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RESEARCH ARTICLE

Experimental relationships between levels of corticosterone in plasma and feathers in a free-living bird

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SUMMARY

Integrated measures of corticosterone (CORT), such as from feathers ($CORT_f$), have intuitive appeal because they incorporate both the duration and amplitude of glucocorticoid secretion. An association between $CORT_f$ and plasma CORT has never been shown in wild birds, and it is unclear as to when and whether these measures should be correlated, given that they are fundamentally different yet related measures of physiology. We hypothesized that $CORT_f$ should correlate with instantaneous measurements of plasma CORT when the latter reflect sustained changes in the activity of the hypothalamic–pituitary–adrenal (HPA) axis. To test this, we experimentally manipulated levels of plasma CORT in wild nestling tree swallows (*Tachycineta bicolor*) using 5 day time-release CORT pellets, and measured plasma CORT and growth parameters before, during and at the end of hormone manipulation (days 7, 9 and 11 post-hatch, respectively). $CORT_f$ and plasma CORT were significantly positively related only when the latter was at its highest and most variable among individuals (day 9). A similar relationship was expected at day 11, but plasma CORT had returned to near-original levels. Nestlings with higher $CORT_f$ were smaller, lighter and less likely to fledge, but we did not detect seasonal effects on $CORT_f$. Our results clearly demonstrate that $CORT_f$ from free-living birds can reflect plasma CORT, but correlations may not always be expected, especially if elevations in plasma CORT are relatively modest and of short duration. Our work suggests that $CORT_f$ is best used to study the activity of the HPA axis over relatively long time frames and can be used effectively to advance avian ecology.

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INTRODUCTION

Ecologists increasingly seek measures of physiology to better understand how and why an individual's state is related to environmental variation and components of fitness. The use of glucocorticoid (GC) hormones in ecological studies has advanced rapidly in this regard because GCs influence energy balance, vary with predictable life-history changes, and are released in response to unpredictable or noxious stimuli (i.e. stressors) (Sapolsky et al., 2000; Dallman and Bhatnagar, 2001; Romero, 2004). Investigations into the causes and consequences of GC secretion have led to productive cross-disciplinary research, including how GCs influence development and behavior, relate to fitness metrics and can benefit conservation (Wikelski and Cooke, 2006; Breuner et al., 2008; Bonier et al., 2009; Busch and Hayward, 2009; Spencer et al., 2010; Crespi et al., 2013; Love et al., 2013).

The majority of ecological studies utilizing levels of GCs have been conducted with instantaneous blood samples. Focusing on GC levels at single points in time can be particularly informative when characterizing the functioning of the GC response and understanding an individual's physiological state at the instant of sampling, but the time frame of such samples is limited. Although stressors are typically discrete events, they are not necessarily short-lived and can overlap in time (e.g. inclement weather, habitat change). Thus, the physiological effects of even short-lived stressors are complex and can be long lasting (Spencer et al., 2010). This makes it challenging to use instantaneous measures to study GCs through time, particularly in free-living animals. Researchers using instantaneous samples must additionally contend with the separate influences of baseline and stress-induced levels of GCs, often with little knowledge of which is more important ecologically (Landys et al., 2006) or how they vary temporally beyond the sampling period. This latter point is important, as the time frame over which GCs are studied is a potential reason why clear evidence of putative GC–fitness relationships has been elusive (for reviews, see Breuner et al., 2008; Bonier et al., 2009; Crespi et al., 2013).

Characterizing the expression of all GC levels within a discrete time period has intuitive appeal because such integrated measurements incorporate the duration, as well as the amplitude, of GC secretion. This is particularly important for ecological factors that operate over longer time periods. Integrated measures also reduce the need to differentiate between baseline and stress-induced GC levels, focusing instead on the expression of individual variation during all periods of GC secretion, including levels intermediate to baseline and stress induced. This emphasizes the total amount of

hormone secreted, which may be a more biologically relevant measure (Dallman and Bhatnagar, 2001; Romero, 2004).

Several integrated measures of GCs have been developed and applied to ecological contexts (Sheriff et al., 2011), notably fecal GC metabolites (Wasser et al., 2000; Möstl and Palme, 2002). Work with birds has shown that feathers contain corticosterone (CORT) (Bortolotti et al., 2008; Koren et al., 2012), the primary avian GC. CORT in feathers ($CORT_f$) has been correlated with variation in parental provisioning (Fairhurst et al., 2012b), nest box microclimate (Fairhurst et al., 2012a), environmental enrichment (Fairhurst et al., 2011), expression of carotenoid-based signals (Bortolotti et al., 2009b; Mougeot et al., 2010; Kennedy et al., 2013), egg mass (Kouwenberg et al., 2013), stable isotopes of carbon (Fairhurst et al., 2013) and components of fitness (Bortolotti et al., 2008; Koren et al., 2012). This biomarker relates to diverse ecological factors, suggesting that it integrates CORT secretion in general, rather than expresses a response to any specific source of environmental variation. Importantly, the time frame of this retrospective measure of CORT physiology is significantly longer than that of blood or fecal GC metabolites.

