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ORIGINAL RESEARCH

Noninvasive assessment of corticosterone and triiodothyronine levels in the endangered Pyrenean Capercaillie (*Tetrao urogallus aquitanicus*)

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Abstract

Endocrine regulation and hormonal responses determine the capacity of an organism to cope with changing environmental conditions, such as variable or extreme temperatures. Hormones can also be regarded as "sensors" for environmental signals and as indicators of an organism's or populations' fitness. The levels of glucocorticoid hormones, such as corticosterone, are generally used as biomarkers for physiological stress and metabolic rate variations, while thyroid hormones (i.e., triiodothyronine—T3) are regarded as indicators of nutritional and thermal stress in birds. The Pyrenean Capercaillie (*Tetrao urogallus aquitanicus*) is an endangered galliform adapted to cold weather, and its population is in steep decline. This study used a noninvasive approach to assess hormone levels in the Pyrenean Capercaillie, in a first attempt to understand how hormone levels may respond to certain environmental variables. We determined the corticosterone and T3 levels in 139 freshly molted covert feathers of 113 Pyrenean Capercaillies (80 from Spain and 33 from France) using enzyme immunoassays. The relationships between hormone levels and several biotic and abiotic variables were assessed. Our findings showed that corticosterone levels were lower in feathers found further away from areas of human leisure activity (i.e., cycling trails). Lower corticosterone levels were also found in certain biogeographical areas (i.e., Northern Pyrenees and Pre-Pyrenees), suggesting that corticosterone levels may respond to certain climate-related variables (such as precipitation or temperature). Conversely, T3 levels were influenced by sex and feather length, but did not correlate with any of the environmental variables. The lack of statistically significant differences in corticosterone and T3 in relation to the types of covert feathers analyzed facilitates the collection process for noninvasive studies. Additional hormonal studies could provide essential data to understand the level of endocrine flexibility of the Pyrenean Capercaillie in response to external variables, which may ultimately define the species' resilience to climate change and other drivers of environmental variation.

Introduction

Endocrine regulation and hormonal responses act as pleiotropic mediators of an organism's coping responses to environmental changes, and ultimately define a populations' health and fitness. Understanding the endocrine flexibility of individuals and species may help to predict their resilience to stochastic environmental events and long-term changes (Little & Seebacher, 2024). Hormone levels may also provide "sensors" for environmental signals, condition epigenetic processes, and modulate phenotypes (Little & Seebacher, 2024; Seebacher & Little, 2024). Nevertheless, the study of endocrine responses to environmental variability is challenging and requires a multidisciplinary approach, including endocrinological, ecological, and evolutionary considerations, mostly because hormones have evolved to be multifunctional and their effects on phenotypes can influence a broad range of ecological functions (Little & Seebacher, 2024; Orr et al., 2020). For example, endocrine flexibility may affect the capacity of a species to cope with the extreme temperatures related to probable future climate change and, therefore, may be a predictor of its resilience (Taff et al., 2024; Zwahlen et al., 2024). Both the hypothalamic–-pituitary–adrenal (HPA) and the hypothalamic–pituitary-thyroid (HPT) axes are major mediators of thermoregulation and have both direct and indirect effects on the responses to changing external temperatures (Orr et al., 2020). For example, the glucocorticoid hormones produced by the HPA axis, can facilitate an animal's behavioral adaptations to extreme thermal conditions (Taff et al., 2024) and optimize the energy available for its thermoregulation. In addition, thyroid hormones, produced by the HPT axis, can affect an animal's thermoregulation by influencing its metabolic rate and respiration (Taff et al., 2024; Zwahlen et al., 2024).

Glucocorticoid and thyroid hormones are both examples of multifunctional hormones. Glucocorticoids have been widely studied in endangered wildlife populations to assess physiological stress and identify biotic, environmental and/or anthropogenic variables influencing population trends (Thiel, Jenni-Eiermann, et al., 2008). Acute responses to stress enable rapid physiological and behavioral changes in unpredictably changing environments (McEwen & Wingfield, 2003) so as to maintain stability, or "allostasis" during times of change (McEwen & Wingfield, 2003). In such situations, the HPA axis is activated, increasing the production of glucocorticoids by the adrenal cortex (Arlettaz et al., 2007; Baltic et al., 2005). Glucocorticoids play a major role in energy mobilization (Jimeno et al., 2018), providing glucose to the blood plasma, hence allowing an organism to recover from a stress response and preparing it to respond to future stressors (MacDougall-Shackleton et al., 2019). As a result, glucocorticoid levels reveal metabolic rate variations (Jimeno & Verhulst, 2023). Conversely, when recurrent, this physiological response may lead to an allostatic overload (i.e., chronic stress) and become physiologically or behaviorally detrimental for an individual, or even a population (Arlettaz et al., 2007; McEwen & Wingfield, 2003). Disturbances leading to chronic stress may affect the fitness of birds through immunosuppression, reducing their reproductive success (Cyr & Michael Romero, 2007), and/or the amount of time dedicated to foraging (Fernández-Juricic & Tellería, 2000), thus predisposing them to higher predation risks or increased energy expenditure (Amo et al., 2006; Cassirer et al., 1992), ultimately affecting their survival (Arlettaz et al., 2007; Hofer & East, 1998; Jenni-Eiermann & Arlettaz, 2008). Thyroid hormones (THs; i.e., the biologically active form triiodothyronine and its precursor thyroxine, which is converted to T3 by deiodinase enzymes) are well-known avian growth promoters (Arancibia et al., 1996; McNabb et al., 1998; McNabb & King, 1993; Ruuskanen et al., 2021) that also modulate metabolism and thermogenesis (Decuypere et al., 2005; Ruuskanen et al., 2021; Shahid et al., 2020). Cold exposure can lead to increased conversion of T4 into T3 (and less frequently to the inactive form, rT3) (Van der Geyten et al., 1999), thus leading to higher circulating T3 levels. THs have also been mechanistically linked to DNA methylation, at least partly by regulating the activity of DNA methyltransferase 3a, one of the main enzymes mediating epigenetic responses and influencing the timing of phenotypic adjustments to environmental change (Seebacher & Little, 2024).

