



Report on monitoring brown bears using non-invasive DNA sampling in the Romanian Carpathians

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Chapter 1

Introduction



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Monitoring large carnivore population sizes and trends is a prerequisite for conservation and management decisions, especially when it comes to highly controversial species such as large carnivores (Ryman *et al.*, 1981). Science-based monitoring schemes on large carnivores should be the foundation of a sustainable decision-making process. This enables implementation of conflict mitigation measures that reinforce the coexistence between humans and large carnivores without destabilizing their long term population viability (Artelle *et al.*, 2014; König *et al.*, 2020). Large carnivores are strictly protected species in the human-dominated landscape of Europe, but their strict protection is often perceived as a burden by the local communities, with the negative attitudes directly related with the number of conflicts (Trajçe *et al.*, 2019). The coexistence between local communities and large carnivores in Europe varies over time and space from acceptance to severe conflict. This applies for the brown bear (*Ursus arctos*), an opportunistic feeder known to cause substantial damages to livestock and crops (Schwartz, Swenson & Miller, 2003; Bautista *et al.*, 2017), and lack of science-based data significantly contributes to suboptimal management decisions in certain parts of the species range (Artelle *et al.*, 2014).

The Romanian Carpathians represent one of the last places in Europe where the entire large mammal community is present in high abundance, with bears co-occurring in the same landscape with Eurasian lynx and wolves (Rozyłowicz *et al.*, 2011). Although it can be considered a good example of a viable bear population in the long term (Zedrosser *et al.*, 2000), the Romanian bear population is surprisingly understudied. Since these bears are facing new threats in the rapidly changing Carpathian environment, there is a clear need for decision making based on scientific knowledge (see Penteriani *et al.*, 2019), management facing new challenges when addressing coexistence (Hartel *et al.*, 2019). Climate change is probably changing bear behaviour in the Carpathians, with denning already being documented as sensitive to climatic variations elsewhere (Delgado *et al.*, 2018), while the shifts in phenology of the plants that form the bulk of the bear diet have impacted the species' food habits elsewhere (Pereira *et al.*, 2021). Bojarska *et al.*, (2019) documented "winter insomnia" as a consequence of intense supplementary feeding during the milder and milder winters. The Romanian bear population was subjected to recent and substantial management changes. A historical, nation-wide and intensive supplementary feeding scheme for trophy hunting purposes was abruptly replaced by reduced feeding after Romania banned trophy hunting in 2016. Then in the past few years, commercial feeding for wildlife watching purposes increased, none of the above decisions following a proper understanding of the effects on bears behaviour (Penteriani *et al.*, 2017, 2021). The expansion of humans into natural areas for recreation happens here much faster than in other countries with no data on potential bear behavioural responses (see Linnell *et al.*, 2010; Morales-González *et al.*, 2020). The development of transport infrastructure too will pose substantial impact on bear population as it will lead to more fragmentation (Fedorca *et al.*, 2019), leading to increased human mortality on the roads as well. Besides ensuring the species conservation status, science-based decision making is a requirement for ensuring coexistence with humans in the benefit of the local communities (König *et al.*, 2020). The current public debate in Romania revolves around the fact that the number of bears is increasing rapidly and exceeding the carrying capacity of the habitat. However, neither the population size nor the carrying capacity were ever quantitatively assessed, rendering the argument in the best case nonsensical and subjective. All these uncertainties contribute at decreasing the social acceptance.

Although population monitoring is a requirement for ensuring the species conservation status in a changing environment, and for a resilient management, science-based population monitoring is scarce in the Romanian Carpathians. Most of the recent research focused on habitat suitability in the context of chaotic habitat losses of the past decades (Roellig *et al.*, 2014; Pop *et al.*, 2018a; Cristescu *et al.*, 2019; Faure *et al.*, 2020; Iosif *et al.*, 2020). At regional scale, Popescu *et al.*, (2017) quantitatively estimated brown bear density, but based it on uncertain track measurements in the snow or mud. Given the complex nature of the problem, a long term monitoring scheme will require a quantitative and collaborative approach that involves stakeholders with different expertise (Melnycky *et al.*, 2021). At national level however, the Romanian bear population is still assessed using minimum counts, population size and structure never being assessed through a science-based scheme. Minimum counts inventories are less robust, their data rarely being enough for sustainable decision making (Linnell *et al.*, 1998). Moreover, Popescu *et al.* (2016) showed that the Romanian bear population growth, as revealed by the official data, is likely overestimated compared to other populations across the species range.

To clarify the uncertainties around brown bear management, the prerequisites are to estimate population size, to understand population trend over years and how it responds to different management decisions. To obtain robust population size estimates, one has to capture, mark and recapture a good fraction of individuals in a population and apply mark recapture statistical models to estimate the total population size (Williams, Nichols & Conroy, 2002; Pollock, 2000). Because physical capture and recapture of brown bear is time and cost intensive, dangerous, and induces stress in captured animals, non-invasive DNA sampling has emerged as

an effective method (Schwartz, Luikart & Waples, 2007). Although genotyping reliability used to be a serious issue in non-invasive DNA sampling, mostly because DNA is of very low quality and quantity in bear hair and scat samples, this have been largely resolved in the recent decades (Taberlet *et al.*, 1996, 1997, De Barba *et al.*, 2010). An additional important methodological step has been made recently through utilization of next generation sequencing (De Barba *et al.*, 2010). This approach reduces subjectivity in genotyping, increases the overall genotyping success, e.g., up to 88% for the Dinaric population of brown bear, and reduces costs as it allows for rapid processing of larger and larger batches of samples (Skrbinšek *et al.*, 2020). To estimate population size from genetic data, scientists have to address the bias induced by the edge effect (i.e., quantified as the exchange of individuals with the highly suitable habitat from outside of the established sampling area, individuals that were sampled occasionally as they entered the sampling area). The continuous forest ecosystem of the Romanian Carpathians is surrounded by a mosaic of human dominated landscape but with significant areas of natural vegetation. This mosaic is permeable to animals' movement, increases the edge effect and poses challenges in getting robust population estimates with mark recapture models (Hupman *et al.*, 2018), especially for species with high movement capabilities like brown bear (Keiter *et al.*, 2017).

In this study, we aimed to demonstrate the feasibility of non-invasive DNA sampling as a monitoring tool for the brown bear in a pilot area in Southern Romanian Carpathians, by using mark-recapture models to estimate density and local population size. We addressed the bias induced by the edge effect by calculating the effective sampling area and correcting the population parameters based on DNA sample-revealed movement of our studied bears. We also recommend study design improvements for future research and monitoring programs in the Romanian Carpathians to allow better interpretation of the multiple potential factors impacting bear population and their coexistence with humans.

Chapter 2

Sampling area



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The sampling area is situated in the Southern Carpathians, Romania, covering 1200 km² in the eastern corner of the Făgăraș Mountains, Piatra Craiului, Iezer-Păpușa and parts of Leaota Mountains. Ranging in altitude between 600 and 2400 m (Figure 1), it includes a national park (i.e., Piatra Craiului National Park), and overlaps with four Natura 2000 sites of community importance. Forests cover most of the area (62%), followed by a mosaic of urban-rural landscape and agriculture with significant areas of natural vegetation (22%), and alpine grasslands and subalpine shrubs (16%).