The potential for $CORT_f$ to advance avian ecology has been hindered by a lack of experimental research into what $CORT_f$ values represent physiologically. Although published evidence suggests that $CORT_f$ reflects plasma levels of CORT (Bortolotti et al., 2008; Lattin et al., 2011), no previous study has explored an experimental connection between the two measures in free-living birds. Plasma and feather levels differ fundamentally in both the time frame over which they quantify CORT (seconds *versus* days) and the aspects of hormone secretion they measure (instantaneous *versus* integrated). This raises concerns about how closely related plasma CORT and $CORT_f$ are likely to be. Here, we address these issues by asking two questions. First, does $CORT_f$ from free-living birds reflect variation in plasma levels of CORT? Second, when during the period of feather growth should we expect such a plasma–feather relationship, given that an instantaneous plasma sample reflects CORT during only a very small fraction of the time it takes a feather to grow?

We hypothesized that $CORT_f$ should correlate with instantaneous measurements of plasma CORT when the latter reflect sustained changes in the activity of the hypothalamic–pituitary–adrenal (HPA) axis. During such instances, CORT levels measured over short time periods may represent a significant portion of the total amount of CORT deposited in the feather and therefore correlate with $CORT_f$. To test this hypothesis, we manipulated levels of plasma CORT during feather growth in free-living tree swallow (*Tachycineta bicolor* Vieillot) nestlings using time-release pellets. We expected treatments to produce a wide range of plasma CORT values and for sufficient time to induce changes in $CORT_f$. Thus, we predicted that $CORT_f$ would positively correlate with plasma levels of CORT sampled during and at the end of the hormone manipulation. We additionally measured morphometrics throughout the experiment, so we were able to determine whether $CORT_f$ reflected individual growth or predicted eventual fledging success.

MATERIALS AND METHODS

Field work

Study area, nest boxes and experimental design

The experiment was conducted at St Denis National Wildlife Area, Saskatchewan, Canada (52°12′N, 106°05′W; average elevation 526 m), from April to July 2012. Tree swallows nest in >200 boxes on this area that has been monitored continuously since the 1990s (R.G.C., unpublished data). Land use consists of agricultural cropland and natural and planted grassland interspersed with wetlands and small groves of trees dominated by aspen (*Populus tremuloides*). Further details on the study area can be found elsewhere (Shutler and Clark, 2003). All nest boxes in the study area were spaced ≥30 m apart and in similar habitat. Boxes followed the 'Long Point' design: floor dimensions were ~14×15 cm, the distance from floor to top was ~28 cm, and the diameter of the entrance hole was ~3.8 cm. Beginning in mid-May, nest boxes were checked regularly to determine clutch initiation date, hatch date of the first-laid egg and brood size. We selected 21 nest boxes with brood sizes that fulfilled requirements for experimental treatments (see below).

Each nest box was visited on days 7, 9, 11 and 15 post-hatch ('day') when we weighed nestlings to the nearest 0.5 g using a Pesola scale, measured wing length to the nearest mm with a ruler, and measured the length of the ninth primary and bill length (distance from nares to bill tip) using dial calipers (to the nearest 0.01 cm). All morphometrics were measured by one person (G.D.F.). Nestlings were banded on day 11 (if large enough) or day 15. On day 15 we collected two to three back feathers from each nestling by grasping the rachis with blunt-ended forceps and gently pulling until the feathers released. Feathers were stored in paper envelopes until subsequent $CORT_f$ analysis (see below). On day 20, we revisited all nest boxes to determine whether nestlings had fledged. If nestlings were still present, their band numbers were recorded and we visited the nest box again 2 days later, repeating this process until we had approximate fledge dates for all individuals.

