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METHODS

Can faecal glucocorticoid metabolites be used to monitor body condition in wild Upland geese *Chloephaga picta leucoptera*?

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Abstract The measurement of faecal glucocorticoid metabolites is used as a non-invasive technique to study stress in animal populations. They have been used most widely in mammals, and mammalian studies have also treated issues such as sample stability and storage methods. In birds, faecal corticosterone metabolite (CM) assays have been validated for a small number of species, and adequate storage under field conditions has not been addressed explicitly in previous studies. Furthermore, while it is well-established that baseline plasma corticosterone levels in birds rise with declining body condition, no study so far investigated if this relationship is also reflected in faecal samples. We here present data of a field study in wild Upland geese *Chloephaga picta leucoptera* on the Falkland Islands, testing different storage methods and investigating the relationship of faecal CM concentrations to body condition and reproductive parameters. We found that faecal CM measures are significantly repeatable within individuals, higher in individuals with lower body condition in both male and female wild Upland geese and higher in later breeding females with smaller broods. These results suggest that measuring faecal CM values may be a valuable non-invasive tool to monitor the relative condition or health of individuals and populations, especially in areas where there still is intense hunting practice.

Keywords Upland goose · *Chloephaga picta leucoptera* · Stress · Body condition · Faecal glucocorticoid metabolites

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Introduction

The measurement of faecal glucocorticoid metabolites (GCM) in faeces has become a valuable tool in conservation biology and ecology to study stress load, enabling researchers to monitor the physiological state of wild animals both non-invasively and repeatedly. Faecal GCM have been used most widely in mammals, and mammalian studies have also treated issues such as sample stability and storage methods. One important question arising especially in studies in remote areas is the appropriate storage of faecal samples from the time of collection until later laboratory analysis. Studies with captive mammals showed that faecal samples are most stable when stored at sub-zero temperatures, followed by cool storage, drying and preservation in ethanol (reviewed in Wasser et al. 1988; Whitten et al. 1998; reviewed in Khan et al. 2002). However, as glucocorticoids are metabolised in a species-specific manner

before excretion and assays need to be validated for each species, we wanted to test whether these findings from studies in primates may be transferred to an avian system. A previous study by Koch et al. (2009) on captive Upland geese showed that the concentrations of corticosterone metabolites (CM) of frozen and dried samples were highly correlated, indicating that the collection of droppings followed by drying might be an easy way to prepare samples for transport during fieldwork. However, Koch et al. (2009) used a freeze-dryer, equipment that is not necessarily available under fieldwork conditions. We thus tested this method under fieldwork condition by air-drying samples. Additionally, we tested whether keeping droppings in ethanol directly after sampling gives comparable results to freezing. This could be especially important for humid regions, where air-drying might not be appropriate.

In our study, we investigated whether CM values in Upland geese (*Chloephaga picta leucoptera*) are related to body condition and reproductive parameters like timing of breeding and number of offspring. Based on the Cort–Fitness Hypothesis (Bonier et al. 2009b), high glucocorticoid (i.e., corticosterone in birds) levels are often interpreted as a sign for individuals or populations in poor condition and linked to reduced fitness. These relationships have mainly been studied using baseline corticosterone concentrations in serum, but studies yield different results, varying within populations and even changing within individuals during their life history (reviewed in Bonier et al. 2009a). Faecal CM measures in birds have been linked to fitness in only a few studies (Kotrschal et al. 1998), although faeces have the advantage to provide a time-integrated measure of hormone levels compared to point measures in serum analyses (Millspaugh and Washburn 2004).

Upland geese belong to the order of the sheldgeese (Tadornini), a group that resembles true geese and shows similar habits but is more closely related to shelducks and ducks. The smaller (migratory) subspecies *Chloephaga picta picta* breeds on the South American mainland, whereas the slightly larger one (*C. picta leucoptera*) is restricted to the Falkland Islands. Their basic breeding biology and life cycle has been studied in the Falklands from 1977 to 1980 (Summers 1983). At the New Island Nature Reserve, numbers of Upland geese have increased since 1973, when all livestock was removed from the island to one of the highest population densities in the Falkland Islands (Quillfeldt et al. 2005). In other parts of their range though they are hunted intensely and there are few data available on their population and conservation status on both the South American mainland and the Falkland Islands. CM analysis in this case could be an easy, non-invasive method to study the effect of stressors like hunting, interactions with humans and habitat changes on important life–history parameters such as timing of breeding and number of offspring potentially affecting population development.

