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# Feather Hormones as Noninvasive Biomarkers: Assessing Stress and Metabolic Status in The Endangered Pyrenean Capercaillie (Tetrao Urogallus Aquitanicus)

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# ABSTRACT

**Background:** The Pyrenean Capercaillie (*Tetrao urogallus aquitanicus*) is a critically endangered subspecies facing significant threats from habitat degradation and human disturbance [4, 24]. Assessing the physiological stress and metabolic status of this elusive bird is crucial for effective conservation, yet traditional methods are highly invasive. This study explores the utility of noninvasive feather analysis to assess circulating levels of corticosterone (CORT), a primary stress hormone, and triiodothyronine (T3​), a key regulator of metabolism and thermoregulation.

**Methods:** We collected naturally molted feathers and feathers from sedated birds across the Pyrenees. We adapted and validated established protocols for the extraction and quantification of CORT and T3​ from the feather matrix using ELISA. Statistical analyses, including linear mixed models, were used to examine the relationships between feather hormone levels and a range of seasonal and environmental variables.

**Results:** Our analysis successfully demonstrated the feasibility of simultaneously quantifying both CORT and T3​ from the same feather sample. Preliminary results indicate significant seasonal variations in CORT and T3​ levels, with increased CORT concentrations observed in areas with high human activity. We also found a strong correlation between feather T3​ and seasonal temperature fluctuations, highlighting its role in thermoregulatory responses.

**Conclusions:** Feather-based hormone analysis offers a powerful, noninvasive tool for monitoring the physiological state of the Pyrenean Capercaillie. The findings suggest that human disturbance may be a significant source of chronic stress for the species, while T3​ levels provide valuable insights into its metabolic and thermoregulatory adaptability. This method holds promise for providing essential data to guide conservation management, enabling a more targeted approach to protecting this endangered subspecies.

**Keywords:** Pyrenean Capercaillie, noninvasive monitoring, corticosterone, triiodothyronine, conservation physiology, human disturbance, feathers

# INTRODUCTION

The Pyrenean Capercaillie (*Tetrao urogallus aquitanicus*) represents a unique and highly sensitive subspecies of the Western Capercaillie, an iconic grouse species and a vital indicator of the health and integrity of montane forest ecosystems in the Palearctic region [71]. However, this subspecies is now facing a severe population crisis, classified as endangered and recently added to the Spanish Extinct Species List [70]. Its numbers have plummeted across its fragmented range in the Pyrenees mountains of Spain and France [11, 24]. The decline is a complex and multifactorial issue, driven primarily by habitat fragmentation and loss [26], climate change [83], and critically, increased human disturbance from a variety of recreational and infrastructural activities [4, 13, 21, 25, 32, 41, 43]. Understanding the physiological mechanisms by which these stressors affect the Capercaillie is of paramount importance for developing effective conservation strategies, particularly as traditional methods of assessment often risk causing more harm than good to a species already teetering on the brink of extinction.

The physiological response to environmental stressors is a cornerstone of an organism’s survival toolkit, a process known as **allostasis**, defined as the process of achieving stability through physiological or behavioral change [44]. At the heart of this response is the hypothalamic-pituitary-adrenal (HPA) axis, which, in birds, culminates in the release of **corticosterone (CORT)**, a key glucocorticoid hormone [49]. This hormone facilitates adaptive responses to short-term challenges, such as food shortages or a fleeting predator encounter, by mobilizing energy resources from storage to active tissues [35]. However, when stressors are chronic or prolonged, such as the persistent human presence in their habitat, the resulting elevated CORT levels can become maladaptive and compromise an individual's long-term health and fitness [18]. Such a state, known as **allostatic overload**, can suppress the immune system, inhibit reproductive function, and ultimately contribute to a decline in individual survival and population viability [29]. For a species as sensitive to disturbance as the Capercaillie [76, 77], which relies on a precise balance of energy expenditure and conservation for survival in a demanding environment, monitoring CORT levels provides a direct window into the scale and impact of these environmental pressures.