Measurement of glucocorticoid levels (e.g., corticosterone) in the plasma of free-ranging animals is an invasive and stressful procedure that may lead to capture myopathy and perhaps even death, as previously reported in several wild bird species (Breed et al., 2019; Höfle et al., 2004; Nicolás Francisco et al., 2022; Ruder et al., 2012). In particular, Pyrenean capercaillies (*Tetrao urogallus aquitanicus*) have been shown to be very sensitive to handling stress (Nicolás Francisco et al., 2022). Noninvasive methods of measuring glucocorticoids have been validated in samples other than plasma, such as fresh droppings (Palme, 2005; Sheriff et al., 2010), and used as an indicator of acute stress (Arlettaz et al., 2007; Baltic et al., 2005; Palme, 2005; Thiel et al., 2005). Corticosterone is deposited in feathers during their growth (Monclús et al., 2017), and both baseline and stress-induced secretion levels have been observed (Bortolotti et al., 2008; Monclús, 2018; Will et al., 2014). Feather samples therefore appear to be ideal candidates for the measurement of hormone levels over the long term (Bortolotti et al., 2008; Monclús et al., 2017; Romero & Fairhurst, 2016). For instance, high feather corticosterone concentrations were used to predict stress during the development of chicks in the nest (Will et al., 2014) and the mortality rate and/or reproductive failure of several free-ranging avian species (Monclús, 2018).

THs have been previously measured and used in several wild bird species (Branco et al., 2023; Hissa et al., 1983; Kitaysky et al., 2005; Pérez et al., 2018; Reinert & Wilson, 1997; Welcker et al., 2013) to indicate food deprivation (Kitaysky et al., 2005), metabolic rates, and thermal regulatory capacity (Decuypere et al., 2005; Shahid et al., 2020). Noninvasive measurement of THs in feces has also been validated for several wildlife species (Wasser et al., 2010). Conversely, there is scarce data regarding the assessment of THs and their validation in feathers in wild birds (Branco et al., 2023).

The Pyrenean Capercaillie is a polygamous forest galliform well adapted to subalpine mountain forests and to their extreme bioclimatic conditions (Canut et al., 2021), and exhibits a complete postnuptial molt (i.e., molts all of its feathers once a year), between the beginning of July and the end of September (Castroviejo, 1975; Leclercq & Ménoni, 2018). This species was recently reclassified as "Endangered" in the Spanish List of Wildlife Species under Special Protection Regime (Spanish Extinct Species List, 2023), based on its currently low reproductive success (Gil et al., 2020) and consequent population decline causes are still unclear. Related grouse populations, including the Western Capercaillie (*Tetrao urogallus*) and other galliform species such as the Black Grouse (*Lyrurus tetrix*) are very sensitive to habitat fragmentation, climate change, and human disturbances (Storch, 2000; Thiel, Jenni-Eiermann, et al., 2008; Yoccoz et al., 2011). Human disturbance may lead to increased corticosterone levels in Western Capercaillies (Thiel et al., 2005; Thiel, Jenni-Eiermann, et al., 2008; Thiel et al., 2008a, 2008b), and can also lead to chronic stress with long term effects on their endocrine status, body condition, home range, habitat, activity level, and overall fitness (Arlettaz et al., 2007; Jenni-Eiermann & Arlettaz, 2008; Thiel et al., 2008a). THs have not been extensively studied in capercaillies (Hissa et al., 1983); however, in other avian species, THs play a key role in growth and thermal regulation and have been shown to serve as indicators of nutritional or metabolic stress (Cogburn & Freeman, 1987; Kitaysky et al., 2005; Klein et al., 2006; Shang et al., 2021).

As a resident boreal species living at the edge of the capercaillie distribution range (Escoda et al., 2023), the Pyrenean Capercaillie is regarded as an "umbrella species," being very sensitive both to stochastic events and long-term environmental changes (Leclercq & Ménoni, 2018). We hypothesized that corticosterone levels in Pyrenean Capercaillie feathers could be positively correlated with certain indicators of the presence of humans (as a proxy for human disturbance) or bioclimatic conditions and could be used as an indicator of higher metabolic demand or higher levels of physiological stress. In addition, we expected to detect some correlations between T3 levels in feathers and several environmental variables potentially related to nutritional or thermal stress—a novel approach to the use of THs evaluation in birds. The aim of this study was to assess the corticosterone and T3 levels in the Pyrenean Capercaillie using a noninvasive approach based on freshly molted covert feathers collected in the Pyrenees, and to see whether there are differences in corticosterone and T3 levels related to various spatial, biological, geostatistical, and human recreational activity variables. The assessment of hormone levels in free-ranging Pyrenean Capercaillies could provide essential data to help understand their capacity to cope with environmental changes and, thus, form an essential part in developing successful conservation strategies.

