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Measuring salivary mesotocin in birds - Seasonal differences in ravens' peripheral mesotocin levels

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ABSTRACT

Oxytocin is involved in a broad array of social behaviours. While saliva has been used regularly to investigate the role of oxytocin in social behaviour of mammal species, so far, to our knowledge, no-one has tried to measure its homolog, mesotocin, in birds' saliva. Therefore, in this study we measured salivary mesotocin in common ravens (*Corvus corax*), and subsequently explored its link to three aspects of raven sociality. We trained ravens ($n = 13$) to voluntarily provide saliva samples and analysed salivary mesotocin with a commercial oxytocin enzyme-immunoassay kit, also suitable for mesotocin. After testing parallelism and recovery, we investigated the effect of bonding status, sex and season on mesotocin levels. We found that mesotocin was significantly more likely to be detected in samples taken during the breeding season (spring) than during the mating season (winter). In those samples in which mesotocin was detected, concentrations were also significantly higher during the breeding than during the mating season. In contrast, bonding status and sex were not found to relate to mesotocin detectability and concentrations. The seasonal differences in mesotocin correspond to behavioral patterns known to be associated with mesotocin/oxytocin, with ravens showing much more aggression during the mating season while being more tolerant of conspecifics in the breeding season. We show for the first time that saliva samples can be useful for the non-invasive determination of hormone levels in birds. However, the rate of successfully analysed samples was very low, and collection and analysis methods will benefit from further improvements.

1. Introduction

Behavioral endocrinology has long depended mainly on blood sampling techniques, which have advanced the knowledge in the field tremendously. However, if performed on untrained subjects, such invasive techniques are often associated with elevated stress levels and welfare issues, and can consequently lead to immediate modulation/change in hormonal concentrations. The consequent need for non-invasive methodologies has put forward alternative approaches such as quantifying hormones out of faeces, urine, saliva, milk, hair or feathers (reviews: Behringer and Deschner, 2017; Palme, 2019). In bird studies the most commonly selected matrices for hormone analysis are blood or droppings. Both techniques have their advantages and disadvantages: While the former entails the before-mentioned invasive approach, it does allow the detection of almost immediate hormonal changes. And while the latter is non-invasive, it does create a time-lag between the hormone release into the blood and the appearance of the hormone metabolites in the droppings, which results from gut passage time (Palme, 2019). Another limitation is that certain hormones, such as oxytocin (and its homologues), or their metabolites, cannot easily be measured in faeces. Consequently, the choice of the appropriate matrix depends on the hormone to be measured, the temporal scale of the research question and the feasibility of sample collection. So far, scientific progress in ornithology has been limited due to the lack of non-invasive techniques which are able to detect immediate or short-term changes in hormone concentrations.

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Oxytocin (OT) is a nonapeptide that exhibits a broad range of central and peripheral effects, from the modulation of neuroendocrine reflexes to complex social behaviours (Gimpl and Fahrenholz, 2001). Depending on the species, the latter include social bonding, trust, maternal and alloparental care, cooperation, consolation, outgroup derogation and sexual behaviour (reviews: De Dreu and Kret, 2016; Goodson, 2013; Quintana and Guastella, 2020; Walum and Young, 2018). Whereas OT occurs mainly in mammals, a homologous form of it, mesotocin (MT), occurs in birds and reptiles. MT has so far, however, been much less investigated than OT, at least when it comes to measuring peripheral concentrations. Manipulation studies administering MT or OT antagonists (Duque et al., 2020, 2018; Kelly, 2019), or studies investigating neural substrates with immunohistochemistry (Goodson, 2013), however, do suggest that, similar to OT in mammals, MT plays an important role in social behaviour in birds. Nevertheless, there is not much information available on naturally occurring peripheral MT levels in birds and how they relate to social behaviour in natural contexts.

