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Health status of Western capercaillie (*Tetrao urogallus aquitanicus*) population in Catalan Nature Reserves by non-invasive approach.

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Genetic approaches to assess the decline of the Western Capercaillie (*Tetrao urogallus aquitanicus*) with a non-invasive sampling

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ABSTRACT

Long-term monitoring of animal populations is essential to measure the impact of anthropogenic and other environmental factors on biodiversity. Until now, most research and population monitoring has been based on direct methods such as counting and Capture-Mark-Recapture (CMR). In the present study, we aimed at developing a non-invasive and integrative approach to monitor an endangered bird species : the Western Capercaillie in the Catalan Natural Reserves (France). During winter, we collected 229 droppings from which we extracted DNA and corticosterone metabolites, in order to assess the 'health status' of the local population. This innovative method, which had never been conducted in the Eastern Pyrenees prior to this study, revealed a low population size (i.e. 67 individuals) with a rather balanced sex ratio as well as low levels of genetic diversity and structuration. Our results also show different levels of stress across the studied areas, suggesting various putative levels of disturbance. All of these data can be implemented in the reserves to improve their conservation plan.

Keywords: Genotyping, Population Structure, Genetic Diversity, Inbreeding, Stress response, Corticosterone metabolites, Faeces, Bird

INTRODUCTION

Anthropogenic pressures and climate change have both been identified as key drivers of biodiversity loss (Solomon, 2007). In this global context, understanding the mechanisms that lead to population decline is an important challenge for conservation biologists (Purvis *et al*., 2000). Environmental change and habitat loss are closely linked as they affect both species ranges and abundance (Fahrig, 2003). Conversion of agricultural land, grazing and outdoor recreation infrastructure have led to the fragmentation of many habitats (Chapin III *et al*., 2000; Rixen & Rolando, 2013; Wilson *et al*., 2016). This is considered to be a negative process for species viability as fragmentation divides an initially continuous habitat into smaller patches and reduces the connectivity between them (Collinge, 2009). In many species, including birds, fragmentation results in a loss of genetic diversity through genetic drift as

can conduct to inbreeding (Frankham *et al*., 2002). Furthermore, global warming is known to induce several changes detrimental to the sustainability of species, especially in mountainous areas (Beniston, 2003). Accelerated snowmelt, trees rising to higher altitudes and the reduction of resource availability are some known examples. The topo-climates resulting from altitudinal gradients create heterogeneous ecosystems with high species diversity and conservation value (Becker *et al*., 2007; Myers *et al*., 2000). Unfortunately, environmental changes involve species migration to higher areas. This migration takes place until a threshold is reached, at which point it is no longer possible to rise further in order to stay within thermal tolerances. These species could therefore be doomed by the evolving environment (Chen *et al*., 2011; de Gabriel Hernando *et al*., 2021; Salvado *et al*., 2022). Although populations of lowland species (e.g. birds and butterflies) have been shown to evolve under climate change scenarios within Europe and North America, (Breed, 2013; Devictor *et al*., 2012), the status of mountain species is generally poorly documented (Scridel *et al*., 2018).

Mountain galliforms are among the species sensitive to environmental changes. The Western Capercaillie (*Tetrao urogallus*, L. 1758) is a keystone species distributed in Central Europe (Duriez *et al*., 2007). Usually associated with old-growth beech and coniferous forests, which are known as high biodiversity spots (Pakkala *et al*., 2003; Suter *et al*., 2002), the Capercaillie is recognised as an umbrella species - which means that its protection extends to other species living in the same area. A subspecies of grouse (*Tetrao urogallus* subsp. *aquitanicus*) has been geographically isolated in the Pyrenees mountains since the last Ice Age and is now endemic to this region (Segelbacher & Piertney, 2007). Since the 90's, a significant population decline has been observed, primarily due to habitat loss. The timber industry, alongside an increased level of human disturbance due to mountain tourism, has led to a substantial reduction of suitable habitats over the last century (Jacob *et al*., 2010; Storch, 1991). These disturbances can be fatal during the winter as this sedentary bird stays within the same area during this period. Breeding, which occurs in spring, may also be limited by these disturbances (Raúl Pimenta *et al*., 2014). Finally, poaching and predation by mammals (e.g. wild boars and foxes) has been an additional detriment to the population (Raúl Pimenta *et al*., 2014).

Currently, the subspecies *aquitanicus* is locally protected by nature conservation laws, Nature Reserves, and by the Natura 2000 framework (Bal *et al*., 2021). In the Eastern Pyrenees, three Catalan Reserves were created during the 1980s to confer suitable environment and conservation management plans for ten renewable years. The territory has a diverse habitat area of 9,352 ha located in high altitude biomes such as willows, beech forests, and alpine terrain (Catalane Natural Reserves WebSite). In the past, studies on bird abundance and dispersal have had to rely on direct methods such as counting or Capture-Mark-Recapture (CMR) (Aleix-Mata *et al*., 2019; Miller *et al*., 2005). These methods require a substantial amount of work, provide limited datasets and cause a relatively high level of disturbance to the birds. This is particularly problematic when dealing with endangered species (Jacob *et al*., 2010). Counting male leks and bioaccoustic have been widely used for estimating the abundance of polygynous bird populations. These estimates can be biased, in particular because the use of lek sites among individuals is age-dependent. Males older than three years tend to be more visible as they defend territories close to leks, while juveniles occupy peripheral territories and may not

show up during the breeding season until they are old enough to replace the elderly males (Storch, 1997). Each year, Western capercaillie population size is locally monitored by several national organisms (e.g. Natural Reserves, the *French Office of Biodiversity* (OFB) and the *Mountain Galliformes Organism* (OGM). The lek counting method has shown a 20% decline in males over the last fifteen years. Less than 2,000 males were counted in the Pyrenees, of which 342 [265 ; 433] were located in the Eastern range. During the last monitoring campaign only about sixty individuals (*i.e.* males and females) were estimated to be present in the Catalan Natural Reserves. Moreover, breeding success is very low (0.6 surviving chicks per hen), which is not sustainable for effective population renewal (Bal *et al*., 2021).

In an evolutionary perspective, a small effective size may have drastic effects on populations as it affects the average fitness of organisms. In general, the maintenance of small effective size enhances genetic drift and reduces adaptive potential, resulting in a loss of genetic diversity (Gibbs, 2001).

In the present study, we proposed an alternative and integrative monitoring approach, using non-invasive faecal sampling to better document the health status of the population of *T. urogallus* in the Eastern Pyrenees. Individual genotyping allows the collection of suitable data for populations or species that are difficult to study via traditional methods, such as endangered or elusive ones. The method to estimate population size through non-invasive genetic sampling consists in collecting and subsequently genotyping a large number of samples from the field. In birds, the most common way to collect DNA is through feather or faecal samples. From phylogeny to population monitoring, non-invasive sampling has been shown to be an innovative and effective method for several capercaillie subspecies (Taberlet & Luikart, 1999). Identifying potential genetic mechanisms involved in population decline has been a key priority for conservation biologists. These are essential parameters for the management of natural areas. Non-invasive genotyping provides a good level of data integration that cannot be achieved through direct methods. Starting from an individual scale, it is then possible to examine quantitative genetic variability and inbreeding rates via a population scale. In addition, kinship and demographic processes can be traced (Primmer *et al*., 1995). Supplementary information on individuals can be determined by sequencing from the same samples (e.g. diet, parasite load).