Materials and methods

Field sampling

The molted covert feathers of Pyrenean Capercaillies were collected from 45 subalpine forests (Table S1) located in Catalonia and Aragon (Spain), and Occitanie (France), in 2019, 2020, and 2021. These feathers are commonly found in the field in late summer and fall, and are generally easily seen and identifiable due to their size and color (Fig. S1); iridescent black in males and brown in females (Leclercq & Ménoni, 2018), hence enabling sex identification.

Feather sampling was opportunistic, performed during census and other fieldwork activities, to avoid causing additional disturbance. Only covert feathers were included in this study, to reduce sampling variability and ensure intraindividual repeatability. Analysis of covert feathers provided the most consistent results in previous noninvasive studies in Cantabrian Capercaillies (*T. u. cantabricus*) (Martínez Padilla & Estrada, 2021). When several covert feathers of one sex were found together, they were assumed to be from the same individual. Pyrenean Capercaillies are confined to their territories (Leclercq & Ménoni, 2018). Thus, in order to minimize duplicate samples, a maximum of five feathers of each sex were collected from each forest during the same collecting year (Table S1). All feathers were georeferenced, identified, and stored in paper envelopes placed in a dark, dry environment at room temperature until their analysis in the lab.

Collected feathers

A total of 139 Pyrenean Capercaillie molted covert feathers from 113 individuals were collected (Fig. S1). All covert feathers were classified into three different types depending on the body area concerned, that is, wing (infra-covert wing feather), tail (uppertail or undertail covert feather), or body (body covert feather—chest, belly, or back areas) (Baker, 1993; Fernandez, 2024). Feathers that could not be classified into one of these three categories were discarded from this study. The birds' sex was recorded, but not their age because it could not be inferred based on the appearance of the coverts in the great majority of cases (Castroviejo, 1975).

Laboratory procedures

The covert feathers were weighted (mg) with a precision scale to the nearest 0.1 mg (mean ± SD: 62.92 ± 24.73 mg) and those weighing between 35 and 155 mg were selected. The type of feather was then classified by body area (i.e., wing, tail, or body). When a single feather was not heavy enough to reach the minimum chosen sample mass of 30 mg per individual, two covert feathers with similar morphological characteristics were lumped together.

An optimized protocol for extracting hormones from feathers was applied (Monclús et al., 2017). Briefly, after removing the calamus, feathers were measured (mm) with a caliper to the nearest 0.1 mm (mean ± SD: 109.40 ± 23.0 mm) and were then mechanically minced in a ball mill (Retsch®, type MM2, Haan, Germany) (Fig. S1). The resulting powder was weighed to the nearest 0.1 mg (53.48 ± 22.38 mg) using a precision scale (Fig. S1) and placed in a 2 mL polypropylene tube. Methanol reagent (1.5 mL of methanol reagent grade 99.9%; Scharlab, Sentmenat, Spain) was added to each tube and samples were incubated overnight at 32°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) for steroid extraction. Samples were then centrifuged in a Hermle Z300K (Hermle® Labortechnik, Wehingen, Germany) and 1 mL of supernatant was transferred to a new aliquot and placed in an oven at 37°C until total dryness. Dried extracts were reconstituted with enzyme-linked immunosorbent assay (ELISA) buffer, shaken for 5 min, and stored at −20°C until analysis.

Validation tests and corticosterone and T3 measurements were performed using commercial ELISA kits (direct competitive Corticosterone ELISA kit; Neogen® Corporation Europe, Ayr, UK, and T3 competitive ELISA kit; IBL International®, Hamburg, Germany). Assay validation was performed using pools of 20 extracts from different individuals (Carbajal et al., 2014). Test precision was assessed by calculating the intra-assay and inter-assay coefficients of variation from all of the duplicated samples analyzed. The specificity was measured using the dilution test, determined by serially diluting the feather pool with ELISA buffer. Finally, the accuracy of the method was assessed using the spike-and-recovery test, measured by mixing different volumes of the pool with different volumes of the pure standard solution provided in the ELISA kit.

Analysis of geospatial, bioclimatic, and landscape anthropization indicators

To describe the landscape features associated with corticosterone and T3 levels, we generated a circular buffer area using ArcGIS Pro 3.1 (ESRI, Redland, CA, USA) that corresponded to the mean home range described for male capercaillies (mean male home range of 688.5 ha kernel 95; Authors' unpublished data) at each sample location. Home ranges were computed using the locations of 38 VHF (Holohil RI-2D, 19 g of weight) radio-tagged Pyrenean Capercaillies (22 males; 16 females) tracked in Catalonia between 2008 and 2018. We used Qgis (QGIS v3.10.12 [QGIS.org, 2019]) to vectorize the point layer and polygons of Kernel raster probabilities (P50, P90, and P95), and the AdehabitatHR Package (v 1.8.20, Clement Calenge 2004) to compute home ranges using the kernel density estimator.

