# Post-print Investigation of Avian Stress Detection Methods

**Authors:** Li Yuezhu, Guo Jinxin, Si Lige, Wang Yu, Sui Jinling

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## Abstract

Glucocorticoids (GC) can be used to reflect stress levels in birds, monitor avian stress conditions, and thereby understand the impact of the environment on bird survival and reproduction. In previous studies, blood, feces, and feathers have all served as materials for glucocorticoid detection in birds; however, currently in China, blood and feces are predominantly employed, while the detection of glucocorticoids in feathers and related ecological research remain largely unexplored, necessitating further improvement of hormone detection methodologies. This article reviews the research progress on these three sampling techniques—blood, feces, and feathers—including the advantages and disadvantages of each technique and factors affecting detection, discusses the importance of feathers as a non-invasive or minimally invasive sampling technique in avian stress monitoring, and suggests that in hormone research, feather sampling techniques should serve as a complementary approach to blood and feces sampling, rather than a replacement. Based on this, this article also explores the applications of feather glucocorticoid detection, with its formation mechanism and ecological significance being key foci for future research.

## Full Text

## Discussion on Methods for Detecting Stress in Birds

**LI Yuezhu, GUO Jinxin, SI Lige, WANG Yu, SUI Jinling*** (College of Nature Conservation, Beijing Forestry University, Beijing 100083, China)

**Abstract:** Glucocorticoids (GC) can be used to reflect stress levels in birds and monitor their stress status, thereby revealing how environmental factors affect avian survival and reproduction. Previous studies have utilized blood, feces, and feathers as materials for glucocorticoid detection in birds. However, current research in China primarily employs blood and feces, while detection

of glucocorticoids in feathers and related ecological studies remain largely unexplored, necessitating further refinement of hormone detection methodologies. This paper reviews advances in three sampling techniques—blood, feces, and feathers—including the advantages and disadvantages of each approach and factors influencing detection accuracy. We discuss the importance of feathers as a non-invasive or minimally invasive sampling technique for avian stress monitoring and recommend that feather sampling serve as a complementary rather than alternative method to blood and feces sampling in hormone studies. Building upon this foundation, we explore applications of feather glucocorticoid detection, suggesting that future research should focus on its formation mechanisms and ecological significance.

**Keywords:** glucocorticoid; stress; corticosterone; feather; blood; feces

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Canadian physiologist Selye [1] pioneered stress research, demonstrating that when confronted with environmental stressors, animals develop stress responses to overcome abnormal external and internal stimuli, thereby maintaining homeostasis under extreme conditions. Subsequent animal stress research has expanded to include indicators such as glucocorticoid levels, immune function, metabolism, and nitrogen balance [2], with avian studies primarily focusing on glucocorticoids. Glucocorticoids are secreted by the adrenal cortex under stimulation of the hypothalamic-pituitary-adrenal (HPA) axis [3] and are considered stress hormones because plasma glucocorticoid levels inevitably rise when animals experience stress [4]. Consequently, glucocorticoid levels serve as important stress indicators. Over 95% of glucocorticoids secreted by birds are corticosterone, and fluctuations in its levels affect the animal' s physiology. Short-term increases in glucocorticoid secretion within normal ranges are beneficial, triggering behavioral and physiological responses that help animals escape life-threatening situations [5-6]. However, chronic stimulation of the HPA axis leading to sustained elevated glucocorticoid levels can severely compromise health by reducing cognitive abilities [7], immune function [3-8], body mass [9], and reproductive capacity [10], ultimately decreasing survival rates and affecting wildlife population dynamics [11]. Hormone measurements help us understand how harsh weather, food scarcity, predator intimidation, interspecific competition, and human habitat destruction impact wildlife survival and reproduction [12-16].

Early glucocorticoid detection primarily relied on blood samples, the most widely applied sampling technique. However, this process itself disturbs and stimulates birds, causing glucocorticoid levels to rise temporarily and preventing measurement of "baseline" levels. Additionally, obtaining blood samples quickly under field conditions is challenging. These limitations have spurred development of new long-term monitoring methods, such as detecting glucocorticoid metabolites in excreta and corticosterone levels in feathers [17]. Currently, avian stress monitoring in China mainly uses blood and feces, while feather glucocorticoid detection and related ecological research remain

virtually unexplored. This paper reviews the current status of glucocorticoid detection across three sampling techniques—blood, feces, and feathers—to provide references for avian stress monitoring and contribute to wild bird conservation efforts.

