Experimental corticosterone manipulation increases mature feather corticosterone content: implications for inferring avian stress history from feather analyses

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ABSTRACT

Feathers incorporate plasma corticosterone (CORT) during their development and, because feathers lack blood supply at maturity, the CORT content of feathers ($CORT_f$) is presumed to represent an integrated average of plasma CORT levels during feather growth. We tested this assumption by quantifying $CORT_f$ in feathers of house sparrows (*Passer domesticus*) that were plucked before and after being implanted with either a corticosterone-filled, metyrapone-filled, or empty ('sham') silastic capsule. Two of the seven flight feathers collected from each bird were fully grown when birds received implants. We found that $CORT_f$ of all seven feathers corresponded with treatment type, and with plasma CORT levels of non-moulting reference sparrows also given these implants. We also found that $CORT_f$ of the two mature feathers of each bird were 4 to 10-fold higher than values measured in new feathers. Given the avascular nature of mature feathers, and the fact that we did not wash the feathers prior to analysis, the most plausible explanation for our results is that CORT was externally deposited on feathers after implant. This outcome emphasises the need for follow-up studies to identify the external sources of CORT that may affect the $CORT_f$ of feathers. We hope this study will stimulate discussions and further much needed studies on the mechanism of CORT deposition in feathers and open exciting opportunities for application of such methods in ecological research, such as measuring multiple time scales in such a non-invasive manner.

IINTRODUCTION

Corticosterone (CORT) is the main glucocorticoid stress hormone in birds and studies have shown that circulating CORT is incorporated into developing feathers via diffusion from the blood during cell differentiation (Jenni-Eiermann *et al.* 2015). Feather CORT content ($CORT_f$) thus varies with plasma CORT levels during feather replacement (Lattin *et al.* 2011; Fairhurst *et al.* 2013) and measurements of $CORT_f$ are presumed to represent an integrated average of plasma CORT levels experienced by birds over the feather's maturation period (Bortolotti *et al.* 2008; Romero and Fairhurst 2016). Consequently, $CORT_f$ has gained prominence as a measure of stress levels experienced by birds over the moulting period (e.g. Koren *et al.* 2012). If these assumptions, that $CORT_f$ represents circulating levels of CORT confined to the period of the feather's development are correct, experimental manipulation of CORT outside the moult period should have no effect on $CORT_f$ of mature feathers.

The seminal study of $CORT_f$ in red-legged partridges *Alectoris rufa* by Bortolotti *et al.* (2008) recognised that the lipophilic property of CORT could result in it being transferred to feather surfaces via preen oil, but discounted this possibility after finding $CORT_f$ to be unaffected by cleansing with a detergent solution. This conclusion was further supported by a study that could not detect CORT in preen oil of starlings (*Sturnus vulgaris*; Lattin *et al.* 2011). These outcomes have resulted in a common practice to not consider the possibility for surface deposition of CORT into mature feathers. However, a subsequent set of experiments by Jenni-Eiermann *et al.* (2015) showed that washing feathers with a dilute detergent solution significantly reduced $CORT_f$ of replacement feathers of captive feral pigeons (*Columba livia domestica*) receiving CORT-enriched pellets, but had no effect on replacement feather $CORT_f$ of control birds. This raises a critical question: Are surface additions of CORT onto feathers of general concern for studies examining $CORT_f$ in free-living birds, or are they an artefact of experiments producing super-physiological elevations of CORT during feather growth?

The aim of our study is to test the assumption that CORT is incorporated in feathers only during the development period. To this end, we quantify the effect of experimental manipulation of CORT on $CORT_f$ of both fully grown (mature) and developing feathers in captive house sparrows (*Passer domesticus*). We expect that if indeed CORT is deposited into feathers only during the development period, $CORT_f$ of mature feathers will not differ

before and after hormonal manipulation, while if surface deposition does occur, $CORT_f$ will vary with hormonal manipulation in both developing and mature feathers.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