In our case, the main objective is to study the health status of the Western capercaille's population located in the Canigó mountain range, which is geographically isolated in the farest Eastern region of the distribution area of the bird within the Eastern Pyrenees. We focused on the Catalan Natural Reserves of Py, Mantet and Prats-De-Mollo-La-Preste. We establisheded the size and sex ratio of the population via the use of polymorphic microsatellites markers. The number of unique Multi Locus Genotypes (MLGs) is recorded from microsatellite dataset and serves as i) estimating a minimum population size (Taberlet & Luikart, 1999) ; ii) examining if genetic mechanisms take part in population decline. We expect that the observed decline in the population size is associated with a low allelic richness, a low expected heterozygosity, and a high inbreeding coefficient as already found in other grouse subspecies (Jacob *et al*., 2010; Syrowitz *et al*., s. d.). Outdoor recreation pressure has also been suspected in the Catalan reserves. Some areas are close to hiking trails. The technicians have detected regular human traces of off-roading close to

the areas where the birds are present. In this context, we also assessed the stress level of a subset of individuals from three main areas diverging in terms of human disturbance level. One of the physiological responses to stress is the activation of the hypothalamo - pituitary - adrenal axis. In vertebrates, this event results in the release of glucocorticoids (GCs) into the bloodstream via the adrenal cortex, which leads to physiological and behavioural adaptations (Wingfield & Romero, 2011). In birds, corticosterone is the main GC, unlike cortisone in mammals. GCs are further degraded into metabolites inside the liver and can be found in the bird's excrement (Palme *et al*., 2005; Taylor, 1971). Nevertheless, the metabolites found in the faeces reflect short-term stress, referring to a physiological stress that occured one to three hours before excretion (Thiel *et al*., 2005). Thus, the stress status on the Capercaillie was achieved by dosing the concentration of corticosterone metabolites (CMs) within the dropping. We expect that the birds present in the most stressful area will have the highest concentration of CMs.

MATERIALS AND METHODS

Study area and field survey

The fieldwork consisted of a systematic screening for indirect evidence of grouse presence. The survey was carried out along transects covering the entire area where capercaillie sightings had been recorded in the reserves. To avoid any disturbances, sampling was carried out by technicians from local reserves. Seven study sectors in Py (i.e P1, P2, P3, P4, P5, P6, P7), six sectors in Mantet (i.e M1, M2, M3, M4, M5, M6) and one in Prats de Mollo (i.e called PR1) were prospected between January and March, 2022. Here, we focused our screening on low branching trees often used for roosting along with root patches or tree stumps (Graf *et al*., 2009), as Capercaillies predominantly use these habitat components in winter (Schroth, 1991). Faeces were collected on the snow during daytime walks, directly put into collection tubes and stored in a freezer at -20 °C until further processing. GPS coordinates were recorded for each sample alongside the date of sampling.

A muscle sample from a Western capercaillie cadaver was provided by Kevin Foulché (French Biodiversity Office, Orlu city, Ariège 09 France, 05/19/2021, SAGIR no. 145327, autopsied at LPL on 07/06/2021) to serve as a positive control for subsequent molecular sexing and genotyping (see sections below).

DNA extraction

We extracted total genomic DNA from 229 faeces samples. Three different DNA extraction kits specific for stool samples were used to extract individual sample DNA, due to delivery issues. We used **E.Z.N.A.® Stool DNA Kit** and **QIAamp® DNA Stool Mini Kit** following the manufacturer's protocol for pathogen's DNA extraction, and extended the incubation time of the proteinase K at 70°C to 2 hours. We deliberately followed the pathogen protocol in order to give the opportunity to use the same DNA extracts to study the parasitic load later on. We also used a **Macherey-Nagel kit** following the manufacturer's protocol. Irrespective of the extraction kit used, the final purified DNA was eluted in 100 µL of elution buffer. DNA extracts were split into two parts : 50 µL were stored at 4°C for experiments and 50 µL were frozen at -20°C as a back-up. Total DNA concentration for each extract was measured with Nanodrop 2000. DNA extracts were then used for genotyping and sexing.

Microsatellites Data production

We used 11 microsatellite markers available from the literature in order to genotype each

faecal DNA extract. The 11 loci were originally designed for several bird species including Black Grouse (BG), Chicken (ADL and LEI), and *T. urogallus* (Tut and StuD). However, they have proved to be suitable for the *T. urogallus* subsp. *aquitanicus* (cf G. Jacob researches, Biology Department, University of Fribourg, Switzerland). Amplification of each microsatellite, either alone or within a PCR multiplex, (Multiplex 1: BG15, BG18, LEI098, TuT1, TuT3 and TuT4. Multiplex 2: ADL142 BG10, BG20, sTuD1 and sTuD3) was first validated using a DNA extract from a muscle sample as positive control. Forward primers were marked using fluorochrome (NED, PET, FAM and VIC) depending on their multiplex. Once the protocol was validated, a PCR was performed on each DNA extract in a final volume of 12.5µL, containing 2µL of DNA extract, 1X of Qiagen Multiplex PCR Master Mix, 0.2 µM of each primer and RNase/DNA free water. We used the following PCR program design : i) Premelt at 95 °C for 15 min, ii) Denaturation at 94 °C for 30 sec, iii) Annealing at 57°C for 2 min, iv) Extension at 72°C for 45 sec and v) Final extension at 72°C for 15 min. Steps ii) until iv) were repeated 38 times. Amplifications were checked on a 2% electrophoresis agarose gel and finally sent to the INRAE Gentyane genotyping platform in Clermont-Ferrand (France).

The raw microsatellite genotyping results were pre-processed using the GeneMarker software (Holland & Parson, 2011) to score alleles at each locus for each sample. Samples with one or more missing loci were genotyped again in an additional PCR replicate to complete the data set. Loci that could not be assessed after this replicated step were coded as missing values.

Gender determination procedure

Each DNA extract was genetically sexed based on the amplification of the *CHD* gene located on the sexual chromosoms within birds. A fragment of the *CHD* region was amplified by Polymerase Chain Reaction (PCR) using the modified primers P3' and 1237L provided by Appendix A of Cayuela *et al.* (2019). The PCR was performed in a final reaction volume of 25µl containing 2µL of DNA extract, 5X of GoTaq Buffer (Promega), 5 unit/µL of GoTaq® G2 DNA Polymerase (ref M7845 Promega), 2µM of each dNTPs, 2µM of each primer and RNase and DNA free water. We used the following PCR Program : i) premelt at 95 °C for 2 min, ii) denaturation at 95°C for 45 seconds, iii) annealing at 55 °C for 2 min, iv) extension at 72°C for 45 seconds and v) a final extension at 72°C for 10 min. A total of 38 cycles were performed from denaturation to extension. PCR products were then run on a 3% agarose gel with TAE 1X in order to assess fragment numbers and sizes. Two alleles differing in size, CHD-Z (244 bp) and CHD-W (269 bp), allowed us to distinguish heterogametic females (Z/W) and homogametic males (Z/Z). According to this molecular sexing procedure, one band is expected for males and two bands for females. Samples with questionable gel results after 3 attempts were associated to a sex using microsatellite data.