Deciduous, coniferous and mixed forests have now equal proportions (22, 21 and 19%). Spruce (*Picea abies*) and fir (*Abies alba*) dominate higher elevations. Mixed forests are dominated by beech-fir or beech-fir-spruce and cover mid slopes. Lower slopes are mostly covered by beech (*Fagus sylvatica*). Transitional woods and shrubs are dominated by *Pinus mugo* and *Vaccinium subsp.* Forest management historically replaced significant areas with spruce monocultures. In the last three decades, the area was affected by chaotic deforestation (Kuemmerle *et al.*, 2009). These clear-cuts are now regenerating into a young forest with abundant understorey vegetation, potentially providing food and shelter for wildlife. The mosaic of traditional agricultural habitats with significant areas of natural vegetation consists of patches of traditionally managed

hayfields, orchards and crops separated by dense forest edges and shrubs providing good connectivity with the compact forest patches. Although bisected by a high traffic national road (DN73) along which localities are distributed, the area is recognized as a corridor for large carnivores' dispersal, with no major barriers outside the mountain ranges. The road network is dominated by unpaved forest roads and temporary logging roads.

The large mammal community is still intact throughout the sampling area, and composed of the three European large carnivores, wolf (*Canis lupus*), brown bear (*Ursus arctos*), and Eurasian lynx (*Lynx lynx*), as well as their main prey wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and chamois (*Rupicapra rupicapra*) in the alpine areas. Hunting of large carnivores and chamois is banned while the rest of the ungulates are hunted regularly only in the northern part of the sampling area and limited to extraction of conflict animals (wild boar) in the southern part. Logging still remains to be an important economic activity and is executed year-round. Grazing is another source of human impact, especially in the alpine areas, whereas lowlands are characterized by small scale traditional farming and tourism development.

Wildlife management is organised into eight different game management units (GMU), four of which are administrated by CARPATHIA (a private conservation initiative for the Făgăraș Mountains, consisting of several legal entities), and the remaining four being under the control of three different hunting clubs. Collaboration, especially in the northern part, was positive, with some of the local hunters participating at sample collection. We also had a good collaboration with Piatra Craiului National Park Administration, park rangers too contributing to sample collection.

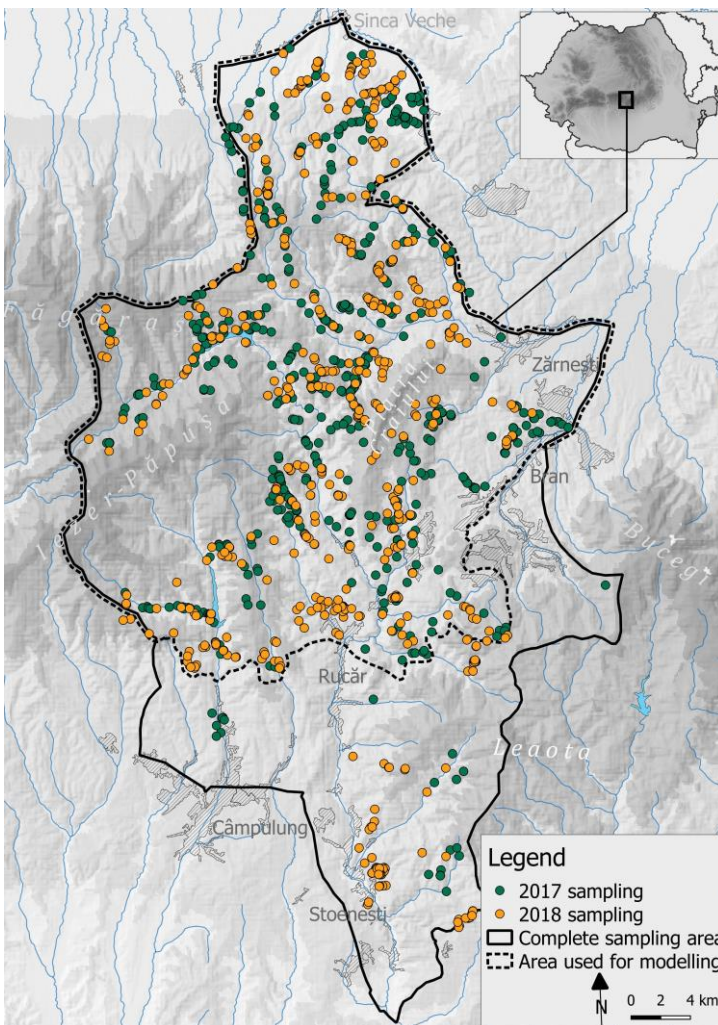


Figure 1. Study area for monitoring brown bear population using non-invasive DNA sampling in the Romanian Carpathians. The sampling took place between August and November 2017 and 2018 respectively. The complete sampling area covers approx. 1200 km² and we used continuous sampling to cover it in both sampling sessions. However, an area in the south we sampled with lower intensity, so we delineated a smaller area that we used for modelling brown bear population parameters. The area used for modelling has approx. 900 km² and had equal sampling effort across both sampling sessions.

Chapter 3

Methods



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3.1. Sampling design

We designed the sampling to be relatively short and before denning when females give birth, during the autumn hyperphagia period, to minimize the violation of the assumption that the sampled population would behave as demographically closed. Studies also show that this period provides samples with the highest analytical success rates (Skrbinšek, 2020). We collected non-invasive DNA samples in two distinct sampling sessions, in August-November 2017 and 2018 respectively. We used a continuous sampling approach proved useful for genetic monitoring of bear populations elsewhere (Skrbinšek *et al.*, 2010; Skrbinešek *et al.*, 2012). We did field trips prior to the sampling session to gain local knowledge on plant phenology forming the main bear diet and understand where the bears were located during our sampling sessions. We started with higher altitudes in August during the wild berries season and sampled the lower parts, orchards and croplands, later during the fruits season. Our continuous sampling had two components: *i.* the continuous intensive sampling with one field team of five wildlife rangers (the “wildlife team”) permanently and systematically involved into searching for bear samples, and *ii.* the continuous opportunistic sampling with 18 more rangers collecting bear samples opportunistically during their daily routine. Across the two sampling sessions, 70% of the samples we collected with the intensive approach and 30% with the opportunistic teams. The wildlife team covered mostly remote

areas, animal paths, ridges, upper valleys and the diversionary feeding points, as well as the fragmented human dominated landscape. The rest of the rangers collected mostly on logging roads during their daily routine in the sampling area.

Since the complete sampling area covers approx. 1200 km² we could not cover it equally with both intensive and opportunistic sampling. An area in the south we sampled with lower intensity and mostly during opportunistic field visits. For this reason, we delineated a smaller area that we used for modelling brown bear population parameters as it had equal sampling effort across both sessions and had a constant intensive / opportunistic sampling ratio between 2017 and 2018. The area used for modelling has approx. 900 km² (Figure 1).

Prior to the start of the sampling, we prepared genetic sampling kits for bear hair and scat samples. The hair kits consisted of a paper envelope closed in a Ziploc bag with 10 g of Silica gel. The scat kits consisted of 8 ml tubes filled with a DETs buffer designed to preserve DNA (Frantzen *et al.*, 1998). The scat kits also contained two wood sticks that we used to collect the samples from the surface of the scat and to mark the already collected scats in the field. The Ziploc bags were fitted with labels where we recorded the collector name, date, GPS coordinates and field-estimated age of the scat. Only samples subjectively estimated to be not more than 5 days old were collected (Skrbinšek *et al.*, 2010). All Ziploc bags, tubes and paper envelopes had stickers with unique numeric codes which were used to identify individual samples and connect the laboratory results with the field data.

We developed a data collection app for mobile devices that allowed us to collect the samples field data consistently and directly in the field, including in places with no GSM coverage by storing the location on the device until the upload option was available. We collected data on GPS location, habitat description, scat content, origin of a collected hair (i.e., from a rub tree, barbwire fence, etc.). We shipped the collected hair samples from the field to the genetic laboratory every month to avoid DNA degradation. We secured the scat samples in the DETs buffer and at -20°C until shipped to the genetic laboratory at the end of each sampling session.

