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NOTE

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Salivary corticosterone measurement in large-billed crows by enzyme-linked immunosorbent assay

Illia AOTA[1,2], Chisato YATSUDA[1], Ei-ichi IZAWA[1]*

[1]Department of Psychology, Keio University, Tokyo, Japan
[2]Japan Society for Promotion of Science, Tokyo, Japan

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ABSTRACT. Salivary corticosteroid measurement, as a surrogate for plasma corticosteroid levels to evaluate an animal's stress or metabolic state, commonly used in mammals. However, the validity of salivary corticosterone (CORT) measurements in birds has not yet been reported. We aimed to measure salivary CORT in crows using a commercially available CORT enzyme-linked immunosorbent assay kit. An adrenocorticotropic hormone (ACTH) challenge experiment using synthetic cosyntropin, an ACTH analogue, was conducted to compare CORT level elevations between the serum and the saliva in a 10–60 min range. Both salivary and blood CORT was significantly elevated 10 min after injecting synthetic cosyntropin. The results supported the validation of salivary CORT as a surrogate for a blood CORT in crows.

KEYWORDS: bird, corticosterone, enzyme-linked immunoassay, glucocorticoid, stress

Corticosteroid hormones, such as cortisol and glucocorticoids, are secreted by the adrenal cortex via the activation of the hypothalamus-pituitary-adrenal axis (HPA axis), which is highly conserved across vertebrates [8]. In veterinary science and animal behavior/psychology, corticosteroids are widely used as biomarkers to evaluate the psycho-endocrinological (i.e., stress) and/or metabolic states of individuals in response to various environmental challenges and stress-related pathology [7, 23, 27]. For measuring cortisol and glucocorticoids in mammals, invasive techniques, such as blood sampling or non-/less-invasive techniques via the collection of saliva, feces, and hair samples are adopted depending on the study field (i.e., experiment, wild, farm, and clinic) as well as the evaluation time of metabolism and/or stress [19, 20, 32].

In birds, previous studies have developed and used non-/less-invasive techniques to measure corticosterone (CORT), a biologically active corticosteroid of avian species, from feces, feathers, and eggs [1, 15, 18, 26, 34]. These non-/less-invasive techniques available so far can measure the basal level of peripheral CORT or the change of that in hours or longer time scale, but fail to measure the acute response of CORT within tens of or even a few minutes. An alternative technique commonly used in mammals is the measurement of salivary corticosteroid (typically cortisol) [5, 6, 12, 13, 21, 31]. The validity of salivary CORT measurements has recently been reported in amphibians [11]. Even though the salivary grands are developed in birds to aid their food habits [14, 17], measurement of peripheral hormone levels from saliva samples has been reported for mesotocin in only one study [29] but not for CORT in birds.

This study aimed to validate the salivary CORT measurement as a less-invasive method to evaluate the acute changes in peripheral CORT levels, for 10 min or longer time range, using saliva samples of large-billed crows (*Corvus macrhorynchos*). Specifically, an adrenocorticotropic hormone (ACTH) challenge was performed to measure peripheral CORT levels in serum and saliva samples at the same time points (10–60 min) after cosyntropin administration from the same individuals and to examine whether a similar elevation of CORT level was observed in serum and saliva samples. Cosyntropin is a synthetic ACTH with a protein sequence (SYSMEHFRWGKPVGKKRRPVKVYP) that greatly matches the ACTH domain in the proopiomelanocortin sequence of large-billed crows (GenBank# BAJ05380). Cortroysyn was confirmed to serve as a stimulant for ACTH challenge in avian species [28]. Therefore, we used cosyntropin as ACTH-stimulant drug for large-billed crows. Hereafter, cosyntropin injection is referred to as ACTH injection in this paper, unless otherwise noted. To validate our method for stress-induced CORT elevation, we examined whether capture stress elevated salivary CORT level, using procedure similar to that used by Astheimer *et al.* [2].

We used six adult large-billed crows, three males and three females, aged between 5 and 8 years, with a body weight of 640–790 g. All crows were caught as yearlings in Tokyo and neighboring regions with permission from the Ministry of the Environment Government of Japan (Authorization No.114001, 301482). Sex was identified using DNA from the blood. The crows were individually housed in steel mesh cages (width 57 cm × depth 93 cm × height 63 cm) in an animal housing room at the Department of Psychology,

*Correspondence to: Izawa E: izawa@flet.keio.ac.jp, Department of Psychology, Keio University, 2-15-45 Mita, Tokyo 108-8345, Japan

Keio University. Housing conditions were set at 21 ± 2°C under a 13 hr light: 11 hr dark photoperiod with light onset at 7:00 am. Food (dried dog food) and water were freely available in the home cages. During the experimental session, the crows were fed only water without food. The study protocols were approved by the Animal Care and Use Committee of Keio University (No. A2022-264).