Extraction and quantification of stress hormones

Using a subset of 96 samples equally distributed among the 8 prospected areas within the reserves of Py and Mantet, we analysed the stress level of the individuals. These analyses were conducted in collaboration with R. Palme and S. Macho-Maschler at the University of Veterinary Medicine in Vienna, Austria (Vetmed). We carried out a spatial study based on short-term stress. Samples were then

selected according to the Reserves's suspicions concerning human disturbances of these sites : 2 suspected "disturbed" sites (P6, M4), 3 sites assumed to be "undisturbed" (P4, PR1, P2) and 3 sites considered as "mid disturbed" (P5, M2, M3).

Two biological replicates per sample were analysed by splitting each faeces into two when possible, or by taking 2 faeces from the same collection tube. We also made 3 technical replicates. We measured the concentration of corticosterone metabolites (CMs) using a cortisone competitive EIA (i.e Enzyme ImmunoAssay) approach that has been validated for Western Capercaillie. This EIA has been demonstrated to be the most efficient to measure CMs targeting the 3,11-dioxoaetiocholanolon (Thiel *et al*., 2005). CMs were extracted from faeces following the recommendations of Palme *et al*. (2013). We weighed 400mg of defrosted samples that we vortexed for 30 minutes in 4 ml of 60% methanol. We centrifuged the mix at 2500g for 15 minutes. The supernatant was collected and put into the freezer at -20°C until the EIA was performed. All results were obtained by measuring absorbance at 450 nm and using the standard curve to trace our initial concentrations. Finally, we made calculations taking into account the dilutions made on our samples.

Data analysis

Multi Locus Genotyping

All statistical analyses were performed using R.4.1.2 software. Using the R package Adegenet (Jombart, 2008) and Poppr (Kamvar *et al*., 2014), we graphically estimated the power of our microsatellite dataset to distinguish different individuals based on an accumulation curve.

The number of unique Multi Locus Genotypes (MLGs), which corresponds to the minimum number of individuals sampled, was determined from the microsatellites dataset (Taberlet & Luikart, 1999). The total obtention of all genotypes will be checked with a rarefaction curve. The cleaned genotypes obtained from each sample were assigned to a unique MLG using the R package 'poppr'. The MLGs were first assigned based on the computation of pairwise dissimilarity distances between each pair of genotypes. Samples were then assigned to a single MLG if they presented less than 3 distinct alleles over the 22 from the 11 loci. Based on this first automated step, we then ran a visual inspection of all genotypes assigned to each unique MLG and cross-checked the results obtained from molecular sex determination.

Once the MLGs are defined, we used *F*- or *G*-statistics (Pegas R Package (Paradis, 2010) and Genodive 3.06 Software (Bertrand *et al*., 2016)), to compute F_{IS} indexes and measure the deviation from panmixia. The F_{IS} coefficient varies between -1 and 1 depending on H_O (observed heterozygote) and H_S (expected heterozygote) values. A positive F_{IS} shows a deficit of heterozygotes (translated by the relationship $H_O < H_S$) and conversely a negative F_{IS} indicates an excess of heterozygotes ($H_O > H_S$). The *P-value* was determined via the implementation of 999 permutations on Genodive.

We then explored levels of genetic diversity and genetic structure amongst individuals and sites by running a Principal Component Analysis (PCA) as implemented in the Adegenet R Package. We also performed a clustering analysis (sNMF) with the LEA R package (Frichot & François, 2015) in order to estimate the most likely number of genetic clusters K (K= 1 to 8) which supports the PCA analysis.

Model averaging approach to assess stress response to human disturbance

The stress response was analysed using a series of Generalised Linear Mixed effects Models (GLMM). GLMMs extend traditional linear models to include a combination of fixed and random effects as predictors. The introduction of random effects offers a significant advantage due to the structure of biological data. This data is often highly structured, containing non-independent and hierarchical groups of units. Mixed models allow us to explicitly model the non-independence of this data. The addition of random effects helps to correctly infer fixed effects depending on the level of the system hierarchy that is being manipulated (Harrison *et al*., 2018). All generalised mixed models were implemented in R via the lme4 package (Bates *et al*., 2014).

We used an information theoretic model averaging approach, testing for the effect of multiple factors and their interactions using the MuMIn package (Grueber *et al*., 2011). This approach allows for multiple hypotheses to be tested in the same analysis in the following steps : (i) from an initial complex model, this approach generates all possible derived models of the predictors of interest (addition or interaction of variables) included in the initial model ; (ii) the models are ranked according to their AIC value (Akaike Information Criterion, 95% confidence level) ; (iii) the average of the predictor estimates across all selected models emerges, and is weighted by the AIC of each derived model (Anderson & Burnham, 2002) ; (iv) the model averaging results are interpreted from the mean estimates and 95% confidence intervals (CIs) of each predictor variable (Turek & Fletcher, 2012). The stress dataset was previously log-transformed. The initial model was chosen for its biological relevance. In our case, two parameters were considered as "fixed" effects, i.e. the sector of sampling collection in respect with its 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 accounted for. The resulting model was written as follow:

lmer(CMs concentration ~ Level of disturbance * Sexe + (1|Replicates)

Hypotheses on the level of disturbance within the sectors were formulated on the basis of the observations by the Catalan Reserves field teams made in relation to hiking trails and off-track activities. Expected disturbance levels were incorporated into the initial model in an ordinal manner.

A value of 1 was assigned to a sector assumed to be "undisturbed", a value of 2 to sectors assumed to be "mid-disturbed" and 3 to sectors assumed to be "disturbed". The lowest ordinal modality is considered as the control, in this case the site assumed to be "undisturbed".

The assumptions of normality and homoscedasticity of the variances of the residuals of the models were checked (Kolmogorov-Smirnov test p-value: 0.5736, Levene test p-value: 0.5883) as well as the consistency between replicates (Pearson correlation test, p-value = 1.374e-14, t = 9.851, df = 66).

RESULTS

Establishing minimum population Size and Sex Ratio

Among the 229 collected dropping samples, 132 were successfully genotyped at at least 11 loci (63.5% amplification success). In this regard, the accumulation curve obtained from our microsatellite dataset indicated that 9 loci

are sufficient to assign all samples to 100% of the MLGs (**Fig 1**). From these 132 genotypes, 67 MLGs were defined. Although the rarefaction curve bends considerably as the number of samples increases, we have not yet reached the plateau (**Fig 2**). Overall each individual was indirectly sampled (via the faeces) from 1 to 7 times (**Fig 3**).

We assessed the sex determination for 203 of the 229 initial samples. Of these 203 samples, 70 were from females, 123 were from male grouse, and 10 had no sex assigned (84.72 % amplification success). Finally, the sexing data was cross-referenced with the MLG results in order to distinguish the sexing of these 10 samples, with the assumption that several samples belong to the same individual. Of the total 67 individuals, we identified 34 females (50.7%) and 32 males (47.8%). Only 1 individual ended without sex determination (1.5%) (**Fig 4**)

Inbreeding and genetic structure

From the MLGs, we studied parameters indicative of the health status within the population. For the Catalan Reserve's population, we obtained a H_O of 0.28, an H_S of 0.36 and a F_{IS} of 0.23 with a *P-value* of 0.001 (CI: [0.1638 ; 0.3096]). This was achieved irrespective of using either R or the Genodive software. These results show a deficit in heterozygotes, and therefore, the presence of inbreeding in the population.
The first two components of the PCA respectively explain 11.70% (PC1) and 8.75% (PC2) (i.e. 20.45%) of the total genetic variance. There is a large overlap between individuals from different reserves, especially between Py and Mantet. An outlier was recorded in the samples collected at the border between Py and Mantet (**Fig 5**). This result suggests that all individuals belong to the same genetic entity with very few if no spatial genetic structuring. The clustering analysis (sNMF) gives us a highest affiliation proportion for K=1 which is in line with the PCA results.