As well as quantifying the Pyrenean Capercaillie sex, covert feather type (i.e., wing, tail or body covert feathers), weight (mg after mincing the feathers), feather length (mm after removing the calamus), and molting year, we described and quantified 12 abiotic and biotic variables in each buffer area. The bioclimatic variables used comprised the average annual temperature (°C), maximum and minimum annual temperature (°C), and average monthly precipitation (mm). The variables related to topography comprised aspect (North, South, East, or West) and vegetation cover (%). Finally, estimates were made of the impact of human presence and recreational activities using data on the human population density, visitor frequency (Strava heatmap data, Appendix S1, Fig. S2), and the distances between sampling locations and cycling and hiking trails and roads (including only main paved roads, located mainly at the valley bottoms). All the time-dependent variables were averaged over the molting year, including the molting season (end of summer–beginning of fall) for each feather. All these variables, units, and sources are summarized in Table 1. The biogeographic region (i.e., Northern Pyrenees, Central Pyrenees, or Pre-Pyrenees) was analyzed as an independent variable.

In order to detect clusters of corticosterone and T3 levels, hot spots of corticosterone and T3 levels were calculated using Getis's Gi* statistic. A calculated value of Gi Z > 1.96 indicated that the sample points within that study area had a risk value rate that is significantly higher than the other points, indicating that it is a hot spot (Getis & Ord, 1992). The study was performed using Spatial statistic tools in ArcGIS Pro 3.1.

Statistical analyses and model selection

Prior to the selection of the putative drivers of hormone variability, we explored the association between the biogeographical assignation of our samples (i.e., Northern, Central, and Pre-Pyrenees) and the bioclimatic characteristics based on our dataset of bioclimatic variables (average annual temperature, maximum and minimum annual temperature, and average monthly precipitation), using a Principal Component Analysis (PCA), including bioclimatic variables. This confirmed that, as expected, these broad biogeographical regions could be treated as consistent, categorical bioclimatic units in the analyses and in the interpretation of the results (Fig. 1).

A Pearson's product–moment correlation test was performed to explore the correlation between the continuous variables (Table 1); and those variables that were highly correlated with other variables were discarded (i.e., mean annual temperature and feather weight). Based on these initial tests, we selected several continuous variables as putative explanatory variables to be included in the model M1 (i.e., vegetation cover, mean monthly precipitation, visitor frequency, distance to the nearest road, distance to cycling trails, distance to hiking trails, and population density), together with the five categorical variables "feather type," "sex," "molting year," "biogeography," and "aspect." Feather length was also added to the models. The two response variables explored were T3 and corticosterone levels.

Although our sampling design maximized the independence of samples as much as possible across a large study area (including 45 subalpine forests from two different countries; Table S1), it was uncertain whether the samples showed a completely independent, random distribution. In order to verify whether there were any underlying patterns of spatial aggregation in our data, autocorrelation semivariograms were performed using the "nlme" package (Pinheiro et al., 2015) to measure the degree of spatial dependence between variables as a function of distance. A visual interpretation of the semivariograms indicated that spatial aggregation was probably not relevant and that our observations were essentially independent. However, a GLS model was constructed to assess whether autocorrelation should be accounted for in the models; this model included the "corSpatial" R function to account for spatial aggregation of our data, in which the coordinates (longitude and latitude) of each sampling point were specified. The initial, most complex GLS model (M1) included all of the explanatory variables listed above and an autocorrelation term. We then simplified the full models by removing as many nonsignificant variables and terms as possible using Likelihood

Table 1 Geospatial, biological, bioclimatic, and landscape anthropization variables included in the Generalized least squares (GLS) non-simplified models (M1) and simplified models (M2) for corticosterone and T3 detected in feathers of the Pyrenean Capercaillie

Variable type	Variables (units)	Source	Included in the M1 models	Included in the CORT M2 model	Included in the T3 M2 model
Samples	Sex (male or female)	Collection dates and feather classification	Yes	No	Yes
	Feather type (wing, tail, or body covert feathers)		Yes	No	No
	Feather length (mm)		Yes	No	Yes
	Molting year		Yes	No	No
Topography	Aspect (North, South, East, or West)	Calculated from Digital Terrain Model (https://pnoa.ign.es/pnoa-lidar/modelo-digital-del-terreno)	Yes	No	No
	Vegetation cover (%)	Hansen et al., 2013, version update 1.10	Yes	No	No
Climatic	Mean annual temperature (°C)	WorldClim (http://www.worldclim.org/)	No[a]	No	No
	Maximum annual temperature (°C)		No[a]	No	No
	Minimum annual temperature (°C)		No[a]	No	No
	Mean monthly precipitation (mm)		Yes	No	No
Biogeographic	Northern Pyrenees, Central Pyrenees, and Pre-Pyrenees	Classified based on climatic variables (mean, minimum and maximum annual temperature, and mean annual precipitation)	Yes	Yes	No
Antropogenic presence and activities	Human population density (humans/km^2)	CNIG, 2018 and data.gouv.fra (https://www.data.gouv.fr/fr/)	Yes	No	No
	Distance to the nearest road (m)		Yes	Yes	No
	Distance to the nearest bicycle or mountain bike trail (m)	Open Street Maps (https://www.openstreetmap.org/#map=6/40.01/-2.49; https://wiki.openstreetmap.org/wiki/Tag:route%3Dbicycle; https://wiki.openstreetmap.org/wiki/Tag:route%3Dmtb)	Yes	Yes	No
	Distance to the nearest hiking trail (m)		Yes	No	No
	Recreational activities: Strava heatmap	Strava heatmap (https://www.strava.com/maps/global-heatmap?style=dark&terrain=false&sport=All&gColor=blue&gOpacity=100&labels=true&poi=true#4.99/42.29/1.7)	Yes	No	No

[a] They were included in the PCA to define the biogeographic regions.