The first two goals of the present study are therefore to test the feasibility i) of training birds to voluntarily provide saliva samples and ii) of quantifying salivary MT in those samples. Since saliva samples allow a non-invasive assessment of the endocrine response to certain stimuli with a delay of only a few minutes, this would open up a suit of opportunities to examine the role of MT in birds more directly. To do so, we tested common ravens (*Corvus corax*), which are well-suited for that endeavour: Because of their relatively large body size we expect them to produce more saliva than a small bird species, and their large beak facilitates saliva collection. Further, ravens have already been shown to be successful in an exchange paradigm (Massen et al., 2015; Müller et al., 2017), which could be used to collect saliva samples by exchanging swabs for a reward. Moreover, they have a relatively complex social life with individuals staying in non-breeder groups until they form pair bonds (Boucherie et al., 2019), and show marked seasonality with regard to breeding and mating, with the latter being accompanied with high levels of aggression (Braun and Bugnyar, 2012; Gwinner, 2003). In particular, in the present paper we make a clear distinction between the mating season, in winter, in which the birds are sexually highly active and the breeding season, in spring, which we refer to as the time period in which the birds usually lay eggs and care for their offspring.

Our third goal is, therefore, iii) to investigate whether salivary MT levels differ seasonally and/or are associated with the ravens' bonding status. In a comparative study on different sparrow species, Goodson et al. (2012) concluded that an increase in MT innervation in certain parts of the brain is important for flocking and may reduce aggression. Moreover, it was shown that the MTergic system is involved in reproductive behaviour (e.g. incubation) in Thai hens (*Gallus domesticus*) (Chokchaloemwong et al., 2013; Sinpru et al., 2017). We, therefore, expected ravens' MT levels to be higher in the breeding season (spring) than in the mating season (winter). Nonapeptides have also been shown to be involved in pair bonding. In zebra finches (*Taeniopygia guttata*) the administration of OT antagonists decreases pair formation (Pedersen and Tomaszycki, 2012) and influences pair maintenance behaviours (Kelly, 2019), and oxytocin-like receptors mediate pair bonding (Klatt and Goodson, 2013). We, therefore, expected pair-bonded ravens to have higher MT levels than group-living ones. Finally, nonapeptide effects are often sex-specific (Goodson, 2013; Kelly and Goodson, 2014), and MT levels seem to differ between males and females in some species. In White Leghorn chickens (*Gallus domesticus*), for instance, males have twice as much MT, at least in their neurohypophysis, as females (Robinzon et al., 1990). Hence, we investigated the potential effect of sex on MT levels in ravens and predicted MT levels in males to be higher than in their female conspecifics.

2. Material and methods

2.1. Animals and housing

The study was conducted on 13 ravens (7 males, 6 females; age: 2 to 6 years). They were housed at Haidlhof Research Station, Bad Vöslau, Austria. Towards the end of the study three pair-bonded subjects were transferred to Cumberland Wildpark in Grünau, Austria. Six ravens were kept in a mixed-sex non-breeder group (aviary ~210m^2) and seven were pair-bonded and kept in separate aviaries (~80m^2 each). All aviaries consisted of several compartments with sheltered areas for weather protection. The subjects were fed twice a day (meat, dairy products, vegetables, fruits and cereals), water was available ad libitum.

2.2. Saliva collection

We trained the ravens to take a saliva swab (~2.5 cm long piece of Salimetrics SalivaBio Children Swab) into their beak, place it in their throat pouch where saliva is accumulating, and return it on command. To achieve this, we followed a training protocol based on positive reinforcement (cf. Massen et al., 2015; see ESM). Saliva samples (max. three/subject/day) were collected opportunistically from whichever bird we could get them from on a given day, either before or at least 1 h after the birds got fed. All samples were stored in Salimetrics Swab Storage Tubes at −20 °C within 10 min after collection. Saliva samples were collected between May and June 2016 and in April and May 2017, representing the breeding seasons, and between November 2016 and January 2017, representing the mating season.