Materials and methods

Study site, field measurements and sampling

The study was carried out in the New Island Nature Reserve, Falkland Islands (51°43′ S, 61°17′ W) from October to December 2004, 2007 and 2008. In 2004, we observed unmarked birds (Quillfeldt et al. 2005), while the individually based data of body condition, hatching date and brood size were obtained in 2007 and 2008. At the start of the 2007 and 2008 field seasons, we mapped nests using GPS. For each nest, we determined clutch size, measured length (L, expressed in cm) and breadth (B, expressed in cm) of each egg to the nearest 0.1 mm using callipers and weighed each egg to the nearest 0.1 g using a digital balance. Egg volume (V in cm^3) was calculated as $V = (L \times B^2 \times 0.507)$ following Furness and Furness (1981). We determined expected hatching dates as described in Gladbach et al. (2010a). Nests were visited at least once a day, starting at the estimated hatching date. All eggs hatched within 0–2 days from the estimated hatch date. In cases where nests were visiting after hatch of the chicks, hatching date was determined from a chick growth curve as described in Gladbach et al. (2010a).

We caught adults during the period when they attended their brood (mean chick age 12±2 days) using a 3×5 m whoosh net. One person herded the family of geese slowly to the catching area, and when they arrived directly in front of the furled net, the other researcher pulled the trigger. Adults were marked with individual metal rings and weighed to the nearest 10 g using a digital spring balance. Head length, culmen length and tarsus length were measured to the nearest 0.1 mm using callipers. Wing length (maximum flattened chord) was measured to the nearest 1 mm using a stopped rule. We estimated a condition index correcting body mass for body size. This is considered to be a measure of nutrient reserves, where reserves are the quantity of utilizable tissues exceeding those required to meet daily nutritional demands. A Principal Components Analysis extracted one principal component (PC1) from measurements of wing, head, bill and tarsus as an overall measure of body size in both males and females. In females, PC1 with an eigenvalue of 1.794 explained 44.844% of the variance. In males, PC1 with an eigenvalue of 2.001 explained 50.018% of the variance. The body condition for males and females was determined accounting for structural size, based on a regression of body mass on PC1. Body condition is expressed as the ratio of the observed body mass to the derived expected body mass according to individual size.

Collection of faecal samples

Faecal samples were collected when families were leading broods (chick age at first sample: 32±2 days). Sampling took place between 10 a.m and 12 a.m., starting at a mean of 20±1 days after capture of adult birds. Families were approached until a dropping of both male and female was observed. If this took longer than 10 min, the attempt was aborted. Droppings were collected and kept in plastic tubes on ice packs in an insulated bag during a sampling session. After returning to the field station (usually within 1 h), droppings were processed immediately. A sample was placed in a petri dish and mixed using a spatula. In 2004, one part was frozen at −20°C and the other part in a sunny window (usually dry within 1 day). In 2007, one part was mixed with ethanol (70%) in a new tube and the other part air-dried. In 2008, one part of the sample was mixed with ethanol in a new tube and of 47 samples, a further part was frozen at −20°C. Tubes of ethanol samples were sealed with a tap and parafilm to prevent leakage. In 2004, we collected a total of 33 samples from 16 males and 16 females. In 2007 and 2008, individuals were sampled repeatedly (three to six times) with an interval of 3 to 5 days between sampling sessions. In 2007, we took a total of 125 samples from 15 males and 14 females. In 2008, we took a total of 132 samples from 13 males and 13 females, of which 47 were partly frozen.