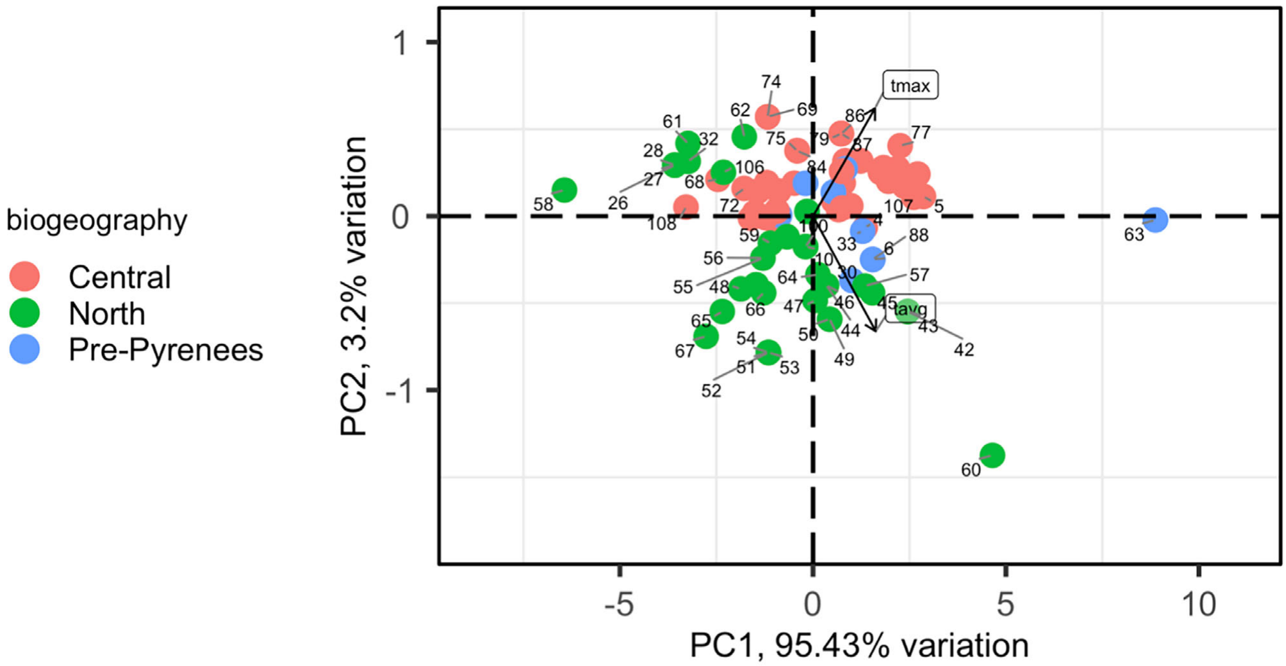

Figure 1 The principal component analysis of the bioclimatic variables (average annual temperature, maximum and minimum annual temperature, and average monthly precipitation), based on the biogeographic classification of the sampling sites.

Table 2 Generalized least squares (GLS) simplified model (M2) exploring the correlations between corticosterone levels detected in feathers of the Pyrenean Capercaillie (log-transformed pg corticosterone/mg feather) and the variables from the M1 model (Table S3) that showed a significant correlation ($P < 0.05$) with corticosterone (included in a dataset called "all_data")

Model M2: gls (log (corticosterone) ~ biogeographic area + distance to the nearest road + distance to cycling trails, data = all_data, method = "ML")

Coefficients	Value	SE	*t*-value	*P*-value
(Intercept)	3.402667	0.06973237	48.79609	0.0000
Biogeographic area-North	−0.198972	0.10032687	−1.98323	0.0495
Biogeographic area-Pre-Pyrenees	−0.383480	0.13468879	−2.84716	0.0051
Distance to the nearest road	0.000034	0.00001838	1.84938	0.0667
Distance to cycling trails	−0.000041	0.00001447	−2.83922	0.0053

The test value (Value) and the standard error (SE), *t*-value, and *P*-value are given. BIC: 177.0671; Degrees of freedom: 132 total; 127 residual.

Ratio Tests, obtaining the simplified models (M2), by selecting the models that showed a clearly lower Bayesian Information Criterion (BIC) score. We used maximized log-likelihood in all cases and checked the normal distribution of the data before running the models. Corticosterone was log-transformed in the models to obtain normally distributed data, whereas T3 was already normally distributed. All statistical analyses were performed in the R studio software v4.3.1 (R Core Team, 2021) with a significance level of $P < 0.05$.

Results

The validation results for the corticosterone and T3 levels in Pyrenean Capercaillie feathers are shown in Table S2. No correlation was found between the corticosterone and T3 levels ($P = 0.58$).

Corticosterone

The mean total corticosterone concentration was 29.80 (±14.43) pg/mg feather. There was no correlation between corticosterone concentration and feather weight ($P = 0.73$) or length ($P = 0.66$) (Fig. S3). The GLS analyses (M1 [Table S3] and M2 [Table 2]) showed that samples from the Pre-Pyrenees and samples from the Northern Pyrenees presented lower concentrations of corticosterone compared with samples collected in the Central Pyrenees ($P = 0.0051$ and $P = 0.0495$, respectively). Finally, of the variables used as a proxy for human disturbance, the distance to roads correlated positively with corticosterone levels ($P = 0.0667$), while the distance to cycling trails was negatively correlated with the various hormone levels ($P = 0.0053$).