Nestlings were marked for individual identification on their thighs using a non-toxic marker on day 7, and again on days 9 and 11 as needed. When nestlings were 7 days old (all >10 g), they were randomly assigned to one of the following seven treatment groups (Table 1 and see 'Implantation of hormone pellets', below) such that all treatments were allocated to each nest box: (i) CORT, an implant treatment intended to elevate plasma levels of CORT; (ii) synthetic GC dexamethasone (DEX), an implant treatment intended to decrease endogenous plasma levels of CORT *via* negative feedback of the HPA axis (Westerhof et al., 1994); (iii) CORT+DEX, an implant treatment intended to suppress endogenous CORT secretion while exogenously augmenting plasma CORT levels; (iv) placebo, a drug-free implant treatment; (v) surgery-only, a control treatment that included all surgical procedures but no implantation; (vi) bleed-only; or (vii) handled-only. We chose day 7 post-hatch to begin 5 day experimental manipulations because we knew that nestling back feathers would emerge by then and complete growth by around day 15 (R.G.C., unpublished observations) (Winkler et al., 2011). We confirmed in all nestlings that back feather growth had begun before beginning experimental manipulations. Thus, all hormone implants occurred during the period of active feather growth.

Blood sampling

We sampled blood from nestlings of all treatments except those in the handled-only group. To reduce the impact of this, each bird was sampled for blood on one occasion only. Nest boxes were randomly assigned to a day that nestlings were sampled for blood (days 7, 9 or 11 post-hatch; henceforth 'age at bleeding') such that seven different nests were sampled on each of those days. Two people bled birds simultaneously. Blood was collected in heparinized capillary tubes following puncture of the brachial vein with a 26-gauge needle. An initial 'baseline' blood sample (~50 µl) was collected immediately after removal of the bird from the nest, and a 'handling-induced' sample (~50 µl) was collected from the same bird 30 min later. All blood samples were kept on ice until centrifugation several hours later the same day, when plasma was

Table 1. Description of treatment groups in the experiment

Treatment group		Handled	Blood sampling	Surgical procedure	Pellet description	Predicted effect on $CORT_f$
CORT		✓	✓	✓	Corticosterone (0.78 mg pellet=8.7 µg CORT g^{-1} body mass day^{-1})	Increase
DEX		✓	✓	✓	Dexamethasone (0.014 mg pellet=0.15 µg DEX g^{-1} body mass day^{-1})	Decrease
CORT+DEX		✓	✓	✓	Corticosterone and dexamethasone pellets	No change; possible small increase
Control	Placebo[1]	✓	✓	✓	Vehicle only	None
	Surgery only[1]	✓	✓	✓	✗	None
	Bleed only[1]	✓	✓	✗	✗	None
Handled only		✓	✗	✗	✗	None

CORT, corticosterone; DEX, dexamethasone; $CORT_f$, corticosterone in feathers.
[1]Because of statistical similarity in baseline plasma CORT values among these groups prior to experimental procedures, birds in these groups were combined into a single control group. See Results for details.

collected and stored at −80°C. We aimed to collect baseline blood samples as quickly as possible, recognizing that samples collected within 3 min may better represent true baseline (Romero and Reed, 2005). However, we needed to sample six nestlings from every nest. To improve the likelihood of collecting accurate baseline samples, we bled nestlings in two 'bleed groups' at each nest. While retrieving nestlings for the first bleed group, we shielded the open nest box from direct sunlight using a black plastic bag. The nest box was kept closed while we bled the birds in the first group. The remaining nestlings (i.e. second bleed group) were left in the box for 10 min before being bled, regardless of how quickly we completed bleeding the first group of nestlings. After removal from the nest box, any nestlings not being measured or bled were kept in cloth bags. When nestlings were outside the nest boxes, we monitored them regularly for signs of distress and ensured that they were kept thermoneutral. All blood sampling was conducted in the morning.

Implantation of hormone pellets

Silastic implants have low permeability to CORT (Kinel et al., 1968) and therefore rely on exposure of crystalline hormone to subcutaneous tissues *via* an opening in the implant (e.g. Newman et al., 2010). Because we were working with nestlings, we wanted to minimize the potential spikes in CORT possible with this approach and maintain consistent dose across individuals. Cholesterol-based dissolving pellets are designed to ensure an even, sustained release of hormones (Meyer et al., 1979; Fusani, 2008). Therefore, we administered CORT and DEX using 5 day time-release pellets (MDD pellets; Innovative Research of America, Sarasota, FL, USA). On day 7, after completion of handling-induced blood samples, nestlings in the CORT, DEX, CORT+DEX and placebo groups received pellets and birds in the surgery-only group received similar surgical procedures but no pellet. Dosages of CORT and DEX (Table 1) were calculated based on previous studies using these drugs and pellets (Rich and Romero, 2005; Franceschini et al., 2008; Müller et al., 2009a) and were intended to alter plasma levels within normal physiological ranges. CORT pellets delivered 8.7 µg CORT g^{-1} body mass day^{-1} (though plasma CORT levels did not remain high for the full 5 day period; see below) and DEX pellets delivered 0.15 µg DEX g^{-1} body mass day^{-1}. All surgeries were conducted in a climate-controlled vehicle. Sterility was maintained by wearing latex gloves, using fresh scalpel tips for each surgery, keeping forceps and the scalpel handle in ortho-phthalaldehyde (Cidex; Advanced Sterilization Products, Irvine, CA, USA) between surgeries, and rinsing surgical tools with sterile water, then drying, prior to use. We avoided sterilization with alcohol as per the pellet manufacturer's instructions.

