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NOTE

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Measurement of urinary mesotocin in large-billed crows by enzyme-linked immunosorbent assay

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ABSTRACT. Mesotocin (MT) is an avian homologue of oxytocin (OT). Behavioral pharmacological studies in birds have suggested the involvement of MT in socially affiliative behavior. However, investigations of peripheral MT levels associated with social behavior are lacking because non-invasive methods to measure surrogate plasma MT have yet to be established. This study aimed to measure urinary MT in crows using a commercially available OT enzyme-linked immunosorbent assay kit. Urine samples were collected after intravenous injection of MT and centrifuged to separate urine and fecal components. We found that urinary MT was significantly elevated 15–30 min after MT injection. These results validate our method for the use of urine samples for the measurement of peripheral MT levels in crows.

KEYWORDS: avian, corvid, enzyme-linked immunoassay, mesotocin, oxytocin

Oxytocin (OT), a neurohypophysial hormone, plays an essential role in conductance regulation in the uterus and milk ejaculation of mammals. In birds, mesotocin (MT) is known to be the avian homologue of OT [1, 10]. It has been suggested that MT in birds is primarily involved in socially affiliative behavior, such as pair bonding and group formation, rather than oviposition [6, 7, 11, 19, 27]. Previous studies on social zebra finches (*Taeniopygia guttata*) reported that systemic administration of OT antagonists impaired pair formation [19] and that central administration of MT facilitated group formation [6, 11]. However, the investigation of peripheral MT levels associated with social behaviors have been lacking in birds because non-invasive methods to assay plasma MT concentration have not been established.

Only one study recently reported a non-invasive measurement of MT concentration using the saliva of common ravens (*Corvus corax*) [26]. However, using this method in the study of various social behaviors of birds may be limited because salivary OT has been suggested to be a weak surrogate measure for plasma OT in mammals [13, 20]. Thus, it is necessary to establish an alternative non-invasive method to assay peripheral plasma MT in birds. An assay measuring urinary MT is a potential alternative because OT concentration in urine has been proven to be a good proxy for peripheral plasma OT levels in mammals [16, 17]. To date, no study has yet measured MT levels using urine samples from birds. This is likely because it is not easy to collect urine from avian excrement as the feces and urine are mixed. However, we can separate these compounds and collect the liquid component of a centrifuged excrement sample [22]. Thus, an assay of MT concentration using urine is possible in birds, similar to that already established in mammals [16, 17].

This study aimed to establish a non-invasive method for the measurement of MT concentration by using urine samples of large-billed crows (*C. macrhorynchous*). These birds have been found to show a variety of socially affiliative behaviors that potentially involve the regulation of MT [9, 15, 28].

We used six male crows aged between 3–5 years and with a bodyweight of between 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.114011). After being group-housed in an outdoor aviary for 3 years, the crows were housed individually in steel mesh home cages (W 57 cm × D 93 cm × H 63 cm) in an animal housing room at the Department of Psychology, Keio University. Housing conditions were set at 21 ± 2°C with cycles of 13L and 11D (light onset at 8:00am). Food (dog food) and water were freely available in the home cages. Only water was available, with food being absent, during the sessions conducted in the home cages. The study protocols were approved by the Animal Care and Use Committee of Keio University (No. 20036).

Over two sessions, dropping samples were collected from all six crows following an intravenous injection of either exogenous MT or saline. Each session began at approximately 7:30am. The MT or saline injection session for the same individual was carried

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out at 1-week intervals and with counterbalancing order among the six birds; specifically, three birds received an MT injection in the first session, and the others received saline injection first, and this was reversed for the second session. In each session, the injection was manually performed by a skilled researcher with 0.3 ml MT (50 pg/ml saline; H-2505, Bachem, Torrance, CA, USA) or saline injected slowly into a wing vein for approximately 3 min without any intervals. We collected dropping samples for 1.5 hr at six time points; Time 0 was defined as the time immediately before injection, and droppings were also collected at a ± 5 min range before and after 15, 30, 60, and 90 min after injection. At each sampling point, a plastic board was placed on the cage floor to catch the droppings.