2.3. Hormone analysis

Salivary MT concentration was quantified using a commercially available enzyme-immunoassay (EIA) kit for oxytocin (Catalog No. K048-H1/H5, Arbor Assays, Michigan, USA), which has been used successfully to measure (salivary) OT in humans and other species (e.g. mice, *Mus musculus* (Ferrer-Pérez et al., 2019); dogs, *Canis familiaris* (Wirobski et al., 2021); gorillas, *Gorilla gorilla gorilla* (Leeds et al., 2018)). This assay has a cross-reactivity of 88.4% with MT and can therefore be used to measure MT. Saliva samples of the ravens were extracted following the instruction of the manufacturer and using the extraction solution provided in the kit. Briefly: To extract the samples the swabs were centrifuged at 1600 *g* and 4 °C for 20 min. Supernatant was pipetted into an Eppendorf tube, diluted with extraction solution (1:1.5), vortexed and incubated for 90 min at room temperature. After centrifugation (1600 *g* at 4 °C for 20 min), supernatant was pipetted into a glass tube and dried-down under a N2-stream. Samples were then resuspended in 210 μl assay buffer and processed following the producers EIA protocol. Final concentrations were corrected for dilution factor. All samples were analysed in duplicates. We calculated the intra-assay coefficient of variation (CV) from concentrations of duplicate aliquots. Samples with an intra-assay CV above 20% were excluded from our analysis and the mean CV of duplicates of all remaining samples was 5.9 ± 4.8% (mean ± SD). Inter-assay CV was calculated by comparing the optical density (OD) of two standards (i.e., at the high and low range of the curve) between assays and were 10.3% and 11.4%, respectively. The detection limit reported by the manufacturer was 22.9 pg/ml.

Prior to analysing the individual samples, we successfully conducted a serial dilution of pooled raven saliva in triplicates (1:1 up to 1:8 dilution) to exclude any possibility of matrix effects (Table 1). The CV of corrected values of the serial dilution was 3.58%. Further, we tested the recovery of known amounts of MT in saliva samples. Recoveries were obtained by spiking a total of 16 aliquots of 50 μl of pooled raven saliva with two concentrations of standard mesotocin (Arbor Assay, Cat.nr. X127) and calculating the recovery in respect to the concentration of unextracted standards, after correcting for the concentration measured for the pooled saliva. Average recoveries for standards of 2000 pg/ml

Table 1
Mesotocin concentrations in serially diluted pooled raven saliva.

Dilution	Mesotocin [pg/ml]	Upcalculated [pg/ml]
1:1	274.85	274.90
1:2	129.90	259.80
1:4	69.18	276.73
1:8	32.34	258.75
	mean	267.55
	SD	9.59
	CV%	3.58

and 1000 pg/ml were 119% and 94%, respectively.

2.4. Statistical analysis

Since the MT concentration of many samples fell below the assay's detection limit, we first fitted a binomial model (*glmer*) to investigate if the detectability of MT within the samples depended on the ravens' bonding status, sex, season and/or on the saliva sample volume (prior analysis showed that sample volume correlated negatively with MT concentrations; see below). This model also comprised subject as random intercept effect and was compared to the null model only including this random effect. Subsequently we investigated the effect of the same factors on ravens' salivary MT concentrations (log-transformed). We computed a linear mixed-effects model (*lmer*), which included bonding status, sex and season as fixed effects, sampling time since sunrise as an offset, and subject as random intercept effect. This main-effects model was compared to the null model, which included only subject as random intercept effect and time since sunrise as an offset. We visually inspected whether the model residuals were normally distributed and homogenous. We detected no multicollinearity issues (max. variance inflation factor = 1.617). Effect sizes were estimated via partial omega squared. Prior to constructing the models, we found that sample volume correlated negatively with MT concentrations (n = 20 samples, $r^2 = -0.49$, $p = 0.027$). Since including saliva volume as fixed effect into our main effects model resulted in singularity issues, we decided not to include this factor in the final model. Instead, we ran a post-hoc analysis, which indicated that sample volume was not driving our main results (see ESM, Post-hoc analysis, Table S1). Statistical analysis were conducted in R (version 3.5.2) (R Core Team, 2018). Further details and R-packages are reported in the ESM.

