

Effects of diet, time since defecation, and drying process of the droppings on corticosterone metabolite measurements in Japanese quail¹

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ABSTRACT The use of noninvasive methods for measuring fecal glucocorticoid metabolites is a useful tool for endocrine assessment particularly in studies where animals cannot be captured, when they should be sampled without disturbing their activities, and/or when welfare needs to be maximized. However, still no complete standardization exists for the methodology, and some confounding variables may play an important role affecting measurements and interpretation of results. The present study focused on whether two different diets (laying feed or seed mixture), the time since defecation (0, 4, 24, or 48 h) and the drying method of those samples (oven-dried or naturally nonoven-dried) may affect concentrations of corticosterone metabolites (CM) measured in male Japanese quail. Half of the birds were provided with plain water (control) and the other half received a corticosterone solution. Birds fed with a seed mixture exhibited

higher values of CM (nanogram/gram) in droppings than quail that received a laying feed diet suggesting that diet should be carefully considered as a potential source of variation. As expected both groups exhibited higher CM concentration after corticosterone treatment. While CM concentrations increased significantly in nonoven-dried samples over time (0 < 4 < 24 = 48 h), oven-dried samples exhibited similar high CM values. At 24 and 48 h postdefecation, nonoven-dried samples had similar CM concentrations as all oven-dried samples. Drying of samples may be considered a reliable method to reduce variations due to water loss over time, facilitating comparisons up to 48 h postdefecation. This finding would allow to enhance the range of application of this noninvasive and welfare friendly method to situations where samples cannot be collected or frozen shortly after defecation.

Key words: diet, stress, corticosterone metabolites, noninvasive methods, Japanese quail, welfare

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INTRODUCTION

The secretion of glucocorticoids (GCs: corticosterone/cortisol) is a classic vertebrate endocrine stress response, exhibiting baseline changes within minutes when animals are exposed to stressors (Moberg, 2000; Sapolsky et al., 2000). Measuring hypothalamic–pituitary–adrenal axis activity is a standard approach to the study of stress and welfare widely used in farm (Mormede et al., 2007; Palme, 2012), free-ranging (Sheriff et al., 2011), and wild-caught animals (Sapolsky et al., 2000; Palme et al., 2005; Schwarzenberger, 2007). Although the reference matrix to quantify GCs is still the use of blood plasma, determinations of urinary or fecal GCs have become useful noninvasive alternatives to overcome the stress related to the blood sampling procedure being that particularly relevant when multiple sampling collections are needed over time. In the

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case of cumulative chronic glucocorticoid evaluations, alternative assessment has also been studied on broiler feathers (Carbajal et al., 2014).

The interest in applying fecal steroid metabolite monitoring to key questions in animal biology has steadily increased over the past decade, and are perhaps the most useful tools for endocrine assessment in studies where animal welfare needs to be maximized. However, still no complete standardization exists for the methodology, and some confounding variables may still play an important role affecting measurements and interpretation of results (Touma and Palme, 2005; Goymann, 2012; Palme, 2012). In laying hen, diet composition (adding 3% of insoluble fiber) affects measurement of hormone metabolites in droppings during rearing (Alm et al., 2014). This could be particularly relevant for comparative studies considering that domestic poultry is not always fed with standard diets, i.e., when accessing to open areas, when reared in home-backyards, etc. Reviews indicate that more information is still needed concerning metabolite concentrations measurements (Klasing, 2005a; Goymann, 2012). Among other influencing factors to be considered, the water content of the samples can clearly be affected by the time elapsed after their evacuation and the concomitant environmental conditions prevailing (Möstl and Palme, 2002; Palme, 2005). Undoubtedly, the best option would be to collect samples shortly after defecation and to freeze them immediately. However, even in controlled environments this is not always possible and gets particularly difficult in uncontrolled and/or outdoor environments (Palme, 2005; Wielebnowski and Watters, 2007).

The present study was conducted 1) to determine whether there are changes in corticosterone metabolites (CM) in male Japanese quail droppings consuming 2 different diets (laying feed or a seed mixture), and 2) to assess the effect of the time elapsed (0, 4, 24, or 48 h) after droppings are evacuated. Aiming to evaluate whether drying of samples may help to reduce variations due to sample water loss over time, half of the samples were sequentially oven-dried and the other half remained in clean trays within the quail room (nonoven-dried). To assess the phenomena in birds showing different hormone concentrations, half of the birds were provided with plain water and the other half with a solution of corticosterone in the drinking water (Shini and Kaiser, 2009).