### 1.1 Blood Sample Hormone Level Measurement

Blood glucocorticoids reflect both free and total glucocorticoid levels in birds, including baseline and stress-induced states [18]. Plasma and serum glucocorticoid levels are equivalent [19] and extremely stable, remaining intact for decades at -20°C [20]. To ensure data accuracy, hormone detection should be completed within 5 minutes to several hours post-capture [21]. Blood samples are typically collected from the brachial vein [22] and stored at -20°C, with centrifugation completed within 24 hours to minimize hormone metabolism [2]. Avian blood glucocorticoid levels are generally several nanograms per milliliter (ng/mL) [23]. Blood sampling offers two advantages: first, it directly measures glucocorticoid levels from the adrenal cortex rather than metabolites [2]; second, it enables comprehensive assessment of avian condition through simultaneous collection of multiple blood components including corticosterone-binding globulin (CBG) [24], palmitate and glucose [25], neutrophil/lymphocyte ratios [3,8], immunoglobulins [26], plasma creatine kinase [27], testosterone [24,28], progesterone [29], and estradiol [30], all of which change in response to stressors. Despite being the most common method, blood glucocorticoid measurement is not always the most effective or even necessary approach [31], as it reflects short-term rather than integrated changes over days to weeks [16].

Seven factors affect blood glucocorticoid detection: (1) Sample reliability. The primary concern is that capture and handling trigger stress responses, potentially confounding results and only reflecting short-term glucocorticoid status [13]. Glucocorticoid levels typically peak 15-30 minutes after stressor application and return to baseline within 60-90 minutes [32]. Studies comparing blood samples collected within 3 versus 4 minutes found minimal differences, both representing baseline levels [33]. Therefore, to obtain "baseline" measurements, blood collection must occur within 3-5 minutes post-capture [28,34-35], which is difficult in field conditions. (2) Sample volume. Blood collection typically requires 50-100 L [35], which is challenging for small birds with limited blood volume. (3) Capture difficulty. For offshore species like seabirds with brief land breeding periods, blood sampling is impractical [16]. (4) Sampling frequency. Frequent capture and blood collection can habituate animals, reducing stress responses [36]. (5) Seasonal (physiological) variation. Baseline and stress-induced plasma corticosterone levels fluctuate seasonally [37]. (6) Diurnal fluctuations. Blood glucocorticoid levels vary with circadian rhythms and short-term disturbances [2]. (7) Photoperiod effects. Serum corticosterone levels are significantly influenced by photoperiod and light stimulation, with gray mannikins (*Lonchura oryzivora*) showing different corticosterone responses to light stimuli under long versus short photoperiods [38].

### 1.2 Feces Sample Hormone Level Measurement

Fecal sampling, a non-invasive technique, is widely applied in hormone detection. Blood glucocorticoids are metabolized in the liver and excreted via kidneys into urine or via bile into the intestine [39], forming fecal glucocorticoid metabolites (FGM). FGM levels closely correlate with baseline blood glucocorticoid levels, reflecting changes in baseline free glucocorticoids [40], making FGM a valuable non-invasive indicator for assessing stress status.

FGM level changes lag behind blood glucocorticoid changes by 1-2 hours in birds [41]. Due to the unique cloacal structure in birds, feces and urine are excreted together. Although previous studies recommended collecting entire fecal and excretory samples, glucocorticoid levels are difficult to distinguish between them [42]. Fecal sampling offers several advantages: first, as a non-invasive technique, it avoids capture and reduces animal disturbance [39]; second, the lag time means glucocorticoids are already present in feces during collection, preventing sampling effects on results [39,41]; third, fecal glucocorticoids reflect average levels over time rather than instantaneous values [43]; fourth, feces allow frequent, continuous, and long-term collection for chronic stress monitoring [39,44]. While feces cannot provide as many indicators as blood, they can also be used for genetic, testosterone, progesterone, estradiol, and immunoglobulin studies [41,44-45]. Research shows fecal glucocorticoid levels can reflect reproductive and immune status and monitor environmental pressures and human disturbance impacts [44,46].

