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Non-Invasive eDNA Reveals the Ecological and Genetic Status of the Western Capercaillie (*Tetrao urogallus aquitanicus*) in the Eastern Pyrenees

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ABSTRACT

Environmental changes and human activities in the Anthropocene era are likely to alter population size, structure, and genetic diversity of many species. Long-term monitoring is therefore essential to assess these impacts and enable informed conservation strategies for threatened species. The Western Capercaillie (*Tetrao urogallus*, L. 1758) is a mountainous species locally threatened by climate change, habitat fragmentation, and human disturbance. Traditional direct monitoring methods, such as lek counts, can be biased and cause disturbance, while molecular approaches are increasingly used for noninvasive population monitoring. Here, we focus on the eastern population of the subspecies *T. u. aquitanicus*, endemic to the Pyrenees, for which no molecular data have been generated so far. We conducted a molecular study based on 229 noninvasive samples (feces) to assess the ecological and genetic status of the local population in the Catalan nature reserves of the Pyrénées-Orientales (Occitanie region, France). At the individual level, we assessed multi-locus genotypes, sexing, inbreeding, stress level (Fecal Corticosterone Metabolites; FCMs) and diet. At the population level, we assessed sex ratio, genetic diversity and structure. We identified 62 individuals and estimated a population size of 79 individuals [95% CI = 68–92]. Of these, we identified 27 females, 18 males, and 17 individuals that we were unable to assign to a sex. Genetic diversity was low ($H_o = 0.279$, mean $H_e = 0.305$) and inbreeding coefficient ($G_{IS} = 0.085$) was significantly higher than expected under random mating at population level. FCM levels were lower in birds of areas considered as disturbed by humans and metabarcoding revealed a geographic structuring of diet at the local scale, with individuals feeding on only one or a few plant species. Our results demonstrate that molecular, noninvasive approaches provide critical ecological and genetic insights, establishing a baseline to support informed conservation management of this threatened population.

1 | Introduction

Mountain species face a dual challenge: the inherent difficulties posed by the topography, and the accelerating effects of climate

change, which periodically push them to the limits of their survival and reproduction capacities. Many cool-climate species have already shifted their range to higher altitudes—often the last areas that meet their ecological needs (McCarty 2001;

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Parmesan 2006; Walther et al. 2005; Willis and Whittaker 2000; Yousefi et al. 2015). Simultaneously, the rapid development of human infrastructures for outdoor recreation, livestock grazing, and timber industry has intensified habitat loss and fragmentation, reducing connectivity for numerous species (Banks-Leite et al. 2020; Wilson et al. 2016). By the late 2010s, nearly 60% of mountainous areas were affected by such pressures (Elsen et al. 2020; Nogués-Bravo et al. 2007; Rixen and Rolando 2013; Schmeller et al. 2022).

Mountain Galliformes are cold-adapted bird species that found refuge at northern latitudes and mountaintop habitats and are particularly threatened by current environmental changes and human activities (McGowan et al. 2012; Chamberlain et al. 2016). Among them, the Western Capercaillie (*Tetrao urogallus*, L. 1758, hereafter Capercaillie) is the largest Galliform found in the contiguous Boreal forests from Siberia to Fennoscandia. It also occurs, albeit in smaller and more fragmented habitats in the mountain ranges of Central and Western Europe, including the Balkans, the Carpathians, the Alps, the Jura, the Pyrenees, and the Cantabrian mountains (Duriez et al. 2007). Globally, the species is classified as “Least Concern” in Europe by the IUCN (BirdLife 2016). However, some local populations are considered threatened in Western, Central, and Southeastern European countries (Storch 2007). In France, recent assessments show that the Capercaillie is declining throughout its distribution range, with conservation status in the Jura and Vosges massifs being considered critical (Bal et al. 2021; Cayuela et al. 2021; Gil et al. 2020; Jiménez et al. 2022; Calenge et al. 2023). As with other parts of Europe, this decline has been mostly associated with destruction of forest habitat and intensive timber industry, excessive hunting, recreative outdoor activities, collisions with cables and fences, increased densities of meso-predators, and consequences of climate change (Alba et al. 2022; Jiménez et al. 2022; Mikoláš et al. 2017; Moss et al. 2001; Rodríguez-Muñoz et al. 2007; Segelbacher et al. 2008; Storch 1991). In winter, the main source of food is pine needles, resulting in a low energy balance for the birds (Leclercq and Menoni 2018). Human disturbance often causes birds to flee, but the low energy balance at this time of year can be fatal and can limit, or even prevent, the likelihood of breeding encounters in spring (Formenti et al. 2015; Rixen and Rolando 2013; Thiel et al. 2011). Simultaneously, climate warming may induce a shift in plant phenology (Inouye 2022) and a mismatch between the peaks of resource availability, in early-spring, and peak of energetic demands by chicks in late-spring (Moss et al. 2001).

Given its status as an umbrella species (i.e., its presence is associated with high diversity of other birds and mammals; Pakkala et al. 2003; Suter et al. 2002), the monitoring of the Capercaillie is a valuable tool to assess population trends and responses to environmental change, thereby supporting management actions.

Traditionally, population size has been estimated through counting at leks (Aleix-Mata et al. 2019, 2025), where males display collectively to attract females. However, this method often underestimates population size, as young non-territorial males can be overlooked (Aleix-Mata et al. 2019, 2025; Jacob et al. 2010; Mollet et al. 2015) as well as females which visit leks only briefly before returning to nesting sites, usually around 300 m from the lek site in the Pyrenees (Ménoni 1997). Thus, it is more likely to

observe organic clues deposited by the individuals (e.g., feathers, feces) than the individuals themselves.

In contrast, molecular approaches using noninvasive samples including feces or feathers deposited by wild individuals in the field as sources of environmental DNA (eDNA) provide a powerful alternative for monitoring elusive species and conduct genetic studies (Ruppert et al. 2019; Sahu et al. 2025; Taberlet and Luikart 1999). These methods have gained wide application in many species across plants and animals, including Capercaillie, allowing robust estimates of population size and sex ratio (Jacob et al. 2010; Mollet et al. 2015; Morán-Luis et al. 2014; Segelbacher et al. 2000, 2003, 2008; Zeni et al. 2024).

Beyond population size, genotyping from noninvasive material enables the estimation of Essential Biodiversity Variables (EBVs), such as genetic diversity, structure, and inbreeding, which are key to assessing population viability (Scott et al. 2020), and identifying both populations at highest conservation risk and potential sources for translocation or genetic rescue (Kyriazis et al. 2021; Supple and Shapiro 2018; Van Der Valk et al. 2019; Whiteley et al. 2015). Several mitochondrial and microsatellite markers, among others, have already been developed for Capercaillie (Jacob et al. 2010; Lucchini et al. 2001; Segelbacher et al. 2000), enabling various studies to be carried out on genetic diversity, habitat fragmentation and phylogeny (Cayuela et al. 2019, 2021; Jacob et al. 2010; Lucchini et al. 2001; Rutkowski et al. 2017; Segelbacher et al. 2003, 2008). These have revealed that boreal and central European populations suffer from low genetic diversity and inbreeding, raising significant concerns about their conservation status (Cayuela et al. 2019; Escoda et al. 2023; Homel et al. 2022; Jiménez et al. 2022; Segelbacher et al. 2008). This genetic vulnerability is partly driven by limited natal dispersal, particularly among males (Leclercq and Menoni 2018), which restricts gene flow between populations. Moreover, males exhibit strong philopatry—the tendency to remain in or return to their natal area (Höglund and Alatalo 1995)—resulting in kin-structured systems at leks (Cayuela et al. 2019; Escoda et al. 2023; Segelbacher et al. 2007). Such kin-structured leks reinforce local inbreeding and can reduce genetic diversity, thereby promoting inbreeding depression and loss of fitness due to increased homozygosity (Cayuela et al. 2019, 2021).