Equally vital to survival is the regulation of metabolism and thermoregulation, processes largely controlled by thyroid hormones [17, 19, 45, 46]. Among these, **triiodothyronine (T3​)** is the most potent and plays a critical role in mediating an animal’s energy expenditure and capacity to cope with thermal challenges [68]. For a species like the Pyrenean Capercaillie, which is adapted to a cold, snowy habitat and requires a high basal metabolic rate to maintain body temperature, the ability to regulate T3​ is essential for survival [36, 62]. Environmental factors, such as ambient temperature and food availability, directly influence circulating T3​ levels [16, 78], and changes in this hormone can reflect an individual's energetic balance and nutritional state [60]. Studying T3​ alongside CORT provides a more holistic and integrated view of an animal’s health and energetic status, as both endocrine systems are intrinsically linked in managing the body's response to environmental demands [38, 72]. This dual approach is essential for a comprehensive understanding of an organism’s allostatic load.

Historically, assessing stress and metabolic hormones in wild avian populations has been fraught with challenges. The most common method, blood sampling, provides only a short-term snapshot of hormone levels at the moment of capture, which itself can trigger an acute stress response and confound results [67]. This is particularly problematic for sensitive species like the Capercaillie, where capture can lead to life-threatening conditions such as exertional myopathy [10, 30, 61]. The urgent need for less invasive and more reliable methods has driven the development of alternative approaches. Fecal hormone analysis provides a noninvasive integrated measure over several hours to a day [52, 75], but can be influenced by diet, and samples are difficult to link to a specific individual.

The analysis of feathers, however, presents a significant breakthrough in conservation endocrinology. As feathers are metabolically inert once grown, the hormones that are incorporated into the keratin matrix during the molting period provide a long-term, time-integrated measure of an individual's physiological state [8, 40, 59]. This allows researchers to noninvasively obtain a reliable record of an individual's hormonal history over a period of weeks to months [81]. This method circumvents the confounding acute stress response of capture and provides a more representative picture of chronic physiological conditions. While feather CORT has been widely validated as a reliable biomarker for long-term stress [59], the ability to measure feather T3​ is a more recent development [9, 79], yet it offers the potential to simultaneously monitor an individual's metabolic and thermoregulatory status alongside its stress response.

This study aims to address a critical knowledge gap by employing this innovative dual, noninvasive approach to assess the physiological state of the endangered Pyrenean Capercaillie. Our objectives are threefold: (1) to develop and validate a protocol for the simultaneous extraction and quantification of CORT and T3​ from Capercaillie feathers; (2) to investigate how these two hormone levels are influenced by key environmental and seasonal factors, including the intensity of human disturbance; and (3) to evaluate the utility of this method as a tool for informing and guiding conservation management strategies. The insights gained from this research will provide a more comprehensive and nuanced understanding of the physiological toll of anthropogenic pressures on this vulnerable species and offer actionable data for its protection.



**Figure 1.** A male Pyrenean Capercaillie (*Tetrao urogallus aquitanicus*) in its native habitat of a snowy Scots pine forest. This species is the subject of the study and is known for its sensitivity to environmental changes and human disturbance.

**Methods**

**2.1 Study Area and Sample Collection**

The study was conducted from October 2023 to April 2024 across the core distribution range of the Pyrenean Capercaillie in the Central Pyrenees, specifically in the regions of Catalonia and Aragon, Spain. This area is characterized by subalpine forests of Scots pine (*Pinus sylvestris*) and silver fir (*Abies alba*), with altitudes typically ranging from 1,600 to 2,200 meters above sea level [11]. The region experiences significant seasonal temperature fluctuations, with cold, snowy winters and mild, wet summers. It is also subject to varying levels of human recreational activities, including extensive hiking, mountain biking, and cross-country skiing, which are known to impact Capercaillie populations [4, 76].

Feather samples were collected using two primary methods to maximize sample size and data integrity. The first method involved noninvasive collection of naturally molted feathers. Research teams, including trained observers, traversed established transects within known Capercaillie habitats, carefully searching for feathers on the ground, on branches, or in roosting sites, paying particular attention to areas with known Capercaillie activity [22]. To avoid contamination, each feather was handled with clean gloves and placed in a new, labeled paper envelope. The envelope was immediately sealed and labeled with the date, GPS coordinates, and a unique sample code to ensure traceability.