Four factors affect fecal glucocorticoid detection: (1) Post-collection storage conditions and sample freshness influence accuracy because microorganisms degrade glucocorticoids, particularly in harsh field environments. Samples should be frozen at -20°C immediately after collection [47-48], though alternative methods include alcohol preservation at room or low temperature [49], air-drying [41,50], and immediate extraction with storage in solid-phase extraction cartridges [51]. (2) Seasonal dietary changes may alter gut microbiota and glucocorticoid metabolism, causing seasonal FGM variation [52], and day length may also contribute [53]. Therefore, complete fecal sample collection is crucial for long-term monitoring studies. (3) Frequent defecation and short intestinal retention times make fecal glucocorticoids susceptible to circadian rhythms [48], necessitating collection at consistent times for stress response comparisons [53]. (4) For accurate measurement, a minimum dry weight of 20 mg is required [54], which is difficult for small birds and can be addressed by multiple daily collections [53]. However, wild bird feces are often scarce and difficult to collect.

### 1.3 Feather Sample Hormone Level Measurement

Bortolotti et al. [35] first demonstrated the feasibility of using feather corticosterone levels to assess avian endocrine status, confirming that feathers can reflect long-term physiological stress. Feathers are regenerative; by molting or plucking feathers from specific locations, new feathers grow and their corti-

costerone levels reflect stress during the growth period. Feather growth cells are highly vascularized and die upon maturation, with hormones deposited via blood vessels during growth and stored after keratinization. Feather corticosterone thus reflects integrated baseline and stress-induced glucocorticoid levels during feather growth [35].

Feathers provide the longest-term glucocorticoid monitoring, with slow growth offering a temporal measurement scale of days, weeks, or months. The prevailing mechanism, proposed by Bortolotti et al. [35] and supported by subsequent research, suggests feather corticosterone originates from blood. Lattin et al. [17] implanted crystalline corticosterone between the scapulae of nine European starlings (*Sturnus vulgaris*) while using eleven controls, finding that plasma corticosterone elevation during feather growth corresponded with significantly higher feather corticosterone levels compared to controls. Fairhurst et al. [33] increased corticosterone levels exogenously in tree swallows (*Tachycineta bicolor*), collecting blood samples at 7, 9, and 11 days of age (within 4 minutes for baseline and at 30 minutes for stress-induced), demonstrating that feather corticosterone reflects both baseline and stress-induced plasma corticosterone but only when plasma levels change significantly and persistently. Although plasma and feather corticosterone correlate, feather levels should be considered an independent physiological measure rather than a direct indicator of short-term plasma changes [55]. Some studies suggest feather corticosterone may relate to surface deposition or enrichment of metals [cadmium (Cd), manganese (Mn), cobalt (Co), copper (Cu), molybdenum (Mo)] and non-metals, which can affect stress axis activity, though specific mechanisms remain unclear [56] and no evidence supports local corticosterone production on avian surfaces [57]. The deposition process appears limited or bottlenecked, as blood corticosterone is typically nanogram-scale while feather corticosterone is picogram-scale [55]. Despite incomplete understanding of formation mechanisms, feather corticosterone measurement is widely accepted as a valuable long-term monitoring tool.

Feather sampling offers several advantages: first, it is a minimally invasive or non-invasive method causing less disturbance than blood sampling and is easier to collect; second, it reflects integrated glucocorticoid levels over the entire stress period, providing the longest monitoring window and reducing the need to distinguish baseline from stress-induced levels while focusing on overall individual variation [33]; third, corticosterone is stable after feather growth [35], so collection does not affect results; fourth, feathers can be stored at room temperature long-term while remaining suitable for analysis. Kennedy et al. [58] analyzed feathers from red-winged blackbird (*Agelaius phoeniceus*) specimens collected in museums between 1964-1966 to examine relationships between pigment deposition and corticosterone. Fairhurst et al. [59] assessed feather corticosterone trends over 143 years (1859-2002). Feather samples stored at -20°C are also analyzable [56], with no evidence of corticosterone degradation over time. Retrospective analysis of corticosterone from preserved specimens, including extinct species, opens new research avenues for understanding historical ecological events [59].