Additionally, the combination of noninvasive eDNA samples with Next Generation Sequencing approaches also provides the opportunity to gather supplementary ecological information about individuals such as diet (Ando et al. 2020; Cabodevilla et al. 2021; Ruppert et al. 2019). Noninvasive samples deposited by wild organisms contain material other than DNA, such as stress hormone metabolites, that can be quantified to evaluate the physiological status of host organisms in response to various stressors (Palme 2019). For instance, by measuring fecal corticosterone metabolites (FCMs), Thiel et al. (2011) established a positive correlation between the practice of outdoor recreative activities during winter and the physiological stress response of *T. urogallus* in the central Alps. However, there is no clear evidence that this stress response is associated with decreased fitness (Moss et al. 2014).

The putative subspecies *Tetrao urogallus aquitanicus* has been geographically isolated during the last Ice Age and is

now endemic to the Pyrenees mountains (Segelbacher and Pierné 2007; Clavero et al. 2024). This taxon is locally protected by nature conservation laws, nature reserves, and by the Natura 2000 framework (Bal et al. 2021). The size of this local population is estimated through periodical monitoring by national organisms, such as nature reserves, the French Office for Biodiversity (OFB), and the Observatory for Mountain Galliformes (OMG) (Calenge et al. 2023). In the Eastern Pyrenees, the Federation of Catalan Nature Reserves (FRNC) is a nonprofit organization that was founded in the 1980s to develop 10-year renewable plans for the management of suitable local habitats and conservation. A baseline size of the Western Capercaillie population was estimated at 57 individuals from data collected during lek counts carried out in 2014 in all known Capercaillie habitats (Pimenta et al. 2014). Despite the efforts engaged to monitor Capercaillie in Catalan nature reserves, methodological difficulties and biases associated with lek counts highlighted the need for complementary approaches (Aleix-Mata et al. 2019, 2025; Jacob et al. 2010; Mollet et al. 2015). Many ecological and evolutionary aspects of the local Capercaillie population remain unexplored, and no genetic studies have been carried out so far in this local area.

The present study aims at supplementing existing data from direct and rather invasive studies to better understand the ecological status of *T. u. aquitanicus* in the Eastern Pyrenees. To do so, we combined microsatellite genotyping, genetic sexing, metabarcoding, and FCMs quantification based on noninvasive samples (feces) collected in three nature reserves within FRNC. First (1), we aimed to estimate a minimum population size and sex ratio. In light of the known decline of the species, ongoing habitat fragmentation, and the effects of climate change on mountain ecosystems, we expected a small population size of *T. u. aquitanicus* in the Catalan nature reserves, potentially with a skewed sex ratio. Secondly (2), we sought to assess population-specific estimated breeding values (EBVs). Given the species' fragmented distribution, we hypothesized that the population would exhibit low genetic diversity and signs of inbreeding, resulting from reduced gene flow due to small population size, limited dispersal capacity, and geographical isolation. Additionally, we expected a higher inbreeding coefficient in males than in females, reflecting male philopatry and sex-biased dispersal. Thirdly (3), we compared individual levels of circulating FCMs across zones characterized by different levels of human disturbance (low, medium, and high). We anticipated that individuals living at more disturbed sites, particularly those affected by winter tourism, would exhibit higher stress levels. Finally, (4) we investigated interindividual variation in diet. As the study was conducted during winter—a period when food resources are limited—we expected the diet to be dominated by pine needles, with relatively low dietary diversity among individuals.

Such a noninvasive method provides a valuable source of information. Collectively, these data contribute to a more comprehensive understanding of the ecological and genetic status of *T. u. aquitanicus* in this region and provide a baseline for future monitoring and conservation strategies within the Catalan nature reserves. On a broader scale, our findings aim to support the conservation of Capercaillie populations throughout the Pyrenees.

2 | Materials and Methods

2.1 | Study Area and Field Survey

The territory of the Catalan nature reserves spans an area of 93.52 km² with diverse habitats, including high-altitude biomes such as willow stands, beech forests, and alpine meadows (see Data S1 for details). Noninvasive sampling of Capercaillie was carried out in the field between January and March 2022, by surveying all the known historical wintering spots ($n = 14$) distributed into three nature reserves (Figure 1A): the Py ($n = 6$), Mantet ($n = 6$), Prats-De-Mollo-La-Preste ($n = 1$) nature reserves and the frontier between Py and Mantet reserves ($n = 1$). Wintering spots correspond to places where capercaillies use to stay and forage in winter, when harsh conditions provoke a decrease in their movements in space. In the Pyrenees, population densities can be locally high because of the limited availability of such areas that are restricted to the surroundings of the few fir trees in the beech forest and at the edge of the mountain pine forest on the upper edge of the beech and fir forest. These wintering spots can be shared by several individuals although males and females tend to occupy distinct spots. As a consequence, it is difficult to accurately estimate the actual number of wintering spots in the Pyrenean context compared to other regions such as Jura and Vosges (Leclercq and Menoni 2018).

Teams of two experienced rangers per reserve searched for indirect evidence (footprints, isolated droppings, feathers) of the presence of Capercaillie. Sampling primarily targeted droppings found under roosting trees, in shelter areas, along forest edges, or on tree stumps (Bollmann et al. 2011; Graf et al. 2009), which are typical habitats during the wintering period. Droppings were collected on the snow during daytime surveys, by limiting as much as possible any disturbances, along vertical and horizontal transects following contour lines (each 5–10 m in elevation), in order to cover the whole of each wintering spot. A total of 229 droppings were collected (one dropping per GPS point, see Figure 1B; Py ($n = 141$), Mantet ($n = 82$), Prats-De-Mollo-La-Preste ($n = 6$)) and stored in collection tubes at -20°C until further processing. We collected the freshest droppings possible. Freshness was determined based on visual criteria (moisture, color, and absence of surface desiccation). GPS coordinates and date of collection were recorded for each sample. A muscle sample from a male individual was provided by Kevin Foulché (French Biodiversity Office, Orly city, Ariège 09 France, SAGIR no. 145327, autopsied the 07/06/2021) and was used as reference genotype and positive control for molecular genotyping.

2.2 | DNA Extraction, Molecular Sexing, and Genotyping

Total genomic DNA was extracted from droppings using three commercial DNA extraction kits specific for stool samples (EZNA Stool DNA Kit, Omega Bio-Tek Inc., Norcross, USA; QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany; NucleoSpin DNA Stool Mini Kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany) (See Data S2 for details). DNA concentration for each extract was quantified using a Nanodrop 2000 spectrophotometer