3.2. Genetic analyses

DNA in non-invasive genetic samples is of very low quality and quantity, and contamination (especially with PCR products) is a serious issue. We used a dedicated laboratory for DNA extraction from non-invasive samples and PCR setup. The laboratory was also used for storage of samples and consumables. All downstream post-PCR stages (PCR, purification of libraries, storage of PCR products) were physically separated on the other side of the building. We enforced strict rules regarding movement of personnel, equipment and material to prevent contamination, and used negative controls throughout.

DNA extraction is a critical part of the genotyping process since it defines the reliability and success of the entire downstream analyses. We used a liquid handling robot (Hamilton Starlet) located in the “non-invasive genetics laboratory” to achieve reliable, error-free and fast DNA extraction. Besides speeding the analyses, the use of the liquid handling robot practically eliminated the possibility of a sample mix-up since all sample handling is done automatically, and sample IDs read and handled through barcodes.

We used the method described by De Barba *et al.*, (2016) for genotyping. The method taps the power of next generation (high-throughput) sequencing (hereafter NGS), solves problems that affected the “standard” approaches (difficulty to compare results between laboratories and subjectivity in genotyping), speeds up analyses and increases amplification success. The PCR conditions, primer sequences, tagging and pooling procedures are described in De Barba *et al.*, (2016).

We multiplex 13 microsatellite markers and a sex id marker in a single PCR. PCR products of all samples from all eight microplates and with all markers are pooled into a single tube (library), purified with a Minelute Purification kit (Qiagen), quantified on a Qbit instrument and sequenced on an Illumina HiSeq sequencer, resulting in approximately 10 million DNA sequence reads per library. Up to 12 or 13 libraries were analysed simultaneously in a single HiSeq run.

Once we obtained the sequences, we used bioinformatics tools to filter out sequences for individual samples and markers and identify individual alleles. We used the bioinformatics tools developed by De Barba *et al.*, (2016), but then programmed our own functions in R for allele calling. We also programmed functionality for management and visualization of these data into our laboratory database application (MisBase) that enabled us to rapidly visually check every genotype for accuracy.

We used a modified multi-tube approach (Taberlet *et al.*, 1996; Adams & Waits, 2007) with up to 8 re-amplifications of each sample according to the sample's quality and matching with other samples. In the first screening we did 4 parallel repeated genotyping runs of each sample. A consensus genotype was produced, and quality index (Miquel *et al.*, 2006) and maximum-likelihood reliability (Miller, Joyce & Waits, 2002) were calculated for each sample. Amplifying samples that needed additional analyses were analysed in additional 4 parallel genotyping runs, after which a decision was made to keep or discard a sample based on the genotype reliability and/or matching with other samples of the same animal.

3.3. Modelling regional population size

The minimum number of animals directly detected as the number of different observed genotypes, while useful, is rarely enough for management purposes. The information difficult to obtain and very much needed is the number of animals that we did not detect during the sampling, and hence the total number of animals in the sampled population. The population size estimate is obtained through mark recapture modelling. However, robust data is a prerequisite with mark recapture modelling. The first requirement is that there are enough recaptures to train the models (White & Burnham, 1999). The second requirement is for the data to reasonably fit model assumptions. In models designed for abundance estimates this usually means that the population must be demographically closed (no immigration/emigration, no births and no undetected deaths during sampling; White & Burnham, 1999). Another important assumption is that each animal has the same probability of being "captured" (in our case this means having its sample collected and successfully genotyped). While these assumptions are violated to a degree in each empirical study, the task of the researcher is to limit these violations as much as reasonably possible to obtain a valid result.

To assume statistical independence of bear captures, we removed the "autocorrelated" samples from further exploration and statistical modelling. We defined the "autocorrelated" samples as the samples collected by the same sampler on the same day and less than 0.5 km apart. With this approach we removed, for example, the samples collected at the same diversionary feeding place in the same day.

We used several mark recapture modelling approaches. We used the Capwire approach (Miller, Joyce & Waits, 2005). The Capwire models assume continuous sampling, which fits with how our data has been collected. An additional advantage of these models is that they are reasonably robust to capture heterogeneity. We also used the generalized linear model approach with the information-theoretic model selection (Burnham & Anderson, 2002) using program MARK (White & Burnham, 1999). We also used the Chao's Mh model (Chao, 1987), which should also be robust to capture heterogeneity and is robust in estimating the lower bound on abundance. We also tested the Darroch Mh (Mt) model (Baillargeon & Rivest, 2009). R package "Rcapture" was used to estimate these two models (Baillargeon & Rivest, 2009). See Appendix 1 for more details on the mark recapture models we used to obtain the regional population size.

3.4. Modelling local population size and density

The sampling area is demographically open without any significant physical obstacles to bear movement and with favourable bear habitat to the west, east and north (Figure 1). This means that the issue of edge effect must be taken into consideration (Royle *et al.*, 2014). It also means that the mark-recapture models describe a regional population of animals that may have a part (or much) of their home range outside of the sampling area, but wander into the sampling area enough that they can be sampled. We used the correction proposed by Wilson & Anderson, (1985) to correct for the edge effect and estimate of the “moment” population size, the number of bears expected in any given moment within the sampling area. We hereafter call it the local population size which alongside with its associated local population density are the main parameters of interest in this study.

Since independent movement data from GPS or VHF telemetry is not available for our bear population, we bootstrapped the moved distance from DNA sample-revealed movement, and corrected the local population estimates (Skrbinšek *et al.*, 2019). We used detected pairwise distances between locations of samples of the same animal to calculate W , the width of the buffer outside of the sampling area where the animals would have a non-negligible probability of being included in sampling. Because of expected differences in habitat use, W was calculated separately for each sex (Figure 2, Table 1). The detected pairwise distance between location of samples pooled across individuals differed significantly between sexes during both 2017 and 2018 ($U_{2017} = 8747$, $P = 0.004$; $U_{2018} = 11605$, $P < 0.001$). Males tend to have larger movements than females, the average pairwise distance was $4539.7 \text{ m} \pm 353.5 \text{ SE}$ for males and $3437.3 \text{ m} \pm 552.7$ for females in 2017, and $4015.3 \text{ m} \pm 257.8$ versus $2682.0 \text{ m} \pm 454.9$ in 2018. To obtain the local population size estimate, we used the A_s / A_t as the correction factor for our regional population size estimate, where A_s is the size of the sampling area, and A_t the total area including the edge buffer (Figure 2, Table 1).

Table 1. Area used for modelling evenly sampled between the two monitoring sessions, the $\frac{1}{2}$ MMDM buffered contributing areas and the edge-effect correction factors. The MMDM estimates have bootstrap-determined 95% confidence interval limits.

Parameter name	Unit	Parameter value	min	max
$\frac{1}{2}$ MMDM F	m	4428.3	3540.1	5316.4
$\frac{1}{2}$ MMDM M	m	6666.5	6225.9	7107.1
Area used for modelling	km ²	898.8	-	-
Males buffer	km ²	2029.8	1950.0	2110.4
Females buffer	km ²	1633.6	1482.6	1788.1
Males correction factor	-	0.443	0.461	0.426
Females correction factor	-	0.550	0.606	0.503

The limited size of the sampling area means that many longer “walks” would be undetected. To correct for this uncertainty, we simulated 100,000 random walks that started at the random location within the area used for modelling (with equal sampling effort across both sampling sessions) and got random length between 0 and 75 km. Then we checked the proportion of walks of certain length that would be detected (would end within the area used for modelling). This proportion was then used as a weight to calculate mean maximum distance moved (MMDM), the parameter used to calculate the correction factors (Figure 2; Table 1). The correction was a buffer of ½ MMDM around the area used for modelling. We removed 1% of the longest and shortest walks as outliers, and bootstrapped the entire calculation of MMDM (by randomly resampling the entire empirical walk dataset with replacement) 1000 times to obtain a more reliable mean value for MMDM and to better understand uncertainty around that mean. Since there are considerable differences in movement between males and females, MMDM is calculated separately for each sex (Table 1). We calculated the final density estimates by dividing the local population size to the corrected effective sampling areas, separately for each sex.