Four factors affect feather glucocorticoid detection: (1) Sample size. Lattin et al. [17] collected 138 feathers from four European starlings, removing quills, cutting them into pieces, grinding to powder, and mixing thoroughly. Testing 3-99 mg samples (3 mg  1/8 primary feather; 99 mg  4 complete primaries) revealed elevated measurements below 20 mg, with stable results above this threshold. Therefore, sample weight should be standardized to avoid small-sample errors, particularly for small birds. (2) Collection site. Flight or body feathers are typically collected. Adjacent feathers from the same bird during the same growth period [17,56] or symmetrical feathers (e.g., fifth secondary on each wing) show equivalent corticosterone levels [56]. Different feather types (flight vs. body) molt at different times, reflecting different life-history stages [60]. (3) Hormone extraction. Feathers are typically cut or pulverized to increase surface area and extraction efficiency [2]. Methanol is commonly used for corticosterone extraction. Kouwenberg et al. [16] found that enzyme immunoassay detection improved significantly when methanol extraction was followed by acetonitrile/hexane purification, which removed interfering compounds, reduced error (non-significant variation in replicates vs. significant variation with methanol alone), and maintained 87% recovery without substantial corticosterone loss. (4) Antibody selection. Lattin et al. [17] detected feather corticosterone levels of 2.4 pg CORT/mm in red-tailed hawks (*Buteo jamaicensis*) to 6.7 pg CORT/mm in European starlings using Sigma antibodies, while Endocrine Sciences antibodies failed to detect feather corticosterone. Sigma antibodies exhibit higher cross-reactivity with steroids like progesterone, deoxycorticosterone, and testosterone, requiring careful antibody selection.

## 2 Evaluation of Glucocorticoid Research Methods

As sampling techniques have expanded from blood to feces and feathers, avian stress research has progressed, though each method has limitations: (1) Animal invasiveness: blood sampling causes the greatest disturbance, followed by feathers, while feces sampling is non-invasive and minimizes interference. (2) Sample acquisition difficulty: except for captive birds, wild bird feces are difficult to collect; blood and feather collection requires capture, which is challenging for small birds, making feather sampling particularly important. Birds naturally molt and regenerate feathers, so collecting fully-grown feathers does not affect flight or survival. Museum specimens and laboratory-frozen carcasses also provide feather samples [61]. However, collecting growing body feathers can affect thermoregulation, and flight feather collection may have drawbacks [62]. (3) Storage conditions: blood requires -20°C freezing, feces need freezing, alcohol preservation, or air-drying, while feathers have no special requirements and can be stored at room temperature, reducing time and costs. (4) Sample utility: blood enables comprehensive physiological and biochemical assessment, directly indicating health and stress status; fresh feces and feathers can be used for genetics and other studies, but blood has broader applications. (5) Monitoring duration: blood glucocorticoids reflect instantaneous changes rather than integrated changes over days to weeks [16], likely due to researcher-induced stress

[13]; fecal glucocorticoids reflect stress over a period, while feather corticosterone reflects the entire feather growth period, providing the longest-term monitoring.

Blood, feces, and feathers represent independent glucocorticoid measurement methods that can complement and corroborate each other. A comparison of the three techniques appears in Table 1 . Overall, feather hormone measurement provides an excellent standard for long-term environmental stress assessment, offering effective individual- and population-level temporal stress detection [35]. When blood samples are unavailable, feathers provide an alternative, though they are unsuitable for short-term, instantaneous monitoring. Blood also allows comprehensive assessment through multiple blood components. We recommend feather sampling as a complement to blood and feces sampling in avian stress studies, not a replacement. For small wild birds where blood and feces are difficult to collect, feather sampling can serve as an alternative. Researchers can also apply all three techniques to the same species, comparing results for more comprehensive conclusions and better understanding of stress status, while also evaluating methodological errors to improve and reduce limitations.

## 3 Application and Ecological Significance of Feather Corticosterone

Feather corticosterone levels can reflect avian physiological stress status and serve as a valuable monitoring tool for challenging research scenarios, including winter pelagic species, eliminating investigator-induced disturbance, and disease analysis of blood hormones [35]. Feather corticosterone measurement reveals how birds respond to environmental perturbations and adapt to different life-history stages [35]. As a minimally invasive or non-invasive technique, feather corticosterone measurement is both feasible and practical. Current research applications include body condition [16,63], feather quality [17], nutritional status [16], environmental enrichment [64-65], reproductive capacity [33,66], nest-box microclimate [67], carbon stable isotopes [68-69], effects of testosterone and parasites on sexual ornaments [70], pigment deposition [58,60,71], oil impacts [72], parasitic infection [73], and urbanization [74].