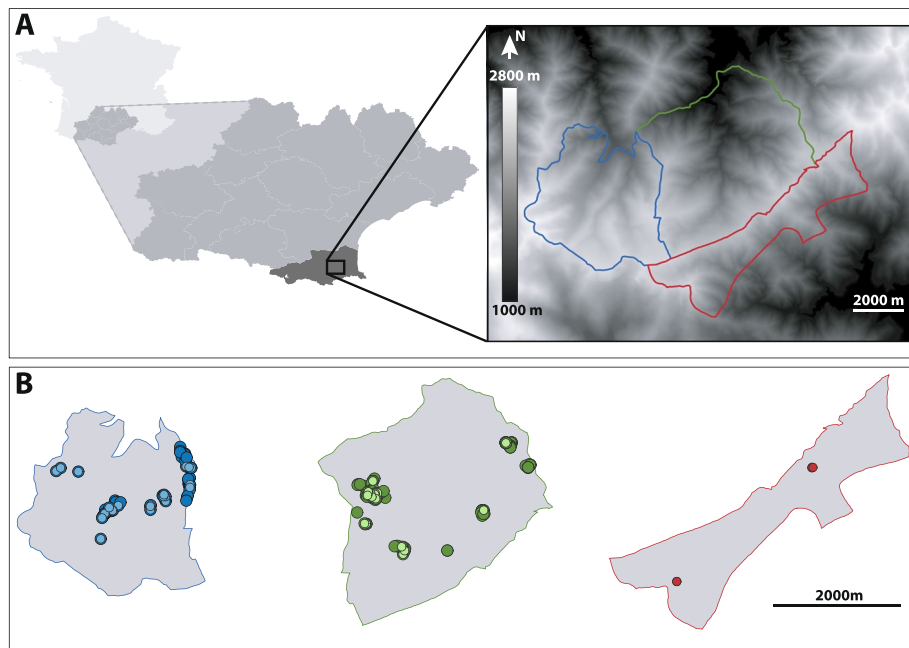


FIGURE 1 | Location of the study area and sampling distribution of the 229 droppings of *Tetrao urogallus* subsp. *Aquitanicus* in the eastern Pyrenees. (A) The droppings were collected in France, in the Occitanie region, more specifically in the Pyrénées-Orientales department, with a zoom on the investigated massif. The topographic background represents elevation (in meters). Colored outlines indicate the boundaries of the three nature reserves: Mantet (blue), Py (green), and Prats-de-Mollo-La-Preste (red). (B) Spatial distribution of sampled droppings within each nature reserve. The large dots correspond to the total sampling while the small dots correspond to the droppings used for diet analyses. To ensure data confidentiality and in accordance with agreements with the Catalan nature reserves, the exact locations of the sampling sites are not displayed.

as an indicative measure of the total amount of extracted DNA. Molecular sexing was performed twice by PCR amplification of a fragment of the *CHD* region using primers P3' and 1237L (Cayuela et al. 2019; See Table S1). PCR was performed in 25 μ L reactions containing 2 μ L of DNA extract, 5 $\times$ of GoTaq Buffer (Promega), 5 unit/ μ L of GoTaq G2 DNA Polymerase (ref M7845 Promega), 2 μ M of each dNTPs, 2 μ M of each primer and RNase and DNA free water. PCR was programmed as follows: initial denaturation at 95°C for 2 min, followed by 38 cycles of [denaturation at 95°C for 45 s, annealing at 55°C for 2 min and extension at 72°C for 45 s] and a final extension at 72°C for 10 min. PCR products were run on a 3% agarose gel with 1 $\times$ TAE. We assigned droppings to a female if we observed two alleles of size 244 bp (*CHD-Z*) and 269 bp (*CHD-W*), and to males if we observed only the *CHD-Z* allele. Droppings providing ambiguous results were amplified in up to three additional PCRs. After the third round of amplification, ambiguous genotypes were left unassigned.

We inferred multi-locus genotypes using 11 microsatellite markers (See Table S1) and initially designed for Black grouse, *Tetrao tetrix* (BG10, BG15, BG18, BG20; Pieltney and Höglund 2001), Chicken, *Gallus gallus* (ADL142, LEI098; Gibbs et al. 1997), and Capercaillie, *Tetrao urogallus* (TuT1, TuT3, TuT4, sTuD1, sTuD3; Jacob et al. 2010; Segelbacher et al. 2000). Microsatellites were amplified into four PCR multiplexes using dye-labeled forward primers (Table S1). PCRs were set in 12.5 μ L reactions, containing 2 μ L of DNA extract, 1 $\times$ of Qiagen Multiplex PCR Master Mix, 0.2 μ M of each primer and RNase/DNA free water. PCR programs were programmed as follows: an initial denaturation step at 95°C for 15 min, 38 cycles of [denaturation at 94°C for 30 s, annealing at 57°C for 2 min, and extension at 72°C for

45 s], and a final extension at 72°C for 15 min. Positive and negative controls were included in each PCR plate used for genotyping all samples. All PCR amplification products, including those from negative and positive controls, were checked on a 2% electrophoresis agarose gel and sent for fragment analysis (INRAE Gentyane genotyping platform, Clermont-Ferrand, France).

2.3 | Individual Identification and Population Size Estimation

Allele scoring was assessed using the GeneMarker software (SoftGenetics, State College, USA; Holland and Parson 2011). Droppings showing ambiguous profiles or non-amplification of alleles at one or more loci were amplified in a second round of PCR. Genotypes that were not unambiguously determined after the second PCR were coded as missing values. We next reconstructed unique Multi Locus Genotypes (MLGs) based on pairwise dissimilarity distances between pairs of genotypes using the R package “poppr” (Kamvar et al. 2014). Droppings were assigned to a MLG if their genotypes differed by less than three alleles. MLGs were crossed with individual genetic sexing to ensure correct assignments. In cases where droppings from the same MLG but differed by sex, the MLG was turned into “unsexed” to account for possible genotyping errors at the *CHD* loci. We graphically estimated the ability of our set of microsatellite loci to distinguish between unique MLGs using an accumulation curve using the R packages “poppr” (Kamvar et al. 2014) and “adeget” (Jombart 2008).

To test for data reliability, we used Micro-Checker 2.2 (Van Oosterhout et al. 2004) to check for the presence of allele dropout

(ADO, i.e., one of the two alleles at a locus of a heterozygous individual is not detected), genotyping errors due to stuttering and null alleles of the markers at the population level. We also manually computed the ADO rates per individual overall markers. We used Genepop 4.7 (Raymond and Rousset 1995; Rousset 2008) to check for Linkage Disequilibrium (LD) between pairs of loci. We used CERVUS 3.0.3 (Kalinowski et al. 2007) to compute the probability that two individuals taken at random shared the same multi-locus genotype (i.e., Probability of Identity (PI)) and accounting for population structuring and the presence of siblings (PI_{sib}), and to assess the discriminatory power of the eight microsatellite markers selected (see results; Kalinowski et al. 2007; Mollet et al. 2015; Taberlet and Luikart 1999; Waits et al. 2001).

We used the R package “capwire” (Miller et al. 2005; Pennell et al. 2013) to estimate population size based on the frequency of recapture of each MLG. Sampling was conducted before the breeding season and in late winter, when mortality is reduced (Cayuela et al. 2021) and individuals still occupied their wintering ranges. We could therefore confidently assume that the population was closed during the sampling window. A Capercaillie population in winter consists of a mixture of individuals differing in their spatial distribution and detection probability. Subadult and non-territorial individuals of both sexes show erratic behavior. Prospections along transects ensure an even sampling effort across the local distribution range. Thus, the spatial distribution of the data should have a minimal effect on the estimation of population size using capwire (Miller et al. 2005). Adult males occupy stable and nonoverlapping home ranges distributed around the lek (Gjerde and Wegge 1989). Females settle into dense forest stands, where they aggregate in small groups (Storch 1995). Given the biology of the species, we can readily assume that individuals differ in deposition rate and/or detection probability. Hence, the assumption of even capture probability seems unrealistic. We therefore used the TIRM model to estimate population size.

2.4 | Estimation of Essential Biodiversity Variables (EBVs)

We inferred the level of genetic structuring of the population within our study area using a Principal Component Analysis (PCA), and determined the most likely number of genetic clusters contributing to the population ($k=1-8$) using a clustering analysis (sNMF) with the “adegenet” (Jombart 2008) and “LEA” R packages respectively (Frichot and François 2015). Genetic diversity and deviation from panmixia across all loci were assessed by calculating the number of alleles (A), expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (G_{IS}) using Genodive 3.06 Software (Meirmans 2020). p values were determined based on 9999 permutations. Analyses were carried out at the population level, and by sex in order to estimate kinship level between males and between females.