Each crow received two 60-min sessions for collecting saliva and blood samples following an intrapectoral injection of ACTH or saline. Three sessions with different individuals were conducted each day consecutively. The 1st and 3rd sessions started at 8:30 am and 11:30 am, respectively. The ACTH or saline injection session for the same individual was carried out at one-week intervals with a counterbalancing order among the six individuals; three birds received an ACTH injection in the first session and a saline injection in the second session. The reversed order was applied to the other three individuals. In each session, an injection of either 0.5 mL of an ACTH (50 IU/kg of BW in 1 mL saline: Cortroysyn, Alfresa Pharma Co., Osaka, Japan) or saline was administered manually by a skilled researcher using a 26-G needle.

To validate the salivary CORT elevation associated with a physical stress, the same six crows were used in the capture-stress experiment one month after the end of ACTH challenge. In this experiment, each crow was captured and restrained for 60 min in a mesh fabric bag (diameter 22 cm × length 33 cm) as a physical stress. The mesh bag did not prevent the bird from breathing. Saliva and blood samples were collected by the same methods and time schedule, where the time-point 0 (i.e., 0 min) was defined as the time immediately before bag restrain.

For sampling, a researcher gently caught the subject from the home cage and held the bird for another skilled researcher to collect saliva and blood subsequently within 3 min after catching. Saliva was collected using a small swab (diameter 6.3 mm × length 30 mm; Infants Swab, Salimetrics, CA, USA) inserted into the bird's oral cavity for 30 sec (Fig. 1). Immediately after swab withdrawal, blood was drawn from the wing basilic vein using a 25-G needle connected to a hematocrit tube (Thermo Fisher Scientific, Columbus, OH, USA) and collected in a serum separator clotting tube (Becton Dickinson Co., Franklin Lakes, NJ, USA). Soon after each session, saliva samples were centrifuged at 12,000 × *g* for 90 sec to separate the liquid components from the swabs (Fig. 1). Blood samples were also centrifuged at 12,000 × *g* for 90 sec to separate the serum component. The extracted samples were stored at −70°C until analysis. In each session, we collected saliva and blood samples every 10 min, including immediately before the ACTH/saline injection (0 min) and, 10, 20, 30, 40, 50, and 60 min after injection. In cases where sampling was not completed within 3 min, the corresponding data were excluded from the analysis to remove the effect of handling stress on circulating corticosterone elevation [33].

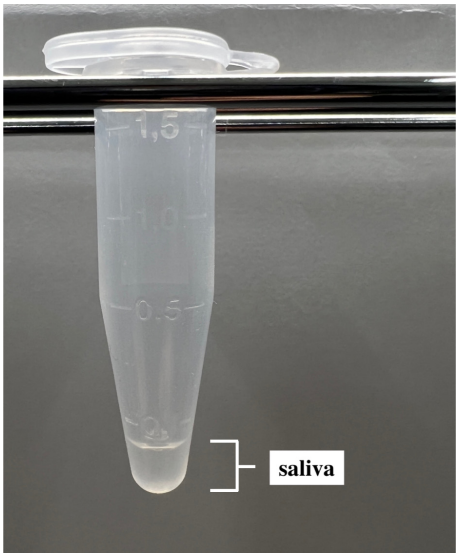

Fig. 1. Representative image of liquid components separated by centrifugation of saliva sample. Inserting a small swab into the oral cavity of crows for 30 sec allowed for collecting 15 µL or more liquid from saliva.

Corticosterone concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit previously described in various bird species (Item No. 501320, Cayman Chemicals, Ann Arbor, MI, USA) [4, 15, 18, 26, 33]. Each saliva sample was centrifuged at 2,000 × *g* for 15 min, and 15 µL of the supernatant was re-sampled in a glass tube. For extraction, 3 mL diethyl ether was added to the glass tube containing 15 µL saliva, stirred for 10 min, and left still for 30 min. The tube was placed in a dry ice ethanol bath to freeze the water layer, and the ether layer was transferred to a new glass tube by decantation. After evaporating the diethyl ether, the extract was stored at −70°C until the assay. A similar method was used to extract serum samples from 15 µL serum. For the assay, extracted saliva and serum samples were reconstituted with 120 µL assay buffer for subsequent processes. All the samples were duplicated for the assay. The intra- and inter-assay coefficients of variation were 6.9% (range; 2.8–8.8%) and 10.0%, respectively.