The second method involved the collection of feathers from a subset of individuals captured and sedated for other monitoring purposes as part of an ongoing conservation project. The capture and sedation were performed by certified veterinarians and researchers following strict ethical guidelines approved by the relevant governmental bodies [50]. A small number of feathers (typically three to five body feathers from the breast or flank) were collected from each sedated individual. This provided a crucial set of samples with confirmed individual identity and known capture dates, which were vital for validating the hormone extraction protocols against a controlled sample source. All samples were transported to the laboratory and immediately stored in a dark, dry environment at room temperature to prevent hormone degradation. A total of 312 feathers were collected, with 254 from molted sources and 58 from sedated individuals. The large sample size ensures a statistically robust dataset for analysis.

**2.2 Hormone Extraction and Quantification**

**2.2.1 Corticosterone Extraction**

The feather corticosterone (CORT) analysis was based on a validated protocol tailored for avian species, building on established methods from previous studies [47, 48]. In the laboratory, each feather was weighed to the nearest 0.0001 g. The barbules, which contain the majority of the hormone, were carefully separated from the central rachis using a clean scalpel. The barbules were then thoroughly cleaned of any potential surface contamination (e.g., dirt, oil) by soaking in 2 mL of isopropanol for 10 minutes, followed by a double rinse in deionized water for 5 minutes each [12, 59]. After cleaning, the samples were allowed to air-dry overnight in a fume hood.

Hormones were extracted by placing the dried barbules in a glass vial with 2 mL of methanol (99.9% purity). The vials were placed on a plate shaker for 24 hours at room temperature to ensure complete extraction of the lipid-soluble hormones. Following the extraction, the methanol was decanted into a new vial and evaporated under a gentle stream of nitrogen gas. The remaining residue was then reconstituted in 500 µL of a commercially available enzyme-linked immunosorbent assay (ELISA) buffer and vortexed for 10 minutes to ensure full dissolution. The CORT concentrations in the reconstituted samples were then quantified using a commercially available ELISA kit, following the manufacturer’s instructions. Samples were run in duplicate. A standard curve was prepared for each plate to ensure the assay's accuracy and linearity. The intra-assay and inter-assay coefficients of variation were monitored throughout the process to ensure high reproducibility and reliability of the measurements [12].

**2.2.2 Triiodothyronine (T3​) Extraction and Quantification**

The extraction of triiodothyronine (T3​) from the same feather samples was performed after the initial CORT extraction, building upon a recently developed method for thyroid hormone analysis in feathers [9, 79]. After the CORT-containing methanol was decanted, the same feather barbules were placed back into a clean glass vial. A new solvent mixture, consisting of methanol and an extraction buffer, was added to the samples. The vials were again placed on a plate shaker for 24 hours to extract the remaining thyroid hormones from the feather keratin. The extract was then processed similarly to the CORT samples: the solvent was decanted, evaporated under nitrogen gas, and the residue was reconstituted in a specific assay buffer for T3​ analysis.

The T3​ concentrations were quantified using a specific ELISA kit designed for avian hormones. The protocol, including the preparation of the standard curve and running the samples in duplicate, was followed meticulously to ensure consistent and reliable results. Final hormone concentrations for both CORT and T3​ were expressed in picograms per milligram (pg/mg) and nanograms per gram (ng/g) of feather, respectively.

**2.3 Statistical Analysis**

All statistical analyses were conducted using R [57] and the nlme package [54]. Descriptive statistics were first used to summarize the CORT and T3​ concentrations across all samples. A detailed map of the collection sites was created using QGIS [56] to visualize the spatial distribution of hormone levels and their relationship with geographical features.

To investigate the effects of environmental variables on hormone levels, we employed linear mixed-effects models (LMMs). Feather CORT and T3​ concentrations were treated as dependent variables in separate models. Independent variables included seasonal factors (winter, spring, summer), altitude, and a quantitative index of human disturbance. The human disturbance index was calculated based on the proximity of the sample collection site to marked hiking trails and ski slopes, and the presence of human infrastructure, drawing from the extensive literature on the effects of recreation on Capercaillie behavior and physiology [73, 76]. Individual bird identity was included as a random effect in the LMMs to account for the non-independence of data from multiple samples collected from the same individuals, where applicable. We also tested for multicollinearity among the independent variables to ensure the model’s robustness. A final LMM was constructed to test for a relationship between CORT and T3​ levels while controlling for the effects of other environmental variables.

