Do glucocorticoids in droppings reflect baseline level in birds captured in the wild? A case study in snow geese

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ABSTRACT

Baseline glucocorticoid (CORT) levels in plasma are increasingly used as physiological indices of the relative condition or health of individuals and populations. The major limitation is that CORT production is stimulated by the stress associated with capture and handling. Measuring fecal CORT is one way to solve this problem because elevation of fecal CORT usually does not occur before 1–12 h after a stressful event in captive animals. However, the effect of capture and handling on fecal CORT levels has seldom been investigated in the wild. In a first experiment, we validated that fecal CORT levels starts to increase in droppings (a mixture of fecal and urinary material) about 1–2 h following injection of CORT-release hormone (ACTH) in captive greater snow geese (Chen caerulescens atlantica). In a second experiment, we investigated whether dropping and plasma CORT were related and if the capture affected fecal CORT levels in wild birds. Baseline CORT was obtained by bleeding individuals within 4 min after capture. No relationship was found between baseline and CORT in droppings shortly after capture (<4 min). In addition, CORT levels in droppings increased linearly with time after capture and was already elevated by a factor two 40 min after capture. The different turnover time of CORT between urine and feces could explain such results. We conclude that droppings cannot provide an index of basal CORT levels in snow geese captured in the wild. Such a result contrast with previous studies conducted on habituated, captive animals. We thus recommend that use of droppings as a non-invasive technique to measure baseline CORT be restricted to non-manipulated individuals in the wild.

1. Introduction

How animals cope with their environment (and its variation) and how environmental perturbations or stressors are perceived can affect survival and reproductive success of individuals [29]. Stress hormones (glucocorticoids, corticosterone or corticosterone metabolites, hereafter called CORT) play a key role in these interactions and thus understanding their effect in natural populations is crucial (reviewed in [7,10]). CORT is increasingly used as an index of relative condition or health of individuals and populations. The Cort-Fitness hypothesis predicts that CORT levels should be linked to some fitness components [7]. Individuals that exhibit higher basal CORT levels are expected to have lower reproductive outputs than individuals with lower CORT levels [2,3,38]. In conservation biology, CORT is increasingly used as a tool to study the response of individuals to disturbance for species of conservation interests [3,47].

Baseline CORT secretion is associated with normal physiological activities and is considered a marker of activities, energetic state and food availability [28]. At baseline levels, CORT is secreted to maintain homeostasis in response to predictable energetic demands [7] and this level should be related to the quality of individuals. Typically, CORT is measured in blood (plasma) but it can also be measured in fecal samples [31,39]. Measuring baseline CORT is difficult because CORT is quickly released during a stressful event, and capturing animals to take blood samples generates stress [37]. Measuring baseline CORT in the plasma thus requires bleeding individuals immediately after capture [36]. In contrast, fecal concentration of CORT should integrate CORT released over a period of several hours prior to collection [19]. This period can also vary according to species or environmental conditions [18].

These two sampling methods have their own limitations. For instance, capture and handling for blood sampling are invasive and CORT concentrations in plasma can start to increase 3 min after capture [36]. Bleeding free-ranging animals within a few minutes after capture may not be feasible [37] or may strongly limit potential sample size. For example, mass captures of flocking birds is often possible but bleeding several individuals shortly after

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capture may be unfeasible. Moreover, increase of CORT concentration may also depend on the capture method used [1,30]. While fecal samples can be collected non-invasively from a large number of individuals, measuring fecal CORT also has some limitations. Associating feces collected in the wild to specific individuals may be impossible in some circumstances such as in flocking birds. Measuring fecal CORT in captured individuals may circumvent these problems. However, Goymann [18] cautioned that correlation between basal CORT (from plasma samples) and fecal CORT may be difficult to obtain due to differences between circulating hormones and their excreted metabolites and because of the time lag between secretion and excretion. To our knowledge, only one study has determined the relationship between plasma and fecal CORT in free living wild animals: Sheriff et al. [41] found a significant positive relationship between CORT levels measured in plasma and fresh fecal samples from shot snowshoe hares (*Lepus americanus*). However, several studies performed on captive animals have found significant relationship between fecal and plasma CORT such as in Northern spotted owl (*Strix occidentalis caurina*) [47] or in human-habituated ring-tailed lemurs (*Lemur catta*) [12]. In the latter studies, bleeding occurred after a fresh fecal sample was obtained [47].