Extraction and measurement of the excreted CM

In the lab, a subsample of 0.25 g of each fresh faecal sample was mixed with 0.75 ml double-distilled water followed by 1.5 ml methanol and vortexed for 30 min. After centrifugation (2,500×*g*, 10 min), the supernatant was transferred to a new tube and used for the analyses with the enzyme immunoassay (EIA) described below.

A subsample of 0.08 g of dried samples was mixed with 1 ml double-distilled water, to compensate for the water loss during drying (see Koch et al. 2009), and 1.5 ml methanol before vortexing for 30 min. Ethanol samples were dried using a STERIS Lyovac GT-2E freeze dryer and then treated like dried samples (see above).

Koch et al. (2009) tested various EIA for CM in Upland geese and found an 11-oxoetiocholanolone-EIA (Möstl et al. 2002) measuring the highest peak values of CM in faeces of Upland geese after adrenocorticotropic hormone (ACTH) injection and this assay was more sensitive than other tested EIAs. After extraction, 20-µl aliquots were transferred into microtiter plates and measured using this assay. Intra-assay variation was 10.9%, inter-assay variation for the low-level pool was 15.6% and 13.2% for the high-level pool. All concentrations are given in nanogram per gram wet weight, assuming a constant water content. Because the actual water content may differ between samples, the final concentration between dry, ethanol and wet samples cannot be compared directly, but a correlation between the corresponding dry, ethanol samples and wet samples would indicate that the methods yield comparative results.

Statistical analysis

Statistical tests were performed in SPSS 11.0. Normality was tested with Kolmogorov–Smirnov tests. Means are given with standard errors. We assessed significance using General Linear Models (GLM). As a measure of effect sizes, we included partial eta-squared values (η^2) in the tables (i.e., the proportion of the effect + error variance that is attributable to the effect). The sums of the partial eta-squared values are not additive (http://web.uccs.edu/lbecker/SPSS/glm_effectsize.htm). Significance level was set to $P<0.05$. Because CM values for the analysis of sample storage methods were not normally distributed we used an ln-transformation. For the analysis of chick age, body condition, reproductive parameters and CM values, we used data from 2007 and 2008 only, while in 2004, birds were not captured. We analysed values of samples stored in ethanol, as this method was used in both 2007 and 2008. To ensure that CM data collected for each year were directly comparable in subsequent analyses, we standardized values of ethanol samples for each year separately (mean=0, SD=1) (see Hõrak et al. 2002; Ochs and Dawson 2008).

For the analysis of the influence of chick age on faecal CM concentrations, we used a GLM for sexes separately with standardised CM values as dependent, individual identity as random factor and chick age as covariate. For the individuals captured in both years, we used CM concentrations from the first year in the overall model to avoid pseudoreplication. To test whether the same trend can be observed in both years, we then ran an additional analysis for each year separately, including all captured individuals (Table 1). As CM concentrations might be related to changes in stressors occurring with increasing chick age, we additionally tested whether CM were related to age of the chick when the first sample was taken. We ran a GLM with CM as dependent, sex as fixed factor and body condition and chick age at first sample as covariates. We also included the interaction between sex and chick age.

For the analysis of body condition and reproductive parameters, we used a mean value for all individuals. We here also included the measurement from the first year if individuals were captured in both 2007 and 2008. We ran GLMs with CM values as dependent, sex as factor, body condition as covariate and the interaction of sex and body condition. As females lose weight during incubation which they regain after hatching of the chicks and there was a time

Table 1 Effect of individual identity and chick age on faecal CM concentrations of wild Upland geese on New Island