Anesthetic (0.005 ml of 8 mg ml^{-1} lidocaine solution; maximum dosage of 0.04 mg) was injected subcutaneously into the site of pellet implantation (between the wings, slightly left of midline) and gently massaged for 3–5 s to aid dispersal. The injection site was marked with a small dot of non-toxic marker. After a minimum of 10 min, the site of pellet implantation was cleaned using povidone–iodine solution (Betadine; Purdue Pharma, Pickering, ON, Canada) applied with a cotton swab and allowed to dry. A small incision was made in the skin using a scalpel, followed by blunt dissection and insertion of the pellet(s) with forceps. The skin was closed using a small dab of tissue adhesive (Vetbond; 3M, St Paul, MN, USA). Upon inspection 2 days after surgery (i.e. day 9), all surgical wounds were completely healed, nestlings had a full range of neck and wing motion, and pellets were visible subcutaneously. All experimental procedures were developed and conducted under the auspices of two licensed veterinarians (C.S. and K.L.M.) and were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20070041).

Hormone measurement

CORT was extracted from feathers using a methanol-based technique (see Bortolotti et al., 2008) that has been used previously with tree swallow nestlings (Harms et al., 2010; Fairhurst et al., 2012a). Feather length was measured, and the calamus cut, removed and discarded. The remaining feather sample was re-measured, placed in a glass scintillation vial, then cut with scissors into very small pieces (<5 mm^2). After the addition of 10 ml of methanol (HPLC grade; VWR International, Mississauga, ON, Canada), samples were capped, sonicated at room temperature for 30 min, then incubated at 50°C overnight in a water bath. We used vacuum filtration to separate methanol from feather bits, and the methanol extract was subsequently allowed to evaporate in a fume hood. Dried extracts were reconstituted in a small volume of phosphate-buffered saline (PBS; 0.05 mol l^{-1}, pH 7.6) and frozen at –20°C until analyzed by radioimmunoassay (RIA). Feathers were extracted in a single batch. To determine the recovery of the extraction, we used an additional three feather samples spiked with a small amount (~5000 c.p.m.) of [^{3}H]corticosterone and assessed the percentage radioactivity recovered [for validation and additional details, see appendix S1 in Bortolotti et al. (Bortolotti et al., 2008)]; 98% of the radioactivity was recoverable in the reconstituted samples.

Plasma was extracted using techniques detailed elsewhere (Wayland et al., 2002). Briefly, plasma was thawed, measured

volumes were extracted twice with 10 volumes of diethyl ether, and the two ether extracts were pooled. Extraction efficiency was calculated using three plasma samples spiked with a small amount (~5000 c.p.m.) of [^{3}H]corticosterone. Plasma samples were extracted in a single batch and 95% of the radioactivity was recoverable.

Feather and plasma levels of CORT were determined by RIA as in previous studies (Wayland et al., 2002; Bortolotti et al., 2008). Measurements were performed on reconstituted extracts and were duplicated. Serial dilutions of both feather and plasma extracts were parallel to the standard curve. Feather samples were measured in two assays with a mean (±s.d.) intra-assay coefficient of variation (CV) of 10.4±3.1%, an inter-assay CV of 10.2% and a mean (±s.d.) limit of detection (ED_{80}) of 10.3±1.6 pg CORT 100 μl^{-1} of reconstituted feather extract. All samples were above the detection limit. $CORT_f$ values are expressed as pg CORT mm^{-1} feather. We corrected assay values by length rather than mass because we wanted to avoid the previously documented effect of mass on $CORT_f$ values (Bortolotti et al., 2008; Bortolotti et al., 2009a; Bortolotti, 2010; Lattin et al., 2011). Deposition of CORT into growing feathers is hypothesized to be time dependent, not mass dependent, and feather length is the most valid estimate of unit time of feather growth (see Bortolotti et al., 2008; Bortolotti et al., 2009a; Bortolotti, 2010). Regardless, we weighed all feathers using an analytical balance (range: 0.0004–0.00097 g) to compute pg CORT mg^{-1}; the two measures (pg mm^{-1} and pg mg^{-1}) were highly correlated (r=0.72; P<0.0001; N=113 individuals). Plasma samples were measured in four assays with a mean (±s.d.) intra-assay CV of 7.7±3.3%, an inter-assay CV of 10.8% and a mean (±s.d.) limit of detection (ED_{80}) of 9.7±1.3 pg CORT 100 μl^{-1} plasma. All plasma CORT samples were above the detection limit. All CORT assays were performed at the Department of Biology, University of Saskatchewan, Canada.