In birds, urine and feces are excreted together as droppings and mixed together in the cloaca prior to excretion [24]. In the present study, we investigated whether CORT measured in droppings can be used to estimate baseline CORT in greater snow geese (*Chen caerulescens atlantica*). During the non-breeding season, mass captures of flocking birds is often possible using cannon nets but bleeding several individuals shortly after capture is logistically challenging. Because capturing birds with cannon-nets can be considered an acute stress, this offers a unique opportunity to study the effect of a stressful capture technique on CORT concentration in wild bird's droppings. We first validated that CORT measured in droppings is a reliable indicator of the adrenocortical activity of geese. We used a standard physiological technique: injection of a CORT-release hormone [18] in captive birds to determine the time lag between injection and the appearance of an elevated CORT level in droppings. Secondly, we investigated whether CORT levels measured in plasma and droppings were related and determined the time-lag between capture and the appearance of an elevated CORT level in both plasma and droppings in wild geese.

2. Materials and methods

2.1. ACTH challenge validation with captive birds

We tested the reliability of CORT measured in droppings as an index of basal CORT using adrenal corticotropic hormone (ACTH) challenges in captive birds [26,44]. Because ACTH is the pituitary hormone that stimulates CORT release by the adrenal cortex, the collection of dropping samples by interval allows a determination of the time required to obtain an elevation of CORT in droppings after an ACTH injection. This physiological validation would also ascertain that CORT measured in droppings is directly issued from plasmatic CORT. We used captive greater snow geese that have been held in captivity (indoor in the winter, outdoor in the summer) at the Zoo Sauvage de Saint-Félicien (Québec, Canada) for ~10 years. We followed the recommendations of Touma and Palme [44] when preparing this experimental set up. Ten days before the experiment, we maintained six adult female snow geese in individual enclosures (15 m^2 each) with *ad libitum* food and water. The experiment was ran over 2 days and droppings were collected every hour from 9 to 16 h both days. At the beginning of the second day, we weighed geese and injected (intramuscular injection into the breast) three females with ACTH (50 IU/kg porcine ACTH, Catalog No. A6303, Sigma–Aldrich, St. Louis, MO) and three females

with saline solution at 1% [46]. For each individual, we calculated the time elapsed between the current dropping collections and the first collection each day. All droppings were mixed and immediately stored at −20 °C until laboratory measurements [18]. This experiment was approved by the Committee of Animal Protection of Université Laval (Authorization No. 2010-071-1).

2.2. Effect of capture on wild birds

The field study was conducted at Ile aux Oies (47°00′N 70°33′W), an island located in the St. Lawrence estuary, 60 km northeast of Québec City (Canada). This site is a major staging area for migrating greater snow geese [5]. Geese were captured with cannon-nets from 29 April to 17 May 2009. All nets were baited with oats following Morez et al. [32]. A total of 22 captures were conducted and, on average, 81 birds were captured each time (range 41–129). Juveniles (i.e. first-year birds) represented an average of 25% of the captured geese. Juveniles were released immediately and adults were retained and sexed by cloacal examination. In this study, we only used adult females. Birds were weighed to the nearest g with an electronic balance and culmen, head and tarsus were measured with a calliper to the nearest 0.1 mm.