We captured 23 House sparrows *Passer domesticus* (11 females, 12 males) at Werribee, (Victoria, Australia 37.9°S, 144.65°E) in autumn 2013. Details of captive conditions, feather plucking, and hormone manipulation methods are detailed in Aharon-Rotman et al. (2017). Briefly, after at least 1 week of adjustment to captivity, birds were anaesthetised before plucking 12 feathers from their wings and tail (five primaries (P), five secondaries (S) and two rectrices (R)) (Fig. 1). Once feathers started to regrow (12 days after plucking), we manipulated corticosterone levels by implanting each bird with either a single corticosterone-filled (Sigma-Aldrich, Sydney, New South Wales, Australia; #27840, 7 birds), metyrapone-filled (Sigma-Aldrich; #M2696, 9 birds), or empty silastic capsule ("sham", 7 birds). A day after receiving implants, birds were transferred from their holding cages to outdoor flight cages (ca. 2 x 3 x 2.5 metres) until replacement feathers were fully regrown. Our experience in maintaining house sparrows found that feathers are least damaged when birds are housed in outdoor aviaries compared to being held in small indoor cages. Food and water were refreshed daily, but birds were otherwise undisturbed to minimise provoking stress responses. Upon completion of feather regrowth, ten replacement feathers were plucked, five to be used in this study (Fig. 1) and 5 analysed and reported elsewhere (Aharon-Rotman *et al.* 2017).

In addition, feathers P2 and S5 from the left wing were plucked only after hormonal manipulation to determine the effect of treatment on the $CORT_f$ of feathers that were mature during the treatment. We used the CORT values prior to the manipulation in feathers P2 and S5 from the right wing as a reference for $CORT_f$ values prior to hormonal manipulation. We assumed that feathers from both sides experience the same levels of CORT and attain similar sizes upon maturation because birds lose and replace flight feathers in bilateral symmetry during moult, thus share temporal development. Comparison of $CORT_f$ in corresponding feathers of starlings confirms this assumption (Lattin *et al.* 2011). This resulted in a dataset of 7 old feathers (Fig. 1A, "pre-treatment"), 5 new replacement feathers ("new") and 2 old feathers (Fig. 1B), which were fully grown (mature) during hormonal manipulation and were plucked after the experiment.

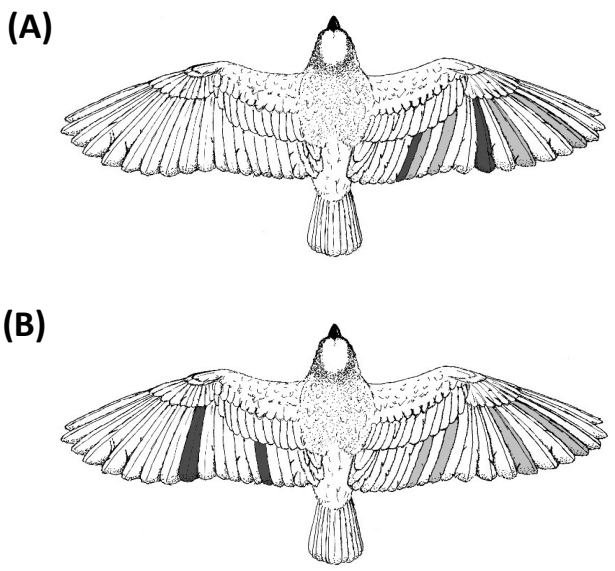

Figure 1. A sketch of a house sparrow (look from top) to illustrate the primary (P), secondary (S), and tail flight feathers plucked for this study and for our earlier study (Aharon-Rotman et al. 2017). Sketch (A) depicts the feathers that were plucked prior to corticosterone (CORT) manipulation and sketch (B) show the feathers plucked after CORT manipulation. Filled dark and light grey feathers were used in this study (right P7, P4, P2, S2, S4, S5 and outer rectrices), and feathers with a grey crisscross pattern on the left wing were used for our earlier study. Dark grey feathers plucked after the experiment (sketch B) were mature at the time of implants (left S5 and P2).