Statistical analyses

All analyses were conducted using generalized linear mixed models (GLMM; PROC GLIMMIX) in SAS version 9.2 (SAS Institute, Cary, NC, USA). All CORT response variables were log-transformed to improve normality. Unless stated otherwise, all GLMMs included nest box identity as a random factor to account for data clustering (i.e. nestlings within boxes), and used a normal distribution and logit link function.

Time of bleeding and measurements of baseline plasma CORT

We began by investigating whether baseline plasma CORT samples from nestlings in the first bleed group differed significantly from those in the second bleed group. We modeled the baseline plasma CORT value as the dependent variable. We were interested in the effect of bleed group, but additionally included as explanatory variables age at bleeding (i.e. 7, 9 or 11 days), treatment and date of bleeding.

We then used three tests to determine whether, prior to implantation (day 7), baseline plasma samples collected after 3 min but within 4:09 min (N=6) were statistically similar to those collected within 3 min (N=27). First, we determined whether those samples collected after 3 min were on average significantly different from samples collected within 3 min. We modeled baseline plasma CORT as the dependent variable, and included as explanatory variables: whether or not samples were collected within 3 min, treatment and date of bleeding. Second, we compared those samples collected after 3 min with the handling-induced samples collected from the same birds (N=6). To do this, we used a GLMM with CORT value as the dependent variable, and included sample (baseline or handled-only), treatment and date of bleeding as explanatory variables. We included individual identity as a random factor to account for the fact that two plasma samples were taken from each bird. Because data in this analysis were not clustered within nest box, we did not include nest identity as a random factor. Finally, we assessed whether baseline CORT samples increased with elapsed time of sampling (i.e. how long it took to collect the baseline sample) and whether the relationship changed by including samples collected after 3 min. Using a GLMM, we included baseline CORT values collected within 3 min (N=27) as the dependent variable and elapsed time, treatment and date of bleeding as explanatory variables. We repeated this analysis including all baseline CORT samples collected within 4:09 min (N=33) to determine whether there was an overall temporal trend in the data.

Effect of treatment on plasma CORT, morphometrics and $CORT_f$

We included control groups for the effects of implants (placebo), surgical procedures (surgery-only) and bleeding (bleed-only) because all three effects were present in our hormone treatment groups and we wanted to assess whether baseline plasma CORT values differed among these groups prior to implantation (day 7). We used a GLMM and included log-transformed baseline plasma CORT values at day 7 as the dependent variable, treatment as a fixed factor, and date of blood sampling to control for temporal effects. To compare among groups, we used Tukey *post hoc* contrasts following assessment of main effects.

To investigate the influence of treatment on baseline and handling-induced plasma CORT throughout the experiment we used two GLMMs, one each for baseline or handling-induced CORT as the dependent variable. Both models included treatment, age at bleeding, a treatment×age interaction and date of bleeding as explanatory variables. Tukey *post hoc* contrasts were used to assess differences among groups.

To analyze the effect of treatment on nestling growth, a GLMM was performed for each of the four morphometrics we measured (wing, ninth primary and bill length, and mass). Explanatory variables for each of these GLMMs included treatment, age at measurement (i.e. days 7, 9 and 11 post-hatch), a treatment×age interaction and date of blood collection. We ran four additional GLMMs that used as separate dependent variables changes (from day 7 to day 11) in each of the four morphometrics. These models included as explanatory variables treatment, age at bleeding and date of bleeding. In all models of morphometrics we included band number as an additional random term because birds were measured repeatedly. All morphometric response variables, including changes in morphometrics, were log-transformed to improve normality.

We assessed the effect of treatment on $CORT_f$ using a GLMM that included as explanatory variables treatment, age at bleeding and date of feather collection to account for potential temporal variation in $CORT_f$.

Relationships between plasma CORT and $CORT_f$

To model the effect of sustained modulation of the HPA axis predicted to result in a relationship between plasma CORT and $CORT_f$, we needed to express, rather than control for, the effect of hormone pellets on CORT. Thus, in our models with $CORT_f$ as the dependent variable, we did not include treatment as an explanatory variable. Because we were interested in the relationship between $CORT_f$ and both baseline and handling-induced plasma CORT, we ran separate models for each of these explanatory variables. We additionally included as explanatory variables date of feather collection, age at bleeding, and a plasma CORT×age interaction to determine whether the relationship between plasma CORT and $CORT_f$ differed throughout the experiment. Because we were interested in understanding whether

$CORT_f$ was more strongly related to baseline or handling-induced plasma CORT, in cases where both were significantly related to $CORT_f$, we used model selection procedures to assess the strength of evidence for the relative influence of those two variables (Burnham and Anderson, 2002; Bolker et al., 2009). We used Akaike's information criterion corrected for sample size (AIC_c) to rank the two candidate models and they were considered as having similar statistical support if the models differed by less than two AIC_c units.