We bled 28 female greater snow geese shortly after capture. Bleeding occurred between 150 and 420 s after firing of the net (mean = 269 s ± 17 SE). Time elapsed from firing of the net to bleeding was measured to the nearest second (this will be referred to as time after capture hereafter). Blood samples were maintained on ice and centrifuged within 3 h after collection and immediately stored at −20 °C until laboratory measurements. We collected droppings both opportunistically during handling at the capture site and systematically on ~10 females per capture that were maintained in individual storage plastic boxes (Rubbermaid Roughneck Latching Storage Box 82 × 50 × 50 cm) for periods ranging from 40 to 115 min. Time elapsed from firing of the net to collection of droppings was also noted. Twenty droppings were collected shortly (between 6 and 10 min) after capture and blood samples were collected <4 min after capture for 9 of these individuals. We collected 189 droppings between 40 and 115 min after capture from birds held in boxes, including a second sample for the 20 individuals for which we obtained droppings shortly after capture. Finally, we collected fresh droppings opportunistically (N = 14) in fields used by undisturbed flocks of geese (mix of males and females, and including young and adults). All droppings were treated as in captive birds. This experiment was approved by the Committee of Animal Protection of Université du Québec à Rimouski (Authorization No. CPA-42-10-78).

2.3. Laboratory analyses

Dropping samples were oven dried (60 °C) during 48 h and homogenized with a pestle. We double extracted 0.10 g (±0.06 SE) of ground droppings following the methods described by Poisbleau et al. [35]. We added 1 ml of distilled water to each 100 mg sample and homogenized the mixture. We took 100 µl of the resulting emulsion for corticosterone analysis. Both plasma and dropping samples were analysed by radioimmunoassay at the Centre d'Études Biologiques de Chizé (CEBC) laboratory. CORT was extracted from the aqueous phase by adding 3 ml of a diethyl-ether solution, vortexing for 2 min and centrifuging for 5 min (at 4 °C and 760 g). The diethyl-ether phase containing the steroids was decanted and poured off after snap freezing the tube in an alcohol bath at −30 °C. We repeated this twice for each dropping sample and the residue was evaporated. The dried extracts from droppings were first redissolved in absolute ethanol and then diluted in a 0.01 M phosphate-buffered saline solution (pH 7.4) containing 0.1% bovine albumin serum (PBS-BSA). Plasmatic

extracts were directly redisolved in PBS-BSA. The dissolved solution incubated overnight at 4 °C with ca. 9000 cpm of the appropriate ³H corticosterone (GE Healthcare, F-91898-Orsay) and a rabbit corticosterone antiserum (Sigma–Aldrich, F-18297-St. Quentin-Fallavier). The bound corticosterone fraction was then separated by addition of dextran-coated charcoal and counted in a Packard liquid scintillation counter. Two assays were performed for each sample. The intra-assay coefficient of variation was 3.1% ($N = 255$). The lowest detectable concentration was 0.5 pg mg^{-1} while the lowest measurement was 50.0 pg mg^{-1}. The laboratory procedure (large sample mass ∼0.1 g and solvent elimination) would likely reduce the potential artificial effect of sample mass on CORT metabolite concentrations [20].

2.4. Statistical analyses

We used Repeated ANOVA to assess the effect of ACTH challenge on CORT in droppings and tested for potential interaction between hours of the day (hours) and treatment for the two days of the experiment. For wild geese, we performed a principal component analysis on morphometric measurements (culmen, tarsus and head lengths). The three variables had loadings ranging from 0.49 to 0.63 on the first axis (PC1), which explained 73% of total variation in the data. We used individual PC1 scores as a measure of body size. We controlled for variation in body size by using the residuals of the relationship between body mass and body size. Relative body mass was defined as the residuals of the regression of body mass on PC1 plus the mean mass of all individuals included in the model [17]. We then used standard regressions to test for relationships between time after capture and CORT measured in plasma, and between basal CORT in plasma and in droppings from same individuals. We examined the effect of time after capture (TIME, in min), period of the capture (HOUR), date (in julian days), body size (PC1, see above) and condition (relative weight, see above) on the CORT in droppings using linear models. CORT concentrations were log-transformed to meet normality and homoscedasticity assumptions. We used a backward stepwise model selection procedure to get a minimal adequate model (Crawley, [14]), starting with the global model and subsequently removing all non-significant terms (P-values > 0.05). Non-significant interaction terms were removed when they did not significantly improve the fit of a model. All means are presented with SE. Statistical analyses were performed in R 2.10.