Model averaging of CMs concentration

The "Sector" is the unique variable contributing significantly in explaining the variation at the level of CMs (**Fig 6 & 7**). The estimated values (-0.40) and effect sizes (CI : [-0.597 ; -0.204]) reveal significant negative effect of disturbance level on the amount of CMs. Individuals are significantly less stressed in the sites *a priori* defined as disturbed compared to the individuals in *a priori* undisturbed control sites. No difference in stress levels was observed between females and males (Estimate = -0.0408 ; CI : [-0.562 ; 0.481]). Finally the level of disturbance in the sites does not affect males and females differently (Estimate = -0.063 ; CI = [-0.460 ; 0.333]). The part of variance explained by the model is 12% (adjusted $R^2 = 0.124$).

DISCUSSION

Non-invasive molecular approaches allow for increasingly innovative and integrative ecological research. In our study, we showed that a substaintial level of integration is possible even when studying sensible and threathened species such as the Western capercaillie, including individual genotyping, inbreeding assessment, sex ratio, population structure and stress status.

Population size and sex ratio

Based on our results, at least 67 individuals are present locally, which is in line with the most recent direct counting campaign achieved in the three Catalan Reserves, during which 60 individuals were reported (11,7% more). However, we would expect to find even more individuals, according to the rarefaction curve obtained. With the further genotyping effort put in place, the number of

assigned individuals should increase to reach a plateau, further demonstrating the robustness of the method.

The use of non-invasive sampling and genetic tagging overcomes the biases of direct counting as seen on leks. Lek use in this species is age dependent. Dominant males defend the territory close to the leks while young males stay back in peripheral areas. Only males aged three years or more are usually present at the singing sites (Raúl Pimenta *et al*., 2014); Young males could therefore be missed during the counting campaigns and the population size underestimated. According to our knowledge, bioacoustic have recently compared male activity on the leks and showed that male activity on leks returns to normal when observers are no longer visible at a great distance from the lek for a while. On the other hand, females only come to the leks for a few days to meet the males. Female counts may also be biased if they are made between two female transits. Importantly however, molecular approaches also display some limitations (Miller *et al*., 2005). Genotyping from non-invasive samples requires special measures to avoid misidentification leading to erroneous inferences (Hausknecht *et al.*, 2010). First, the robustness of the non-invasive genetic approaches relies on the sampling effort. In this regard, and despite an important sampling (i.e. 229 droppings sampled) only 132 were exploitable at the end. The quantity and quality of DNA extracted from this kind of sample is generally low, especially in faeces. The quality of faeces DNA extracts mainly depends on sample freshness. Sampling was carried out by taking as many fresh samples as possible, but this is not always possible in field surveys. The faeces' freshness is difficult to determine. The time of collection was not recorded in order to know how much time had elapsed between the collection of the sample and its storage in the freezer. At low DNA concentrations, allelic dropouts are more frequent during the PCR reactions. One of the two alleles at a locus of a heterozygous individual may not be detected (Gagneux *et al.*, 1997), even if the fragment of interest is amplified. Caution is therefore required in the assignment of consensus genotypes. In this respect heterozygous samples affiliated with identical MLGs that contained homozygous samples were preferred to make the consensus genotypes in order to compensate for the allelic dropout.

Assigning each DNA extraction to unique MLGs, or individuals, also depends on the genetic resolution of the marker used. Here, and despite low variability among loci, 9 out of the 11 loci used in this study were sufficient to reliably obtain distinct MLGs. It is however necessary to repeat the genotyping to check the initial allele assignments and to fill in missing data for samples that were doubtful. Testing the efficiency of the extraction kits on the same samples confirmed that the 3 kits were equivalent and perfectly suited for genotyping. For MLGs identification, we chose to favour a mismatch threshold of 3, but we also compared with a mismatch threshold of 2 for complicated genotypes. This allowed us to better estimate the number of individuals.

Regarding sex determination, 84.72% of the samples were successfully amplified. We consider that our method worked. For this study, we expected that there would be more females than males in accordance with the estimates of the Reserves. However, the results show an almost equal sex ratio with 34 females and 32 males. The fact that males were found more often than via direct observations can be explained that males are those that are more easily missed on leks. Our results thus indicate that males are probably more common than previously thought even if

those males most likely do not participate in reproduction. The gel sexing data were cross-referenced with the MLG to assign a sex to each genotype. Some samples from the same MLG could have a different sex. In such a case the affiliation "female" was preferred. This choice is based on the potential bias of the CHD gene amplification (e.g. a single band at 269 bp, the position of the W allele, or a bar between 244 and 269). Importantly however, and according to Gwenael Jacob's previous work, the capercaillie population in Belarus (*T.u. subsp. pleskei*) is composed of 13 males and 7 females (20 individuals) while the population in the Vosges (*T.u. subsp. major*) has 72 males and 60 females (132 individuals). It is in our interest to increase the genotyping effort in order to obtain more concrete data and to check if this trend of balance persists or if our population looks like the others.

Genetic structure and diversity.

The F_{IS} value supports the hypothesis that the local population of *T. u.* subsp. *aquitanicus* suffers from *inbreeding* and suggests that the population is not renewing itself sufficiently. Compared to our population, grouse from Belarus ($H_O = 0.623$; $H_S = 0.636$; $F_{is} = 0.021$), the Pyrenees ($H_O = 0.389$; $H_S = 0.403$; $F_{is} = 0.036$) and the Vosges ($H_O = 0.351$; $H_S = 0.395$; $F_{is} = 0.112$) were also inbred although to a lower extent. In fact the inbreeding index of the Catalan populations is much higher than that of other populations. Although these values are likely to change with increased sampling effort, this puts the capercaillie in a delicate position from a genetic point of view. With a balanced sex ratio, one would expect a positive degree of reproductive success and genetic diversity but that doesn't seem to be the case here.

This deficit of genetic diversity can be explained by the high sedentarity of this species. Migration in this species is described as low (Raúl Pimenta *et al*., 2014). Some pairs stay in the same area and may spend several winters together. In all capercaillie populations, a variable proportion of males parade solitarily. The first case observed is that of juvenile roosters. If they survive, they integrate a lek during their third year. Their crowing location is generally not stable. Their display activity is reduced and irregular, inducing poorer detection. As seen in another example, it is possible to find solitary roosters because their home range is too small to accommodate others. Roosters using mountainous areas with high relief are found in partitioned habitats where it is difficult to recruit new males and females. A common belief is that the situation in a singing place is unchanging. If a population is at equilibrium in a stable habitat, the singing place remains stable too (Bernard & Menoni, 2018). On the contrary, if the population is reduced, the numbers on the singing places decline and it is more common to find solitary males. This may explain the low recruitment rate and the loss of genetic diversity.