Immediately after collection, the droppings were centrifuged at 14,000 rpm for 15 min or longer to separate the urine and fecal components with a very small portion of urate component (Fig. 1). We collected only the liquid urine component from the centrifuged sample using a pipet to exclude the urates and fecal components. The collected urine samples were stored at −70°C until analysis, which occurred within 60 min after collecting the droppings. If no droppings were collected at a time point, we considered this a missing value for the analysis.

Urinary MT concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for OT which was confirmed to be 100% cross-reactive to MT (Item No. 500440, Cayman Chemicals, Ann Arbor, MI, USA). Due to the high variance of urine MT concentrations between individuals, dilutions of urine samples varied from no dilution to between 5 and 30 times dilution to ensure that readout values fell within the detection range of the kit (minimum=23.5 pg/ml, maximum=750 pg/ml). The optimal dilution for each individual was determined using pilot analyses. We applied an aliquot of 100 µl mixture composed of 10 µl of diluted urine samples and 90 µl of assay buffer to each well and incubated for 18 hr at 4°C. All samples were analyzed in triplicate. The intra- and inter-assay coefficients of variation (CV) were 8.9% and 9.6%, respectively. To confirm the reliability of the Cayman OT ELISA kit to measure the MT concentration using crow urine, we tested the serial dilution of pooled crow urine in triplicates (1:1 up to 1:8 dilution) and recovery of known MT in crow urine samples. Recoveries were obtained by spiking the pooled urine with a known concentration of MT (H-2505, Bachem AG, Bubendorf, Switzerland). The estimated and actual concentrations were compared. In both tests, there were significant and strong correlations between the estimated and the actual values (Fig. 2).

For the analysis, MT concentration was represented as a ratio of MT to creatinine in order to control for the quantity of water in the urine samples, which enables the comparison of MT levels between individuals [4, 18, 24, 29]. The creatinine concentration of each urine sample was measured using a commercially available kit, which was previously used for droppings of other avian species [5, 8], based on the Jaffe reaction method (Item No. 500701, Cayman Chemicals). In the creatinine assay, all samples were diluted five times. The plate was read at an optical density of 490 nm using a microplate reader. Urinary creatinine concentration ranged from 0.9 to 13.34 (median 6.84) mg/dl. The intra- and inter-assay CVs for creatinine were 6.8% and 1.0%, respectively (duplicate, r=0.99, $P<0.001$).

The effects of the MT injection on MT levels were compared between MT and saline treatment within individuals using a non-parametric Friedman analysis of variance (ANOVA), which included '*treatment*' and '*time*' factors. For missing values due to no droppings at a certain time point of a condition, a mean concentration among the data of the corresponding time point and the condition was assigned to run the Friedman ANOVA. If the factor(s) was significant by the Friedman ANOVA, the non-parametric Wilcoxon test or Mann-Whitney test was performed as a post-hoc test for paired or non-paired (including missing values) data, respectively, to compare the two conditions at each time point. Statistical analyses were conducted using software R v. 3.6.0 [21] at a significance level of 0.05.

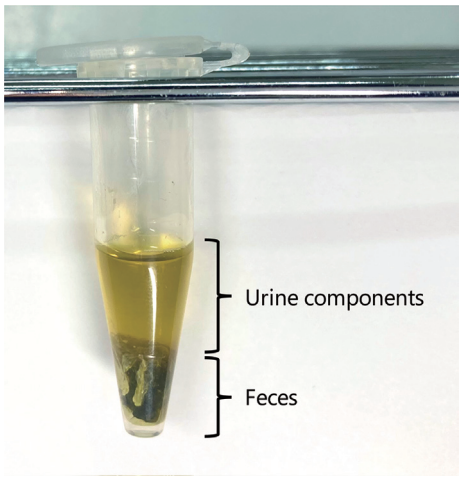

Fig.1. A centrifuged dropping sample in a 1.5 ml tube. After being centrifuged, the sample was separated into the upper liquid urine and the lower solid fecal components with a very small proportion of urates. The liquid urine component was collected for the assay.