## 3. Results

3.1. ACTH challenge experiment

During the first day, the CORT measured in droppings of the two groups (control and treated birds) was not different (Repeated ANOVA: $F_{1,37} = 0.55$; $P = 0.50$) and CORT did not vary according to the hours of the day (Repeated ANOVA: $F_{1,36} = 0.56$; $P = 0.46$). CORT levels were 14.6% higher the second day compared to the first one in control birds (Repeated ANOVA: $F_{1,37} = 5.41$; $P = 0.03$). During the second day, we also found a strong effect of the ACTH challenge on the level of CORT in droppings (interaction between time and treatment: $F_{1,38} = 18.79$; $P = 0.0001$). An increasing trend started after 1 h 30 min and a sharp increase after 3 h (Fig. 1). This effect was not significant when time after injection was <250 min (i.e. 4 h 10 min: $F_{1,17} = 2.08$; $P = 0.08$).

3.2. CORT from plasma and droppings in wild birds

Plasma CORT was related to the time elapsed after capture ($\beta = 0.12 \pm 0.05$; $F_{1,27} = 7.24$; $P = 0.01$; Fig. 2). This relationship
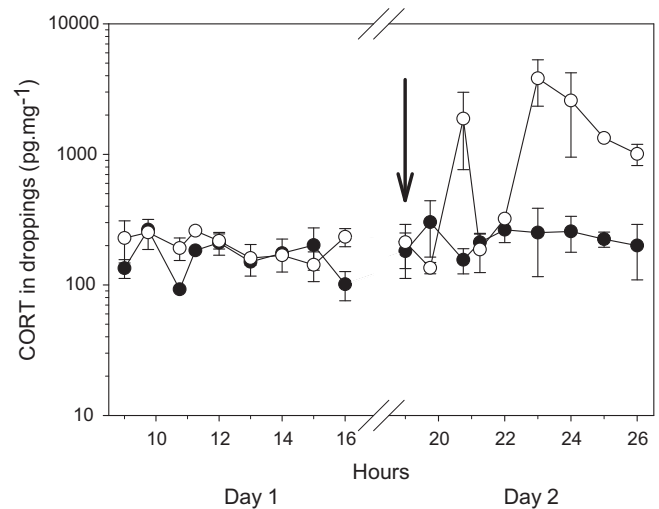

Fig. 1. Mean corticosterone (CORT) metabolites measured in droppings (±SE) of six adult female snow geese sampled at hourly intervals during the daytime over two consecutive days. On the second day, geese were split in two groups and injected once (black arrow) with either ACTH (open dots) or a saline solution (black dots). One individual presented an increase of CORT 1 h 30 min after ACTH injection (this individual did not defecate during the next 2 h which explains the apparent decrease of CORT afterward).
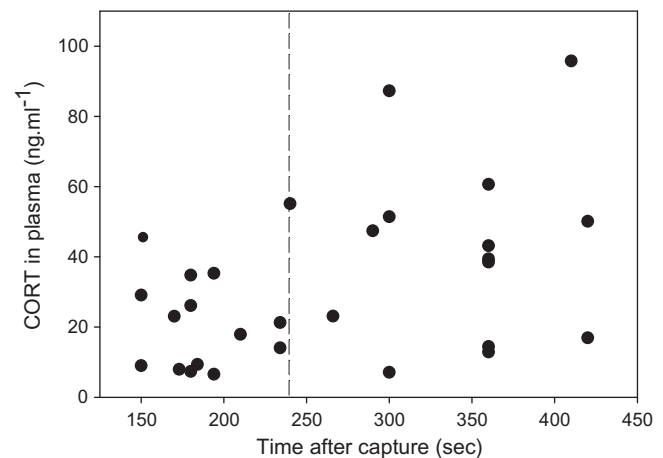
Fig. 2. Relationship between plasma corticosterone (CORT) concentration of greater snow geese and time elapsed after capture in spring. The dashed line indicates the threshold below which the slope of the relationship becomes quasi-null (see text).