CORT elevation after ACTH injection across the time (~60 min) in serum and saliva samples was measured by comparing the CORT levels among the *conditions* (ACTH, saline) × *time point* (0, 10, 20, 30, 40, 50, and 60 min) using a two-way ANOVA with repeated measurement on individuals, separately for serum and saliva samples. For analyzing CORT elevation induced by capture stress, we used one-way repeated ANOVA separately for serum and saliva samples to compare the CORT levels among *time points* (0, 10, 20, 30, 40, 50, and 60 min). If significance was detected, *post-hoc* multiple comparisons with Tukey test were conducted to compare the CORT concentrations between saline and ACTH at each time point. For ANOVA analysis, CORT concentration data were log-transformed to meet the normal error distribution. These analyses were conducted using the packages lme4 [3], car [9], and lsmeans [16] of free-software R v. 4.0.5 [22].

In the ACTH challenge, ANOVA on serum CORT showed a significant interaction *time point* × *condition* ($F_{6,\ 61.045}$=10.103, P<0.001). Multiple comparisons by Tukey test revealed that serum CORT at 10 min and all subsequent time points after ACTH injection were significantly higher than those at 0 min (0 min *vs*. 10 min, t_{61}= −8.014, P<0.001; *vs*. 20 min, t_{61}= −8.024, P<0.001; *vs*. 30 min, t_{61}= −9.496, P<0.001; *vs*. 40 min, t_{61}= −9.421, P<0.001; *vs*. 50 min, t_{61}= −8.858, P<0.001; *vs*. 60 min, t_{61}= −9.878, P<0.001; other time-point pairs, $-1.863<t_{61}<0.09$, *n.s.*; Fig. 2A). On the other hand, in the saline condition, no significant difference in serum CORT level was found between any time-point (all time-point pairs, $-2.113<t_{61}<1.6$, *n.s.*). The CORT levels in the ACTH condition were significantly higher than those in the saline condition at 10 min and subsequent time points (10 min, t_{61}=4.858, P<0.001; 20 min, t_{61}=5.268, P<0.001; 30 min, t_{61}=7.761, P<0.001; 40 min, t_{61}=7.552, P<0.001; 50 min, t_{61}=5.439, P<0.001; 60 min, t_{61}=6.260, P<0.001)

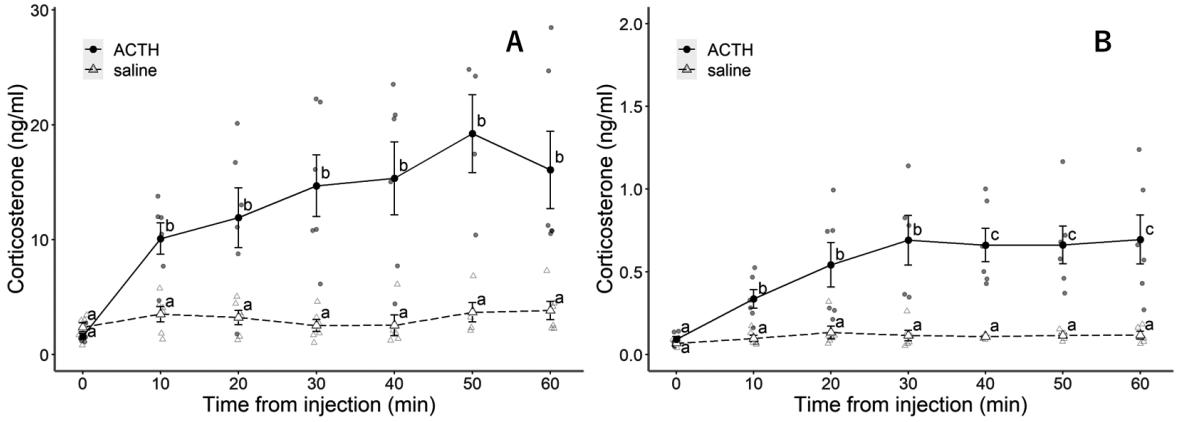

Fig. 2. Temporal changes in serum (**A**) and sarivary (**B**) corticosterone levels after adrenocorticotropic hormone (filled circle) and saline (open triangle) injections. Different letters indicate the statistically significant differences in corticosterone levels between the time points (P<0.05). Data are represented as mean ± SEM.