In the present study, we used an enzyme immunoassay (EIA) suitable for measuring CM in droppings of chickens (Rettenbacher et al., 2004), which has also been successfully validated and applied to measure adrenocortical activity in Japanese quail (Busso et al., 2013; Dominchin et al., 2014) as well as in other galliformes (Arlettaz et al., 2007; Thiel et al., 2011; Stöwe et al., 2013). The Japanese quail was used in this study because it is considered an important poultry species used for farm and home meat and egg production (Caron et al., 1990; Jones, 1996) as well as a suitable

experimental model exhibiting similar dietary, anatomical, and physiological characteristics as other relevant poultry (meat- and egg-type chickens, partridges, etc).

MATERIALS AND METHODS

Animals and Husbandry

Male Japanese quail were used in this study. After hatching, birds were reared in wooden boxes (90 cm long × 90 cm wide × 60 cm high) until 4 wk age. Each box had 2 feeders covering the front part and 16 automatic nipple drinkers (8 on each side). A wire-mesh floor (1-cm grid) was raised 5 cm to allow the passage of excreta and a lid prevented the birds from escaping. Brooding temperature was 37.5°C during the first wk of life, with a weekly decline of 3.0°C until room temperature (24 to 27°C) was achieved (Nazar and Marin, 2011; Nazar et al., 2012). Birds then were sexed by plumage coloration, and wing-banded to ensure further identification. To ensure appropriate individual sample collections and standardize the technique, 36 males were individually and randomly housed in two 5-tier cage batteries, each battery comprising 20 cages. Fully visual and auditory contact was allowed among birds to minimize potential isolation stress. Each cage measured 45 cm length × 20 cm width × 25 cm height. At this time, birds were switched to a laying feed, with plain water provision ad libitum. Laying feed (Marcelo E. Hoffman e Hijos S.A., Entre Ríos, Argentina) contained corn meal, soybean meal, wheat shorts, sunflower meal, limestone, sodium chloride, dicalcium phosphate, vitamins, and minerals with 21.5% CP and 2,750 kcal ME/kg. Quail were subjected to a daily cycle of 16L:8D (300 to 320 lx) during the study, with light on at 0600. Temperature was kept at 25 ± 2°C during the rest of the study. Daily maintenance and feeding chores were performed at the same time each day (0800).

All studies were carried out in accordance with local Argentinian laws and following the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 80 23, revised 1978).

Treatments and Dropping Collection

Diet effects on CM concentrations At 11 wk age all quail from each cage were randomly assigned to one of 4 treatment combination groups (9 birds/treatment). The study treatments were as follows:

Treatment 1 (**T1**): laying feed + plain water (control group).

Treatment 2 (**T2**): laying feed + corticosterone (treated group).

Treatment 3 (**T3**): seed mixture feed + plain water (control group).

Treatment 4 (**T4**): seed mixture feed + corticosterone (treated group).

Birds assigned to T1 and T2 (laying feed treatments) continued receiving the same feed (see above) until the end of the study. Birds assigned to seed mixture feed treatments (T3 and T4) were switched to a ratio of seed mixture containing equal percentages of corn, sorghum, canary (*Phalaris canariensis*), millet (*Pennisetum* spp.), and oats (*Avena sativa*) seeds during the next 14 d. Corticosterone treatments started 10 d after assignment to diet treatment and lasted 4 d. Birds from T1 and T3 were provided with plain water (control group) and birds from T2 and T4 were provided with water containing a corticosterone solution (10 mg/L water; approximately 1.2 mg/bird/d; treated group). Similar corticosterone doses and administration procedure were previously found to increase plasma corticosterone measurements within bird's physiological range (Post et al., 2003; Shini and Kaiser, 2009; Wall and Cockrem, 2010). Corticosterone (Sigma-Aldrich, St Louis, MO) was dissolved in a 1 mL ethanol solution per 1 L drinking water. The corticosterone treatment in drinking water was provided manually through bottle-drinkers that were protected from light (because the hormone is photosensitive) and located on the rear part of the cages. The volume of water added every day was recorded so the total volume consumed over the treatment period and the mean daily water intakes were calculated. Mean daily water intakes were multiplied by corticosterone concentrations to obtain an estimate of mean daily corticosterone consumptions for each bird. At the end of the treatment birds were again weighed to ensure that groups did not differ in this variable.