**3.1 Correlation Between Feather Corticosterone Levels and Avian Body Condition** Feather corticosterone level variations affect reproductive success, including breeding capacity, fledging rates, and clutch size. Birds with lower reproductive success exhibit higher feather corticosterone levels [66], and elevated feather corticosterone reduces fledging rates and delays fledging dates [33]. High corticosterone levels in females affect current-year clutch size but not subsequent years, while the number of black feathers in males—a social rank signal—positively correlates with corticosterone levels [35].

Feather corticosterone reflects health status and affects growth and development. Elevated corticosterone during molting reduces feather quality [17]. Nestlings with high feather corticosterone have smaller body size and mass and lower fledging probability [33]. In raptors, negative correlations between feather corticosterone and body size suggest larger birds experience fewer homeostatic

challenges or better cope with environmental stress [75].

Feather corticosterone captures variation in life-history stages and individual stress responses. López-Jiménez et al. [75] found that feather corticosterone was highest in the youngest black kites (*Milvus migrans*), gradually declining with age until reaching lowest levels at 7-11 years, then slightly increasing in the oldest individuals ( 12 years), revealing the most vulnerable and challenging periods in avian life histories. Captive red-legged partridges (*Alectoris rufa*) showed pronounced escape responses when humans approached before breeding, but exhibited greater tolerance during incubation with correspondingly lower feather corticosterone levels [35].

Feather corticosterone can predict individual survival rates. Koren et al. [76] captured house sparrows (*Passer domesticus*) in autumn, collected flank feathers, and tracked winter survival, finding an inverse relationship: individuals with higher feather corticosterone had significantly lower survival rates.

### 3.2 Ecological Factors Affecting Feather Corticosterone Levels

Feather corticosterone correlates with numerous ecological factors including food availability, nutrition, weather, climate, and habitat conditions, offering new perspectives for research.

Food scarcity creates harsh survival conditions that affect feather corticosterone. Will et al. [66] found that feather corticosterone in rhinoceros auklets (*Cerorhinca moncerata*) correlated with weight gain, with restricted diets elevating levels. Kouwenberg et al. [16] demonstrated that nutritional status affected corticosterone levels in Atlantic puffin (*Fratercula arctica*) chicks, with well-nourished chicks showing lower levels and food-deprived chicks showing higher levels that may promote begging behavior.

Climate change influences feather corticosterone. Legagneux et al. [15] monitored common eiders (*Somateria mollissima*) for five years using temperature, precipitation, and North Atlantic Oscillation as environmental factors, finding that feather corticosterone was strongly affected by external conditions rather than reproductive investment, timing, or pre-breeding body condition. Environmental conditions were the primary factor affecting stress responses during molting, with foraging conditions in summer and early autumn influencing corticosterone levels during this energy-replenishment period for females.

Urbanization differentially affects species, reflected in physiological stress level changes. Burrowing owls (*Athene cunicularia*) showed similar tail feather corticosterone levels between urban and rural populations, indicating that urban living does not increase physiological stress and suggesting non-random individual distribution across habitats [74].

### 4 Research Prospects

Feather corticosterone detection serves as a long-term monitoring tool for assessing impacts of weather, climate, food resources, and habitat changes on birds. However, unresolved issues remain: (1) The diffusion mechanism of corticosterone into feathers. While corticosterone diffuses from blood into feathers through a limiting factor or bottleneck, the specific mechanism remains unclear. Future research should comprehensively understand factors influencing and limiting corticosterone deposition. (2) Representative sampling. Studies typically collect flight or body feathers, but whether samples from specific locations represent whole-individual hormone levels is uncertain. Some waterfowl molt all flight feathers simultaneously after breeding, but for non-synchronous molters, timing differences may affect results. While adjacent or symmetrical flight feathers from the same growth period show equivalent corticosterone levels, differences between primary, secondary, and tertiary feathers are unknown. Furthermore, the ecological significance of feather corticosterone requires further investigation.

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