Genetic data were prepared in our laboratory database (MisBase), which we used also to keep the record of the field data. The data were exported into QGIS software (QGIS Development Team) to determine spatial characteristics of each data point (inside/outside of the modelling area locations). All non-GIS analyses were run in R (R Development Core Team 2018), with the exception of the mark-recapture analysis in program MARK (White & Burnham, 1999).

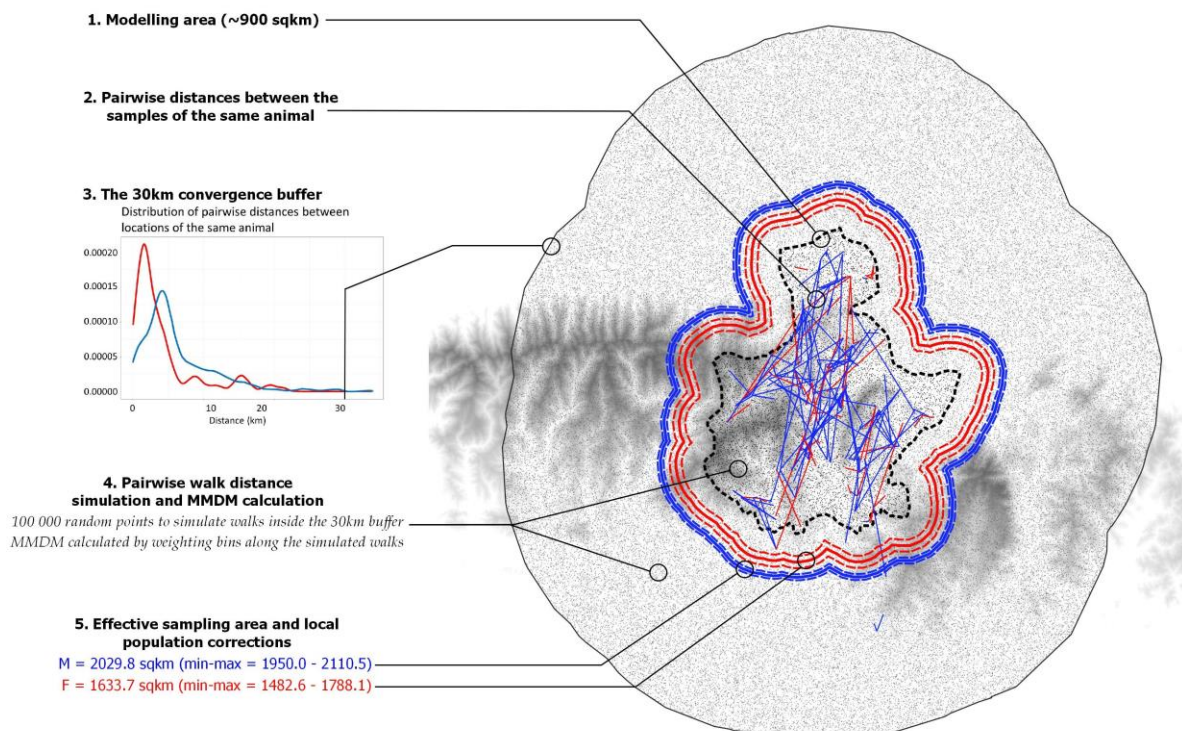


Figure 2. We applied correction factors separately for males and females, to transform regional population size into local population size and to estimate the local density. Within the area used for modelling (step 1) we reconstructed the pairwise distances between the samples of the same animal (step 2). The distribution of these distances converges to zero at around 30 km (step 3). The resulted 30 km edge strip was used to simulate 100 000 random walks. For calculating the MMDM, we binned the simulated walks into 500 m bins and weighted each walk by the number of bins inside (detected during sampling session) and outside of the area used for modelling (undetected during sampling session).

Chapter 4

Results



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4.1. Amplification success

From the total 1426 samples collected across the two sampling sessions, 63% were samples that gave reliable genotype or matched on many loci another reliable genotype (hereafter genotyped samples), 33% were samples that were of insufficient quality to provide a useful genotype (hereafter poor samples), and 4% were samples which returned mixed genotypes – where DNA of two or more individuals was collected in the same sample (hereafter mixed samples). Amplification success for the hair samples collected at the rub trees was comparable between 2017 and 2018 with 70.4 and 71.0%. Amplification success for the scat samples dropped from 2017 to 2018 from 63.5 to 54.4%. Amplification success by sample type is detailed in Table 2.

Mixed genotypes were mostly detected in the hair samples, with 10.3% in 2017 and 3.4% in 2018 (Table 2). This is not unexpected since we often collected more than 1 hair with each sample at the rub trees, where different individuals might rub on top of each other during a short time. The problem with poor sample (DNA) quality is expected in non-invasive samples, which are exposed to the environment and DNA degradation, and is the key issue affecting the amplification success. The loss of useful samples because of that was higher in the scat samples, especially in the 2018 sampling (Table 2). Given the lower amplification success than

expected for scat samples in 2018, we further explored possible factors affecting the success rates. In 2017 the sample age (range = 1 - 5 days) estimated in the field explained the amplification success well, with scat amplification success decreasing from 77.9 to 33.9% from 1-day to 5-days old samples. In 2018 however, the relation to field-estimated sample age is not clear, with 5-days old samples having higher average amplification success than 3-days old samples (Appendix 2).

Table 2. Amplification success by sample type during the 2017 and 2018 sampling sessions. There are three types of samples: i. 'genotyped' refers to samples that gave reliable genotype or matched on many loci another reliable genotype, ii. 'mixed' refers to samples which returned mixed genotypes, and iii. 'poor' refers to samples that were of insufficient quality to provide a useful genotype.

Session	Sample type	N genotyped	N mixed	N poor	Total	% genotyped	% mixed	% poor
2017	hair	157	32	43	223	70.4	10.3	19.3
	scat	321	1	183	505	63.5	0.2	36.3
2018	hair	209	10	75	294	71.1	3.4	25.5
	scat	220	1	183	404	54.5	0.3	45.2

To better understand the drop in scat amplification success in 2018, we explored the influence of the food items found in the scat and the month of sampling. We were not able to find a clear effect of any of these factors. The details are presented in Appendix 2.

4.2. Detected individuals

In 2017 we identified 184 unique genotypes, 87 females and 97 males. In 2018 we identified 163 unique genotypes, 65 females and 98 males. The genotype overlap between the two monitoring sessions was 33.1%, when looking at the modelling area with comparable sampling effort (16 females and 45 males found in 2017 were recaptured in 2018 as well, 17.9% and 47.3% respectively). When looking at the complete sampling area, across the two sessions, we identified a total number of 283 unique genotypes, 137 females and 146 males (Figure 3 and 4).

When looking at the modelling area with comparable sampling effort, our genotype dataset revealed a sex-capture bias with a lower capture probability for females. In the 2017 sampling session, sex capture probability in hair samples was biased towards males with the probability of capturing males being 0.78 while for females being only of 0.22. The sex bias is less pronounced in scat samples, with the probability of capturing females increasing to 0.42 in 2017 and to 0.39 in 2018.

After removing the autocorrelated samples, we were left with 430 samples in 2017 and 307 in 2018 within the area used for modelling (Figure 3 - 300 scat and 130 hair samples in 2017, and 161 versus 146 samples in 2018). The maximum number of recaptures we recorded per individual was 7 amongst females and 13 amongst males in 2017, and 5 versus 16 in 2018. The number of days between the first and the last time an animal was "seen" in our dataset ranged up to 455 days for male EK.18YL and up to 430 days for female EK.1A1Y. When looking at the individuals recaptured across both sessions, some individuals had clustered

recaptures on the map, others appear to move long distances while others had only scattered recaptures (Figure 5).