		Ring	Chick age
♀	Overall	$F_{21,71}=1.666$, $P=0.058$, $\eta^2=0.330$	$F_{1,71}=9.391$, $P<0.001$, $\eta^2=0.169$, $t=-3.803$
	2007	$F_{13,44}=1.256$, $P=0.276$, $\eta^2=0.271$	$F_{1,44}=9.391$, $P=0.004$, $\eta^2=0.176$, $t=-3.064$
	2008	$F_{12,47}=2.207$, $P=0.027$, $\eta^2=0.361$	$F_{1,47}=4.056$, $P=0.049$, $\eta^2=0.079$, $t=-2.014$
♂	Overall	$F_{21,76}=3.706$, $P<0.001$, $\eta^2=0.506$	$F_{1,76}=11.323$, $P=0.001$, $\eta^2=0.130$, $t=-3.365$
	2007	$F_{13,46}=3.495$, $P=0.001$, $\eta^2=0.497$	$F_{1,46}=9.300$, $P=0.004$, $\eta^2=0.168$, $t=-3.050$
	2008	$F_{11,45}=3.910$, $P=0.001$, $\eta^2=0.489$	$F_{1,45}=3.319$, $P=0.075$, $\eta^2=0.069$, $t=-1.822$

GLMs with standardised CM values as dependent, individual identity as random factor and chick age as covariate. Partial eta-squared values (η^2) denote the effect size and t values the direction of the effect

lag in our study between the capture of animals and faecal sampling, we ran a second analysis for females. In 2008, we recaptured ten females with a mean of 35 days after the first capture. We found a mean daily mass increase of 3 g. Using this information, we calculated a hypothetical body condition for each female (adding a hypothetical mass to the measured mass by multiplying the days between capture and first hormone sample with 3 g). This resulted in a mean increase in body condition of 0.02 points. We ran the tests again using this hypothetical body condition for females and the results remained qualitatively the same.

As we sampled pair partners, we analysed the relation of CM values and hatching date or brood size for males and females separately using Pearson correlation to avoid pseudoreplication of hatching date and brood size.

Results

Comparison of sample storage methods

CM values of frozen and air-dried samples were significantly correlated (Pearson correlation, $R=0.740$, $P<0.001$, $N=31$, Fig. 1a). CM contents of ethanol samples and frozen samples were significantly correlated ($R=0.506$, $P<0.001$, $N=47$, Fig. 1b), as well as CM measured in ethanol samples and dried samples ($R=0.691$, $P<0.001$, $N=125$, Fig. 1c). In 2008, we analysed 18 ethanol samples twice (subsamples were created at the weighing stage). The correlation between both subsamples was highly significant ($r=0.85$, $P<0.0001$), which indicates that storage in ethanol gives reliable results.

Influence of chick age within individuals

The overall model showed that faecal CM concentrations decreased with increasing chick age in both males and females and there were significant individual differences (Table 1). This trend could be found in both 2007 and 2008 (Table 1). The effect size for inter-individual differences in