3. Results

We collected 151 saliva swabs (n = 13 subjects; 11.62 mean ± 2.45 SEM samples per subject). Collected saliva volume after centrifugation of the swabs ranged between 2 and 200 µl with an average of 30.36 µl ± 37.70 SD. 73 swabs did not contain saliva, 7 contained less than 5 µl and, thus, only the remaining 71 samples which contained at least 5 µl saliva volume (n = 11 subjects) were analysed. MT could be detected in 28 samples (n = 9 subjects), but only samples, which had a duplicate CV below 20% were considered in the statistical analysis. Consequently, for the MT concentration model we had 20 samples (n = 7 subjects; see ESM: Table S2), whereas for the MT detectability model we had data on 62 samples (n = 10 subjects).

3.1. Detectability of MT

Overall, we found a clear effect of season (Fig. 1a) and saliva volume on the detectability of MT concentrations (binomial full-null model comparison: ΔAIC = 4.147, $\chi^2 = 12.147$, df = 4, $p = 0.016$). MT was less likely to be detected in samples collected during mating season (mean probability = 0.23 ± 0.17 SD) and in samples of low volume (not detected: mean = 28.00 µl ± 27.95 SD) than in samples collected during breeding season (mean probability = 0.53 ± 0.12 SD) and in samples of high volume (detected: mean = 40.40 µl ± 46.84 SD; Table 2).

3.2. Effects of bonding status, sex and season on MT levels

Season also had an effect on the ravens' salivary MT levels themselves (main effects-null model comparison: ΔAIC = 5.372, $\chi^2 = 11.372$, df = 3, $p = 0.010$), with lower MT concentrations occurring during mating season (mean = 355.44 pg/ml ± 226.75 SD) than during breeding season (mean 812.22 = pg/ml ± 471.83 SD; $p = 0.015$; Fig. 1b, Table 2 and ESM: Table S2). Neither the subject's bonding status nor sex had a significant impact on MT concentrations (Table 2).

4. Discussion

In the present study we could show i) that it is possible to collect saliva from birds in a non-invasive manner, based on the voluntary collaboration of the subjects with the experimenter, ii) that MT is detectable in ravens' saliva using a commercial enzyme-immunoassay, and iii) that ravens' salivary MT levels are linked with a biologically relevant parameter, i.e., mating vs. breeding season. This opens up new