Our experiment involved three treatment types: (1) CORT-filled implants to achieve hyper-physiological CORT levels, (2) metyrapone-filled implants to attain hypo-physiological levels, and (3) empty implants (sham group) to sustain physiological levels. In our previous study using RIA (Aharon-Rotman et al. 2017), we confirmed that $CORT_f$ values in new feathers that developed following plucking varied in response to the hormonal manipulation as detailed above. To minimise disturbance and consequent stress-reactions of aviary-housed birds, we measured plasma CORT in a reference group of non-moulting sparrows given the same implants to validate the efficacy of the implants. Plasma CORT measurements of the reference group showed persistent hyper-physiological levels in CORT-treated birds, initially low and then hyper-physiological levels in the metyrapone-treated group (probably due to the depletion of the metyrapone from its implant), and sustained physiological levels in sham-treated birds (see Aharon-Rotman et al. 2017 for details).

LC-MS/MS QUANTITATION

We used liquid chromatography coupled to tandem mass spectrometry to eliminate potential antibody cross-reactivity and ensure that only corticosterone is quantified. Details of the MS method are provided in the Supplementary Information. Briefly, each feather had the calamus removed, was weighed, and then extracted in a 13 × 100 mm borosilicate tube in 5 mL of methanol pre-chilled to 4˚C (LC/MS grade, Fisher Scientific). Extractions occurred at 4˚C for 20 h. The calibration line used the area ratio between the sample and the matched deuterated

IS, fit with 1/x weighting. All r2 were > 0.995. Low and high QC yielded 90% and 93% precision over 5 runs, with a coefficient of variation of 10% and 7%, respectively.

STATISTICAL ANALYSIS

Before statistical analyses, concentrations in ng/mL were converted to pg/mg based on calamus-free feather weight before extraction, rather than length, following Will *et al.* (2019). We analysed the effect of treatment on $CORT_f$ in both regrown feathers ("new") and feathers which were fully grown at the time of receiving implants and were collected at the end of the experiment ("mature"). For this purpose, we ran a general linear mixed effect model with individual as random effect. At first, $CORT_f$ (pg/mg feather) was set as the dependent variable in relation to treatment (CORT, sham or metyrapone), $CORT_f$ of feathers plucked prior to implants ("pre"; to use as reference $CORT_f$ values), sex and feather type ("new" or "mature"). Reference $CORT_f$ (of the corresponding feather plucked prior the experiment) was entered as a covariate as individuals with high pre-treatment $CORT_f$ have been shown to display higher $CORT_f$ post treatment (Aharon-Rotman *et al.* 2017). Since CORT levels may differ between sexes (but see Koren *et al.* (2012)), we included sex in the model. The initial model contained the main effects and all possible interaction terms. To evaluate differences between treatment groups, we used estimated marginal means post hoc test for multiple comparisons with Tukey method for adjusted p-value. Because there was a significant interaction between feather type and treatment, suggesting a different slope for the different feather types ($F_{2,132}$= 3.51, p<0.05), we separated the models by feather type. We therefore report the values for two separate models, with $CORT_f$ of new and old feathers as the dependent variable, respectively. The explanatory variables remained the same: treatment (CORT, sham or metyrapone), $CORT_f$ of feathers plucked prior to implants ("pre") and sex.

Secondly, we analysed the relation between $CORT_f$ of new, replacement feathers and those of mature feathers that were plucked after the experiment. We again used a general linear mixed effect model with individual as a random effect. $CORT_f$ (pg/mg feather) in the replacement feathers ("new") was set as the dependent variable in relation to $CORT_f$ in the mature feathers and treatment (CORT, sham or metyrapone).

Variables were excluded from models using ANOVA Type III, based on a threshold significance level set to p=0.05. We confirmed the use of random effect in the models by comparing the AIC of the best model with and without random effect using the REML method (Zuur *et al.* 2009). R-function *lme* in R- package "nlme" was used to perform mixed

effect models and R-function *emmeans* in the R package "emmeans" was used to compute the post-hoc test. All statistical analyses were conducted using R version 3.3.0 (R Development Core Team, 2019).

RESULTS

All sparrows retained their implants throughout the experimental period, with no evidence of broken skin at the site of the wound when implants were removed. All metyrapone implants were completely depleted when removed, whereas all CORT implants had considerable residual content. Plasma CORT measurements of a reference group of non-moulting sparrows given these implants showed persistent hyper-physiological levels in CORT-treated birds, initially low and then hyper-physiological levels in the metyrapone-treated group, and sustained physiological levels in sham-treated birds (Aharon-Rotman *et al.* 2017).