2.5 | Stress Response to Human Disturbance

Individual levels of stress hormones were assessed from a subset of 96 droppings collected during the initial sampling survey

across the three nature reserves. The freshest droppings available were selected in order to minimize hormone degradation. The droppings were selected according level of disturbance at wintering spots classified a priori as “disturbed” (in Mantet $n=15$ and in Py, $n=16$), “mid-disturbed” (in Mantet $n=19$ and in Py $n=12$) and “undisturbed” (in Py, $n=28$ and in Prats-De-Mollo-La-Prete, $n=6$) in order to quantify potential differences in FCMs. Levels of human-induced disturbance were categorized by nature reserve agents, based on the intensity of outdoor activities, including hiking trails and off-piste skiing. These droppings were also genotyped and sexed. We quantified the concentration of excreted FCMs using a competitive cortisone Enzyme Immuno-Assay (EIA), specifically targeting metabolites with a 3,11-dioxo structure (for details see Rettenbacher et al. 2004). This EIA has been validated for assessing FCMs from droppings in Capercaillie (Thiel et al. 2005). Analyses were conducted at the University of Veterinary Medicine in Vienna, Austria (Vetmeduni), where FCMs were extracted in duplicate for each sample (i.e., replicates from two pieces of the same droppings by MLG) in accordance with the recommendations set forth by Palme et al. (2013). Extracts were analyzed in the cortisone EIA in duplicate. The analyses were repeated when CVs (i.e., coefficients of variation) of duplicates exceeded 10%. Interassay CVs of a low and high concentration pool sample were 6.7% and 5.1%, respectively.

Difference in FCMs concentration was analyzed using a series of Generalized Linear Mixed Models (GLMM) implemented in the “lme4” package in R (Bates et al. 2015). As the likelihood of the different models tested could not be differentiated based on Akaike Information Criterion (difference in AIC <2), an information theoretic model averaging approach was adopted using the “MuMIn” package (Grueber et al. 2011). The model implemented in MuMIn was chosen for its biological relevance. In our case, two parameters were considered as “fixed” effects, that is, the expected level of human disturbance (“disturbed”, “mid-disturbed”, “undisturbed”) and the sex of the individual (male or female). The “biological replicate” was set as a random effect. The interaction effect between these two fixed variables was also taken into account. The resulting model was coded as follow: `lmer(FCMs concentration ~ Level of disturbance*Sex + (1|Replicates))`. A positive effect size suggests that the variable is positively associated with the stress level—an increase in stress levels. A negative effect size suggests that the variable is negatively associated with the stress level, that is, it is linked to a decrease in stress levels (FCM concentration). The assumptions of normality and homoscedasticity of the variances of the residuals of the models were checked (Kolmogorov Smirnov test p value: 0.743, Levene test p value: 0.6245) as well as the consistency between replicates (Pearson correlation test, p value = $1.374e-14$, $t=9.851$, $df=66$, Figure S1).

2.6 | Assessment of the Bird's Diet

We described individual diet following a metabarcoding approach based on a fragment of the chloroplastic trnL gene, amplified using the trnL-F (CGAAATCGGTAGACGCTACG) and trnL-R (GGGGATAGAGGGACTTGAAC) primers pair (Taberlet et al. 2007) and Universal Illumina sequencing adapters. Amplifications were performed in 25 μ L reactions

containing 2 μ L of DNA extraction, 5 $\times$ of GoTaq Buffer (Promega), 5 unit/ μ L of GoTaq G2 DNA Polymerase (ref M7845 Promega), 2 μ M of each dNTPs, 2 μ M of each primer, and RNase and DNA-free water. The PCR program consisted in a first denaturation step at 95°C for 2 min, followed by 40 cycles of [denaturation at 95°C for 45 s, annealing at 52°C for 2 min, and extension at 72°C for 45 s] and a final extension at 72°C for 10 min. PCR products were run on 1% agarose gel to visually assess the PCR efficiency. Out of 229 droppings amplified, 49 showed clear amplification patterns and were sent for library preparation and 2 $\times$ 150 pb paired-end sequencing on a MiSeq 2000 (Illumina) at the BioEnvironment platform (University of Perpignan Via Domitia, France). These 49 samples corresponded to droppings from a total of 34 individuals (as defined by the obtained multilocus genotypes; see Results), of which 19 were sexed as females (11 from Mantet and eight from Py), six as males (three from Mantet and three from Py), and nine remained unsexed (seven from Mantet and two from Py). Consequently, between one and three droppings per individual were analyzed to characterize diet.

Sequencing reads were processed using the FROGS (Find Rapidly OTU with Galaxy Solution) bioinformatic pipeline implemented in the Galaxy platform (Escudé et al. 2018). After a preprocessing step, we clustered sequences into Operational Taxonomic Units (OTUs) using the swarm algorithm and an aggregation clustering distance of three. The resulting dataset was filtered out for chimeras using VSEARCH (Rognes et al. 2016), and rare clusters (relative abundance $< 5 \times 10^{-5}$) were removed. Droppings showing less than 5000 reads were removed from the subsequent analyses. We assigned OTUs to taxa using the NCBI Blast program (nonredundant nucleotide collection, updated in Oct. 2023). OTUs not assigned to *Plantae* taxa were excluded, as well as OTUs showing $< 97.5\%$ identity with the reference sequence. Since most OTUs were affiliated to several reference sequences from different species, we chose the best hit based on the Blast *e*-Value score and on the “Total score” in the case of same *e*-Values. We also checked whether the assignments with

similar *e*-Values and total score values belong to the same species or genus, which was always the case. For sake of conservatism, we interpreted the resulting assignments at the taxonomical level at which all the best hits converged. The obtained raw OTU abundance matrix was rarefied based upon the smallest total abundance observed over droppings ($n = 5735$; see Section 3). Using the final OTU abundance dataset, we performed a Non-Metric Dimensional Scaling analysis (NMDS) based on pairwise Jaccard similarity indices to visualize and interpret diet composition similarity between droppings. A permutational multivariate analysis of variance (PERMANOVA; Anderson 2017) was run to assess the effect of the nature reserve (Py versus Mantet) and individual sex on the observed diet. *p* values were calculated based on 9999 permutations. The NMDS and the PERMANOVA were computed in R using the “Vegan” package (Dixon 2003). We also ran an indicator taxa analysis based on 9999 randomized permutations, as implemented in the “indicspecies” package (Cáceres and Legendre 2009), to detect associations between OTUs and the studied nature reserves.

3 | Results

3.1 | Reliability of the Data

Out of the 229 collected droppings, 179 (78.2%) were successfully genotyped and 203 (88.7%) were unambiguously sexed. We found no evidence for possible allele dropout, null alleles, or errors due to stuttering for most microsatellite markers overall samples at the population level. Excess homozygosity, possibly suggesting null alleles, was observed in loci BG10 and TuT1. Linkage disequilibrium was observed between loci BG10 and TuT1. We therefore excluded BG10 and TuT1 from subsequent analyses, as well as LEI098 (monomorphic). The eight remaining microsatellite markers were polymorphic, with the number of alleles ranging from two to four alleles (Table 1). At the individual level, we found an average ADO rate of 5.9, ranging from 0% to 18.2%.

TABLE 1 | Estimation of genetic diversity and deviation from panmixia across all loci, analyzed at the levels of the overall population, males, and females.

Locus	Global ($n = 62$)				Males ($n = 18$)				Females ($n = 27$)			
	A	H_O	H_E	G_{IS}	A	H_O	H_E	G_{IS}	A	H_O	H_E	G_{IS}
TuT3	3	0.097	0.123	0.216	3	0.111	0.212	0.477	2	0.037	0.037	0.000
TuT4	2	0.419	0.401	−0.045	2	0.500	0.435	−0.150	2	0.370	0.392	0.055
BG15	3	0.557	0.672	0.171	3	0.389	0.683	0.431	3	0.654	0.678	0.036
BG18	3	0.452	0.424	−0.065	2	0.278	0.441	0.370	3	0.481	0.417	−0.154
StuD3	3	0.393	0.415	0.051	3	0.471	0.535	0.120	3	0.333	0.352	0.053
StuD1	3	0.267	0.305	0.126	2	0.235	0.375	0.373	2	0.269	0.237	−0.136
BG20	2	0.016	0.016	−0.000	1	0.000	0.000	NA	1	0.030	0.000	NA
ADL142	4	0.311	0.389	0.199	4	0.235	0.434	0.458	3	0.333	0.389	0.143
Overall	2.667	0.279	0.305	0.085	2.333	0.247	0.346	0.288	2.333	0.275	0.278	0.009

Note: The analysis includes calculations of the number of alleles (A), expected heterozygosity (H_E), observed heterozygosity (H_O), and the inbreeding coefficient (G_{IS}). Significant estimates are highlighted in bold (*p* value < 0.05).