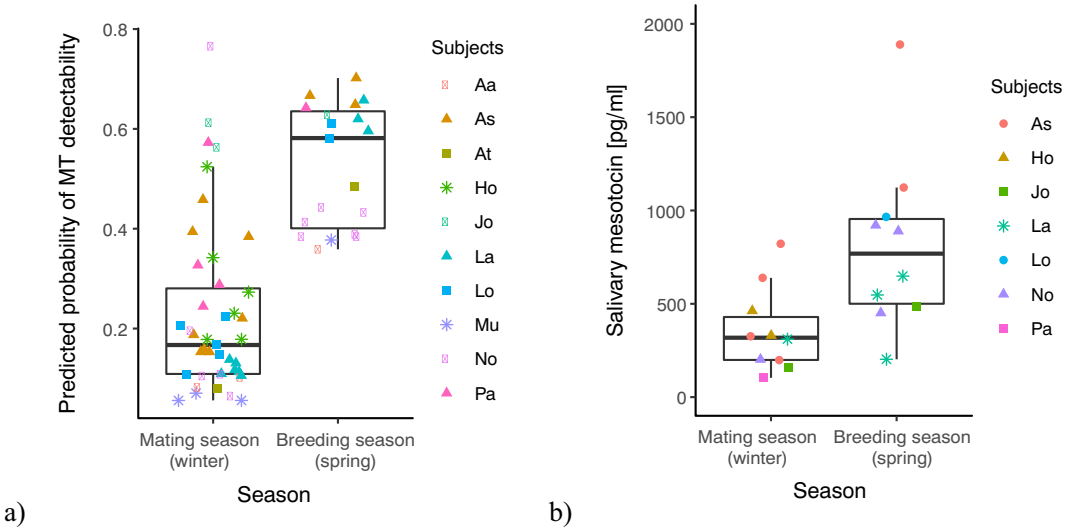

Fig. 1. a) Predicted probability of MT detectability and b) salivary MT levels in mating (winter) and breeding season (spring). Boxplot: bold line represents the median, the boxes represent 1st and 3rd quartile, and the whiskers show the largest and smallest value within 1.5 times the interquartile ranges respectively.

Table 2
Given are estimates, standard errors (SE), confidence intervals (CI), degrees of freedom (df), z- (binomial model) or t-values, *p*-values, and partial omega squared (ω^2) for each parameter of the (linear) mixed-effects model.

Parameter	Estimate	SE	CI	df	z/t	p	$\omega^{2\,a}$
Binomial model: detectability of MT							
(Intercept)	0.176	0.716			0.246	0.806	
Sex (male)	0.441	0.708			0.623	0.533	
Season (mating)	−2.281	0.789			−2.892	**0.004****	
Status (non-breeder)	−0.811	0.737			−1.100	0.271	
Saliva volume	0.020	0.010			2.105	**0.035***	
Main effects model: effects on MT							
(Intercept)	6.664	0.392	(5.726, 7.539)	12.956	16.984	<0.001***	0.94
Sex (male)	−0.349	0.271	(−1.037, 0.361)	1.215	−1.289	0.390	0.04
Season (mating)	−0.906	0.278	(−1.502, −0.236)	7.896	−3.260	**0.012***	**0.33**
Status (non-breeder)	−0.334	0.287	(−0.972, 0.496)	1.586	−1.163	0.390	0.02
Time since sunrise	0.034	0.062	(−0.096, 0.168)	5.970	0.546	0.605	−0.05

Bold p values indicate statistical significance ($p < 0.05$).
[*] $p \leq 0.05$.
[**] $p \leq 0.01$.
[***] $p \leq 0.001$.

opportunities to study peripheral MT in birds. Measuring naturally occurring MT levels could particularly be useful in studies related to sociality or animal welfare and can be easily applied in research institutions and zoos.

Although we were able to measure ravens' salivary MT levels, the effectiveness rate shows that some improvements are needed. To begin with, it was difficult to estimate the saliva volume soaked up by the swab during the ongoing collection procedure, resulting in many empty swabs. Additionally, we encountered constraints due to the enzyme-immunoassays detection limit. In any immunoassay, low volume samples need to be diluted to reach the required assay volume. This causes a reduction of the hormone concentration, which can fall below the assay detection limit. In the present study, samples of low saliva volume and samples that were collected during the mating season, during which MT concentrations were generally lower, were thus more likely to have a MT concentration below the detectable limit. Another issue was that several samples had a variation coefficient (CV) of duplicates higher than 20% and hence could not be used for further analysis. Future studies, thus, should focus i) on increasing the saliva volume gained by improving and intensifying the training of the birds as well as the training of the trainers/experimenters, and ii) on developing more sensitive assays. Improving the methodology is unquestionably crucial for future applications.

The third goal of our study was to investigate whether we can detect biologically relevant differences in salivary MT levels. We found that MT concentrations differed between mating (winter) and breeding season (spring), but that there was no evidence for an effect of bonding status or sex. As expected, MT levels were higher in the breeding season, a time in which ravens are less aggressive than in the mating season (Braun and Bugnyar, 2012; Gwinner, 2003). A study on sparrow species suggests that an increase in MT innervation in certain parts of the brain plays an important role for flocking and might lower aggression (Goodson et al., 2012). Furthermore, studies on Thai hens (*Gallus domesticus*) suggest that the MTergic system is involved with reproductive behaviour (Chokchaloemwong et al., 2013; Sinpru et al., 2017). Therefore, MT in our ravens might have increased in preparation for breeding.