We found a significant effect of treatment type ($F=3.46_{2,19}$, $p=0.05$) on $CORT_f$ values in the new, replacement feathers, which grew during hormonal manipulation. We also found a significant effect of treatment type ($F=10.64_{2,20}$, $p<0.001$) on $CORT_f$ values in the old feathers, which grew before capture, and were mature during hormonal manipulation. Post-hoc tests revealed significant differences between $CORT_f$ values of the sham and CORT-treated birds ($t=-2.63$, $p=0.04$) in the new feather group. In the mature feather group, post hoc tests revealed significant differences between both sham and metyrapone-treated birds compared with CORT-treated sparrows ($t=-4.6$, $p<0.001$ and $t=-2.72$, $p=0.03$), respectively (Fig. 2 and Table 1). A summary of the raw values is presented in Supplementary Table A1.

Contrast	Feather	Estimate	SE	df	t-ratio	p-value
sham-mety	New	-0.459	0.322	19	-1.425	0.3489
sham-cort	New	-0.911	0.346	19	-2.631	0.0416
mety-cort	New	-0.451	0.326	19	-1.384	0.369
sham-mety	Mature	-0.89	0.419	20	-2.128	0.109
sham-cort	Mature	-2.03	0.441	20	-4.599	0.000
mety-cort	Mature	-1.14	0.419	20	-2.722	0.033

Table 1: Results of post-hoc estimated marginal means test for multiple comparisons with Tukey method for adjusted p-value. Post-hoc test was performed following a significant effect of treatment on corticosterone (cort) levels in feathers in both new and old feathers, using ANOVA type-3 test, to evaluate the differences between all treatment groups.

DISCUSSION

We have demonstrated for the first time that feather corticosterone content of mature feathers can be affected by a bird's circulating CORT outside the moult period. Although it has been shown that CORT can be synthesised locally in mammalian skin and that hair follicles possess a fully functional equivalent of the hypathalmo-pituitary-adrenal axis (HPA axis; Ito *et al.* 2005; Slominski *et al.* 2007; Taves *et al.* 2011), these processes have not been identified in birds (Romero and Fairhurst 2016). Our results show that $CORT_f$ values in feathers that were completely mature at the time of CORT manipulation were similarly affected by hormonal manipulation as feathers that grew during the experiment, but we were particularly surprised by the extent to which this occurred. If, as is generally assumed, CORT were only deposited during feather synthesis (Bortolotti *et al.* 2008), there should be no difference between fully grown flight feathers plucked before CORT manipulation versus corresponding flight feathers which were completely mature during CORT manipulation.