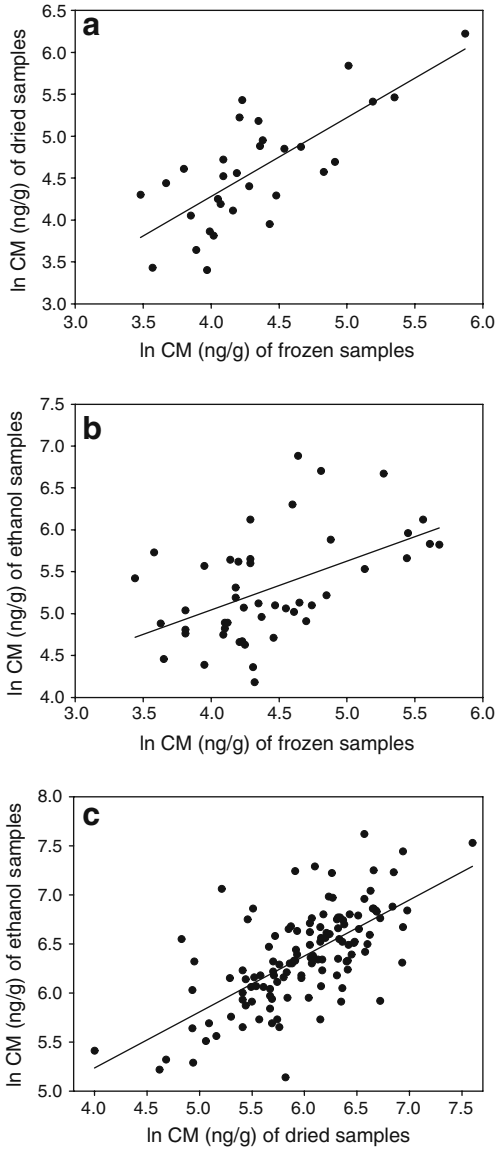

Fig. 1 Pairwise comparisons of different storage methods on corticosterone metabolite (CM) concentrations in faecal samples of Upland geese on New Island. **a** frozen–air-dried, samples from 2004; **b** frozen–ethanol, samples from 2008; **c** air-dried–ethanol, samples from 2007

CM concentrations was higher than for intra-individual differences with increasing chick age (Table 1). Chick age at first sample had no effect on CM concentrations (GLM, factor sex: $F_{1,45}$=1.182, P=0.185, body condition: $F_{1,45}$=5.905, P=0.020, chick age: $F_{1,45}$=2.388, P=0.130, interaction of sex × chick age: $F_{1,45}$=2.469, P=0.124).

We found faecal CM concentrations within individuals to be significantly repeatable for both males and females (males: r=0.332, P<0.001, N=28; females: r=0.158, P=0.005, N=29).

Influence of sex, body condition and reproductive parameters

There was no significant difference in CM values between males and females ($F_{1,45}$=0.600, P=0.443) during the time when they were leading chicks. We found tendency of CM values to be related in pair partners (partial correlation, controlling for year: R=0.348, P=0.089, df=23).

CM values were significantly negatively related to body condition in both males and females with individuals in better condition showing lower values (GLM, factor sex: $F_{1,45}$=0.002, P=0.966, η^2<0.001, body condition: $F_{1,45}$=10.993, P=0.002, η^2=0.211, t=−2.467, interaction of sex × body condition: $F_{1,45}$=0.004, P=0.948, η^2<0.001, Fig. 2a). In females, these results remained qualitatively the same using the hypothetical body condition (GLM, hypothetical body condition: $F_{1,22}$=12.257, P=0.002, η^2=0.280, t=−3.501).

Hatching date was positively related to CM values in females, with individuals with lower CM values starting to reproduce earlier in a season (Pearson correlation: R=0.686, P<0.001, N=22; Fig. 2b). CM values were not related to hatching date in males (R=−0.199, P=0.362, N=23; Fig. 2b).

The brood size (number of chicks per brood) was negatively related to CM values in females (R=−0.543, P=0.009, N=22; Fig. 2c) but not in males (R=−0.374, P=0.079, N=23; Fig. 2c).

Discussion

Comparison of sampling methods

We found a significant correlation between all pairwise compared sampling storage methods. The highest correlation coefficient was observed between frozen and air-dried samples. The correlation coefficient is lower than the one reported by Koch et al. (2009), who dried samples directly in the laboratory using a freeze dryer, but the highly significant result in our case still supports the use of air-drying under fieldwork conditions. Oven drying of faecal samples in field sites without electricity or organic solvents has been used for instance in primate studies (Brockman and Whitten 1996; Whitten et al. 1998) and proved to be a useful preservation technique there as well. However, caution is necessary here as well as also drying temperature may affect hormone levels (e.g., Terio et al. 2002).

The correlation between ethanol and frozen samples was also highly significant in our study. However, the correlation coefficient was lower than for air-dried samples, indicating that storage in ethanol might increase variation in CM measures more than air drying. The way the storage of faecal samples in ethanol affects hormone concentrations is still discussed in the literature. For example, Whitten et al. (1998) found that the majority of steroids are extracted into the solvent when faeces are kept for prolonged periods in 90% ethanol at ambient temperatures and Khan et al. (2002) suggested that immunoreactive metabolites might have a higher extraction efficiency when stored in ethanol for several months at ambient temperature, caused by the deposition of metabolites on the surface of the faecal