Moreover, the lek breeding system leads to difficulties in finding a mate (Gascoigne *et al*., 2009) or in performing cooperative behaviour. Related to age-dependency, the dominant breeding of old males does not favour genetic mixing. Studies have shown that birds from the same lek are more frequently related to each other than to birds from neighbouring locations. This tendency to return to or remain at the place of birth (i.e. philopatry) is known for many species (Bernard & Menoni, 2018). From all these perspectives, it exposes the population to a high risk of genetic drift, and later extinction (Courchamp *et al*., 1999). These consequences have already been observed in *Tetrao urogallus* (Swiss Alps) by Cayuela *et al*., (2021) who showed a high inbreeding rate and low allelic diversity.

Added to this perspective, PCA and clustering analysis reveals that the individuals appear to belong to a single genetic entity. Only one individual leaves the block, probably due to a microsatellite reading error. This individual has an allele for the LEI098 marker that is not found in any other sample before MLGs affiliation. It would be interesting to genotype it again to validate or not the appearance of this new allele. The population dynamics of the capercaillie can be linked to a metapopulation system (Segelbacher & Storch, 2002). If a favourable area becomes available, or a previously less favourable area has evolved, new females colonise this area to raise chicks. A few years later, a new singing place was created nearby. Here, we did not detect any migrant individuals from a genetically different population. It does not mean that inter-population breeding cannot occasionally occur. The metapopulation structure does not stand out either because there is sufficient mixing to obtain a homogenisation of the population. Or because the geographical scale is too small to allow it to be seen. In a further perspective, the study should be extended to other populations (e.g. Pyrenees, Vosges, Belarus) to see if new PCA patterns emerge on a larger scale. From a conservation perspective, it would be important to see whether there is sufficient inter-habitat connectivity to allow individuals from different areas to interbreed.

Stress Status

Our study serves as a 0-state and gives answers to the Catalan Reserves regarding their suspicions about the different levels of stress in different sectors. Modelling on CMs concentration tends to be the opposite that we expected. It revealed a trend towards lower stress levels among individuals present in areas with high frequentation. Several hypotheses can be put forward to explain this result. Perhaps anthropogenic pressures are not as involved in grouse stress as expected.
The model averaging explains a small percentage of the variance in the stress level of individuals, which means some variables not considered here are involved in the unexplained variance (e.g. predation, mate finding…). It should be remembered that the study was conducted on a spatial and not a temporal scale. A temporal scale would have required a larger sampling effort (e.g. 10 days renewable) and therefore a higher probability of disturbance of the bird. The spatial scale was favoured to overcome this counterproductive bias. But the spatial scale cannot reveal trends beyond the date of sampling. Thus our results cannot predict whether at a different date than our sampling, the state of stress would have been more or less important.
According to the observations of the Reserves, the areas classified as "disturbed" are also the ones with the most individuals. It can be assumed that there is a balance : the pressure to find a mate decreases when population density is high but competition for resources and reproduction can occur. In the next perspective, it will be interesting to repeat the study during the breeding season, to test the stress analysis on a time scale in order to follow the evolution of the trend.

CONCLUSION

Our study has completed the understanding of the Western Capercaillie in the Catalan Nature Reserves by providing new knowledge on the general state of the sub-population of *T. urogallus* subsp. *aquitanicus*. The sex-ratio, the population size, genetic diversity, as well as the status of stress within the birds were successfully determined, although adding more samples would increase the reliability of the results. These results will give the reserves the opportunity to adapt their conservation plan by focusing on decreasing the level of inbreeding within the population,

and hopefully increase the number of grouse. Our perspective moving forward would be to assess the parasitic load of the birds as well as their diet from the same non-invasive DNA samples, in order to go further in the integration of data.

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ILLUSTRATIONS

Figure 1 : Graphical representation (genotype_curve R function) showing that 9 loci are sufficient to obtain 100% of the MLGs within Western Capercaillie population (*Tetrao urogallus subps. aquitanicus*). A maximum of 2 missing loci per sample was thus accepted.

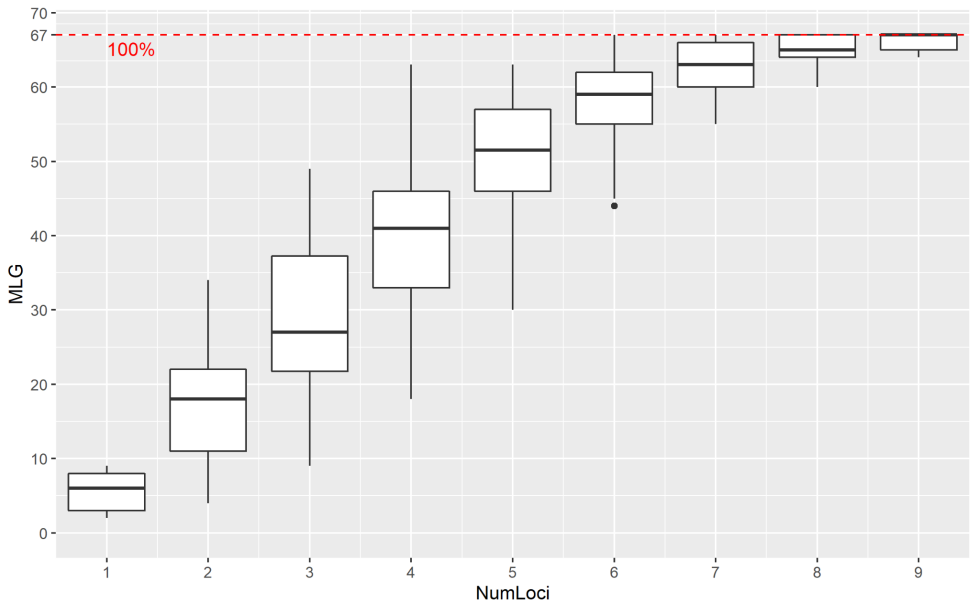


Figure 2 : Rarefaction curve which shows the estimated number of individuals according to the number of drops sampled. This curve aims to reach a plateau illustrating the minimum number of drops sampled necessary to obtain all individuals present in the reserve.

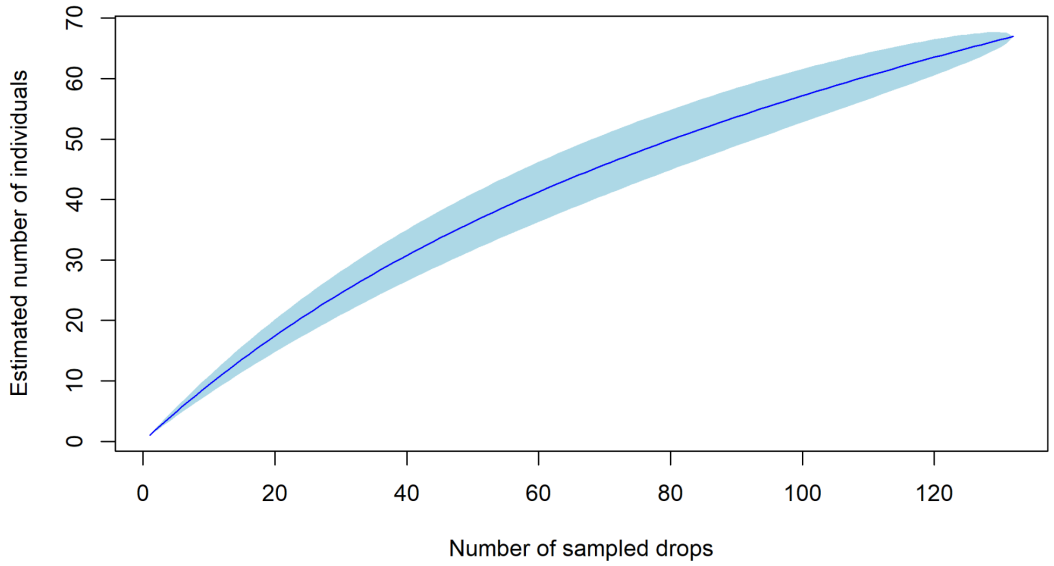


Figure 3 : Frequencies of the number of sampled faeces per individual. The number of samples belonging to the same individual varies from 1 to 7. Most of the individuals were genotyped once.