Table 3 Generalized least squares (GLS) simplified model (M2) exploring the correlations between T3 levels detected in feathers of the Pyrenean Capercaillie (pg T3/mg feather) and the variables from M1 (Table S4) that showed a significant correlation ($P < 0.05$) with corticosterone (included in a dataset called "all_data")

Model M2: gls (T3 ~ sex + feather length, data = all_data, method = "ML")

Coefficients	Value	SE	*t*-value	*P*-value
(Intercept)	22.730591	1.5602012	14.569013	0e+00
Sex_male	−2.659424	0.6648244	−4.000190	1e−04
Feather length	−0.073952	0.0131483	−5.624409	0e+00

The test value (Value) and the standard error (SE), *t*-value, and *P*-value are given. BIC: 736.2911; Degrees of freedom: 132 total; 129 residual.

Thyroid hormones

The mean total T3 concentration was 12.96 (±4.25) pg/mg feather. The GLS analyses (M1 [Table S4] and M2 [Table 3]) revealed that the T3 concentration (pg T3/mg feather) was significantly affected by the covariate "feather length" (negative correlation, $P = 0.0001$), and by sex, being significantly lower in males than in females ($P = 0.0001$), but it did not correlate with the rest of the variables. Feather length did not differ significantly between males and females ($P = 0.452$).

The hotspot analyses identified significant clusters and hotspots for corticosterone and T3 levels (Fig. 2a,b, respectively), corresponding to two different areas: Hotspots for corticosterone were located in the middle of the study area (Pallars Sobirà and Val d'Aran counties in Catalonia, Spain), while the hotspots for T3 values mostly corresponded to the western side of the study area (Aragon [Spain] and Occitanie [France]). The results of the correlations between T3 and the rest of the variables are shown in Table S4.

Discussion

In this study, we validated a method to determine corticosterone and T3 levels in Pyrenean Capercaillie covert feathers; while covert feathers have been widely used for noninvasive hormone determination of corticosterone, they have not often been used to measure T3 in wild birds (Branco et al., 2023; Martínez Padilla & Estrada, 2021). The ELISA validation assays gave reliable results that demonstrated the assay's precision, specificity, and accuracy in measuring concentrations of both hormones in Pyrenean Capercaillie feathers (Carbajal et al., 2014; Monclús et al., 2017). An ELISA validation of the T3 measurements in feathers was only recently published for the Purple Martin (*Progne subis*) (Branco et al., 2023). It is well-known that, regardless of the time of the year in which feather sampling is performed, hormone levels in feathers correlate with blood hormone levels during the feather growth period (Monclús et al., 2017), and vary according to feather length (Mallet-Rodrigues, 2012), with lower rates in smaller (Wolf et al., 2003) and faster rates in larger feathers (Ingolfsson, 2008). In addition, the complete postnuptial molt of capercaillie means that its feathers provide suitable samples to estimate physiological stress levels during the feather growth period, between July and September; females start molting about a month and a half later than males, but finish at approximately the same time (Castroviejo, 1975). In addition, covert feathers are known to grow faster than their corresponding flight feathers (Castroviejo, 1975) so that their growing period is shorter and their hormone levels reflect conditions over a shorter specific time period, helping to minimize confounding factors (Hardy et al., 2009; Monclús et al., 2017).

To the best of our knowledge, this is the first determination of corticosterone and T3 levels in Pyrenean Capercaillies, an endangered umbrella species known to be very sensitive to handling stress, and which can die suddenly due to capture myopathy during field studies (Nicolás Francisco et al., 2022). Martínez Padilla and Estrada (2021) measured corticosterone in the feathers of another capercaillie subspecies, the Cantabrian Capercaillie, and concluded that physiological stress was higher in the wild than in captive individuals due to nutritional stress (i.e., the wild diet is less optimal than that in captivity) (Martínez Padilla & Estrada, 2021); however, these authors did not measure T3 levels, which are reportedly related to food deprivation in some avian species (Kitaysky et al., 2005; Schew & Ricklefs, 1998).

Our samples from the Northern Pyrenees and Pre-Pyrenees showed lower concentrations of corticosterone compared with samples from the Central Pyrenees. The Northern Pyrenees are colder and more humid than the Pre-Pyrenees, and are considered to offer more suitable bioclimatic conditions for the Pyrenean Capercaillie (Poirazidis et al., 2019). Conversely, the Pre-Pyrenees is the biogeographic area where most of the recent local extinctions of the Pyrenean Capercaillie have occurred (Canut et al., 2021). Thus, lower corticosterone levels have been detected both in areas with theoretically favorable climatic conditions for the species and areas where the population seems to be struggling. A possible explanation is that the corticosterone levels found in the Northern Pyrenees and the Pre-Pyrenees may respond to certain characteristics of these biogeographic regions that have not been explored in this study (e.g., lower prevalence of certain diseases or internal parasites, higher habitat fragmentation, lower density of Pyrenean Capercaillies, and thus, less opportunities to interact and breed).