3.2 | Estimated Size and Sex Ratio of the Local Capercaillie Population

The probability that two individuals shared the same multi-locus genotypes was low ($PI=0.004$) even when considering population structuring and the presence of siblings ($PI_{sib}=0.0736$). Our set of microsatellites provided enough power to distinguish individuals based on their multi-locus genotypes. Indeed, complementary analyses showed that a minimum number of seven loci was required to unambiguously assign genotypes to one of the MLGs (See Figure S2).

Pairwise dissimilarity analyses suggested the occurrence of 62 MLGs (see Figure S3), of which 27 were identified as females, 18 as males and 17 individuals whose sex we were unable to assign. Maximum likelihood estimator (MLE) using the two innate rate model (TIRM) was 79 individuals [95% CI=67–92]. Relative differences in capture probability between types of individuals (harder vs. easier to capture, $\alpha=4.0$) confirmed our hypothesis of capture heterogeneity between individuals. MLEs by sex were 29 female individuals [95% CI=24–33; $\alpha=2.9$] and 24 male individuals [95% CI=18–33; $\alpha=4.4$]. MLE were estimated from an average capture frequency of 2.89 per individual, 3.57 per females and 2.50 per males.

3.3 | Essential Biodiversity Variables (EBVs)

In terms of genetic diversity and structure, the first two dimensions of the Principal Component Analysis explained 13.4% (PC1) and 12.3% (PC2, sum = 25.7%) of the total genetic variance (Figure 2). Overlapping distribution of individuals from the three nature reserves in the two dimensions of the PCA space based on their genotypes shows no or low levels of spatial genetic structuring at the scale of the study area. This is in line with the output of the clustering analysis (sNMF), which shows that

individual genotypes originated from a single gene pool ($k=1$). At the population level, we observed low levels of heterozygosity (mean $H_O=0.279$, mean $H_E=0.305$), a deficit in heterozygotes, and a significant positive inbreeding coefficient ($G_{IS}=0.085$, p value <0.05). At the sex level, we found a mean H_O of 0.275, a mean H_E of 0.278, and a non-significant G_{IS} of 0.009 for females. For males, we observed a mean H_O of 0.247, a mean H_E of 0.346, and a significant positive G_{IS} of 0.288 (p value <0.05).

3.4 | Individual Levels of FCMs Assessed From Droppings

Regarding FCMs, we initially analyzed a subset of 96 droppings, of which 19 were excluded because of ambiguous results at molecular sexing. The analyses were carried out on 77 droppings, of which 54 were from females and 23 from males. The level of human disturbance was the only variable that significantly affected FCM concentrations (Figure 3). A priori level of human disturbance was negatively correlated with individual levels of FCMs (Estimate = -0.332 ; [95% CI = -0.50 ; -0.165]; p value = $3.272e-05$). In other words, individuals had lower FCM levels at sites a priori defined as disturbed (mean = 170.8 ng of FCMs/g of droppings) compared to the individuals at a priori undisturbed (mean = 320.2 ng of FCMs/g of droppings; Figure 4). We neither observed a difference in levels of FCMs between females and males (Estimate = 0.079; [95% CI = -0.464 ; 0.6625]; p value = 0.2648), nor regarding the interaction between sex and disturbance levels (Estimate = 0.127; [95% CI = -0.207 ; 0.463]; p value = 0.4509). Overall, our model explained 14% of total variance (adjusted $R^2=0.139$).

3.5 | Assessment of the Bird's Diet

Out of 229 droppings analyzed, 49 (21.4%) showed enough amplification product when visualized and were sequenced,

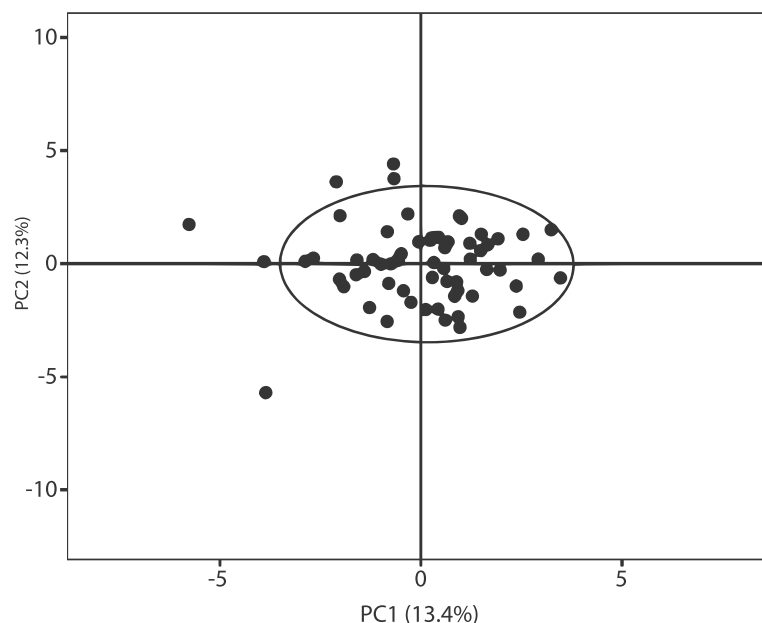


FIGURE 2 | Principal Component Analysis (PCA) obtained from the 62 MLGs present in the three Catalan nature reserves. The first two principal components (PCs) jointly accounted for 25.7% of the total variance. The analysis shows that the Capercaillies from the different nature reserves form a single entity of genetic structure.

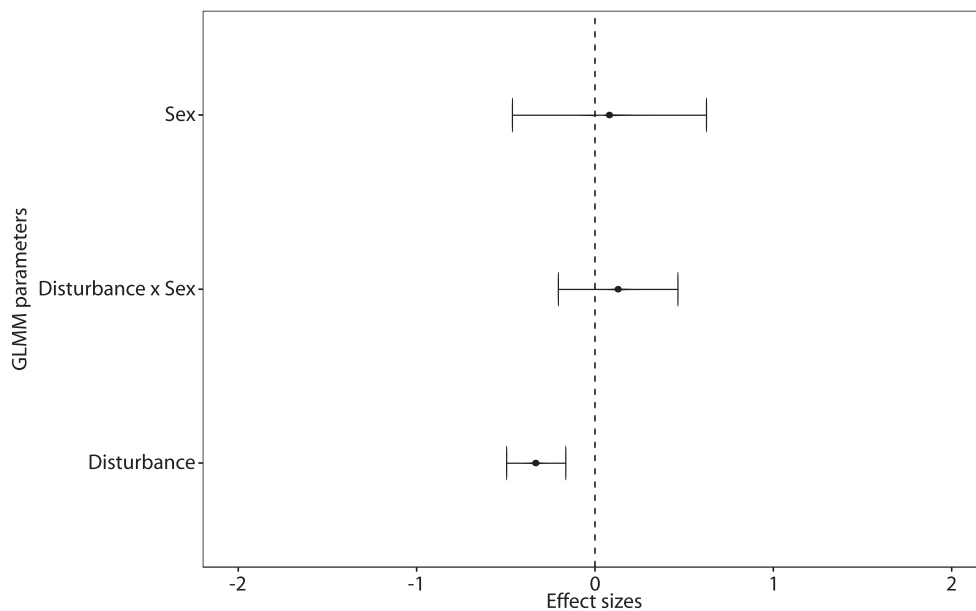


FIGURE 3 | Effect sizes ($\pm 95\%$ confidence intervals) of the fixed effects obtained from model averaging (based on AICc weights) of generalized linear mixed models (GLMMs). The model included level of disturbance and sex (female vs. male) as fixed effects, and replicates as a random intercept. Estimates are averaged across all candidate models using AICc weights. Effects whose confidence intervals do not overlap zero can be considered statistically robust.