became non-significant when time after capture was <300 s ($\beta = 0.17 \pm 0.09$; $F_{1,15} = 3.89$; $P = 0.07$) and the slope was quasi-null when time after capture was <240 s ($\beta = -0.01 \pm 0.12$; Fig. 2). There was no relationship between basal CORT in plasma and CORT in droppings of the same individuals (Fig. 3), either when considering only feces collected shortly (<10 min) after capture or all samples ($F_{1,7} = 0.03$; $P = 0.88$ and $F_{1,15} = 0.29$; $P = 0.60$, respectively). Individual variation in CORT level in droppings was best explained by the time elapsed after capture, date and an interaction between date and time of day (Table 1). Body size and body condition were not retained in our model selection. Overall, CORT significantly decreased over the season but the interaction between date and time of day revealed that it increased with date when sampling occurred in the afternoon but tended to decrease when sampling occurred in the morning. CORT concentration in droppings also increased with time elapsed after capture (Fig. 4). Furthermore, CORT in droppings randomly collected in the field (mean = 147.7 ± 14.6 pg mg^{-1}) was also slightly lower than in droppings collected between 6 and
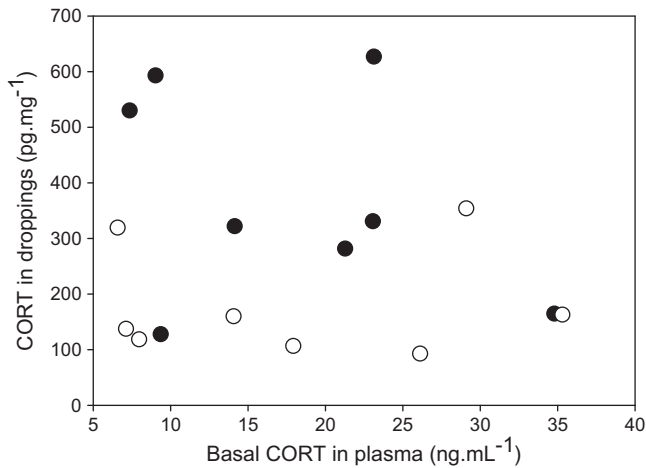
Fig. 3. Relationship between basal CORT concentration from blood samples (plasma) taken <4 min after capture and CORT concentration in droppings of the same individuals. Black dots represent droppings collected in plastic boxes (68.6 ± 1.2 min on average after capture; see text) and open dots represent droppings collected opportunistically from individuals shortly after capture (mean = 7.9 ± 0.4 min).

Table 1
Parameter values of the linear model examining the effects of time elapsed after capture (TIME), time of day (HOUR), date of the season (DATE), body size, body condition and two-way interactions on CORT in droppings. Only the terms retained from a backward selection procedure are shown.

Parameter	Slope	SE	t-value $_{(df)}$	P-value
Intercept	5.376	0.295	$t_{(1)} = 18.23$	<0.001
HOUR	−0.018	0.017	$t_{(1)} = −1.06$	0.292
DATE	−0.055	0.020	$t_{(1)} = −2.80$	0.006
TIME	0.0106	0.002	$t_{(1)} = 6.47$	<0.001
HOUR × DATE	0.0042	0.001	$t_{(1)} = 3.17$	0.002

Model statistics: $F_{4,204} = 13.38$; $P < 0.001$; $R^2 = 0.21$.
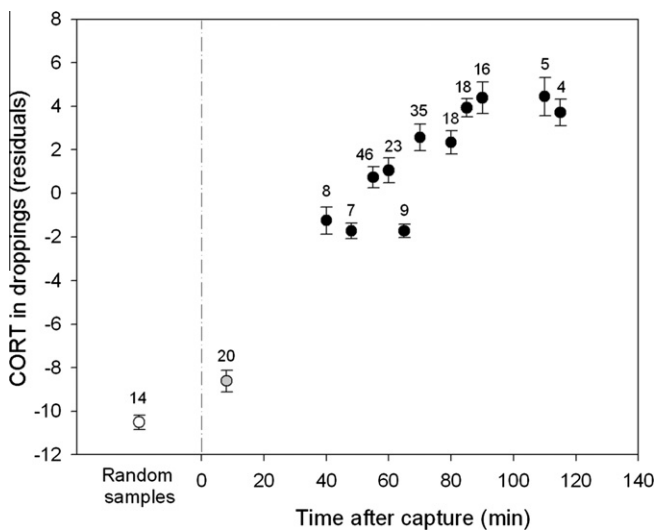
Fig. 4. Relationship between CORT measured in droppings (mean ± SE residuals from a model including DATE × HOUR; Table 1) and the time elapsed after capture. Black dots represent droppings collected from individuals placed in plastic boxes after capture (see text), the grey dot represents droppings collected opportunistically from individuals shortly (<10 min) after capture and the white dot represents fresh droppings randomly collected in the field (undisturbed birds).