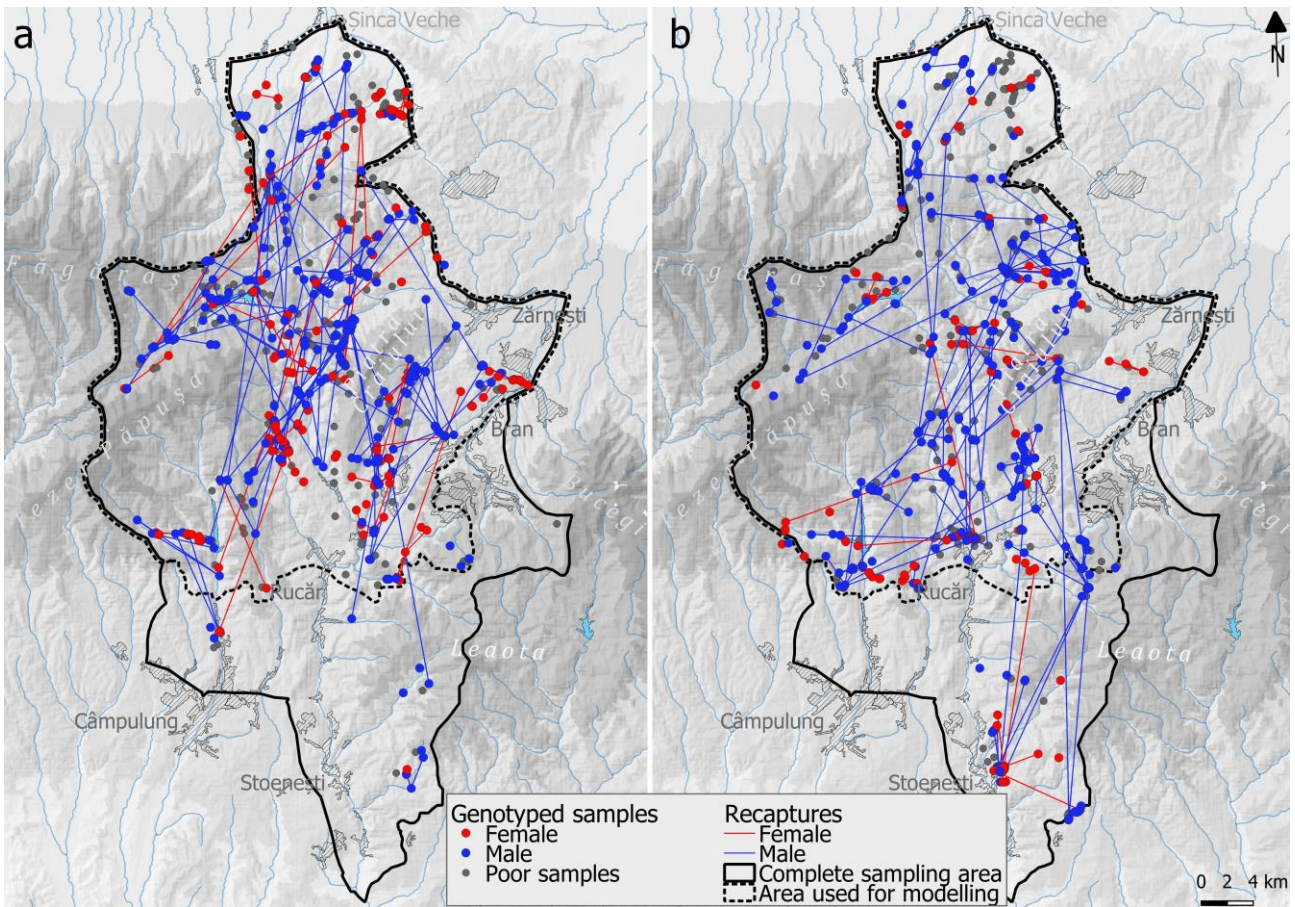


Figure 3. Genotyped brown bear samples and recaptures of individual genotypes. Panel (a) shows the 2017 sampling session and panel (b) shows the 2018 sampling session. The complete sampling area covers approx. 1200 km² and we used continuous sampling to cover it in both sampling sessions. However, an area in the south we sampled with lower intensity, so we delineated a smaller area that we used for modelling brown bear population parameters. The area used for modelling has approx. 900 km² and had equal sampling effort across both sessions.

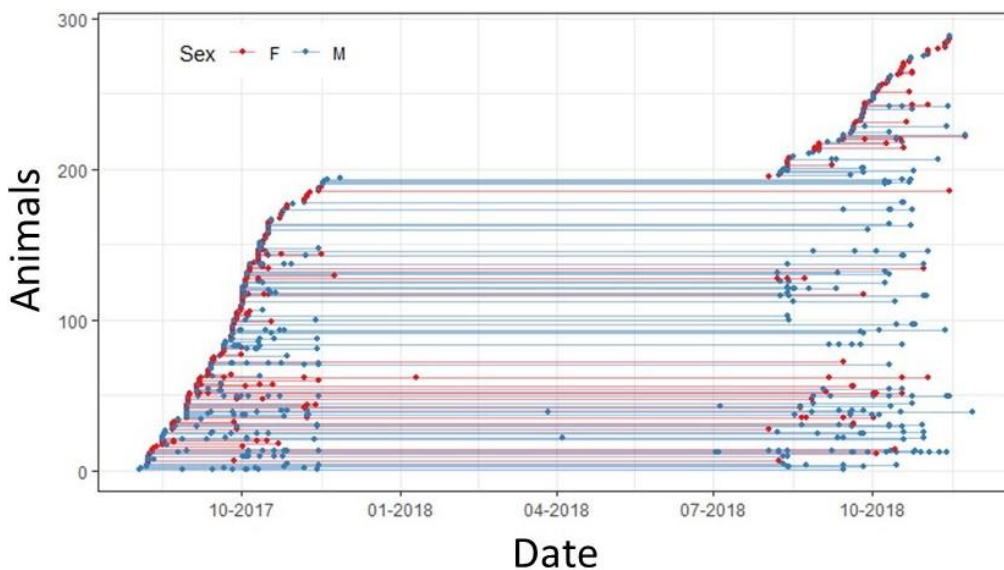


Figure 4. Capture mark recapture saturation graph showing all identified genotypes and their recaptures across both 2017 and 2018 sampling sessions and within the complete sampling area. Each point is a genotyped sample, each line an individual animal (coloured by sex). We identified a total of 283 individuals, 137 females and 146 males.