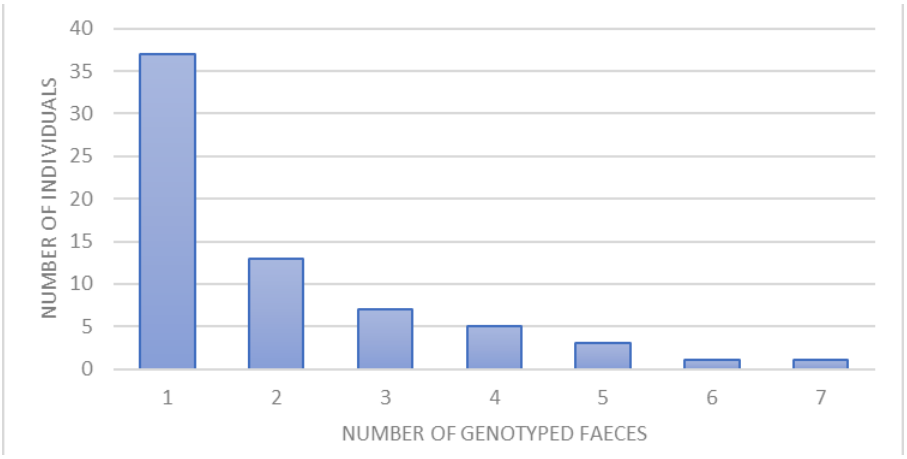


Figure 4 : Global sex ratio of the Reserve of Py, Mantet and Prats-de-Mollo. The total number of individuals considered is 67, with 32 males (47.8%), 34 females (50.7%) and 1 unidentified (1.5%). This result is the combination of the sex-ratio obtained with the amplification of a CHD gene region and the result of the microsatellites study, giving us the MLGs.

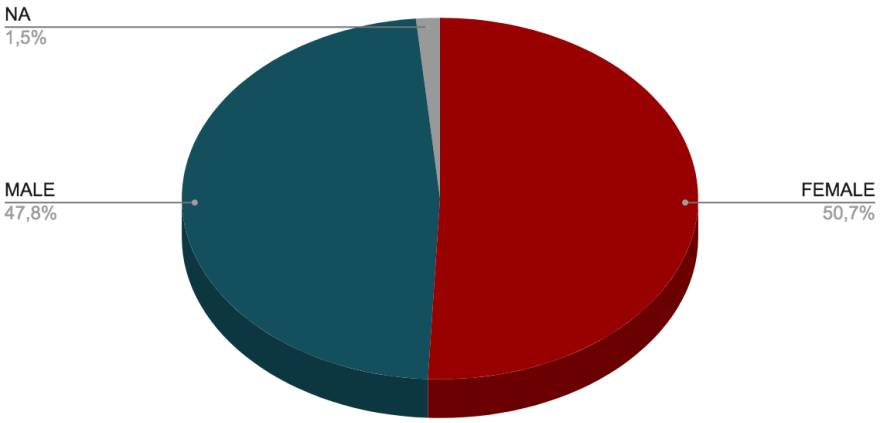


Figure 5 : Principal Component Analysis (PCA) obtained after the analysis of the different MLG present in the reserves of Py, Mantet and Prat-des-Mollos, as well as the section of PY-Mantet, which is composed of samples that were recovered at the border of the reserves. The analysis shows that the samples from the different reserves come together to form a single entity of genetic structure.

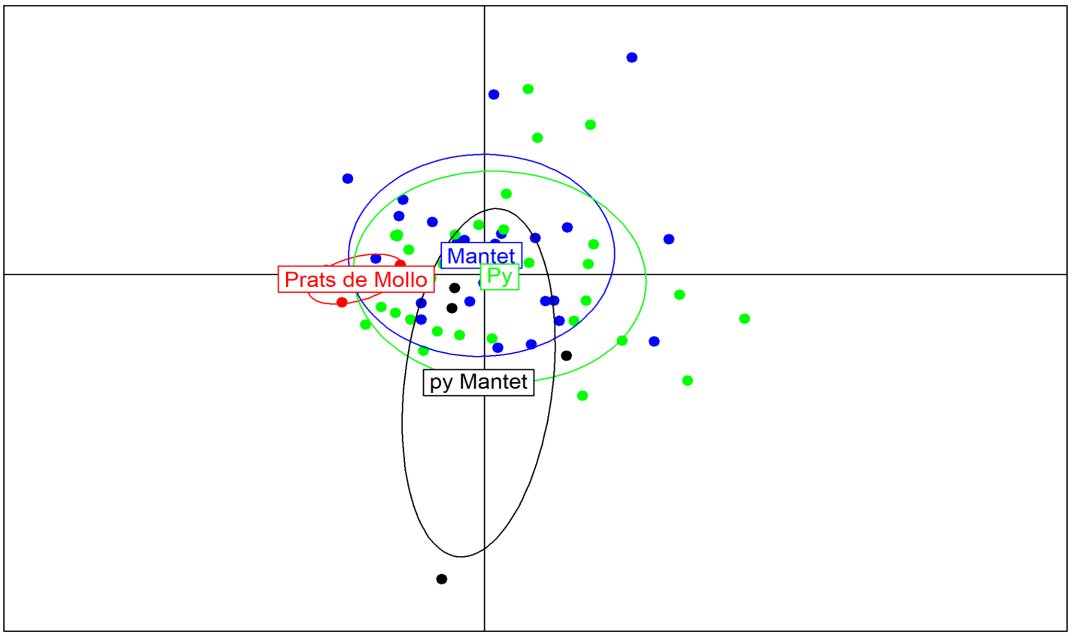


Figure 6 : Boxplot of CM concentration (ng/g faeces) as a function of sectors. The average concentration of the results for each disturbance condition is highlighted by a circle. The "disturbed" sites show a trend of lower stress levels.