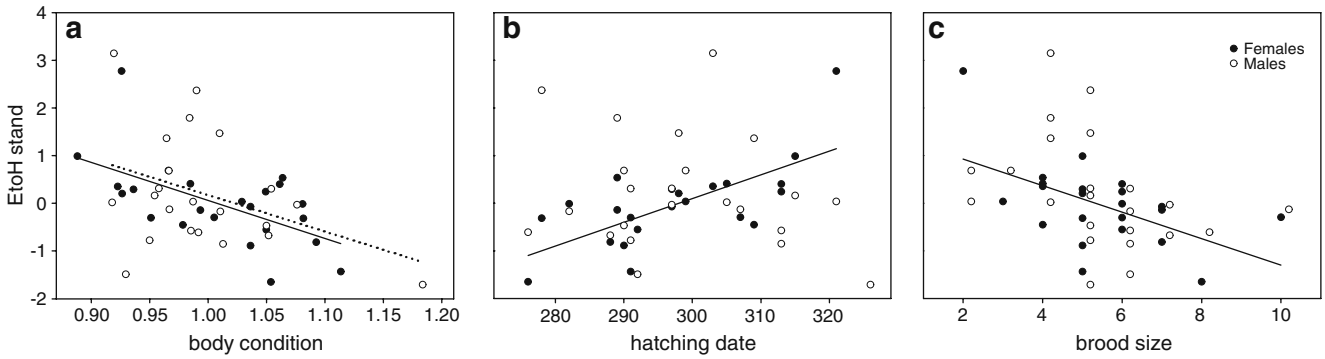

Fig. 2 Relationship between (**a**) body condition, (**b**) hatching date and (**c**) brood size and corticosterone metabolite (CM) values in faecal samples of female (*black dots*, *solid line*) and male (*white dots*, *dotted line*) Upland geese on New Island. CM values were standardized for each year separately to ensure that data collected were directly comparable. Regression line only for significant relationships. See text for statistical analysis

material during ethanol evaporation and freeze-drying, which then go more easily into solution during extraction. Based on our empirical results, we would therefore recommend the use of air-drying if possible. Storage in ethanol is also valid, but less preferable. Dried samples are easier to transport as they take less space and do not need to be sealed to avoid leakage of alcohol.

Repeatability and influence of chick age within individuals

Studies in other goose species suggest that the faeces contain an integrated, proportional record of the plasma corticosterone levels approximately 1 h before defaecation (Kotrschal et al. 1998; Hirschenhauser et al. 2000; Mostl et al. 2005); however, metabolites are excreted faster in urine (after 1–2 h) than in the faeces (3 h) (Mostl et al. 2005). In birds, the cloaca serves for the excretion of both faeces and urine (uric acid) and in some species, urine in droppings can be sampled separately from the faeces (Wasser et al. 1997; Klasing 2005). In Upland geese, the urinary part is hard to distinguish from the faecal part (personal observation). The study by Koch et al. (2009) showed a peak 2–3 h after the injection of an ACTH analogue, indicating that metabolites mainly excreted in the faeces were measured by the assay. The time span is especially important to determine, when a specific stressful event took place. However, in our study, we were interested in baseline levels. In both males and females, faecal CM concentrations from consecutive samples were highly repeatable indicating that we measured baseline levels and that there is considerable individual variation. This is further supported by the higher effect size of individual identity in our model testing the influence of chick age and identity. Variation in baseline corticosterone levels has been found to be repeatable in geese before (Kralj-Fiser et al. 2007) and partly heritable in several other avian species (Satterlee and Johnson 1988; Evans et al. 2006), thus possibly providing an honest signal of individual quality with lower quality individuals perceiving their environment as more challenging (Bonier et al. 2009a).