Relationships between $CORT_f$ and nestling growth and fledging

To model the relationship between $CORT_f$ and nestling growth, we needed to express, rather than control for, the effect of hormone pellets on $CORT_f$. Thus, when we modeled the effect of changes in morphometrics between day 7 and day 11 on $CORT_f$, we did not include treatment as an explanatory variable. To avoid including collinear explanatory variables in the same model, we ran four models, each using as an explanatory variable changes in one of the four morphometrics we measured (see above). Additionally, we included date of feather collection and age at bleeding as explanatory variables to control for potential temporal effects.

We assessed the relationship between $CORT_f$ and whether or not an individual fledged by using the latter as a binomial response variable in a GLMM with a logit ink function. The model included age at bleeding and date of feather collection to control for temporal effects. We further considered only individuals that successfully fledged and calculated a relative fledge date by subtracting the date of the earliest fledged bird(s) from the fledge date of each individual. We addressed the relationship between $CORT_f$ and relative fledge date using the latter as a dependent variable in a GLMM that incorporated effects of $CORT_f$, age at bleeding and date of feather collection to control to temporal effects.

RESULTS

Effects of bleed group and time of bleeding on baseline plasma CORT

Baseline plasma CORT samples did not differ between birds bled in the first and second bleed groups ($F_{1,60}$=0.01, P=0.93), after controlling for the effects of treatment, age at bleeding and date of bleeding. Therefore, data from the two bleed groups were pooled and this variable was not considered in subsequent models.

Baseline plasma CORT samples collected within 3 min were statistically similar to those collected after 3 min but within 4:09 min ($F_{1,20}$=0.00, P=0.97). After controlling for the effects of treatment and date of bleeding, baseline CORT samples collected after 3 min but within 4:09 min were significantly lower than handling-induced samples collected 30 min later from the same birds ($F_{1,4}$=11.74, P=0.03). There was no relationship between time to collect baseline plasma samples and CORT value for samples collected within 3 min ($F_{1,14}$=0.73, P=0.41). When we included all baseline plasma samples collected within 4:09 min, there was still no effect of time on CORT ($F_{1,20}$=0.54, P=0.47). Therefore, subsequent analyses using baseline plasma values include all samples collected within 4:09 min.

Baseline plasma CORT values of birds bled on day 7 did not differ among treatment groups ($F_{5,21}$=1.21, P=0.34) and there was no effect of date ($F_{1,21}$=1.04, P=0.32). Despite a non-significant treatment effect, we used *post hoc* contrasts to confirm that baseline plasma CORT values did not differ among the placebo, surgery-only and bleed-only groups (unadjusted Tukey tests: all P>0.14). Thus, to increase power of statistical tests, these groups were combined into a single 'control' group for all subsequent analyses. We recognize that this is a statistical, not biological, grouping. However, using the bleed-only group as the sole control in models of baseline plasma CORT did not change the significance of any model results described below (all P<0.003). Birds in the handled-only group were not included in the control group to provide a reference group that was not sampled for blood.

Plasma CORT

The effect of treatment on plasma CORT was similar for baseline and handling-induced CORT. The interaction between treatment and age when birds were bled on CORT was significant (baseline: $F_{6,55}$=7.92, P<0.0001; handling-induced: $F_{6,54}$=3.74, P=0.004), so we analyzed the effect of treatment separately by age at bleeding. There was a significant effect of treatment on CORT for birds bled during (day 9; baseline: $F_{3,20}$=26.37, P<0.0001; handling-induced: $F_{3,20}$=7.66, P=0.001) but not before (day 7; baseline: $F_{3,23}$=1.54, P=0.40; handling-induced: $F_{3,23}$=0.84, P=0.49) or at the end of (day 11; baseline: $F_{3,12}$=0.41, P=0.75; handling-induced: $F_{3,12}$=0.49, P=0.70) the implantation period (Fig. 1). Date of sampling was not significant in any of these models (all P>0.15). Tukey *post hoc* contrasts revealed that, at day 9, plasma CORT values from birds in the CORT (baseline: t=7.61, P<0.0001; handling-induced: t=3.26, P=0.02) and CORT+DEX (baseline: t=6.38, P<0.0001; handling-induced: t=4.19, P=0.002) groups, but not the DEX group (baseline: t=–1.41, P=0.51; handling-induced: t=–0.73, P=0.88), were significantly elevated relative to those of birds in the control group. Baseline plasma CORT values did not differ between CORT and CORT+DEX groups (baseline: t=1.10, P=0.69; handling-induced: t=–0.82, P=0.84).