Many human recreational activities have detrimental impacts on wildlife species (González et al., 2006; Marion et al., 2020; McClung et al., 2004) and trigger stress responses in them (Thiel et al., 2011; Thiel, Jenni-Eiermann, et al., 2008; Thiel et al., 2008a). This study found no correlations between corticosterone levels and human population density or visitor frequency, but there was a statistically significant negative correlation of corticosterone levels and the distance between sampling points and

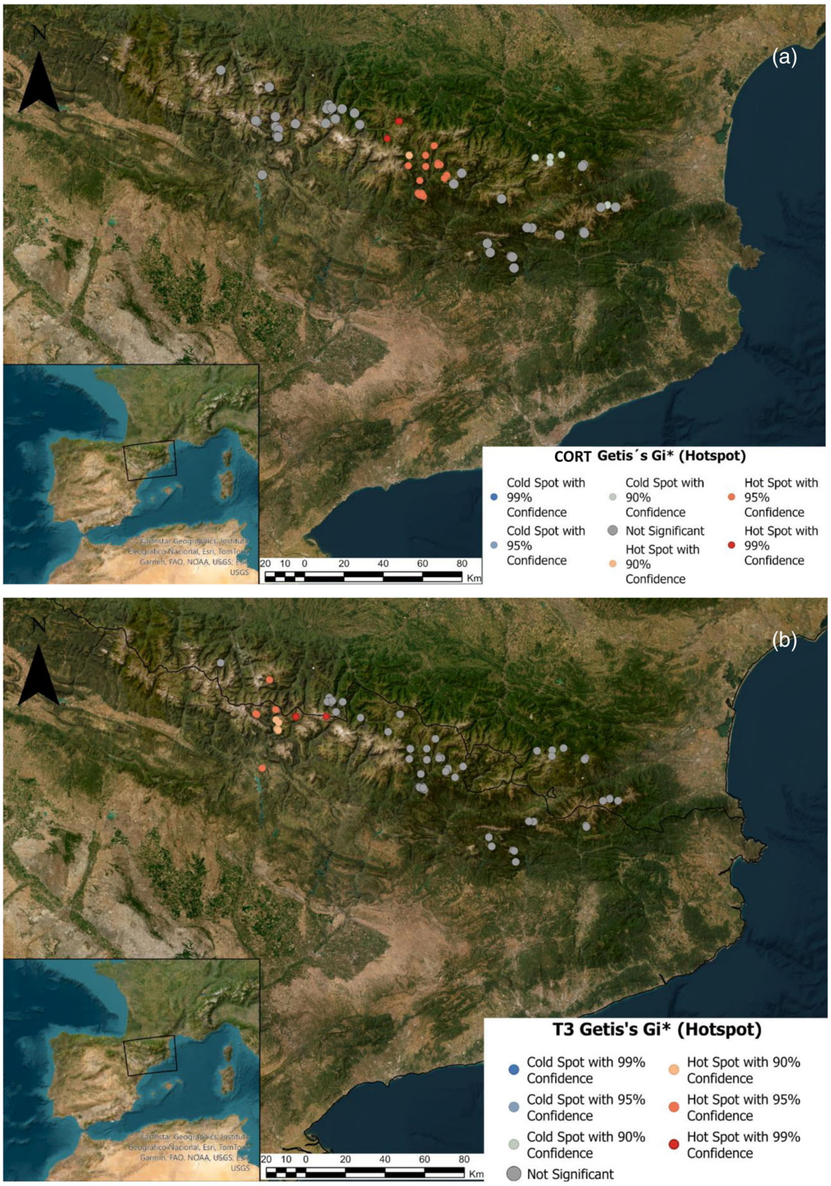

Figure 2 Hotspot analyses for (a) corticosterone (CORT) and (b) triiodothyronine (T3) detected in feathers of the Pyrenean Capercaillie. The hotspot analyses identified significant clusters and hotspots for corticosterone and T3 values (Fig. 1a,b, respectively), corresponding to two different areas. Hotspots for corticosterone were located in the middle of the study area (Pallars Sobirà [Cuco, Esterri de Cardós, Lo Calbo, Orri, Virós and Baiasca forests] and Val d'Aran [Montgarri forest] counties in Catalonia, Spain), while hotspots for T3 values were mostly found on the western side of the study area (Costadue, Tabernes and Trigoniero forests in Aragon [Spain] and Viela d'Aura, Cauteret and Sarrat de Saubat in Occitaine [France]).

cycling trails that, in some cases, cross the habitat of the Pyrenean Capercaillies. Thus, it appears that this last variable could be a better indicator of human presence and the impact of leisure activities. A positive correlation was also found between corticosterone levels and the distance between sampling points and the main paved roads. Physiologically, this correlation is difficult to explain, but it is important to note that most of the subalpine Pyrenean Capercaillie forests are not crossed by proper roads but by unpaved roads, paths, and trails. The linear distances from sampling points to main paved roads ranged from approximately 0.5 km to over 10 km, with a mean distance of 4.12 km, suggesting that this variable may not truly indicate the degree of human presence because roads do not really provide access to the mountain forest areas included in this study.