Although the seasonal difference in MT concentrations in our ravens could be associated with social factors (related to aggression in the mating season or behavioral changes associated with reproduction in the breeding season), it could also result from abiotic environmental factors, such as temperature. Several studies show that the MT system is involved in thermoregulation (McConn et al., 2019; Robinzon et al., 1988). In chicks (*Gallus domesticus*) central injections of MT led to increased cloacal temperature and reduced water and food intake, suggesting that MT plays an important role in avian metabolism (McConn et al., 2019). Accordingly, changes in MT levels, like the ones predicted by the present study may facilitate metabolic adaptations to the different seasons (accompanied by different environmental temperatures).

The effects of nonapeptides are often sex-specific (Goodson, 2013; Kelly and Goodson, 2014), and there is evidence for differences in nonapeptide levels between males and females in birds (Robinzon et al., 1990). However, in our study MT levels did not differ between the sexes. Neither did we find evidence for MT levels to differ between pair-bonded and group-living ravens. These results parallel a recent study in another corvid species, pinyon jays (*Gymnorhinus cyanocephalus*), which showed that intranasally administered MT neither affects the formation of pair bonds nor their maintenance (Duque et al., 2020), although administration of MT in the same species has been found to increase pro-social food-donations (Duque et al., 2018). However, the effects of administered hormones rely heavily on the available receptors. Studies on zebra finches *Taeniopygia guttata*) showed that oxytocin-like receptors do in fact mediate pair bonding (Klatt and Goodson, 2013) and that the administration of OT antagonists decreases pair formation (Pedersen and Tomaszycki, 2012). In the present study, it should be noted that the number of group-living subjects included in the model that investigated effects on MT concentrations was very low ($n = 2$), which may have hampered the detection of potential differences between them and the pair-bonded subjects. Moreover, the group-living subjects might have already started to form pair-bonds within their non-breeder group, which might have been reflected in similar physiological activity in the group-living birds as in the pair-bonded ravens. Finally, it is important to keep in mind that we explored bonding status as a general factor and did not investigate the effect of social interactions in a way that would allow us to ascribe causality to it (cf. e.g. Lürzel et al., 2020). To be able to study a causal link between MT and certain behaviours in a non-invasive way, we recommend specific sampling regimes as well as matched controls. The herein described procedure of salivary MT determination would allow for that more easily and precisely than existing methods, like analysing droppings.

We do acknowledge that it is debated whether peripheral OT/MT concentrations reflect central concentrations, which affect the above described social correlates (Neumann, 2008). Recent studies, however, have identified a pathway through which peripheral OT can cross the blood-brain barrier and thereby affect social behaviour (Higashida et al., 2019; Yamamoto et al., 2019; Yamamoto and Higashida, 2020). This possibly explains why many of the reported differences in peripheral OT/MT have been linked to social factors (Crockford et al., 2014). In line with this, Lefevre et al. (2017) found that in primates, simultaneously collected (peripheral) plasma OT and (central) cerebrospinal fluid OT

correlated positively, suggesting that peripheral OT can be used as a proxy for central OT. (For further discussion about the relationship between central and peripheral OT levels and challenges for measuring OT, please, see among others Grinevich and Neumann, 2021; Higashida et al., 2019; Lefevre et al., 2017; MacLean et al., 2019; Yamamoto and Higashida, 2020.)

In sum, using positive reinforcement, birds can be trained to voluntarily provide saliva samples, from which biologically meaningful variation in MT can be analysed.

Ethical statement

This study complies with the Austrian Animal Experiments Act (§ 2, Federal Law Gazette No. 114/2012).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2021.105015.

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