At 13 wk age, the cage trays were cleaned (8:30 AM) and 60 min later, droppings were collected. Sixty min were considered the minimum time-interval needed to ensure enough dropping mass for the study (see below). Immediately after collection, samples were homogenized and total weight was recorded; then a fraction of 0.4 g droppings was labeled and stored at -20°C until steroid metabolite measurement for evaluating diet effects. The remaining collected material was used in Study 2.

Effects of the time since defecation on CM concentrations Droppings taken for Study 1 were also used to determine the effects of the time since defecation on CM concentrations. Besides diet composition and corticosterone treatment, 4 periods since defecation (0, 4, 24, and 48 h) and 2 dropping conditions (nonoven-dried and oven-dried; see below) were also considered. To ensure enough dropping material for each of the treatment combinations, samples from 3 individuals were pooled, labeled, and maintained on a clean large tray within the quail room (exposed to the same environmental conditions as the birds). Sequentially, at the end of each of the 4 periods since defecation, samples from each of the corresponding periods were weighed, half of them were immediately frozen for later steroid extraction and CM analysis (the nonoven-dried sample condition), and the other half were immediately taken into the oven and dried at 60°C during 24 h (oven-dried

sample condition). The dried samples were also immediately weighed and frozen for later steroid extraction and CM analysis.

Steroid Extraction and CM Measurements

Samples were extracted with a 60% aqueous methanol solution in a relation of 1 g sample per 10 mL solvent (Palme et al., 2013). Suspension was shaken during 2 min on a vortex and then centrifuged (1,000 g, 15 min). Aliquots of the supernatant (after a 1:10 dilution with assay buffer) were measured with a cortisone enzyme immunoassay (a group-specific EIA measuring CM with a 3,11-dione structure). Details of the EIA are given elsewhere (Rettenbacher et al., 2004). Interassay CVs of a high and a low concentration pool sample were 6.5 and 6.2% for the cortisone EIA, and sensitivity was 25 ng/g. This EIA has been successfully validated for Japanese quail (Busso et al., 2013). Concentrations of corticosterone metabolites are expressed as nanogram/gram droppings.

Statistical Analyses

Two-way ANOVAs were performed to evaluate the effects of diet (laying feed vs. seed mixture feed), the 2 hormone concentrations induced with the administration of corticosterone in drinking water (control vs. treated groups), and their interaction on CM. To assess the effect of time since defecation on the CM measurements, a repeated measure ANOVA was performed to evaluate the same factors mentioned above, the effect of the 4 periods since defecation (0, 4, 24, and 48 h; the repetition factor) and the effect of dropping conditions (nonoven-dried vs. oven-dried) as well as their interactions. CM data were transformed to ranks (Shirley, 1987) to better fit ANOVA assumptions. Changes in body and dropping weight were also evaluated by ANOVA (data transformations were not required in these cases). Fisher's least significant difference test was conducted for posthoc analyses. A P -value of ≤ 0.05 was considered to represent significant differences.

RESULTS

Diet Effects on CM Concentrations

Quail subjected to different treatments produced similar quantities of fresh droppings ($F_{3,32} = 5.01$; $P = 0.56$). In addition, no differences were detected in the weight of the dried dropping samples between birds that received laying feed or a seed mixture feed (0.09 ± 0.02 vs. 0.10 ± 0.05 g, respectively). Analysis of variance showed a significant main effect of diet on CM ($F_{1,32} = 5.01$; $P = 0.03$). Quail fed on a seed mixture exhibited higher values of CM than birds that received a laying feed (Figure 1). As expected, CM