10 min after capture (mean = 224.73 ± 21.69 pg mg^{-1}) (t-test, $t = −2.89$, df = 30.6, $P = 0.007$, $N = 34$). Finally, in individuals for
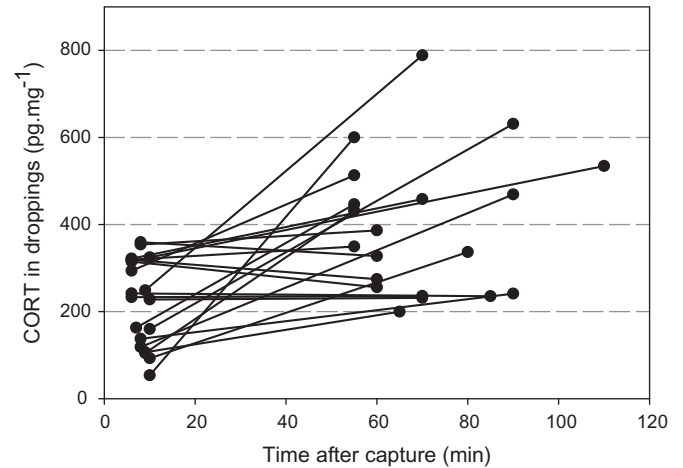
Fig. 5. CORT measured in droppings shortly after capture (<10 min) and later on (70.3 ± 1.6 SE on average). Lines connect the same individuals.

which droppings were collected shortly after capture and later in boxes (62 min after capture on average) CORT also increased over time (Repeated ANOVA: $F_{1,19} = 16.02$; $P < 0.001$; $\beta = 2.71 ± 0.64$; Fig. 5).

4. Discussion

The effect of capture on CORT levels measured in droppings has seldom been considered in wild birds (but see [22], presumably because it was assumed that metabolites would take a relatively long time to increase in feces following the stress of capture. However, this study revealed that CORT levels in droppings increased rapidly after capture with canon-nets in female greater snow geese. CORT levels collected 6–10 min after capture were apparently much already 52% higher than in droppings randomly collected in the field at the same sites. Moreover, the absence of a positive relationship between basal CORT measured in plasma and in droppings was unexpected and likely resulted from a rapid change in CORT in droppings following capture, at least in some individuals. We are confident that the CORT levels measured in the plasma was a reliable indicator of basal (or near baseline) value because we restricted our analysis to samples collected prior to capture-induced increase in CORT level [36] (i.e. <240 s after capture; see Fig 3). Moreover, our value of basal CORT measured in snow geese (mean = 18.6 ng ml^{-1} ± 2.9 SE) is almost identical to the value reported in Common eider (*Somateria mollissima*; 18.4 ng ml^{-1}) [9].

The rapid increase was not observed in our ATCH challenge CORT validation experiment. Manipulation of captive individuals induced an increase of CORT in droppings but this effect was very slight. After hypothalamo–pituitary–adrenal (HPA) axis activation through ACTH injection, one individual presented an increase after 1 h 30 and this was the case after 3 h for the other individuals. Such validation experiments have been used in captive birds to infer the time lag required to detect a sharp increase in CORT measured in droppings (see for example [16] in starlings *Sturnus vulgaris*, [46] in mourning doves *Zenaida macroura*, [47] in the Northern spotted owl *Strix occidentalis caurina*, [25,26] in two goose species, see also [44] for a review). Injection of ACTH increased levels of CORT in droppings after ~1 h in the upland goose *Chloephaga picta* [25] and after ~2 h in the greylag goose *Anser anser* [21,26,40] which is consistent with our results in the greater snow goose.