Morphological measurements and $CORT_f$

Interactions between treatment and day were significant for all morphometrics (all P<0.0001), so we analyzed the effect of

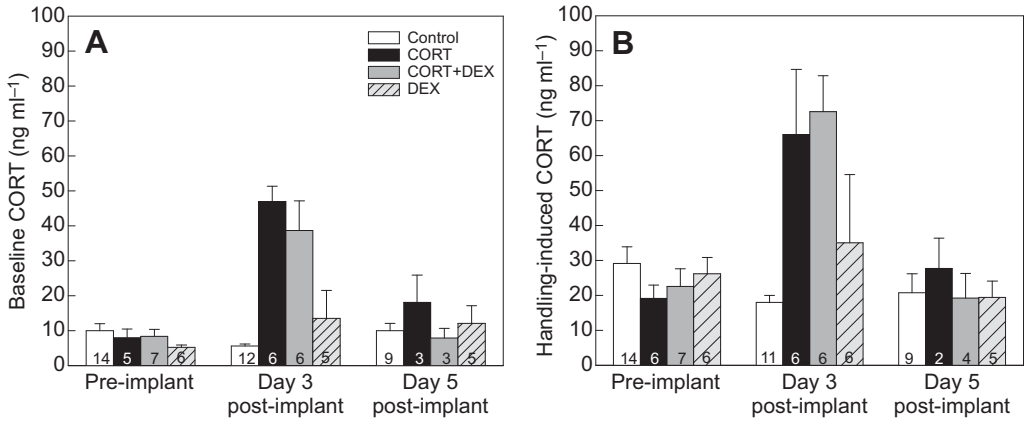

Fig. 1. Baseline (A) and handling-induced (B) levels (means+s.e.m.) of plasma corticosterone (CORT) collected prior to (day 7 post-hatch), during (day 9 post-hatch) and at the end of (day 11 post-hatch) the hormone implantation period. Birds were implanted with time-release pellets containing CORT, dexamethasone (DEX) or both hormones (CORT+DEX); controls received no hormones. See Table 1 for further description of treatments. Samples sizes are displayed on each bar.

Table 2. Results of GLMMs assessing the effects of treatment, date of blood collection and period of the experiment (day 7, 9 or 11) when birds were bled on changes in nestling tree swallow growth from day 7 to day 11

Response variable	Model terms	*F*-statistic (d.f.)	*P*-value
Wing length	Treatment	97.08 (4,82)	<0.0001
	Date	1.68 (1,82)	0.20
	Period	0.51 (2,82)	0.60
Ninth primary length	Treatment	35.03 (4,84)	<0.0001
	Date	1.19 (1,84)	0.28
	Period	0.00 (2,84)	0.99
Bill length	Treatment	13.42 (4,84)	<0.0001
	Date	5.24 (1,84)	0.02
	Period	0.19 (2,84)	0.82
Mass	Treatment	33.19 (4,84)	<0.0001
	Date	1.08 (1,84)	0.30
	Period	0.81 (2,84)	0.45

Table 3. *Post hoc* comparisons of changes in morphometrics during the experiment in nestling tree swallows treated with hormones relative to controls

Response variable	Treatment	*t*-value (d.f.)	*P*-value
Wing length	CORT	−13.79 (1,82)	<0.0001
	CORT+DEX	−15.42 (1,82)	<0.0001
	DEX	2.82 (1,82)	0.05
Ninth primary length	CORT	−7.51 (1,84)	<0.0001
	CORT+DEX	−9.77 (1,84)	<0.0001
	DEX	1.62 (1,84)	0.49
Bill length	CORT	−5.03 (1,84)	0.01
	CORT+DEX	−6.14 (1,84)	0.007
	DEX	1.96 (1,84)	0.29
Mass	CORT	−7.31 (1,84)	<0.0001
	CORT+DEX	−9.13 (1,84)	<0.0001
	DEX	2.82 (1,84)	0.05

All *P*-values were corrected for multiple comparisons. Birds were implanted with time-release pellets containing CORT, DEX or both hormones (CORT+DEX); controls received no hormones. See Table 1 for additional descriptions of treatments.

treatment separately by visit (supplementary material Table S1). None of the morphometrics differed among treatments at day 7 (supplementary material Table S1). At days 9 and 11, nestlings in the CORT and CORT+DEX groups had significantly shorter wings, ninth primaries and bills, and were significantly lighter than controls (supplementary material Tables S1, S2). The effect of DEX on morphometrics was neither as strong nor as persistent as it was for the CORT and CORT+DEX groups (supplementary material Table S2). Relative to controls, DEX-treated birds had significantly shorter wings at days 9 and 11, ninth primary length and mass were significantly reduced only at day 9, and bill length did not differ significantly in any sampling period (supplementary material Table S2). In the three models where date of sampling was significant (bill length at day 11; mass at days 9 and 11), parameter estimates were all negative, potentially indicating developmental effects.