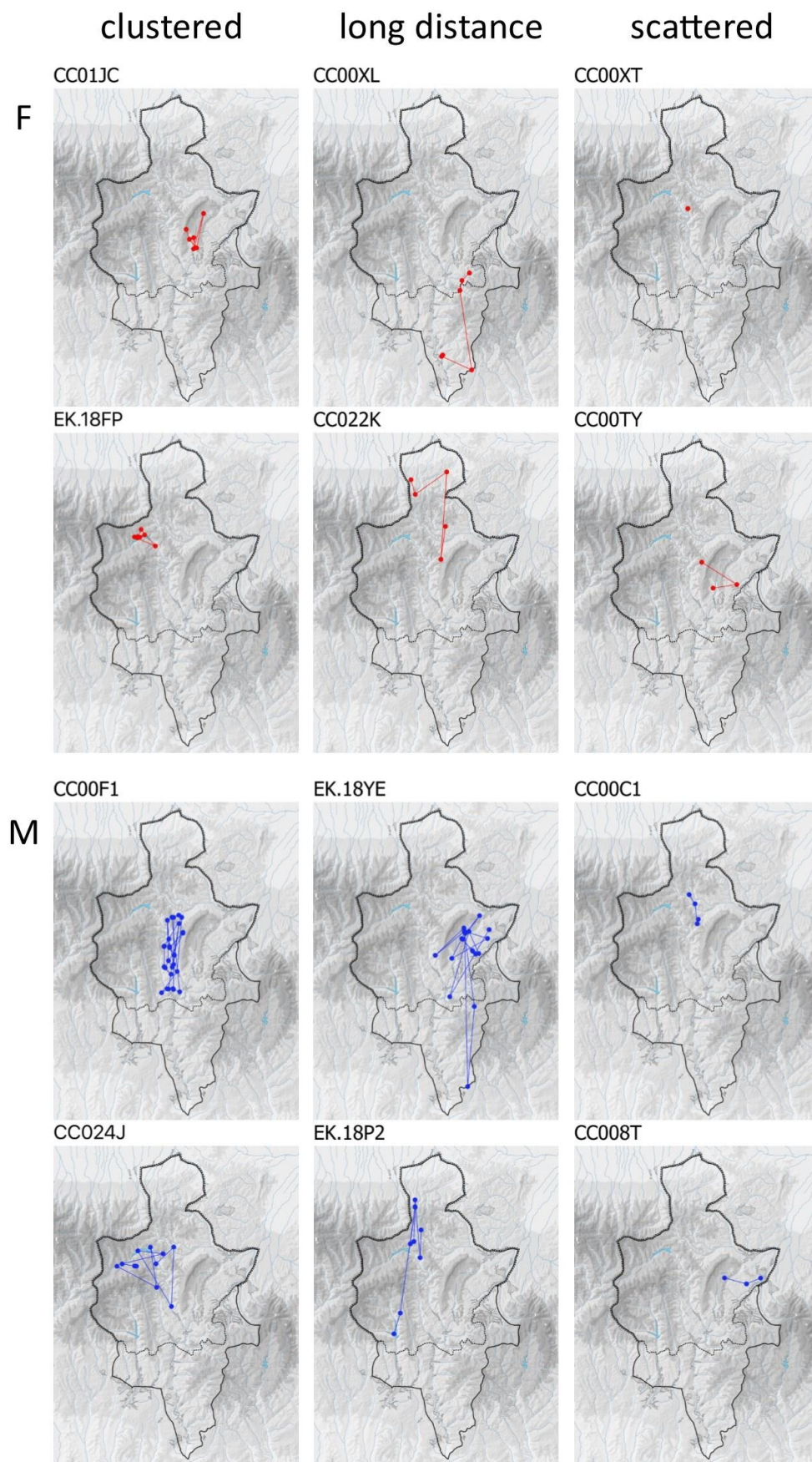


Figure 5. Examples of sample-revealed movement patterns of individuals recaptured across both 2017 and 2018 sampling sessions.

4.3. Population size and density estimates

With the autocorrelated samples removed, in 2017 we recaptured the females used for modelling 1.64 times \pm 0.11 SE, while the males we recaptured 2.71 times \pm 0.24 SE. Similarly, in 2018 we recaptured the females used for modelling 1.64 times \pm 0.15 SE, while the males we recaptured 2.75 times \pm 0.33 SE (Figure 6). The capture mark recapture saturation graph shows that males used for modelling start saturating (approaching the asymptote with less and less new animals detected) after three months of sampling while we still detected many new females towards the end of both sampling sessions (Figure 6).

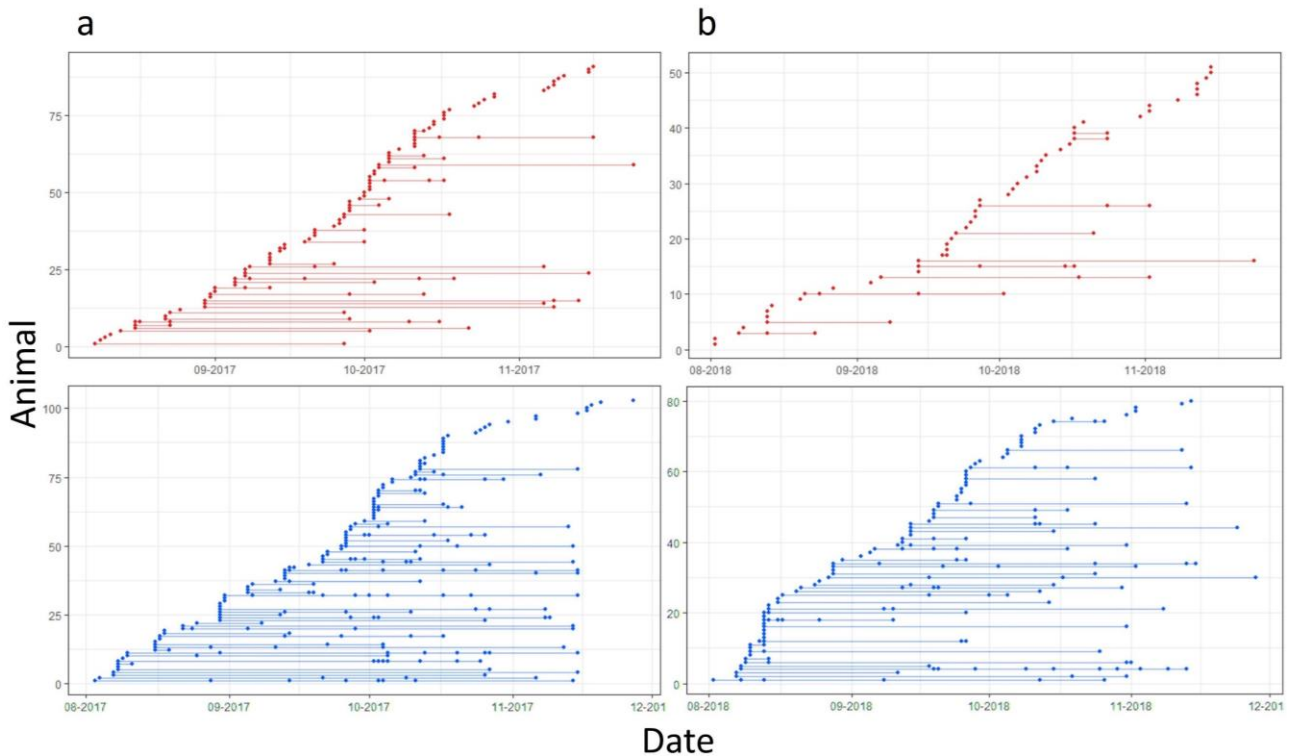


Figure 6. Capture mark recapture saturation graphs showing the genotypes used for modelling brown bear population parameters. These genotypes were detected and recaptured in the area used for modelling with similar sampling effort between 2017 (panel a) and 2018 sampling (panel b). Females are coloured in red and males in blue.

Different mark recapture models returned similar regional abundances (Figure 7; Appendix 1) but we calculated the final population parameters based on the Capwire TIRM model which assumes capture heterogeneity and was shown to be robust with smaller datasets. Males have comparable predictions in both sampling sessions with similar and robust CIs, i.e., 148 in 2017 (95%CI = 135 - 168) and 121 in 2018 (112 - 143) (Table 3). Since the capture probability for females in 2018 was lower (Appendix 1), we extrapolated the 2018 estimates for females, and the estimates for all individuals subsequently, from the 2018 estimates for males by keeping the sex ratio as estimated in 2017. This makes an implicit assumption that the sex ratio did not change in one year, which seems reasonable. The total number of bears for 2018 was obtained as the sum of males and females estimates, taking into account the additional uncertainty introduced by the extrapolation for females (Table 3, Appendix 1). The females estimate for 2017 was 185 (170 - 250) and the extrapolated number of females for 2018 was 150 (113 - 265), driving the regional abundance to 312 (303 - 398) in 2017 and to 271 (225 - 408) in 2018 (Table 3, Figure 7).

