0
34	June	0	0	0	0.0	0.0	0.0
35	July	1	0	1	16.7	0.0	16.7
36	July	0	0	1	0.0	0.0	16.7
37	July	0	0	0	0.0	0.0	0.0
38	July	0	0	0	0.0	0.0	0.0
39	July	0	0	0	0.0	0.0	0.0
40	July	0	0	0	0.0	0.0	0.0
41	July	0	0	0	0.0	0.0	0.0
42	July	0	0	0	0.0	0.0	0.0

		Number of STR markers			Success rate (%)		
Sample	**Month**	**Outside**	**Inside**	**Piece**	**Outside**	**Inside**	**Piece**
43	July	0	1	1	0.0	16.7	16.7
44	July	0	1	0	0.0	16.7	0.0
45	July	0	0	0	0.0	0.0	0.0
46	July	0	0	0	0.0	0.0	0.0
47	August	8	8	8	100.0	100.0	100.0
48	August	7	0	8	100.0	0.0	100.0
49	August	3	0	8	50.0	0.0	100.0
50	August	0	0	0	0.0	0.0	0.0
51	August	8	8	8	100.0	100.0	100.0
52	August	8	8	8	100.0	100.0	100.0
53	August	8	8	7	100.0	100.0	100.0
54	August	8	4	8	100.0	66.7	100.0
55	August	8	6	8	100.0	100.0	100.0
56	August	0	0	1	0.0	0.0	16.7
57	August	7	8	8	100.0	100.0	100.0
58	August	8	8	8	100.0	100.0	100.0
59	August	3	0	8	50.0	0.0	100.0
60	August	8	1	8	100.0	16.7	100.0
61	August	0	0	2	0.0	0.0	33.3
62	August	8	8	8	100.0	100.0	100.0
63	August	8	8	8	100.0	100.0	100.0
64	August	8	8	8	100.0	100.0	100.0
65	August	0	0	1	0.0	0.0	16.7
66	August	8	8	8	100.0	100.0	100.0
67	August	6	8	8	100.0	100.0	100.0
68	August	8	8	8	100.0	100.0	100.0
69	August	8	7	8	100.0	100.0	100.0
70	August	2	8	7	33.3	100.0	100.0
71	August	8	8	0	100.0	100.0	0.0
72	September	8	8	8	100.0	100.0	100.0
73	September	0	0	8	0.0	0.0	100.0
74	September	2	1	1	33.3	16.7	16.7
75	September	7	7	8	100.0	100.0	100.0
76	September	8	8	8	100.0	100.0	100.0
77	September	8	6	3	100.0	100.0	50.0
78	September	8	8	8	100.0	100.0	100.0
79	September	8	5	7	100.0	83.3	100.0
80	September	0	0	1	0.0	0.0	16.7
81	September	8	8	0	100.0	100.0	0.0
82	September	0	0	0	0.0	0.0	0.0
83	September	7	8	8	100.0	100.0	100.0
84	September	8	7	8	100.0	100.0	100.0
85	September	5	8	8	83.3	100.0	100.0
86	September	8	8	2	100.0	100.0	33.3
87	September	8	8	8	100.0	100.0	100.0
88	September	5	8	8	83.3	100.0	100.0
89	September	8	8	5	100.0	100.0	83.3
90	September	0	1	1	0.0	16.7	16.7