Among all of the variables tested, T3 levels (pg T3/mg feather) were influenced by sex and showed a negative correlation with feather length; the simplified GLS model revealed lower T3 values in male Pyrenean Capercaillie feathers than in female feathers. T3 concentration in birds generally decreases as a result of stress related to food deprivation and nutritional deficits (Schew & Ricklefs, 1998), lowering the basal metabolic rate and resting energy expenditure (Rosen & Kumagai, 2008), and activating energy saving mechanisms (Cherel et al., 2004; Nelson, 2005) as previously reported in the Tufted Puffin (*Fratercula cirrhata*) (Kitaysky et al., 2005) and King Penguin (*Aptenodytes patagonicus*) (Cherel et al., 2004). During the molting period, Pyrenean Capercaillies segregate according to sex; males and females use their habitat differently during this period and show distinct trophic variability and a corresponding dietary segregation (Blanco-Fontao et al., 2012). Thus, the difference in T3 levels according to sex in the Pyrenean Capercaillie may be a result of their dietary segregation during the molting period, leading to different nutritional stress levels. An alternative hypothesis may be related to thermoregulation; thyroid hormones decrease when temperatures rise above, and increase when temperatures fall below, the species' thermal neutral zone (Silva, 2006; Silva et al., 2023). Hissa et al. (1983) detected seasonal changes in blood plasma thyroid hormone levels in captive capercaillies (in both sexes) in Finland, with a marked drop in May–June, the time in the year when mean temperatures tend to increase. T3 plasma levels also showed a negative correlation with environmental temperatures in other avian species (such as Barn Owl [*Tyto alba*], chickens [*Gallus gallus*], Asian Short-toed Lark [*Alaudala cheleensis*]) (Cogburn & Freeman, 1987; Collin et al., 2003; Klein et al., 2006; Shang et al., 2021). Therefore, an alternative hypothesis is that the thermoregulatory capacity of male Pyrenean Capercaillies is more challenged than that of females, due to their larger body mass (Leclercq & Ménoni, 2018), thus decreasing their thyroid hormone production during the molting period, which coincides with the European summer months (in July–September) (Abhay et al., 2015).

The only previous noninvasive study on T3 levels in an avian species (Branco et al., 2023) did not elucidate the correlations between the variables studied and T3 levels. Herein, T3 levels did not correlate either with environmental variables, as it was observed in other previous studies about THs blood levels in birds (Decuypere et al., 2005; Kitaysky et al., 2005; Shahid et al., 2020). We hypothesize that this lack of correlation may be due to: (a) the lack of information regarding, for example, physiological stress data, food availability, and bioclimatic characteristics of the areas studied, all of which should be examined in future studies, and (b) the knowledge gap regarding the process of THs deposition in feathers, which is essential to the full understanding of the information that the noninvasive T3 measurement can offer. Only with this deeper knowledge regarding the accumulation of T3 in feathers, we will be able to judge whether this noninvasive hormonal assessment is truly a good proxy for evaluating certain physiological conditions or for its potential correlation with certain environmental variables. For example, we hypothesized that the negative correlation observed between T3 levels and feather length could indicate a dilution effect of T3 when it deposits in longer feathers. We can probably conclude that, despite the available information on how T3 responds to certain environmental variables in avian species (Decuypere et al., 2005; Kitaysky et al., 2005; Shahid et al., 2020), interpretation of T3 levels in feathers and their correlation with environmental variables is actually more complex that we assumed before this study.

This study provides valuable and novel data regarding corticosterone and T3 levels in the feathers of Pyrenean Capercaillies, and the correlations between corticosterone levels and anthropogenic or bioclimatic variables. Contrary to previous studies on capercaillie (Martínez Padilla & Estrada, 2021) and Purple Martin (Branco et al., 2023), where no significant correlations were found between hormone levels and environmental variables, our results support the notion that measuring hormone levels in feathers is a valuable tool to assess the suitability of certain environmental conditions for the Pyrenean Capercaillie. Further longitudinal noninvasive studies could provide additional data to understand the THs deposit in feathers, the correlations detected between corticosterone levels and certain bioclimatic and anthropogenic variables, and the tolerance thresholds of the Pyrenean Capercaillie toward environmental changes and anthropogenic variables, such as rising environmental temperatures and leisure activities in subalpine forests, so as to improve management and conservation actions.

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Author contributions

ONF, AC, and IAJ conceived the ideas and designed the methodology; ONF, AB, and IAJ collected the data; OG, IS, AB, AM, IAJ, MFM, JN, AM, and CS analyzed the data; ON, OG, ACE, CS, and AM led the writing of the manuscript; AC, OG, ACE, MLB, EM, AM, and CS did the manuscript review and editing. All authors contributed critically to the drafts and gave final approval for publication.

Conflicts of interest

The authors declare no conflict of interest.

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Data availability statement

Data will be made available upon request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Forest of origin (forest name, municipality, county, or department, region, and country) and sampling years of the samples included in the study.

Table S2. ELISA kit assay validations for the corticosterone and triiodothyronine (T3) analyses; the results show the precision (intra- and inter-assay coefficients of variation [CV]), specificity (dilution test), and accuracy (spike-and-recovery test) of the assays.

Table S3. Generalized least squares (GLS) non-simplified model (M1) exploring the correlations between corticosterone levels and the selected variables explained in section 2.5 (in a dataset called "all_data"). The test value (Value) and the standard error (SE), *t*-value, and *P*-value are indicated.

Table S4. Generalized least squares (GLS) non-simplified model (M1) exploring the correlations between T3 levels (pg T3/mg feather) levels and the selected variables explained in section 2.5 (in a dataset called "all_data"). The test value (Value) and the standard error (SE), *t*-value and *P*-value are given.

Figure S1. A female Pyrenean Capercaillie molted covert feather being prepared for mechanical mincing using a ball-mill (left); feather dust being weighted on a precision scale (right).

Figure S2. Textbox showing the Strava heatmap extraction data process. A Strava heatmap generated as a raster image using Qgis (French sampling points in white, and Spanish sampling points in blue).

Figure S3. A scatterplot showing corticosterone values (-pg/mg feather) in relation to feather weight (mg, Fig. S3a) and length (mm, Fig. S3b). No correlation was detected between either the corticosterone level and feather weight, or length. In both plots, male values are shown in red and female values are in black.