In sharp contrast with these results, we found that CORT levels in droppings started to increase as early as 6–10 min after capture in wild greater snow geese and was considerably elevated 40 min

after capture. This prevents the use of CORT in droppings as an index of basal CORT levels unless it is collected from undisturbed birds. The very rapid increase in CORT could be due to at least two factors. First, the capture of wild birds with canon-nets is presumably perceived by geese as a major threat and induces a maximal stress response compared to an injection in habituated captive individuals. Second, we followed the recommendations of Millspaugh and Washburn [31] of not separating uric acid from the fecal portion of bird droppings. The turnover time of CORT differs between these two fractions as urine is excreted more rapidly than feces [23,33,42,44]. Kalliokoski et al. [23] recently showed in mice that CORT content was about 50% higher in urine than in feces. In livestock, Palme et al. [34] infused radioactive cortisol into mammals and found that a significant amount of cortisol metabolites was excreted in urine after an extremely short time interval. It is also possible that the confinement of geese in boxes increased the amount of urine produced (an increase in urine excretion is often observed in stressed birds [45], thereby increasing the contribution of CORT coming from this fraction to the whole droppings. However, this can hardly explain the observed difference between samples collected few minutes after capture and those randomly collected in the field.

Droppings collected in the field will not come only from adult females but also from males and juveniles. Möstl et al. [33] examined the effect of sex and found that the increase of CORT after ACTH injection was more pronounced in females than in males, probably because progesterone, a precursor of CORT, also increased after ACTH injection [4]. The antibody used in this study (polyclonal rabbit antiserum, Sigma, USA) cross-reacted with progesterone (15.7%). This important cross-reactivity of the antibody with progesterone would partly explain the difference observed between droppings from captured females and randomly selected in the field. If we had sampled young and males, the differences between random and samples collected few minutes after capture could have been reduced. However, this potentially confounding factor does not apply to droppings collected in boxes and to the rapid increase seen in those samples after capture.

The increase in CORT in droppings was variable among individuals (Fig. 5). In response to a threatening event, some birds may have dramatically increased their CORT secretion whereas others may have only slightly increased this secretion. Such variance is likely to be related to the high variability in the sensitivity of the HPA to stress between individuals [13]. Snow geese also have social hierarchies [6] that are known to have an impact on HPA axis reactivity to the physiological stress of capture [15,27]. Dominants and subordinates can respond differently to a threat [48]. Capture stress protocol (i.e. blood samples taken from 2 to 60 min from birds maintained in cloth bag) is a classical method to investigate stress response in birds [10,48]. Since our results indicate that CORT in droppings rapidly increase after capture, measuring CORT from repeated droppings could be a less invasive alternative to investigate individual heterogeneity and stress response. However, this possible approach needs future validation to determine if capture stress protocols using blood and droppings lead to similar results. This would be the case only if CORT metabolites measured in droppings collected in nature are repeatable over time [8].

5. Conclusion

Measuring CORT in bird's droppings can possibly allow researchers to assess basal CORT levels using a non-invasive method and investigate the effect of CORT on various life history traits [7,41]. However, we clearly showed that CORT levels in droppings can be highly sensitive to capture and handling time in wild birds, as found in blood samples [36,37]; this study. The type of stressor (ex: life threatening *vs.* others) can influence the magnitude of the stress response [11] and it remains to be seen if potentially less stressful capture methods (e.g. mist-nets) would also elicit a similar response in wild birds. Indeed, baseline CORT can indeed be affected by the capture methods [1,30,37]. An alternative could be to separate the marking and feces collection process in time, for instance by collecting dropping in individually-marked individuals that would be tracked after their release, once capture stress has disappeared [43]. While we acknowledge that future studies on other species are needed, we recommend that use of droppings as a non-invasive technique to measure baseline CORT be restricted to non-manipulated individuals in the wild or to separate the marking and dropping collection process in time.

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