Sample	Month	Number of STR markers Outside	Inside	Piece	Success rate (%) Outside	Inside	Piece
91	September	8	8	8	100.0	100.0	100.0
92	September	0	0	1	0.0	0.0	16.7
93	September	8	8	8	100.0	100.0	100.0
94	September	0	3	4	0.0	50.0	66.7
95	September	8	8	0	100.0	100.0	0.0
96	September	7	7	0	100.0	100.0	0.0
97	September	8	8	8	100.0	100.0	100.0
98	September	8	8	6	100.0	100.0	100.0
99	September	8	8	0	100.0	100.0	0.0
100	September	8	8	0	100.0	100.0	0.0
101	September	8	8	0	100.0	100.0	0.0
102	September	8	8	8	100.0	100.0	100.0
103	September	3	5	0	50.0	83.3	0.0
104	September	6	7	7	100.0	100.0	100.0
105	September	1	0	7	16.7	0.0	100.0
106	September	8	8	0	100.0	100.0	0.0
107	September	8	8	0	100.0	100.0	0.0
108	September	8	8	8	100.0	100.0	100.0
109	September	1	1	6	16.7	16.7	100.0
110	September	8	8	0	100.0	100.0	0.0
111	October	8	8	8	100.0	100.0	100.0
112	October	1	1	8	16.7	16.7	100.0
113	October	8	8	8	100.0	100.0	100.0
114	October	7	8	8	100.0	100.0	100.0
115	October	4	8	8	66.7	100.0	100.0
116	October	1	2	7	16.7	33.3	100.0
117	October	8	2	2	100.0	33.3	33.3
118	October	8	8	1	100.0	100.0	16.7
119	October	8	8	0	100.0	100.0	0.0
120	October	8	8	8	100.0	100.0	100.0
121	October	8	8	8	100.0	100.0	100.0
122	October	8	8	8	100.0	100.0	100.0
123	October	8	8	8	100.0	100.0	100.0

***Table A2**. Number of collected feces samples and approximately maximum exposure time of the collected feces, i.e. the time the marked individual brown bear entered the cluster until sample collection, per month in 2020.*

Month	*N*	Exposure Hours	Days
May	5	142.0	5.9
June	29	234.3	9.8
July	12	69.7	2.9
August	25	367.6	15.3
September	39	209.9	8.7
October	13	83.1	3.2
Mean	*20.5*	*184.4*	*7.6*
SD	*12.7*	*111.3*	*4.7*