Urinary MT was significantly elevated 15–30 min after MT injection (Fig. 3). Post-hoc comparisons between the treatments according to the Friedman ANOVA (*treatment* × *time*, $\chi^2=53.19$, $P<0.001$) revealed significant differences at time points 15 min ($W=33.00$, $P<0.05$) and 30 min ($W=33.00$, $P<0.05$) after injection. In contrast, no significant difference was found before injection (Time 0, $W=27.00$, *n.s.*), 60 min ($U=6.00$, *n.s.*), and 90 min ($U=-2.00$, *n.s.*). The minimum concentration of urinary MT at Time 0 of saline treatment ranged between 9.52 and 120.72 pg/ml, which suggests considerable individual variability. This result indicates that the intravenous MT injection elevated urinary MT. Therefore, MT measured by ELISA in this study reflects the activation of renal metabolism of MT in crows.

The present study showed that MT is measurable in the urine of crows using a commercial ELISA. The highest concentration of urinary MT occurred 15–30 min after intravenous injection of exogenous MT, which indicates that it is important to measure the change in urinary MT between 15 and 30 min after an MT-relevant stimulus to the crow. The peak time after intravenous injection occurred earlier in crows than in mammals, where the peak occurs around 30–90 min (humans; [2], common marmosets; [25]; dogs; [14]). This might be because the blood pressure of the glomerular capillaries (intraglomerular pressure) in birds is higher than in mammals due to higher arterial pressures in birds [3, 23]. In birds, this may be important.

There was a large variation in the MT concentrations, as shown in Fig. 3. Such large variations are likely to reflect the inherent individual differences of

peripheral MT concentrations and are not thought to be due to errors in the measurement process for the following reasons. First, the reliability of the ELISA kit used to measure the MT concentration in crow urine was confirmed by the strong correlations found in both the dilution and spike tests. Second, the inter-assay CV was sufficiently small in both the creatinine and MT measurements. Third, a statistically significant difference was confirmed by the comparison of the same individuals, namely within-subject comparison using the Wilcoxon test, between MT and saline treatments even though the MT data points seemed to strongly deviate. These facts support the present assay method as being sufficiently valid and less likely to contain methodological factors leading to measurement errors. Thus, it is reasonable to consider the large variation of MT concentrations that were measured as the inherent individual differences. However, more precise measurement may be possible by performing an extraction process,