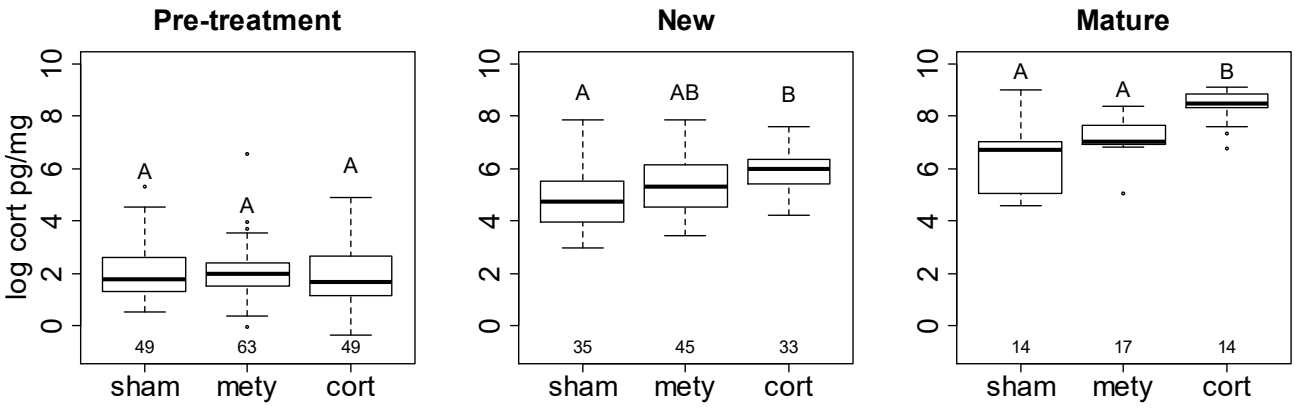

Figure 2: Boxplot of log corticosterone (cort) values in pg/mg feather in old feathers pre-treatment ("Pre-treatment"), re-grown feathers post treatment ("New") and old feathers post-treatment ("Mature"), in relation to type of implant: sham, metyrapone ("mety") and corticosterone ("cort"). Feathers used for the "pre-treatment" and "new" analysis are P4, P7, S2, S4 and rectrix from the right wing (P= primary, S= secondary). Mature feathers post treatment are P2 and S5 from the left wing (Supplementary Fig. 1). Numbers below each bar depict the number of feathers in the analysis. Total number of individuals=23. Solid line depicts median values. Different letters above each box and whisker depict significant differences between treatments.

There is no possibility for diffusion of plasma CORT into mature feathers, as anatomical studies show that the growing feather undergoes a retrograde resorption as it matures, resulting in an avascular, non-living tissue at the end of moult (Lillie 1940). Given the latter, and the fact that we did not wash feathers prior to analysis, the most plausible explanation for the significant $CORT_f$ increases we found in mature feathers was a result of external

deposition. However, the mechanisms for this transfer are yet to be identified. Although birds shared an outdoor flight cage after receiving implants, we consider that surface deposition was self-administered. This is based on our finding that the $CORT_f$ in mature feathers always aligned among birds within each treatment group.

Without exception, the $CORT_f$ of the mature feathers sampled after the implant period were significantly higher than values measured for corresponding feathers collected before the implant insertion and also higher than values determined for new, replacement feathers (Fig. 2). All of the birds in our study received implants at first emergence of replacement feathers from their sheaths and were transferred to an outdoor flight cage the following day. One of the routine maintenance activities of birds involves preening their feathers through mandibulation. Preening is typically preceded by wiping the bill on the preen gland. Because steroids are highly lipophilic, preen oil is a potential source of extravascular CORT. Although previous studies did not find CORT in preen oil (Bortolotti *et al.* 2008; Lattin *et al.* 2011; Will et al. 2019), there is an opportunity for preen oils to acquire cutaneous steroids during preening, because epidermal tissues surrounding the calamus are highly vascularised during feather growth. In addition, as a bird manipulates its feathers using its bill it also exposes them to saliva, a fluid known to reflect plasma steroid content and routinely used to evaluate glucocorticoid levels in mammals (Sheriff *et al.* 2011). External steroid transfer from preening oils or saliva may also explain the higher values of $CORT_f$ in the mature feathers in comparison to the new replacement feathers. Presuming that grooming activities persisted throughout the post-implant period, mature feathers would present a larger surface area for a longer period than the emerging feathers. This creates a greater potential for fully grown feathers to accumulate surface deposits of CORT over the 3-week period following CORT manipulation. However, the supra-physiological levels of cort experienced by the cort-implanted birds may cause exceptional cort excretion in preen oil or saliva, which would not happen naturally. We did not test for CORT in preen oil, and given the role of preen oil as waterproofing agent this hypothesis invites further speculation.

The pertinence of our findings and the applicability to ecological work in free-living birds remains uncertain. Our experimental procedure did not resemble natural response, but we have identified a potential mechanism of circulating CORT surface deposition in mature feathers, which its extent needs to be further investigated. Importantly, although two of our hormone treatments produced hyperphysiological CORT levels in reference birds, plasma

values for birds receiving empty implants are a consequence of endogenous CORT secretion and could occur under free-living conditions. Indeed other studies have found house sparrows to be very sensitive to caging and to show elevated capture-stress responses even following 9-weeks in captivity (Love *et al.* (2017).

What is critically needed is to further investigate the routes of entry of CORT into feathers and to identify possible external sources of CORT deposition that could affect the $CORT_f$ of mature feathers. We hope this letter will stimulate discussions and further much needed studies on the mechanism of CORT deposition in feathers and open exciting opportunities for application of such methods in ecological research, such as measuring multiple time scales in such a non-invasive manner.

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