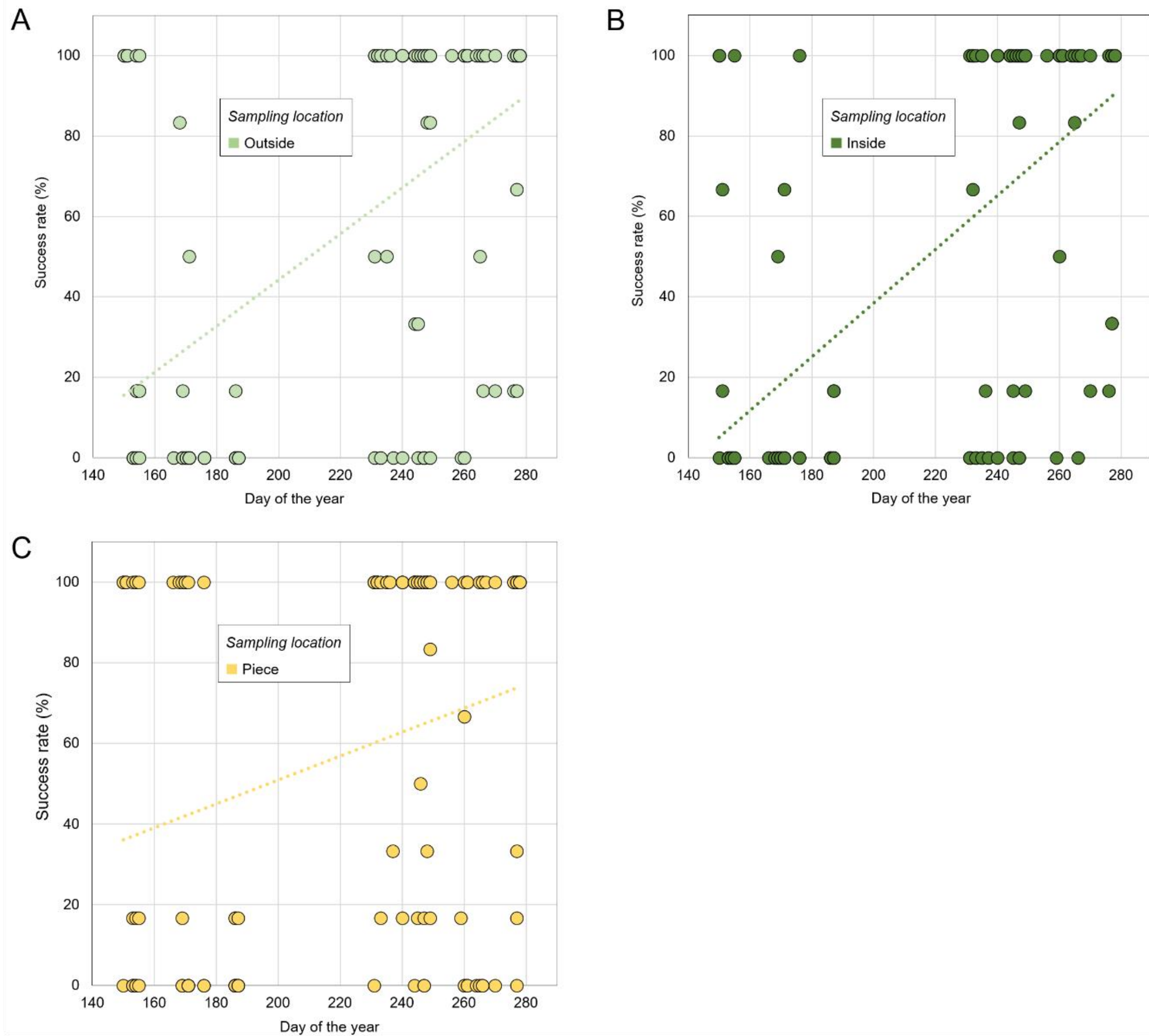

***Figure A1**. Every sample and its success rate and day of the year the brown bear feces was sampled from the outside, b=0.57 (CI 0.41, 0.74), R^2=0.28, p<.001 (A), inside, b=0.67 (CI 0.51, 0.82), R^2=0.37, p<.001 (B) and the additional collection of a piece on silica, b=0.30 (CI 0.11, 0.48), R^2=0.07, p<.01 (C).*

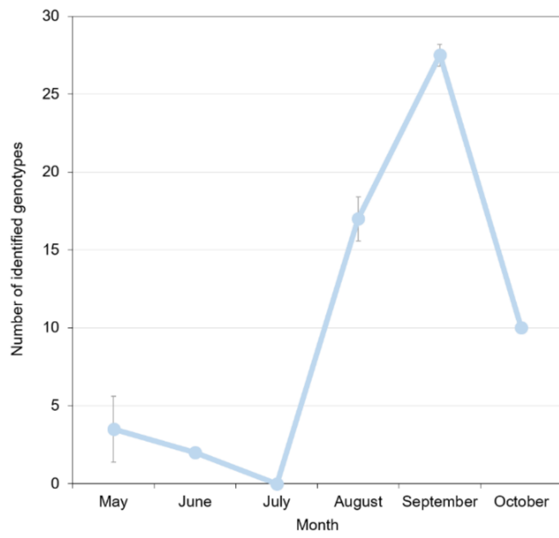

***Figure A2**. Average number of individual genotypes (incl. standard deviation) identified over all methods and study period.*

***Appendix A1**. Sampling instructions for the professional field personnel.*

INSTRUCTIONS FOR BROWN BEAR FECES SAMPLING

STUDY

Outside vs. inside vs. regular (silica) fecal sampling

Comparison of DNA extraction success from brown bear fecal samples when a) sampled from random spots and stored on silica, b) sampled from the inside (without silica, and stored in lysis buffer) and c) sampled from the outside (without silica, and stored on lysis buffer).