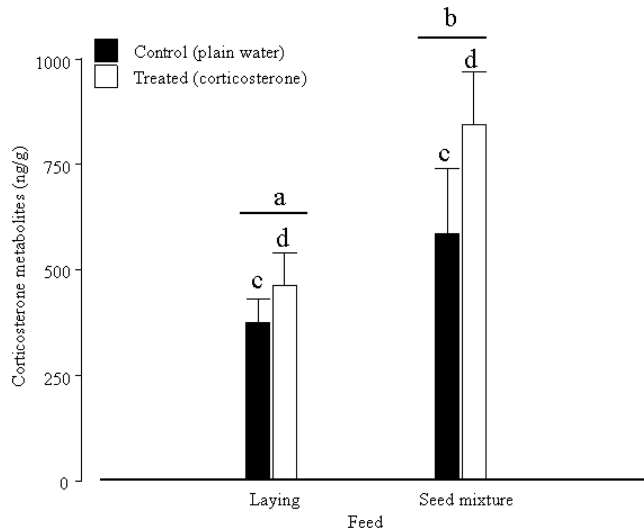


Figure 1. Mean (\pm SEM) of corticosterone metabolite concentration of male Japanese quail that received a laying feed or a seed mixture feed and were provided with either plain water or corticosterone (10 mg/L) in the drinking water. Groups with different superscripts (i.e., a, b or c, d) differed at $P \leq 0.05$.

measurements in droppings were significantly higher in quail that received corticosterone in the drinking water than in those that received only plain water (Figure 1; $F_{1,32} = 5.04$; $P = 0.03$). The ANOVA performed on BW data showed that neither diet nor corticosterone treatment affected this variable (data not shown).

Effects of the Time since Defecation on CM Concentrations

Dropping weight was affected by time since defecation and drying condition ($F_{3,56} = 88.9$; $P < 0.001$). No significant effects of feed and corticosterone treatment on dropping weights were detected. Posthoc test showed that all oven-dried samples exhibited the same

low mean weight values, regardless of the time elapsed before drying, feed, or corticosterone treatment. On the other hand, nonoven-dried droppings had significantly lower weight 4 h after they were excreted, reaching similar low values compared to the oven-dried samples 24 h after excretion. No further decrease was observed in the samples weighed 48 h after defecation (Figure 2).

There was an interaction between the effects of the time since defecation and the effects of dropping condition ($F_{3,56} = 11.01$; $P < 0.001$). As expected, a significant effect ($F_{1,16} = 83.29$; $P < 0.001$) of corticosterone treatment was also found (T2 and T4 > T1 and T3). No significant interactions were detected between feed treatment and the other variables (time since defecation, corticosterone treatment, or dropping condition). Posthoc test showed that nonoven-dried samples exhibited a significant ($P < 0.05$) increase in CM over time ($0 < 4 < 24 = 48$ h). On the other hand, oven-dried samples exhibited similar high mean values of CM, regardless of the time elapsed before drying and of feed treatment. At 24 and 48 h since defecation, nonoven-dried samples had high CM measurements, similar to the oven-dried samples (Figure 3). At all sampling times (0, 4, 24, and 48 h), droppings from quail that received corticosterone in water showed significantly higher ($P < 0.03$) CM measurements than droppings from their counterparts that received only plain water.

DISCUSSION

The use of noninvasive methods for measuring fecal glucocorticoid metabolites is perhaps one of the most useful tools for endocrine assessment in studies where animal welfare needs to be maximized. The present study evaluates whether two different feed compositions (laying or seed mixture), the time since defecation, and the drying method of droppings

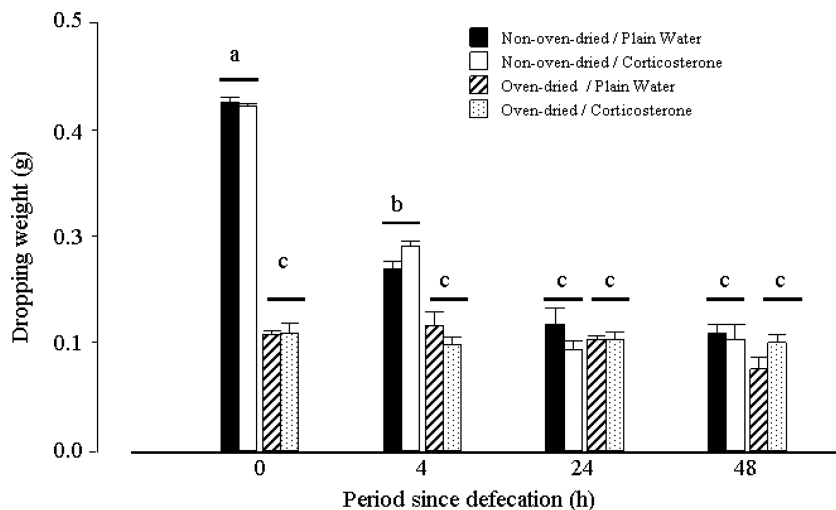


Figure 2. Dropping weight (mean \pm SEM) of samples that were kept under control condition or were oven-dried after different periods since defecation. Birds received either a laying feed or seed mixture feed (data from those groups are shown pooled) and were provided with either plain water or corticosterone in the drinking water (10 mg/L). Groups with different superscripts (i.e., a–c) differed at $P \leq 0.05$.