We found that in both males and females, faecal CM concentrations decreased with increasing chick age. We propose three possible and not mutually exclusive explanations for this pattern:

1. With increasing chick age, chicks become more independent and have a lower risk to be taken by the main predators, Striated caracaras (*Phalcoboenus australis*) and Falkland Skua (*Catharacta antarctica*). They are also less vulnerable to be killed during territorial fights by neighbouring ganders, which regularly kill goslings by picking or even throwing them (personal observations). Parents can thus decrease their vigilance and concentrate more on foraging and self maintenance.
2. Both males and females regenerate their body condition after the high investment at the beginning of the breeding season. Females are known to lose weight during incubation which is regained after hatching (Summers and McAdam 1993). Males on the other hand invest heavily into the establishment and defence of the breeding territory. With advancing season, these fights become less violent as territory boundaries are established and only few geese (presumably first breeders) try to establish a new territory and start breeding (personal observation). In both males and females, this could affect individual stress load and condition.
3. Environmental conditions change with advancing season. While October and November are usually relatively dry, precipitation starts to increase in December, improving the freshwater supply that Upland geese depend upon and the amount of vegetation available for foraging. These more favourable conditions may relax individual perceived stress levels.

Relation to body condition

We found that in both males and females, faecal CM concentrations were negatively related to body condition, with individuals in better condition showing lower values. As pointed out by Husak and Moore (2008), stress hormone levels have been linked to body condition in a variety of animals (Moore and Jessop 2003) based on the role of glucocorticoid hormones in energy mobilisation, although cause and effect remain unclear (Husak and Moore 2008). Studies in avian systems often measure baseline corticosterone levels in plasma samples and report a condition dependence of corticosterone levels (Perez-Rodriguez et al. 2006). To our knowledge, no study so far investigated if this relationship can also be found using faecal samples, as normally faecal samples are specifically used as a non-invasive technique without capturing focal animals. The use of faecal samples might be advantageous when studying the relationship between corticosterone and individual condition because they provide a time-integrated measure of hormone levels compared to point measures in blood plasma analyses. Individuals showed a clear correlation of CM values and body condition despite the time lag of 20 days between the two measurements. Our data thus suggest that Upland geese in good body condition conserved their good condition throughout the chick-rearing season.

Relation to reproductive parameters

We found that only in female Upland geese CM values were related to hatching date and brood size, with higher

CM values in later breeding individuals and in individuals with smaller broods. A recent study by Schoech et al. (2009) found a similar pattern in Florida scrub-jays *Aphelocoma coerulescens*, where corticosterone levels were also positively associated with timing of reproduction in females. In Upland geese, males and females differ in their specific parental roles, with males establishing and intensely defending the territory and females incubating and brooding (Summers and McAdam 1993; Gladbach et al. 2010b). Our present findings thus fit well into this pattern, as number of offspring and timing of breeding depend mainly on female resources and condition (Gladbach et al. 2010a). Early nesting birds often lay bigger clutches; their offspring grow more rapidly and have a higher chance of survival and recruitment than late nesting birds (Drent and Daan 1980; Hochachka 1990; Blums et al. 2002; Sockman et al. 2006), which results in a decrease in offspring value within a season. The earlier hatching dates and larger brood sizes of females with lower CM values may be explained by differences in individual quality. Low quality individuals might secrete corticosteroids at higher levels (Wingfield and Sapolsky 2003; Husak and Moore 2008; Bonier et al. 2009a) either because they perceive certain environmental circumstances as more challenging per se or because these circumstances are more challenging to them due to their lower condition. As elevated corticosterone levels are assumed to reallocate resources away from reproduction (Bonier et al. 2009a), onset of incubation (and thereby hatching date) might be postponed in low quality individuals. Brood size could then be smaller either because the expected offspring value is assumed to decline during a breeding season or because lower quality individuals are not able to invest as heavily into reproduction as high quality birds. The observational nature of this study cannot clarify causality, but our empirical results can be a good basis to be used in conservation practice.

Summary

We found a significant correlation between all pairwise compared sampling storage methods, with air-dried samples being best correlated to frozen samples followed by ethanol-stored samples. Faecal CM measures are significantly repeatable within individuals, higher in individuals with lower body condition in both male and female wild Upland geese, and higher in later breeding females with smaller broods. The results were strong despite the fact that ethanol storage might not have been optimal compared to drying. Our results suggest that measuring faecal CM values may be a valuable non-invasive tool to monitor the relative condition or health of individuals and populations, especially in areas where there still is intense hunting practice.

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