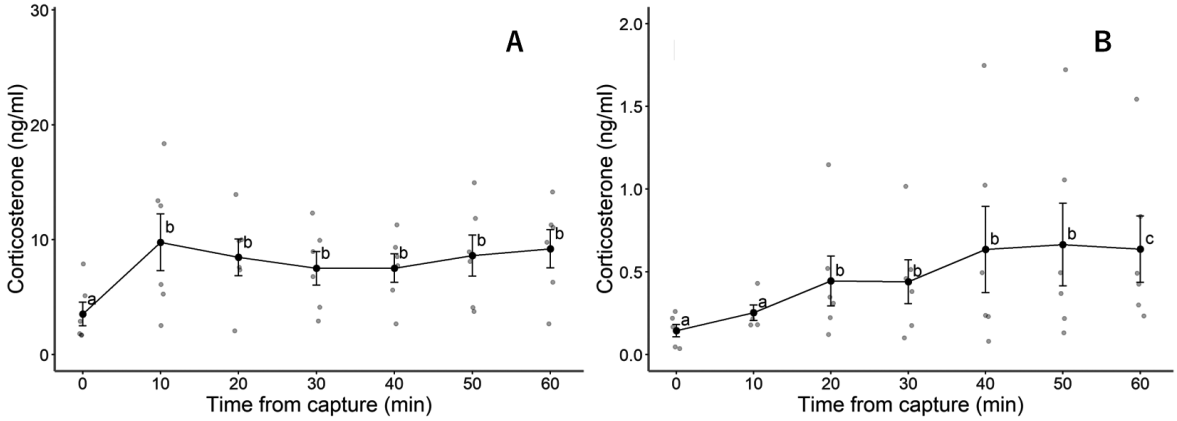

Fig. 3. Temporal changes in serum (**A**) and salivary (**B**) corticosterone levels after birds were captured in a mesh bag. Different letters indicate the statistically significant differences in corticosterone levels between the time points (P<0.05). Data are represented as mean ± SEM.

but not at 0 min (0 min, t_{61}= −1.505, *n.s.*). A similar elevation in CORT levels after ACTH injection was found in the saliva. ANOVA on saliva CORT produced a significant interaction *time point* ×*condition* ($F_{6, 61.077}$=6.346, P<0.001). *Post-hoc* multiple comparisons by Tukey test for the ACTH condition showed that saliva CORT levels at 10 min and subsequent time points were significantly higher than those at 0 min (ACTH condition, 0 min *vs*. 10 min, t_{61}= −6.026, P<0.001; *vs*. 20 min, t_{61}= −7.77, P<0.001; *vs*. 30 min, t_{61}= −8.517, P<0.001; *vs*. 40 min, t_{61}= −9.242, P<0.001; *vs*. 50 min, t_{61}= −9.196, P<0.001; *vs*. 60 min, t_{61}= −9.163, P<0.001; Fig. 2B). Significant differences in salivary CORT levels were found between 10 and 40 min (t_{61}= −3.216, P<0.032), 10 and 50 min (t_{61}= −3.170, P<0.032), and 10 and 60 min (t_{61}= −3.137, P<0.040) in the ACTH condition. No significant difference was found between any time-point pair in the saline conditions (all time-point pairs, −2.572 <t_{61} <0.73, *n.s.*). Comparisons between the ACTH and saline conditions at each time-point showed significant differences at 10 min and subsequent time points (10 min, t_{61}=5.761, P<0.001; 20 min, t_{61}=6.321, P<0.001; 30 min, t_{61}=7.835, P<0.001; 40 min, t_{61}=7.411, P<0.001; 50 min, t_{61}=7.750, P<0.001; 60 min, t_{61}=7.539, P<0.001) but no significant difference at 0 min (t_{61}=1.123, *n.s.*).