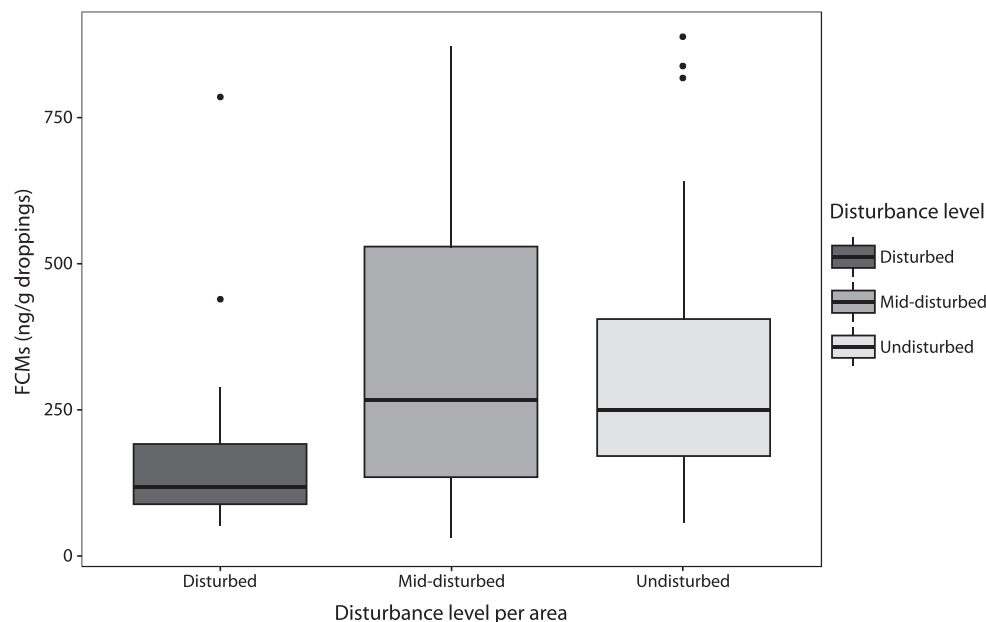


FIGURE 4 | Boxplot of FCMs concentration (mean of two replicates in ng/g droppings) as a function of the GPS positions of the droppings and the estimated level of disturbance. The droppings from a priori “disturbed” sites show a trend of lower stress levels than droppings from a priori “mid-disturbed” and “undisturbed.”

providing on average 86,011 reads (range: 69–145,785 reads). After filtering and processing (FROGS pipeline), excluding operational taxonomic units (OTUs) not assigned to taxa within *Plantae* or showing $< 97.5\%$ identity with reference sequences, and excluding droppings with low amplification success (number of reads < 5000), we obtained a matrix of 29 OTUs and an average of 81,827 reads per sample (range: 5796–141,564) from 48 out of the 49 samples analyzed. Three OTUs assigned to *Arabidopsis thaliana* and *Allium* sp. may have resulted from cross contamination during the laboratory processing and were

excluded. OTUs matching multiple species were assigned at the family level. Our final dataset thus comprised 26 OTUs belonging to 16 plant families and a rarefied number of reads per sample of 5735. The number of OTUs detected within droppings ranged from 1 (12.2%) to 6 (8.2%) with most droppings containing 2 (38.8%) or 3 (26.5%) OTUs.

Based on the NMDS analysis (Figure 5) and PERMANOVA analysis, the nature reserve (Py, Mantet, Figure 1) explained variation in individual diet ($F = 5.69$; p value = 0.002) while

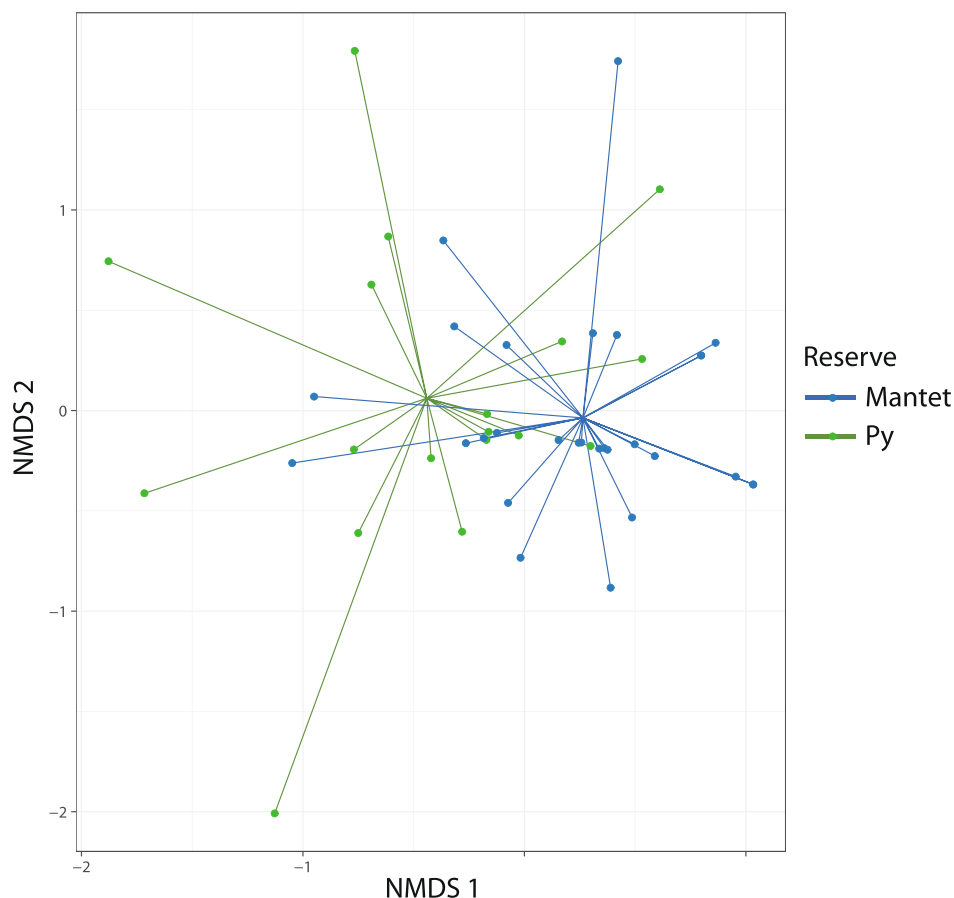


FIGURE 5 | NMDS analysis showing the difference in diet composition similarity between droppings among Py, Mantet, and at the frontier of Py-Mantet nature reserves.

the sex of Capercaillie individual did not (p value=0.24). The OTU1 (Pinaceae) was found to be an indicator of the Mantet nature reserve (p value=0.0139) while the OTU15 (Betulaceae) was exclusively found in the Py nature reserve (p value=0.005).

4 | Discussion

The relationship between species and their habitats strongly influences abundance estimates and the effectiveness of monitoring programs, largely due to imperfect detectability (Kellner and Swihart 2014). This can lead to inaccurate threat assessments in rare and elusive species and hinder conservation decision-making. For Capercaillie, traditional approaches, such as lek counts, although informative, may be biased, logistically demanding, and invasive (Aleix-Mata et al. 2019, 2025). Noninvasive eDNA sampling combined with molecular techniques offer a valuable complement by providing more accurate estimates of demographic and genetic parameters, thereby reducing the limitations of field observations (Royle et al. 2005; Schwartz et al. 2007). Beyond these, eDNA can also capture ecological and physiological information, such as diet composition, stress indicators, and interactions with other species, thus broadening the scope of conservation assessments (Ando et al. 2020; Palme 2019; Ruppert et al. 2019). In the present study, we combined such approaches to assess the ecological

status of the Capercaillie in the Catalan nature reserves. Our study estimated the size and provided genetic characteristics of a single, genetically cohesive population distributed across the Catalan nature reserves in which the presence of Capercaillie had been confirmed, and also assessed individual stress-related hormone levels and diet selection.