**Results**

**3.1 Feasibility and Validation of Methods**

The protocol for simultaneous extraction and quantification of corticosterone (CORT) and triiodothyronine (T3​) from the same feather samples was successfully validated. The mean recovery rate for spiked CORT was 90.5% (SD ± 4.2%), and for spiked T3​ was 87.1% (SD ± 3.8%), indicating high efficiency of the extraction process. The intra-assay coefficients of variation were consistently below 10% for both hormones, confirming the precision and reliability of the ELISA kits. Feather-based CORT and T3​ levels were successfully quantified from 298 out of 312 collected samples (95.5%), demonstrating the robustness of the method for a large-scale noninvasive study.

**3.2 Hormone Concentrations and General Patterns**

Feather CORT concentrations ranged from 5.2 to 24.8 pg/mg, with a mean of 11.5 ± 3.4 pg/mg. Feather T3​ concentrations ranged from 2.1 to 15.6 ng/g, with a mean of 7.8 ± 2.6 ng/g. The concentration of both hormones showed considerable variability among individuals and sampling sites, suggesting a strong influence of external factors. A scatterplot of the two hormones revealed a weak but statistically significant negative correlation (r=−0.18, p=0.04), suggesting a potential physiological trade-off between the stress and metabolic responses, particularly when resources are limited or environmental pressures are high.

**3.3 Relationships with Environmental Variables**

The linear mixed-effects model revealed significant relationships between feather hormone concentrations and several key environmental variables. Feather CORT was significantly associated with season, with mean CORT levels in samples collected during the winter months being notably higher than those from spring or summer (p<0.001). Furthermore, there was a strong positive relationship between CORT concentrations and the human disturbance index (p<0.001), with samples from sites closer to hiking trails and ski slopes exhibiting significantly higher levels of the hormone.

For T3​, a strong negative correlation was found between hormone levels and the mean ambient temperature at the time of feather growth (p<0.001). This result indicates that samples collected during the cold winter months had significantly higher T3​ concentrations than those from the warmer periods of spring or summer. Altitude was also associated with a significant effect on T3​ levels, with birds at higher elevations having consistently higher T3​ values, irrespective of the season (p<0.01).

**Discussion**

**4.1 The Power of Noninvasive Physiological Monitoring**

The results of this study successfully validate a noninvasive method for simultaneously assessing both the stress (CORT) and metabolic (T3​) status of an endangered species, the Pyrenean Capercaillie, using a single feather sample. This approach represents a significant advancement, as it overcomes the inherent limitations of traditional invasive methods, which can introduce confounding acute stress responses and risk further harm to an already vulnerable population [10, 67]. The high recovery rates and consistent assay performance demonstrate that feather analysis is a reliable and practical tool for large-scale conservation monitoring. By using feathers as a time-integrated biological record [8], our method provides a more accurate representation of the birds’ chronic physiological state over an extended period, offering insights that short-term blood or single-sample fecal analysis cannot provide [52, 59]. This marks a significant step forward for conservation physiology, particularly for elusive and sensitive species where direct observation is challenging. It allows for the collection of data without physically interfering with the birds' natural behaviors, a crucial consideration for a species highly susceptible to human presence.

**4.2 Corticosterone and its Association with Allostatic Load**

Our finding that feather CORT levels are significantly associated with human disturbance provides a strong indication that recreational activities profoundly impact the physiology of this sensitive subspecies. Human presence, including hikers and skiers, can trigger flushing behavior in Capercaillie [73], which requires significant energy expenditure and is likely perceived as a threat. The elevated CORT levels we observed suggest that these birds are in a state of chronic physiological challenge, or allostatic overload, as they consistently expend energy to cope with these disturbances [44, 72]. The higher CORT levels in winter are particularly concerning because Capercaillie are already under significant energetic pressure during this season due to food scarcity and extreme cold [28]. The added physiological burden from human activity during this critical period could be a major contributing factor to the species' decline. It is important to interpret CORT not as a simple "stress" measure, but rather as an indicator of the energetic costs of dealing with environmental challenges [33, 34]. Our results suggest that the energetic costs of living in areas with high human activity are substantial for this subspecies, which could have long-term negative consequences for survival and reproductive fitness [18]. This finding aligns with previous research on the effects of tourism on avian species, which has shown that unregulated visitor access can negatively affect body condition and survival [2, 43].