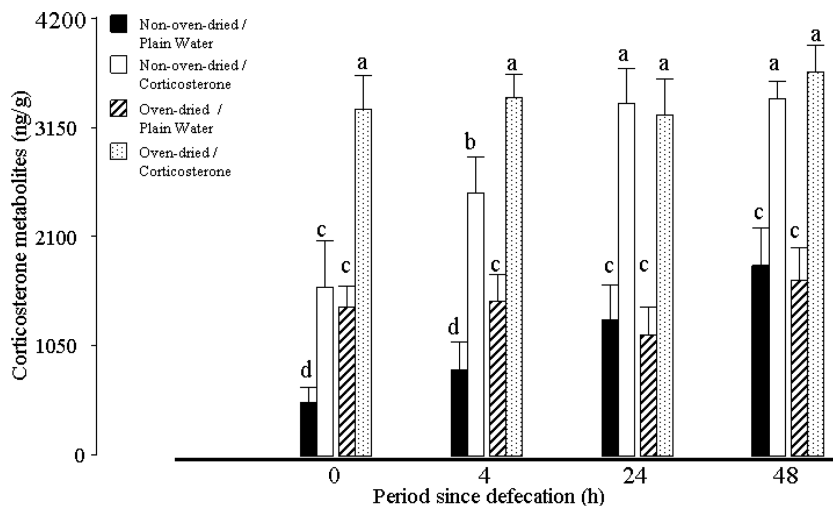


Figure 3. Corticosterone metabolites (mean \pm SEM) in droppings that were kept under control conditions or were oven-dried after the different periods since defecation. Birds received either a laying feed or seed mixture feed (data from those groups are shown pooled) and were provided with either plain water or corticosterone in the drinking water (10 mg/L). Groups with different superscripts (i.e., a–d) differed at $P \leq 0.05$.

(nonoven-dried or oven-dried) affect CM concentrations in male Japanese quail. To induce 2 hormone levels within each diet group, quail were provided with either plain water (control group) or a corticosterone solution (treated group). This treatment mimicked a stress response in poultry within normal physiological ranges according to other reports (Post et al., 2003; Shini and Kaiser, 2009). Accordingly, in our study CM concentrations were higher in droppings from quail that received corticosterone in the drinking water than in their counterparts that received only plain water.

The potential impact of diet on hormone metabolites in excrement of mammals and birds have been analyzed in some studies (Lepschy et al., 2010; Goymann, 2012). In mammals, diet manipulations have shown to affect fecal steroid metabolite measurements (Wasser et al., 1993; Morrow et al., 2002). Goymann (2012) pointed out that in birds the high-fiber diet probably led to an increase in gut passage time, and to an increase in excrement mass, both factors that could affect hormone metabolite measurements (Goymann, 2005; Lepschy et al., 2010). Accordingly, Alm et al. (2014) have recently proved in laying hens that the addition of just 3% crumbled straw pellets in the feed can increase both fecal CM concentration and excretion rate, showing that rather small changes in diet and fiber content can influence fecal CM levels. In our study, although fiber percentages in the administered diets were not determined, it is clear that the diets have different fiber percentages (seed mixture > laying feed) (Klasing, 2005a). Nevertheless, we found that both feed treatment groups produced similar amounts of droppings; similarly to data from experiments with stonechats being fed with or without cellulose (Goymann, 2005). Therefore, considering that quail subjected to different treatments weighed the same, produced similar quantities of fresh droppings within an hour sampling time,

and that no differences were detected after the samples drying, it seems that at least in this study, expressing CM as excreted amounts (Goymann, 2005; Hau et al., 2011) instead of nanogram/gram (Lepschy et al., 2010) would not change the outcome of this study. It should also be mentioned that neither undigested seeds nor different amount of water in droppings from the diet treatments were detected; thus, it may be considered that CM were not “diluted” in the samples. We hypothesize that the differences in the levels of CM observed between birds that received laying feed or seed mixture are presumably due to changes induced during the digestion process. Probably a lower reabsorption of steroids from the intestine in the fiber rich seed diet might be an explanation. The average time that diet components spend in the gastrointestinal tract may be affected by several characteristics. For example, it has been reported that corn meal, soybean meal, and sunflower meal in balanced food exhibit lesser amounts of fiber than whole grains of the seed mixture (Klasing, 2005b). Regardless of the mechanism responsible for the CM differences observed, our results clearly are consistent with Alm et al. (2014). They suggest that poultry diet should be carefully considered as a potential confounding factor during noninvasive CM monitoring.