4.1 | Estimating Population Size From Noninvasive Samples

The high level of genotyping success observed in our study confirmed the efficacy of combining noninvasive sampling and microsatellites genotyping for the monitoring of populations, and especially for species that are difficult to observe like Capercaillie (Aleix-Mata et al. 2025; Cayuela et al. 2021; Jacob et al. 2010; Mollet et al. 2015; Zeni et al. 2024). Noninvasive samples, and especially droppings, generally contain amounts of highly degraded DNA, which increases the risk of non-amplifying one of the alleles of a heterozygous individual and overestimating the proportion of homozygotes (Gagneux et al. 1997; Taberlet 1996). Our microsatellite control process pointed toward such a problem at only two among the 11 loci used. These two loci were excluded from subsequent analyses. Despite the low polymorphism of the eight remaining loci, the probability of two individuals sharing the same multi-locus genotype (MLG) was low. Moreover, the combination of seven loci was sufficient to

unambiguously attribute the droppings to MLGs. Thus, individuals could be safely identified from their MLG.

Our estimate of 79 [68–92] individuals was 1.5 times higher than the figure of 57 individuals reported in the most recent survey based on lek counts (Pimenta et al. 2014). However, given the use of different methods and the temporal gap between estimates, the results are not directly comparable. Using capwire for overall population size (N) in the range of 100 individuals, a mean frequency of observation of 2.89, as observed in our study, generally provides estimates within 10% from the true N and CIs of width $< 1/3N$. Heterogeneity in capture probability between individuals has been shown to further reduce bias and narrow CIs (Miller et al. 2005). This population size remains relatively small and consistent with figures from other studies in France and Europe in fragmented habitats (Cayuela et al. 2021; Jacob et al. 2010; Mollet et al. 2015; Zeni et al. 2024). Our study thus provided the first genetically based estimate of Capercaillie population size in the Catalan nature reserves. This baseline allows managers to adapt the monitoring scheme to achieve the desired level of accuracy, whether for estimating population size or assessing the direction of population demographic trend.

4.2 | Sex-Ratio in the Local Population of Catalan Nature Reserves Is Biased Toward Females

Within sexed MLGs, we observed a sex ratio favorable to females, although the estimated population size suggests a largely similar number of males and females in the study area. It is also surprising that females were on average captured more often than males. This pattern contrasts with the male-biased ratios reported in other declining *T. urogallus* populations in the Vosges (70 males and 59 females in Cayuela et al. (2019); 66 males and 50 females in Cayuela et al. (2021)), Central Switzerland (77 males and 46 females; Mollet et al. 2015), in East Tyrol (Austria, 55 males and 28 females; Zeni et al. 2024), and *Tetrao urogallus* subsp. *cantabricus* in the Cantabrian Mountains (32 males and 19 females; Morán-Luis et al. 2014). However, the mechanisms driving this trend remain unclear (but see Donald 2007). A counterintuitive result of sex-specific demographic parameters was reported in Augustine et al. (2020) during a study of Capercaillies from East-Central Switzerland. The monitoring showed a higher number of males than females but a declining male population while the female population remained fairly stable. The number of males fell from 78 to 59 and the number of females from 46 to 50 between 2009 and 2015. The authors suggested that the recruitment of females is higher than that of males and that sex-specific recruitment could be the product of an imbalance in the sex ratio at birth, sex specificity in asymmetrical natal dispersal rates and/or sex-specific survival to adulthood, in this case of females, due to the lower energetic requirements of smaller female chicks (Wegge 1980). However, no recent study has demonstrated this. In addition, an anterior study suggests that males have the most strict habitat requirements (Wegge and Rolstad 1986), so they may be the first to respond to a decline in habitat quality. Based on our current data, and given that the available reference studies focused on different geographical regions and different periods, it is difficult to draw conclusions. Further monitoring would be useful to assess more accurately the demographic dynamics of the sex ratio in the study area. In addition, these results should be interpreted with caution,

as 17 of the 62 MLGs could not be sexed. Ideally, sex determination should have been performed using sequencing-based methods and sex-specific primers (Aleix-Mata et al. 2025), which offer greater sensitivity and reduce the risk of false negatives in the detection of sex-specific fragments.

4.3 | The Presence of Inbreeding Suggests a Lack of Gene Flow With Neighboring Populations

EBVs estimates revealed low genetic diversity and evidence of inbreeding, as reported in other studies (Cayuela et al. 2021; Escoda et al. 2023; Segelbacher et al. 2003; Zeni et al. 2024). The level of heterozygosity at population level ($H_0=0.279$; $H_E=0.305$; $G_{IS}=0.085$) was lower in the Catalan nature reserves compared to levels observed in other *Tetrao urogallus* populations throughout Europe, while inbreeding coefficient follows the same trends (Cayuela et al. 2021; Escoda et al. 2023; Segelbacher et al. 2003). In contrast, higher inbreeding levels were detected in 14 individuals from the endangered *T. u. cantabricus* population inhabiting the Cantabrian Mountains (mean $F_{IS}=0.10$, range: 0–0.34; Escoda et al. 2023).

The low genetic diversity observed in the Eastern Pyrenees population is consistent with low genetic connectivity to neighboring populations and habitat fragmentation at large scale, owing to the occurrences of topographic barriers and discontinuous forest cover, and at smaller scale through human-induced disturbance (settlements and recreational activities) (Bal et al. 2021; Escoda et al. 2023; Mikoláš et al. 2017; Segelbacher et al. 2003). The Segre Valley separates the central and eastern Pyrenees and has been identified as a putative barrier to gene flow causing genetic differentiation between rock ptarmigan populations (*Lagopus muta*; Bech et al. 2009, 2013). Capercaillie and rock ptarmigan occupy the same mountain ranges and are both characterized by short natal dispersal distances (i.e., ranging from 0.2 to 5.9 km for males and 5.2–17.7 km for female rock ptarmigan respectively; Brenot & Novoa, unpubl. data; Novoa et al. 2005) and 1–2 km, median 5–10 km for Capercaillie (Leclercq and Menoni 2018). It is plausible that large-scale topographic features similarly restrict dispersal in Capercaillie populations and may contribute to population isolation and limited gene flow. However, sampling at broader geographic scale would be required to test this hypothesis.

Reduced genetic variability and increased inbreeding may also be influenced by male philopatry and sex-biased dispersal (Höglund and Alatalo 1995; Pimenta et al. 2014). Typically, females are the primary dispersing sex, while males establish territories close to their natal areas (Dunn and Braun 1985). The spatial structure of Capercaillie leks is shaped by female home range distribution, with leks forming near the geometric center of these ranges (Menoni 1997). During winter, males and females from nearby leks form mixed-sex flocks, which disband at the onset of the breeding season as males return to leks and females visit them to mate (Höglund and Alatalo 1995; Leclercq and Menoni 2018). While females typically disperse before their first breeding season, males tend to remain within their natal groups, leading to lek structures composed of closely related males—a pattern observed in the Vosges Mountains (Cayuela et al. 2019; Segelbacher et al. 2007). This behavior results in higher inbreeding coefficients among males, consistent with kin selection theory. Although females

have higher dispersal potential, fragmentation can reduce their ability to move across the landscape, increasing the likelihood of mating among relatives. Small total and effective population sizes, combined with low levels of gene flow, accelerate genetic erosion through genetic drift and increase the probability that offspring carry potentially deleterious mutations in the homozygous state (Pekkala et al. 2014).

Overall, these findings suggest that the isolated population of Western Capercaillie in the Catalan nature reserves warrants special conservation attention. Translocation of individuals from neighboring genetically diverse populations may induce genetic rescue and increase genetic variability. Translocations were successfully integrated into the management strategy of the Pyrenean population of rock ptarmigan (Novoa et al. 2020) and should be considered for the Capercaillie in the Catalan nature reserves.