In the capture-stress experiment, ANOVA on serum CORT levels showed a significant interaction *time point* ($F_{6, 30}$=7.4, P<0.001). Multiple comparisons by Tukey test revealed that serum CORT at 10 min and all subsequent time points were significantly higher than those at 0 min (0 min *vs*. 10 min, t_{30}= −5.370, P<0.001; *vs*. 20 min, t_{30}= −4.933, P<0.001; *vs*. 30 min, t_{30}= −4.432, P<0.002; *vs*. 40 min, t_{30}= −4.570, P<0.002; *vs*. 50 min, t_{30}= −5.137, P<0.001; *vs*. 60 min, t_{30}= −5.473, P<0.001; other time-point pairs, −1.040 <t_{30} <0.938, *n.s.*; Fig. 3A). Elevation in salivary CORT levels as well was found after exposing the birds to capture stress. ANOVA on salivary CORT levels produced a significant interaction *time point* ($F_{6, 29}$=8.578, P<0.001). *Post-hoc* multiple comparisons by Tukey test showed that salivary CORT levels at 20 min and subsequent time points were significantly higher than those at 0 min (0 min *vs*. 20 min, t_{29}= −4.476, P<0.001; *vs*. 30 min, t_{29}= −4.428, P<0.002; *vs*. 40 min, t_{29}= −5.012, P<0.001; *vs*. 50 min, t_{29}= −5.617, P<0.001; *vs*. 60 min, t_{29}= −6.078, P<0.001; Fig. 3B). Significant differences in saliva CORT were also found between 10 and 60 min (t_{29}= −3.215, P<0.045). No significant difference was found between other time-point pair (other time-point pairs, −2.778 <t_{29} <0.048, *n.s.*).

Results from ACTH challenge experiment showed that serum and salivary CORT levels were elevated 10 min after ACTH stimulation and were maintained for at least 60 min, indicating a similar change in serum and salivary CORT levels over time. Capture stress caused similar elevation of serum CORT at 10 min and salivary CORT after 20 min following the capture, suggesting that stress-induced CORT elevation in saliva was measurable 20 min or later after a stress-relevant event. Our findings report, for the first time, the validity of CORT measurements using saliva samples as a proxy for peripheral CORT.

Both serum and salivary CORT levels showed similar elevation 10 min after the ACTH challenge. However, the time to peak CORT levels differed between the serum and the saliva. Serum CORT reached its highest level in 10 min and was maintained thereafter, whereas salivary CORT was significantly elevated at 10 min from the baseline (0 min) but reached its highest level at the 40 min time-point. The difference in the temporal patterns of CORT elevation between serum and saliva suggests that elevation of CORT levels from the baseline is detectable within 10 min after ACTH stimulation in both serum and saliva, but that the time to peak of salivary CORT could be delayed approximately 30 min to that of serum CORT in crows. The temporal characteristics of serum and salivary CORT in response to ACTH challenges, such as the time of elevation, peak, and recovery to baseline, have been suggested to vary depending on the injection method, dose, and species [2, 10, 21, 24, 25, 30, 35]. Thus, the variation of temporal characteristics in serum and salivary CORT in crows, and even in other avian species, should be further investigated in future studies.

The salivary CORT measurement established in this study employs the collection of saliva with swab and is relatively easy and less-invasive technique that could help to assess psycho-endocrinological and metabolic states in birds such as domestic fowls, geese, and turkey. The application of this method to various bird species might be challenging, especially for small birds with small bill and slow or little secretion abilities of saliva. Such morphological and physiological characteristics might cause difficulty in inserting a swab into the bill and collecting a sufficient volume of saliva (e.g., 15 µL for crows) within a few minutes.

We also should note that in ACTH challenge experiment, several saliva samples were below the measurement range of the ELISA kit used in this study (8.2–5,000 pg/mL according to the manual). Specifically, 4 out of the 12 samples (approximately 30% of samples: 2 samples in ACTH group, and 2 samples in saline group) at the time-point 0 showed CORT levels below the lower limit (i.e., 8.2 pg/mL). These samples were obtained from 4 different birds, indicating that low levels of baseline CORT around measurement limit in saliva samples may occur not in an individual-specific manner but at a certain probability (e.g., 30%). Such low levels of baseline salivary CORT might make it challenging to evaluate CORT elevation compared with baseline in stressful events.

Capture stress experiment showed that the present method validates the measurement of salivary CORT elevation in response to a stressful event. However, as seen in Fig. 3, CORT elevation to statistically significant levels was delayed by 10 min in saliva samples compared to that in serum samples. This delay might be due to low levels of salivary CORT. However, it is a possibility that capture procedure may not be so stressful for crows. Given such temporal characteristic of delayed CORT elevation in saliva, for evaluating acute CORT response to a stressful event/stimulus, using the saliva samples 20 min after the event/stimulus or later may be reliable.

To conclude, salivary CORT measurement as a surrogate of blood CORT assessment in birds could be advantageous. This is, particularly, so in situation where blood CORT needs to be measured repeatedly from the same individuals, with salivary CORT as an alternative to blood collection with cannulation into the vein.

CONFLICT OF INTEREST. The authors declare no competing interests.

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