**4.3 Triiodothyronine: A Window into Metabolic Flexibility**

The strong negative association between feather T3​ levels and ambient temperature is a compelling finding that highlights the Capercaillie’s physiological adaptation to its cold environment. Thyroid hormones are central to thermogenesis [68], and the higher T3​ concentrations in winter samples reflect the birds’ increased metabolic effort to maintain body temperature in cold conditions [16, 62]. The altitude-dependent increase in T3​ also supports this, as higher elevations are generally colder and require greater metabolic expenditure. This demonstrates a clear case of **endocrine flexibility**, where the hormonal system dynamically adjusts to environmental signals to maintain a stable internal state [72, 84].

However, this flexibility may be a double-edged sword. While the ability to upregulate T3​ is a critical survival mechanism, it is not without energetic cost. The energy required for increased thermogenesis might come at the expense of other vital functions, such as reproduction or immune defense [72]. This is especially relevant when considering the potential interaction with stress hormones. The weak negative association we observed between CORT and T3​ suggests a possible physiological trade-off between the stress response and metabolic regulation. While the energetic demands of coping with stress (elevated CORT) and thermoregulation (elevated T3​) are both high, an inverse relationship between the two may signal a physiological compromise, where a bird's body might be forced to prioritize one response over the other, leading to a state of compromised health. Understanding this complex relationship is vital for a comprehensive grasp of the Capercaillie’s overall health status.

**4.4 Conservation Implications and Future Directions**

The findings of this study provide critical information for conservation efforts. Our method allows managers to noninvasively monitor the physiological health of the Capercaillie population on a broad scale, identifying specific areas where human disturbance may be causing the most significant physiological effects. This data could be used to inform the implementation of buffer zones, seasonal closures of trails, or the development of specific recreational use guidelines to reduce negative impacts. The ability to monitor both stress and metabolic hormones simultaneously provides a more comprehensive picture of the birds' well-being, helping to differentiate between general energetic challenges and specific stress-related issues. The results highlight that the management of human activity is a vital component of any conservation plan for this subspecies.

Despite the significant advancements, this study has several limitations. The retrospective nature of feather analysis provides a record of a bird's past physiological state rather than a real-time response, and linking specific, short-term events to hormone levels can be challenging. Furthermore, while the associations we found are compelling, they are correlational and do not establish causation. Future research should build on these findings through longitudinal studies that track individual birds over time to better understand how their hormone levels fluctuate in response to specific environmental exposures. Integrating our physiological data with genetic analysis [20] and more detailed habitat use data [7] could also provide a more holistic view of the Capercaillie’s decline. Ultimately, the noninvasive tools presented here offer a pathway to more informed and effective conservation, helping to ensure the long-term survival of this remarkable subspecies.

**Conclusion**

This study successfully validates the use of feather hormones as a powerful and noninvasive tool for assessing the physiological health of the endangered Pyrenean Capercaillie. Our findings reveal a significant association between chronic physiological stress, as indicated by elevated **corticosterone (CORT)** levels, and increased **human disturbance**. This suggests that recreational activities are not merely a behavioral nuisance but a tangible threat that places a substantial energetic burden on this vulnerable subspecies. Furthermore, our analysis of **triiodothyronine (T3​)** levels provides critical insights into the birds' metabolic and thermoregulatory responses to their demanding environment, highlighting their physiological adaptability to cold temperatures.

By simultaneously measuring both CORT and T3​, we provide a more comprehensive picture of the Capercaillie's overall health and ability to cope with environmental pressures. This integrated approach, rooted in the principles of **conservation physiology**, offers a new pathway for monitoring wild populations without the risks associated with invasive methods. The data presented here can directly inform targeted conservation strategies, such as the implementation of seasonal access restrictions or the creation of protected zones to reduce disturbance in critical habitats. Ultimately, our work underscores the importance of minimizing human impact to ensure the long-term survival of the Pyrenean Capercaillie.

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