The $\frac{1}{2}$ MMDM calculation was 4428.3 m for females (min-max = 3540.1 - 5316.4 m) and 6666.5 m for males (6225.9 - 7107.1 m). This estimated the effective sampling area for the regional abundance at 2029.9 km² for males (1950.0 - 2110.4 km²) and at 1633.7 km² for females (1482.6 - 1788.1 km²; Map from the methods). The resulting correction factor for the local population size was 0.443 (min = 0.461, max = 0.426) for males and of 0.550 (min = 0.606, max = 0.503) for females. After accounting for the edge effect in our population, we estimated the local population size at 168 bears in 2017 and at 160 bears in 2018 (95%CI = 165 - 197 and

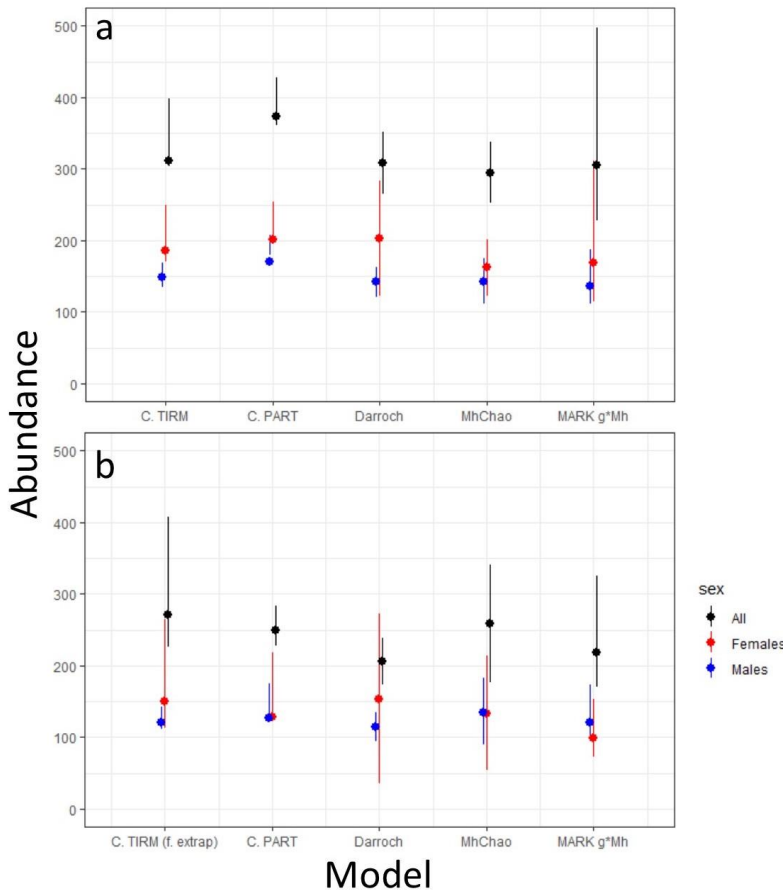


Figure 7. Mark recapture models used to estimate the regional abundance of bears in the studied population. Panel (a) shows comparison between different models in 2017 and panel (b) in 2018 sampling session. We used several mark-recapture modelling approaches. We used the Capwire approach with the R-package Capwire, with C. TIRM and C. PART referring to the Capwire class models. We used the robust models that include capture heterogeneity such as Chao's Mh model and Darroch implemented in R package Rcapture. We also used the generalized linear model approach with the information-theoretic model selection as applied in program MARK. Note that the 2018 sampling session shows the result from the female extrapolated scenario of the Capwire TIRM model - C.TIRM (f. extrapol). We extrapolated the Capwire TIRM 2018 predictions for females and for all individuals from the estimate for males by keeping the same sex ratio as in 2017, under the reasonable assumption that the sex ratio did not changed in our population during one year.

138 - 231 respectively; Table 3). The number of males and females are detailed in Table 3. The local density, a population parameter comparable with other project areas, we estimated at 18.66 bears / 100 km² in 2017 (18.39 - 21.94) and at 17.76 (15.40 - 25.74) in 2018 (Table 3). Derived sex ratio, another population parameter that can be compared with other project areas, is

weighted towards females (e.g., 7.34 males versus 11.32 females / 100 km² in 2017; Table 3).

Table 3. Population parameters for a brown bear population in Southern Carpathians, Romania, as derived from Capwire TIRM models. The 2018 results show a females extrapolated model, a model in which we extrapolated the Capwire TIRM 2018 predictions for females and for all individuals from the estimate for males, assuming the same sex ratio as in 2017. The regional abundance is predicted without acknowledging the edge effect in our population living in an area without natural or artificial boundaries for dispersal. In this respect we applied correction factors, separately for males and females, to transform regional abundance into local population size and to estimate the local density (see Methods).

Sampling Session / Model	Parameter	N	Cid	Ciu
2017	Regional abundance	312	303	398
2017	Regional abund. Males	149	135	168
2017	Regional abund. Females	185	170	250
2018 (females extrapolated)	Regional abundance	271	225	408
2018 (females extrapolated)	Regional abund. Males	121	112	143
2018 (females extrapolated)	Regional abund. Females	150	113	265
2017	Local Population Size	168	165	197
2017	Local Population Males	66	62	72
2017	Local Population Females	102	103	126

2018 (females extrapolated)	Local Population Size	160	138	231
2018 (females extrapolated)	Local Population Males	66	64	75
2018 (females extrapolated)	Local Population Females	93	74	156
Population Density [bears/100 km²]				
2017	Total Density	18.66	18.39	21.94
2017	Density Males	7.34	6.92	7.96
2017	Density Females	11.32	11.47	13.98
2018 (females extrapolated)	Total Density	17.76	15.4	25.74
2018 (females extrapolated)	Density Males	7.39	7.12	8.4
2018 (females extrapolated)	Density Females	10.37	8.28	17.34
Derived Sex Ratio				
2017	%Males	39.33%		
2017	%Females	60.67%		
2018 (females extrapolated)	%Males	41.60%		
2018 (females extrapolated)	%Females	58.40%		

Chapter 5

Discussion



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We provide a large scale science-based monitoring scheme to estimate brown bear population parameters in the Romanian Carpathians. For the first time in this part of the species range we provide objective population estimates using mark-recapture modelling and noninvasive genetic sampling. We obtained a good genotyping success >70% for the hair samples and a moderate success for the scat samples, i.e., 63% in 2017 which dropped to 54% in 2018. Both hair and scats had an amplification success comparable with other studies across species range such as for the bear populations in Greece (Tsaparis *et al.*, 2015), northern Italy (De Barba *et al.*, 2010), the Russian Far East (Latham *et al.*, 2012) or North America (Sawaya *et al.*, 2012), all studies using similar genetic protocols to extract DNA from non-invasive samples. The second session had a lower amplification success in scats even though the DNA extraction protocols were already streamlined for this species (Skrbinšek *et al.*, 2012). We explored possible interactions with factors that might have affected amplification success of scats, but did not find a statistically meaningful explanation. Such variations in the amplification success are not totally unexpected since environmental factors, storage and extraction methods may have an influence on faecal DNA studies (Waits & Paetkau, 2005). This result highlights the importance of doing pilot studies and calibrations of the genetic protocols until better results are obtained for a long term monitoring scheme (Skrbinšek *et al.*, 2019).