The goal of this project is to investigate two different sampling techniques specifically the spots (outside vs. inside in comparison to the current method, a random sample stored on silica) on the feces sample to potentially improve sample quality and DNA-yield per sample. Additionally, we would like to investigate the effect time has on the success when sampling from these spots. Here the Scandinavian Brown Bear Research Project offers a unique opportunity to collect such samples during the regular scat collection for diet analysis and then to assess the time when a particular, marked individual has been at the sampling location. Therefore, you can use the same sample numbers you assign for the samples collected for the diet analyses and simply add a letter indicating the spot where the sample was taken from (O, I or S; see below). We aim to collect a minimum of 100 samples from different feces (not different individuals necessary) throughout the season, from spring to autumn. The duration of the project is one season and results will be ready by the end of this year. The project is funded by the Norwegian Environment Agency.

EQUIPMENT NEEDED (and provided by us) for each feces sample found:

1

1. Two sterile swabs
2. Two tubes containing DNA lysis buffer
3. One tube containing silica
4. Three blank stickers to label the sampling tubes
5. One pair of rubber gloves
6. Field protocol forms of the "food monitoring with fecal samples"
7. Field manual (this document)

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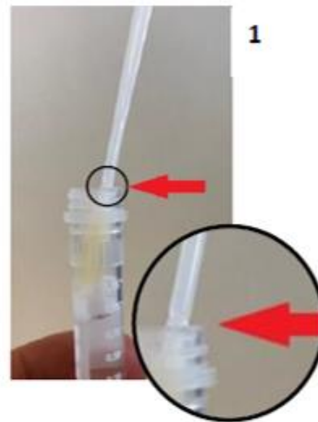
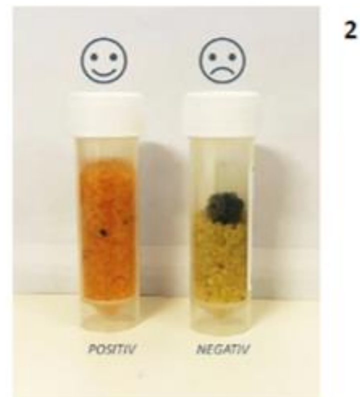
SAMPLE COLLECTION



1. **O-SAMPLE:** Take the **first sample** only **from the outside, the surface** of the scat (labelled with **"O" for outside** aside from the assigned sample number) using the provided swab. Swab the swab back and forth on the surface of the scat and do this on a large portion of the surface as possible (this to increase the probability of getting the bear's DNA). If the surface of the scat is dry, moist the swab with liquid from the tube with buffer. After you swabbed the scat, put the tip of the swab into a new **tube with lysis buffer** and crack the tip gently (that it did not spill) so that the tip falls into the tube (Figure 1). The picture illustrates the process and the predetermined breaking point is of the swab. Store the sample at room temperature (**Do not freeze!**).

2. **I-SAMPLE:** For the **second sample**, open up the middle of the scat to take another **sample from the inside** (labelled with **"I" for inside**). Once open, take a new swab and scrub into the inside of the scat with the swab. Moist it with the buffer before, if the scat is dry. Put the swab into a new **tube with lysis buffer**, as described above.



3. **S-SAMPLE**: The **third sample** you simply take a **pea size part of the feces** and put it into the provided tube containing the **silica** (Figure 2; and labelled with **"S" for silica**). This tube can also be stored in room temperature.

The overall rule of thumb is that **less is more**. If you swab the surface (O-samples) and also inside (I-samples) gently, it should be sufficient. Try not to put too much sample material into the sample tubes. E.g. too much, especially moist material such as leaves, may be too much to keep the sample dry on silica, which then leads to a negative success of the sample (Figure 2, notice the different color of the silica pellets; those should stay orange).

SAMPLE STORAGE

Store the collected samples in room temperature, but if possible, in a dark, dry and cool room, until shipment to the laboratory. We suggest to send us the samples when you have collected, in batches of 50 samples.

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The Norwegian Institute for Nature Research, NINA,
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