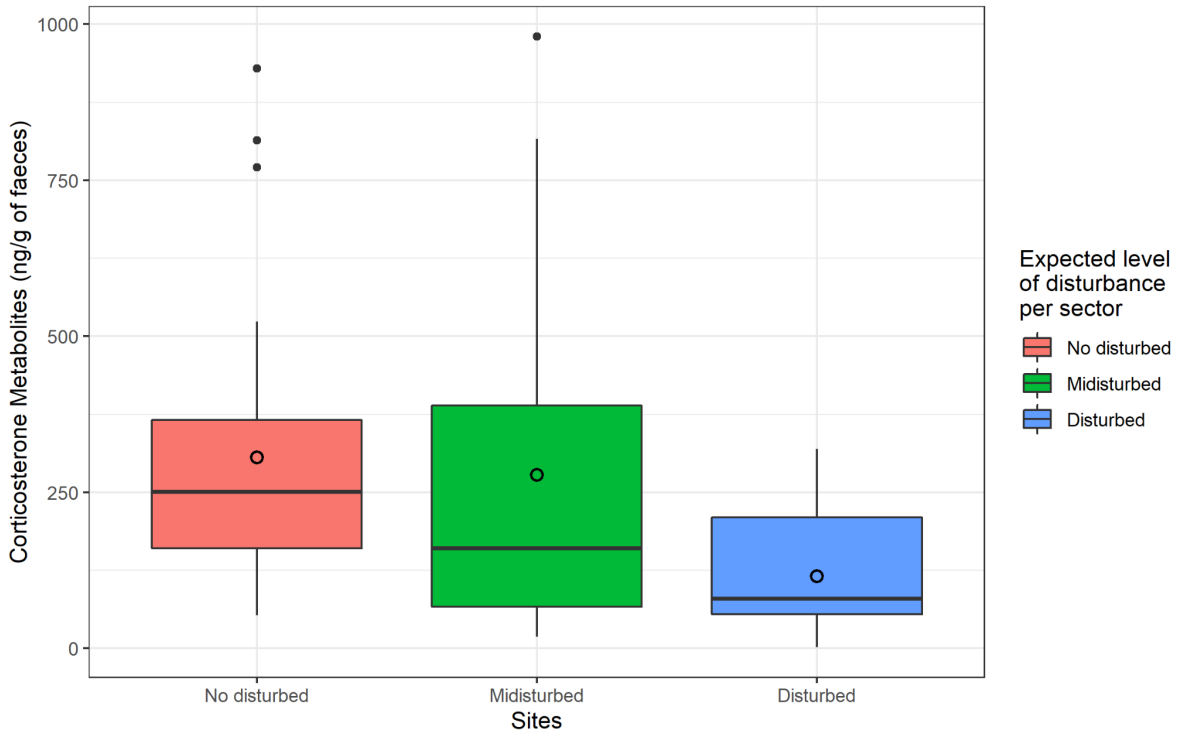
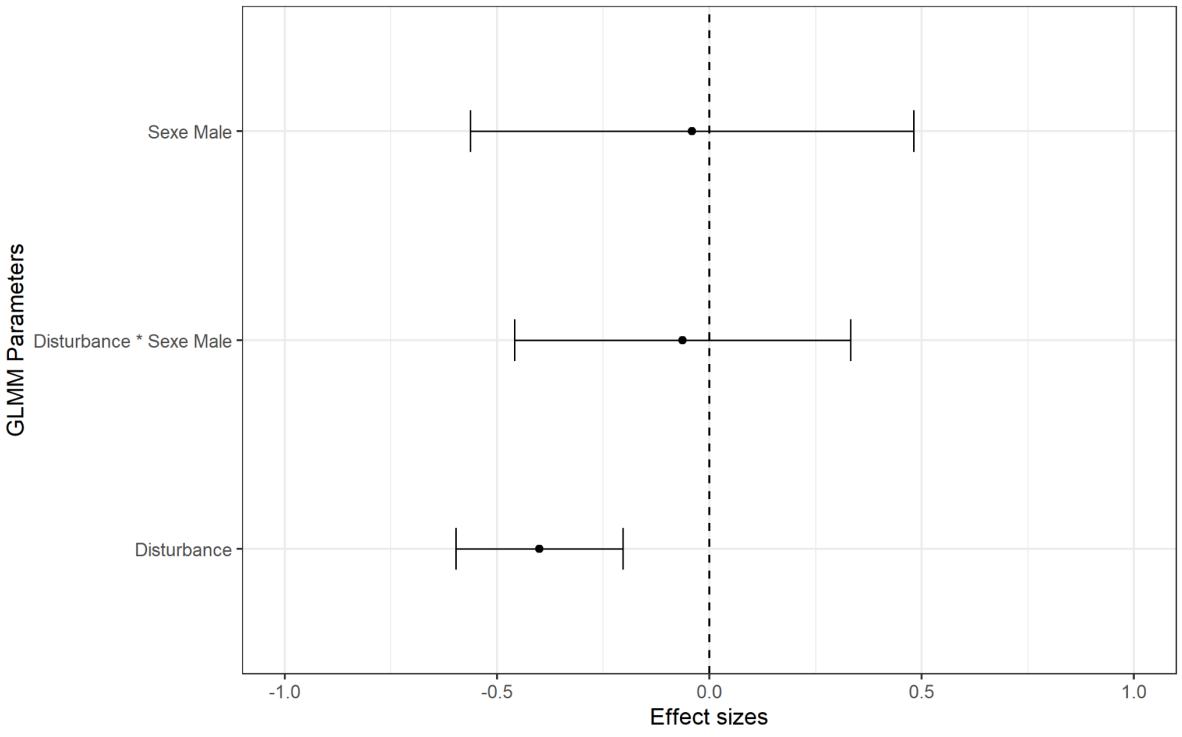


Figure 7 : Estimates and CI values of the stress level by model averaging. Overall, 12% of the variance is explained by the model (R^2 adj = 0.124). The "Sector" is the only variable that contributes significantly to explain the variation of the CMs. Individuals were significantly less stressed in the supposedly disturbed sites compared to the undisturbed control sites (Estimates = -0.40; CI : [-0.597; -0.204]).