The lower amplification success of the scat samples and the high rate of hair samples in 2018 resulted in a lower recapture rate for females in that year. For this reason, different mark-recapture models had to be constructed for each sex, and we were not able to produce an acceptable direct estimate of the number of females in the 2018 session. Under the reasonable assumption that the sex ratio remained unchanged during the two years, we derived the 2018 estimate of females, and the total population size, from the number of males estimated in 2018 using the 2017 sex ratio. This approach increased the estimated uncertainty as it also included the uncertainty of the sex ratio estimate, and resulted in disproportionately larger CIs from one year to the next, but provided comparable estimates. Our DNA-revealed bear densities are 50% higher than bear densities obtained through integrating sign surveys and telemetry data in the Eastern Romanian Carpathians (Popescu *et al.*, 2017). Our densities are comparable with north American brown bear populations living in a similar habitat mosaic with different forest types and stand ages created by forest fires, logging, mining, energy exploration and development (Boulanger, Nielsen & Stenhouse, 2018). On the other hand, our density values are lower than the ones obtained in the Dinaric Mountains where a maximum of 40 bears / 100 km² was recorded even though the Slovenian bear population was subjected to a similar management scheme as the bears in Romania, with trophy hunting and intense supplementary feeding spanning over decades (Jerina *et al.*, 2013). However, our density values are significantly higher when compared to the Apennine or Pindos populations. Despite the strict protection there, habitat loss and fragmentation led to density values 4 times lower than in our sampling area (Ciucci *et al.*, 2015; Karamanlidis *et al.*, 2015). When compared to another biodiversity hotspot, the Caucasus, with a history of hunting and habitat loss, our densities are again 3 times higher (Burton *et al.*, 2018).

An interesting outcome of this study is the considerable difference between males and females capture probability. This particularly applies to hair samples, where capture probability of males is considerably larger than that of females. The capture probability difference between males and females is less pronounced in scat samples. In 2018 for example, the capture probability for females was 0.17 in hair samples and 0.39 in scat samples. The lower capture probability for females supports the idea that there are more females that remained undetected (reflected in the higher difference between the actually detected individuals and the number determined through mark recapture modelling) and this is visible on the mark recapture saturation graphs as well (Figure 6). We consider the sex bias in hair samples from the rub trees as not random, males rubbing behaviour contributing to this bias. Sawaya *et al.*, (2012) in a similar non-invasive DNA study, found that bear rubs had higher detection rates for male grizzlies compared to manufactured hair traps installed in the forest which detected more females. We can also speculate that the capture probability in hair traps is not random between individual males either, as it can be different for different age categories and/or social status. When it comes to the sex bias in scat samples this may be an artifact of the low genotyping success, unless it is affected by the behavioural differences between the sexes during sampling, e.g. more sampling in the areas that females avoid due to the permanent presence of males (Wielgus & Bunnell, 1994). For example, females with cubs of the year may avoid diversionary feeding points in the area because of male infanticide (personal observation, June 2021). More scat samples collected of males than of females can be also explained by the larger regional population for this sex caused by larger home ranges (Pop *et al.*, 2018b). While hair samples from rub trees are a useful source of samples and were important in this study, the main focus should be on collecting scat samples, as a high proportion of samples from rub trees can provide severely biased results and/or undersampling of females. A scat sample require less participation of the animal, and are less biased by sex, age and behaviour of different animals. This gives scat much better statistical properties for mark-recapture analyses, something to be aware of in future studies in the Romanian Carpathians.

Another interesting outcome of this study was that the genotype overlap between the two sampling sessions was only 33.1% when looking at the modelling area with comparable sampling effort (16 females and 45 males

found in 2017 were recaptured in 2018 as well, 17.9% and 47.3% respectively from the total number of captured females and males). The low overlap in the genotypes detected between the two consecutive years raises the question of where did a high proportion of the animals go in such a short time? The first explanation is that females are under sampled in 2018, their lower detectability causing a low overlap between genotypes of the two sampling sessions. However, the overlap of male genotypes can also be considered low. This low overlap of both sexes may also suggest a shift in the space use by brown bears in our sampling area. Latham *et al.*, (2012) suggested that shifts in seasonal habitat reduced bear capture probability despite the increases in sampling effort in the Russian Far East. Pop *et al.*, (2018a) applied resource selection functions on GPS collared bears in Romania and documented range shifts from one season to another with significant differences between females and males. Females consistently selected for mixed forest habitat during all seasons while males had a generalist approach, selecting between regenerating forest, mixed and coniferous forest stands (Pop *et al.*, 2018a). However, the sampling sessions were short in our case covering only hyperfagia season. This may suggest that brown bear population dynamics in our fragmented landscape are driven by changes in the food productivity from one year to another. McCall (2011) found similar explanations for a black bear *Ursus americanus* population monitored over four years in Idaho, USA. She found evidences of violating the geographic closure assumption due to temporary migrations on and off the study area in search for food with high production variability between years. Similarly, we hypothesize that in our sampling area, in 2018 the fruit production in the orchards at the interface with the forest habitat was by far lower than in 2017, probably determining the bears to concentrate in areas less accessible for sampling (e.g., alpine shrubs). A high mortality rate can be another explanation for the low genotype overlap between the two sampling sessions. Subadult mortality rate is expected to be high, documented at 30-40% in Japan for example (Shimozuru *et al.*, 2017). Since it's not possible to estimate an animal's age from its genotype, we cannot assess the number of subadults we sampled.

A particular challenge of our study is the very open sampling area, with the bear population extending its borders on all sides, particularly towards west, east and north. While the population closure test indicates that by having a short sampling season we managed to attain a reasonable demographic closure of the population, the obtained mark-recapture estimate actually applies to the wider area around the sampling area. Since males have larger home ranges than females (Pop *et al.*, 2018b), we estimated the correction separately for each sex. We observed the edge effect and the different capture probabilities between males and females have an interesting impact on the sex ratio. In a (until recently) trophy-hunted bear population, one can expect the sex ratio to be skewed in favour of females. However, in this study we counter-intuitively detected more males than females. But after obtaining the actual mark-recapture estimates and deriving the sex ratio from the calculated population densities, the estimates were skewed towards females, nearly identical to what was observed in Slovenia and Croatia (Skrbinšek *et al.*, 2019). A better way of dealing with this problem is through use of spatially explicit capture recapture (SECR) modelling. This class of models implicitly considers the edge effect in the estimates by reconstructing animals' activity centres (Royle *et al.*, 2014). Unfortunately, these models are designed for trap-grid type of study designs and are unsuitable for the data collected in our study (a combination of continuous-intensive and opportunistic sampling). For future sampling on Romanian brown bears, if the sampling area cannot be increased above 1000 km², we recommend recording sampling effort in space and using a grid study design to estimate local density through a SECR approach (López-Bao *et al.*, 2018). This approach will not necessarily provide significantly different estimates, but has proven to enhance the confidence intervals around the predictions (Boulanger *et al.*, 2018).

Management implications: our study provides a solid foundation for long-term monitoring of this species, while the technical experience gained here is certainly influencing brown bear monitoring in other pilot areas across the Carpathians. We provide here a first starting point to define the approximate sampling size per area

for future sampling. We strongly suggest the importance of increasing the effective sampling area as much as possible (to an extent of a major mountain unit with natural barriers) in order to deal with the edge effect and increase the robustness of the population predictions. We also suggest sampling according to plant phenology and bear movement and the importance of a spatially continuous sampling across the entire study area, rather than focusing on diversionary feeding sites only or on focusing on areas with road accessibility. By targeting the sampling effort only to accessible areas or the feeding sites, there is a major risk of missing the detection of a significant proportion of a population such as females with cubs of the year and creating capture heterogeneity, resulting in bias estimates that can have negative consequences in decision making. We suggest a higher proportion of scat samples instead of collecting hairs from rub trees, which have proved to be detection-biased towards males. However, scat collection poses higher risks for poor DNA material, which requires extra precautionary measures to avoid DNA degradation or contamination in the field stages. This highlights the importance of optimising the genetic protocols until better results are obtained for a long-term monitoring scheme. We suggest that this first robust genetic population study of brown bears in Romania and the non-invasive DNA monitoring scheme applied here have a potential for advancing species monitoring in the Romanian Carpathians, clarifying the uncertainties around official data on this species and eventually advancing species management towards improvement of humans-bears coexistence.

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