Deep-freezing fecal samples does not seem to completely inactivate bacterial enzymes that metabolize steroids. If these enzymes are not inactivated before storing the samples by drying, heating or alcohol addition, bacterial metabolism can continue after thawing and lead to biased results (Hunt and Wasser, 2003; Möstl et al., 2005). Another confounding factor when we apply non-invasive hormone monitoring is the water content in fecal samples. At present, drying is one of the approaches used to minimize imprecision in hormone measurements due to bacterial metabolism and/or water content (Palme, 2005; Wielebnowski and Watters,

2007). It is expected that CM measurement would increase after the drying process, but it is not clear if different corticosterone measurements of fresh samples are correlated to values detected in dried samples. In our study, as expected, the weight of non-oven dried dropping samples decreased with time after defecation. Postdefecation (24 h), dropping samples were found to reach the lowest weight values, most probably due to an extreme loss of water. No further weight changes were observed in the samples 48 h after defecation. When samples of the same droppings (0, 4, 24, or 48 h) were placed in the oven for complete drying, in all cases they exhibited the same low weight values regardless of the time elapsed before drying. These findings suggest that both oven-dried and nonoven-dried (24 h or more) resulted in equal minimal water content of the samples. When CM were measured in those droppings, nonoven-dried samples exhibited increasing CM measurements over time ($0 < 4 < 24 = 48$ h). Interestingly, no differences were detected between those nonoven-dried samples during 24 or 48 h and all oven-dried samples. This result suggests that the drying process allowed us to minimize the CM measurement bias due to water content variations that otherwise would have been originated by the different times of sample collections. It is important to note that the drying process did not affect detection of different CM measurements induced by the presence of corticosterone in drinking water. One major problem associated with fecal steroid metabolites is that they may undergo further bacterial metabolism once evacuated, leading to changes in CM measurements, depending on bacterial metabolism rate and antibody cross-reactivity (Möstl et al., 2005). Even though in our study, we did not measure bacterial activity directly, it is assumed that the oven-drying process was able to minimize bacterial activity. No differences were detected in CM measurements among samples that were immediately oven-dried after defecation (0 time) and those that were maintained during 48 h under the quail room environmental conditions (nonoven-dried). These findings suggest that bacterial metabolism did not play a role in our study, either because there was no further bacterial activity or because the bacterial enzymes do not affect amounts of measured CM (those with a 3,11-dione structure).

Chromatographic studies that detect potential CM differences between dropping conditions over time would be useful to elucidate the actual process involved. Regardless of the underlying biological phenomena, drying of samples has the advantage of reducing sample variations, as the water content, due to collecting feces that do not have the same time of excretion and therefore may present different water contents. More importantly, CM measurements between 24 and 48 h since defecation were not affected by natural drying compared to oven-dried droppings at 0, 4, 24, and 48 h. These results suggest that CM concentrations in naturally dried samples are stable over time at least up to 48 h, which is consistent with findings reported

in a capercaillies study (Thiel et al., 2005) where no changes were found in droppings stored at 8 or 21°C for 7 d. These results are relevant from an application of this noninvasive method since immediate freezing of the samples does not seem really necessary. However, more studies would be needed to evaluate whether the same pattern is observed in other environmental conditions and birds species.

In conclusion, the seed diet affected CM in droppings of male Japanese quail suggesting that diet should be carefully considered as a potential source of variation during noninvasive monitoring of adrenocortical activity in birds. Time elapsed after droppings are evacuated only affected CM measurements in fresh samples. Thus, drying of samples may be considered a reliable method to reduce variations due to water loss of samples over time, facilitating comparison of CM measurements in droppings up to 48 h postexcretion. This finding would allow researchers to enhance the range of application of this noninvasive and welfare friendly method to situations where samples cannot be collected or frozen shortly after defecation.

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