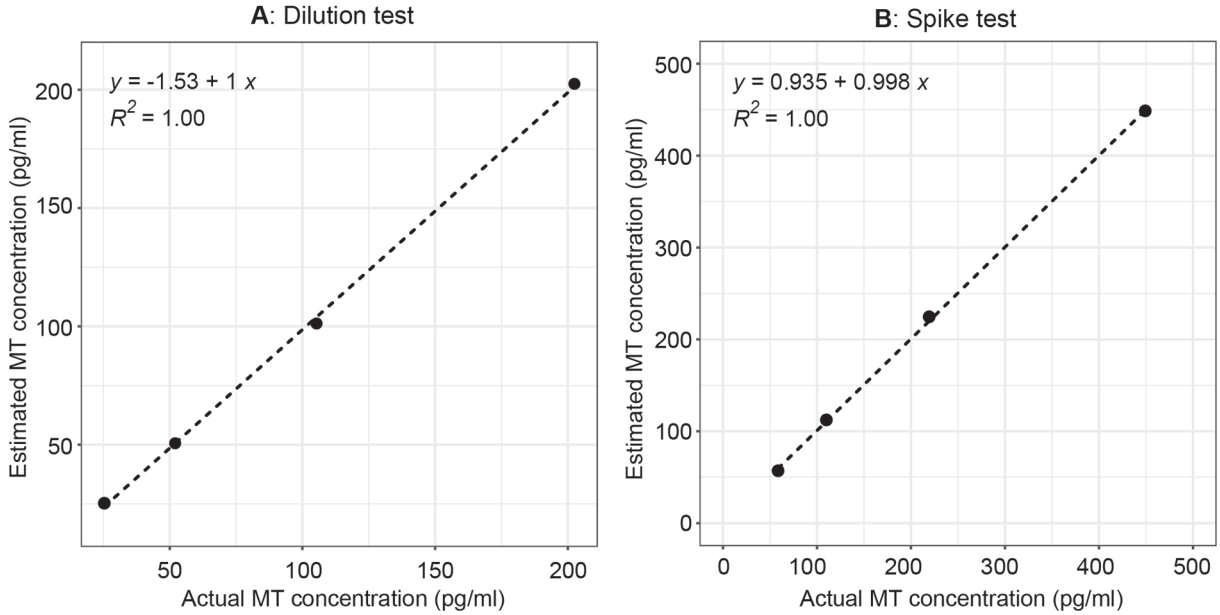

Fig. 2. Dilution test (**A**) and spike test (**B**) to confirm the reliability of measuring mesotocin (MT) levels with the oxytocin ELISA kit. In both tests, there were strong correlations (dilution: $y = -1.53 + 1x$, R^2=1.00. spike: y=0.935 + 0.998x, R^2=1.00).

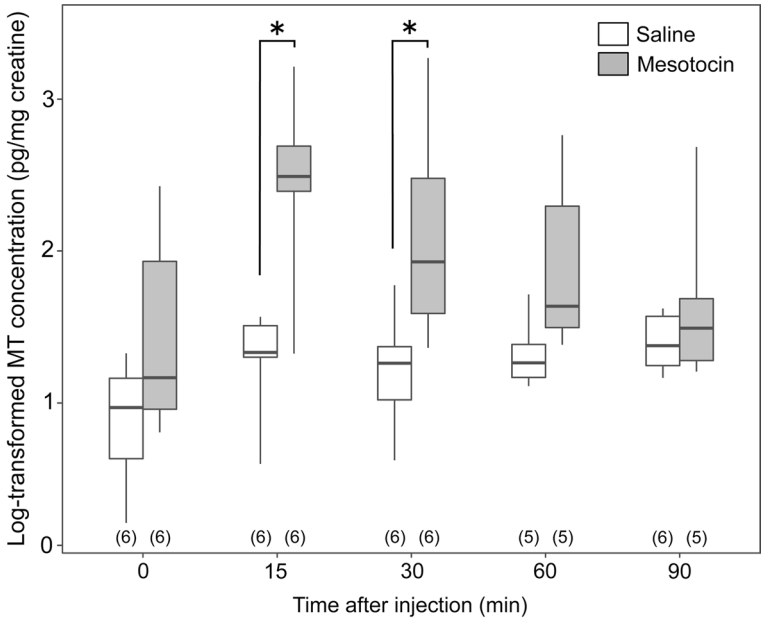

Fig. 3. Temporal changes in mesotocin (MT) levels after MT (shaded) and saline (open) injections. Asterisks indicate the statistically significant difference in MT concentration between the conditions (P<0.05). The upper, median, and bottom of the boxes show the 75, 50, and 25 percentiles, respectively, with whiskers representing the maximum and minimum values. Numbers in the brackets below the boxes denote the number of samples. Note that at 60 and 90 min, data was not completely paired due to the lack of dropping samples.

which was not undertaken in our assay, to remove interfering proteins often used for hormonal assays [12].

In this study, we used only urine components for the MT assay but did not separate the components of the excretion, such as feces. However, the separated fecal components might contain MT that originated from the feces *per se* or a small portion of unseparated urine, or both. Given that the previous reports in mammals have suggested that OT in feces is immeasurably small (e.g. [25, 30]) and that potential urine liquid contained in the separated feces was proportionately small in the present study, the potential MT concentration in the separated fecal component is likely to be very low and insufficient to invalidate the current results. However, future studies should measure how much or whether MT is contained in the separated fecal component after centrifugation.

The use of urine samples offers several advantages. Urine can be collected very easily, and the sampling is feedback-free because there is no need to capture and handle the animal. Therefore, repeated sampling of the same individual is possible without affecting the animal's behavior or endocrine status. This method allows the monitoring of hormonal changes in response to specific situations, social encounters, or treatments.

Only one study recently reported a non-invasive measurement of MT concentration in the saliva of common ravens (*C. corax*) [26]. However, saliva samples collected at one time from birds are too small to reach a sufficient amount above the detection limit for the assay [26]. Given the weakness of salivary samples, our assay using urine is advantageous as a sufficient volume can be collected.

Our method reported here may be useful not only for the measurement of peripheral MT levels but also for other biomarkers of birds, both in captivity and in the wild.

CONFLICT OF INTEREST. The authors declare no competing interests.

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