4.4 | Individual Levels of Circulating Corticosterone Metabolites (FCMs)

The observed trends in individual levels of FCMs were contrary to our initial expectations. Based on the findings of Thiel et al. (2011), we had anticipated that individual Capercaillie would react to disturbance from winter recreational activities by increasing their corticosterone and thus FCM levels. In light of the levels of human activity documented by nature reserves technicians, it seems implausible that disturbance levels were not high enough to induce the production of FCMs by Capercaillie. It is possible that the individuals have become accustomed to, or even benefit from, human activities, particularly if human presence deters predators (Berger 2007; Moss et al. 2014). In previous studies the overall levels of FCMs in grouse were found to be lower than those observed in our study (Thiel et al. 2008, 2011), with a notable difference between the sexes (Thiel et al. 2008). However, the lowest levels observed in our results were within the range of those previously reported, while others were slightly higher (though they did not reach the maximum levels observed after an ACTH challenge; Thiel et al. 2005). Our model incorporated few explanatory variables and explained little variance, which suggests that other variables, such as predation pressure, mate finding or abiotic factors, may play a role. However, estimating predator and Capercaillie densities either qualitatively or quantitatively is notoriously challenging. Appropriate design would require a comparison between specific sites, including control sites in which relevant factors are estimated. Additionally, longitudinal studies involving the same animals in different situations would provide valuable insights. Dietary factors, in particular, have the potential to influence FCM levels (Palme 2019), making them an intriguing avenue for further investigation.

4.5 | Variation in Individual Diet Between Nature Reserves

Metabarcoding analyses have enabled the detection of 26 plant OTUs that are likely to compose Capercaillie diet in the Eastern Pyrenean Mountains. Pinaceae were found in most feces, which confirmed previous knowledge on Capercaillie feeding behavior

during winter and spring (Leclercq and Menoni 2018). We also observed differences in diet composition at the scale of a few kilometers within Catalan nature reserves. This pattern may result from differences in altitude and slope across the two nature reserves, two forces shaping plant communities. In particular, species of the Betulaceae family were almost exclusively present in the Mantet nature reserve. The Pinaceae OTU (OTU1) found mainly in the drops collected in Py nature reserve could rather reflect a genetic differentiation of Pinaceae trees between the two nature reserves. Our findings are in line with recent studies based on metabarcoding, which identified 122 plant species from Capercaillie feces, and showed that the number of consumed plant species differed between European populations and seasonally between autumn and spring (Chua et al. 2021). A complementary approach would be to analyze the available habitat in relation to the Capercaillie's diet. This would allow us to see whether the plants consumed were selected or whether they were chosen "at random" given their high availability, using a selection ratio as seen in Manly et al. (2002).

At the individual level, most droppings contents indicated a feeding behavior upon only one dietary resource (*Pinus* sp.) while few drops contained several, yet at most 6 OTUs. Most plants other than *Pinaceae* that were identified in this study, including *Vaccinium* sp., *Quercus* sp., or *Cupressus* sp., were already identified as being part of Capercaillie diet and in particular in neighboring populations in the Pyrenees or in the Cantabrian mountains (Blanco-Fontao et al. 2010; Chua et al. 2021). It would be interesting to know whether the different birds established locally in the Catalan nature reserves that make up the same genetic entity behave as different ecological populations using specific trophic niches over time, or whether the birds exhibit opportunistic feeding behavior depending on where they are found. This could be studied by characterizing the diet of birds for which several droppings could be collected from different areas. Unfortunately, this could not be done in this study most likely because of the rather poor quality of plant DNA extracted from droppings. A specific plant DNA extraction kit should be considered to improve plant DNA yields and the success of the metabarcoding approach.

Diet did not differ between males and females, although females were expected to select more energy-rich resources than males to compensate for resources allocated into egg production. Sex-biased difference in diet selection was detected at a local scale, but not at a European scale, with females largely preferring *Alnus* compared to males (Chua et al. 2021). The Betulaceae OTU that could potentially be assigned to *Alnus* sp. (OTU47) was detected in feces droppings from only one male and two females. The other Betulaceae OTU (OTU15, possibly *Corylus avellana*) that was predominantly (although not exclusively) found in the Py nature reserve was found in drops from three males, three females, and six unsexed individuals. Together, these results suggest that *Betulaceae* may be rare habitats used by Capercaillie in the Catalan nature reserves.

4.6 | Methodological Considerations

Molecular approaches provide powerful and complementary information to traditional monitoring methods, including

estimates of population size, sex ratio, physiological stress, and diet, as illustrated in this study. However, genetic analyses based on noninvasive samples present specific methodological limitations that must be considered when interpreting results. In particular, genetic outcomes strongly depend on DNA quality from both the host and dietary components present in feces and DNA contamination with nontarget material, complicating PCR amplification and increasing the risk of genotyping errors such as allelic dropout and false alleles (Taberlet 1996; Taberlet and Luikart 1999; Panasci et al. 2011). Here, we maximized DNA preservation by systematically targeting the freshest droppings and selecting samples deposited on snow, which likely provided a form of natural DNA conservation prior to collection (Bergan et al. 2016). Nevertheless, DNA quality assessment was limited to total DNA quantification using a Nanodrop spectrophotometer and to PCR amplification success of host microsatellites and diet markers. More refined approaches, such as fragment size analysis or quantitative PCR, would allow a better evaluation of DNA integrity and increase confidence in downstream genetic results (Hayward et al. 2020).

In addition, due to logistical constraints, microsatellite loci were amplified only once or twice per sample, with additional replicates performed only when genotyping quality was poor. Studies based on fecal or environmental DNA generally recommend multiple genotyping replicates per sample to minimize genotyping errors and more particularly allelic dropout, and to obtain reliable consensus genotypes (Taberlet and Luikart 1999). To reduce the risk of overestimating population size from our genotypes, we adopted a conservative analytical strategy by allowing a relatively high number of genotyping mismatches when reconstructing multilocus genotypes. This precautionary framework, combined with potentially underestimated allelic diversity resulting from genotyping uncertainty, may indirectly contribute to the relatively high probability of identity observed and to conservative estimates of population genetic parameters. Increasing the number of genotyping replicates per sample would therefore improve the robustness of individual identification and genetic inferences in future studies.

5 | Conclusion

This study provides the first comprehensive ecological and genetic assessment of the Capercaillie in the Catalan nature reserves using a combination of noninvasive and molecular approaches. We obtained a first estimate of population size, revealed a female-biased sex ratio that requires confirmation, and documented low genetic diversity with evidence of inbreeding, linked to the species' behavior and suggesting isolation from neighboring populations. In addition, we investigated individual stress physiology and diet composition, highlighting both methodological opportunities and future challenges. Together, these findings provide essential baseline data for the conservation of this isolated population. Continued genetic and demographic monitoring, combined with management actions to enhance connectivity or consider genetic rescue, will be critical to safeguard the long-term viability of the Capercaillie in the Catalan nature reserves. Extending this approach to neighboring populations would allow assessment of genetic connectivity

across the Pyrenean range and guide coordinated management actions, including potential translocations.

Author Contributions

P.B., C.P., M.C., M.T., O.R., J.A.M.B., and V.D.-H. made major contributions to the study design. P.B., C.P., M.C., M.T. mainly contributed to the acquisition, analysis and interpretation of microsatellite genotyping and stress data, with the help of O.R., J.A.M.B., and V.D.-H. O.R. fully analyzed the metabarcoding data from the diet part once the data was generated. P.B. and O.R. primarily drafted the manuscript, with assistance from J.A.M.B., V.D.-H., S.D., R.P., G.J., and A.G. for reviewing the final version. R.P. and S.M.-M. hosted and supervised the stress response part of the study. C.L. provided help and technical support during laboratory manipulations. A.G. provided assistance and technical support for the analysis of stress data. G.J. brought his knowledge of the Capercaillie, provided microsatellite sequences and his skills in calculating the census population size. Finally, S.D. designed the field teams, provided help and support during the sampling campaigns and knowledge of the study area and the Capercaillie.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Datasets associated to this work are available on Zenodo (Id. 19347104).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370265-sup-0001-Supinfo.docx.