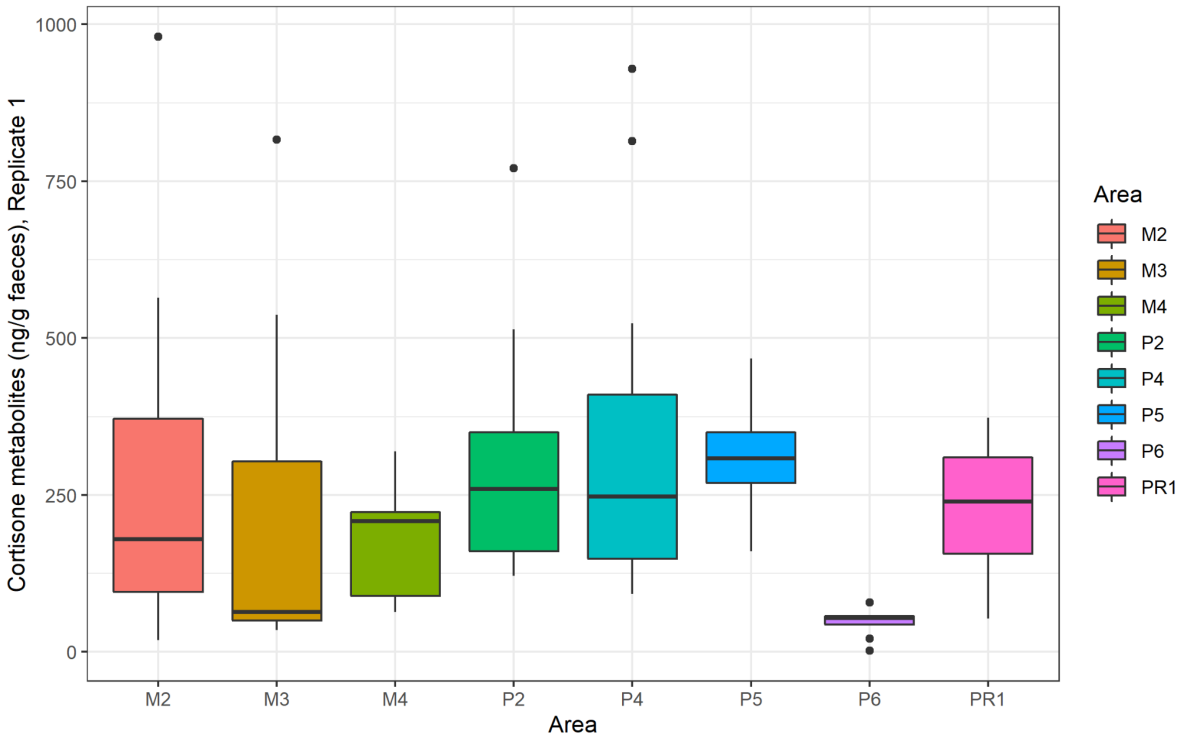


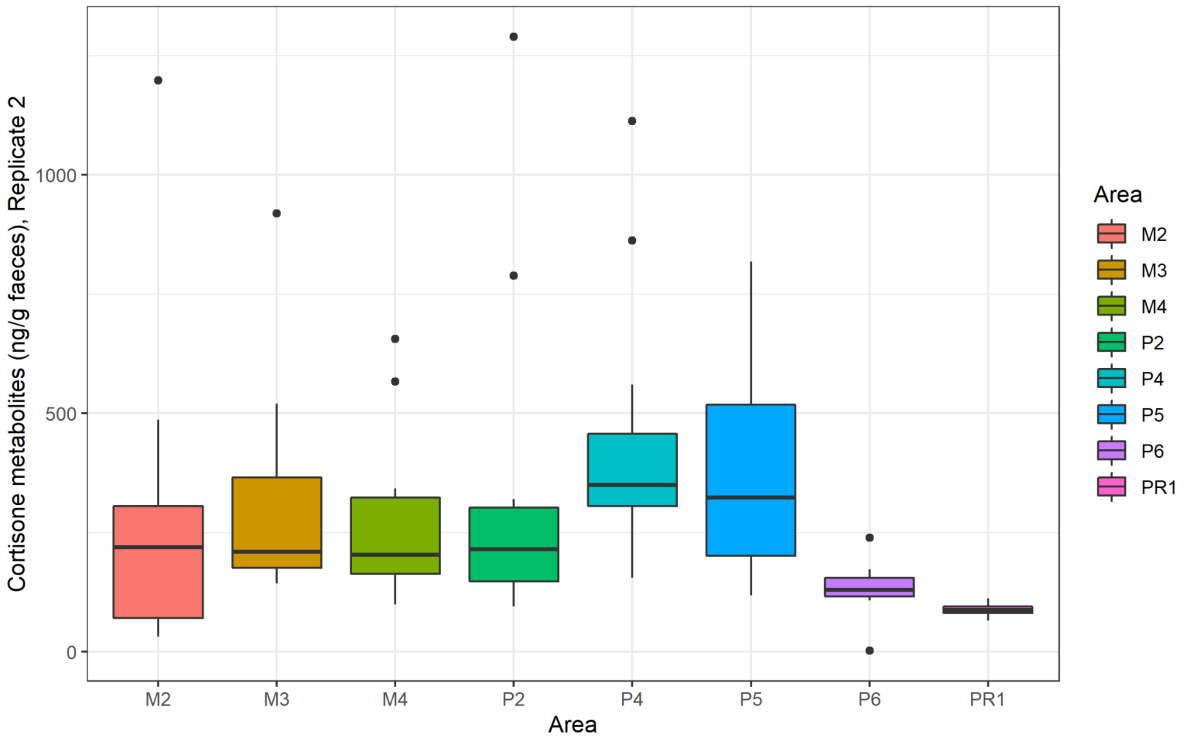
SUPPLEMENTARY FILES

Appendix 1 : Eleven microsatellites and a CHD-gene fragment that were amplified for sexing and genotyping. We indicate locus name, number of alleles, size and range, forward and reverse sequences, fluorescent dye (Blue: FAM, Yellow: VIC, Red: PET , Green: NED), GenBank accession number and reference.

Locus	#Allele (size-range)	Sequences	Dye	GeneBank #accession	Reference
1237	Z(244) W(269)	F: RATGAGAAACTGTGCAAAACAG	Na	Na	Cayuela et al. 2019
P3	Z(244) W(269)	R: GGARTCACTATCAGATCCAGAATATC	Na	Na	Cayuela et al. 2019
BG15	3 (139-143)	F: AAATATGTTTGCTAGGGCTTAC	NED	AF381549	Piertney & Hö̈glund 2001
		R: TACATTTTTCATTGTGGACTTC	Na	AF381549	Piertney & Hö̈glund 2001
BG10	4 (195-207)	F: ATGTTTCATGTCTTCTGGAATAG	VIC	AF381546	Piertney & Hö̈glund 2001
		R: ATTTGGTTAGTAACGCATAAGC	Na	AF381546	Piertney & Hö̈glund 2001
BG18	3 (186-194)	F: CCATAACTTAACTTGCACTTTC	NED	AF381551	Piertney & Hö̈glund 2001
		R: CTGATACAAAGATGCCTACAA	Na	AF381551	Piertney & Hö̈glund 2001
LEI098	1 (148)	F: CAGTTAGCAGAGATTTTCCTAC	FAM	X82860	Gibbs et al 1997
		R: TGCCACTGATGCTGTCACTG	Na	X82860	Gibbs et al 1997
TuT1	4 (198-226)	F: GGTCTACATTTGGCTCTGACC	FAM	AF254653	Segelbacher et al 2000
		R: ATATGGCATCCCAGCTATGG	Na	AF254653	Segelbacher et al 2000
TuT3	3 (150-158)	F: CAGGAGGCCTCAACTAATCACC	VIC	AF254655	Segelbacher et al 2000
		R: CGATGCTGGACAGAAGTGAC	Na	AF254655	Segelbacher et al 2000
TuT4	2 (172-176)	F: GAGCATCTCCCAGAGTCAGC	VIC	AF254656	Segelbacher et al 2000
		R: TGTGAACCAGCAATCTGAGC	Na	AF254656	Segelbacher et al 2000
ADL142	5 (195-217)	F: CAGCCAATAGGGATAAAAGC	NED	G01567	Cheng unpublished
		R: CTGTAGATGCCAAGGAGTGC	Na	G01567	Cheng unpublished
BG20	2 (124-133)	F: AAGCACTTACAATGGTGAGGAC	VIC	AF381553	Piertney & Hö̈glund 2001
		R: TATGTTTTCCTTTTCAGTGGTATG	Na	AF381553	Piertney & Hö̈glund 2001
sTuD1	4 (144-158)	F: ATTTGCCAGGAAACTTGCTC	FAM	AF254644	Jacob et al 2010
		R: CCTTTGCCTCCTTATGAAATCC	Na	AF254644	Jacob et al 2010
sTuD3	4 (144-158)	F: CAAGGGGAAAATATGTGTGTG	FAM	AF254646	Jacob et al 2010
		R: TGTCAAGATATTTCAAGCCTTTG	Na	AF254646	Jacob et al 2010

Appendix 2 : Boxplots of CMs concentration per sector for replicates 1 & 2.





Appendix 3 : Correlation between Replicate 1 & 2 values. (R^2 = 0.771, cor.test : p-value = 1.374e-14, t = 9.851, df = 66).