Treatment significantly influenced the change in all morphometrics from day 7 to day 11 (Table 2). Relative to controls, nestlings in the CORT and CORT+DEX groups had significantly smaller changes in all morphometrics (Table 3, Fig. 2), but for DEX-treated birds only wing growth and mass gain were significantly reduced.

The effect of treatment on $CORT_f$ was significant ($F_{4,84}$=23.46, P<0.0001; Fig. 3), but neither day post-hatch that birds were bled ($F_{2,84}$=1.13, P=0.33) nor date of feather collection ($F_{1,86}$=0.03, P=0.86) was significant. Compared with birds in the control group, birds in the CORT (t=6.82, P<0.0001) and CORT+DEX (t=6.83, P<0.0001) groups had significantly elevated $CORT_f$ levels, whereas $CORT_f$ levels of birds in the DEX group did not differ significantly from those of control birds (t=0.33, P=1.00). Values of $CORT_f$ did not differ significantly between the CORT and CORT+DEX groups (t=–0.49, P=0.99).

There were no significant differences between the control and handled-only groups in any of the morphometrics in any period of the experiment (all P>0.19), or in changes in morphometrics between days 7 and 11 (all P>0.79). Likewise, values of $CORT_f$ did not differ significantly between control and handled-only groups (t=0.02, P=1.00). These results suggest that bleeding, surgical procedures and placebo implants did not affect birds physically or physiologically any more than did handling alone.

Relationships between plasma CORT and $CORT_f$

There was a significant interaction between baseline plasma CORT and day of the experiment ($F_{2,59}$=4.57, P=0.01) on $CORT_f$. Therefore, we ran separate models for each of the three days (7, 9 and 11) that we sampled plasma. There was no relationship between baseline plasma levels and $CORT_f$ at day 7 ($F_{1,24}$=0.06, P=0.82) or day 11 ($F_{1,14}$=2.49, P=0.14), but there was a significant positive relationship at day 9 ($F_{1,22}$=21.78, P<0.0001; AICc=–36.71; Fig. 4). Date of feather collection was not significant in any of these models (all P>0.32).

A similar interaction with day occurred in the model analyzing the effects of handling-induced plasma CORT on $CORT_f$ ($F_{2,62}$=3.72, P=0.03), so we ran separate models for each day. There was no relationship between $CORT_f$ and handling-induced plasma CORT at day 7 ($F_{1,26}$=1.85, P=0.19) or day 11 ($F_{1,14}$=0.87, P=0.37), but there was a significant positive relationship with handling-induced plasma CORT sampled on day 9 ($F_{1,22}$=14.37, P=0.001; AICc=–31.39; Fig. 5). Date of feather collection was not significant in any of these models (all P>0.39).

Relationships between $CORT_f$, nestling growth and fledging success

There were significant negative relationships between $CORT_f$ and changes from day 7 to day 11 in wing length ($F_{1,90}$=70.00, P<0.0001), length of the ninth primary ($F_{1,90}$=71.73, P<0.0001), bill length ($F_{1,90}$=11,65 P=0.001) and mass ($F_{1,90}$=30.75, P<0.0001; Fig. 6). Neither age at bleeding nor date of feather collection was significant in any of these models (all P>0.33).

After controlling for the age at which birds were bled ($F_{2,88}$=0.21, P=0.81) and date of feather collection ($F_{1,88}$=0.77, P=0.38), birds with lower levels of $CORT_f$ were more likely to fledge than those with higher $CORT_f$ values ($F_{1,88}$=7.73, P=0.007). Of the birds that did fledge, there was a significant positive relationship between $CORT_f$ and relative fledge date ($F_{1,83}$=27.20, P<0.0001); age at bleeding ($F_{2,83}$=1.45, P=0.24) and date of feather collection ($F_{1,83}$=1.13, P=0.29) were not significant in this model.

DISCUSSION

We demonstrate experimentally for the first time in a free-living bird that CORT from feathers ($CORT_f$) can reflect plasma CORT on an individual level. Two days after implantation (day 9), birds that received time-release CORT implants had significantly elevated baseline and handling-induced (30 min later) plasma levels of CORT relative to controls. The effects of CORT treatment were

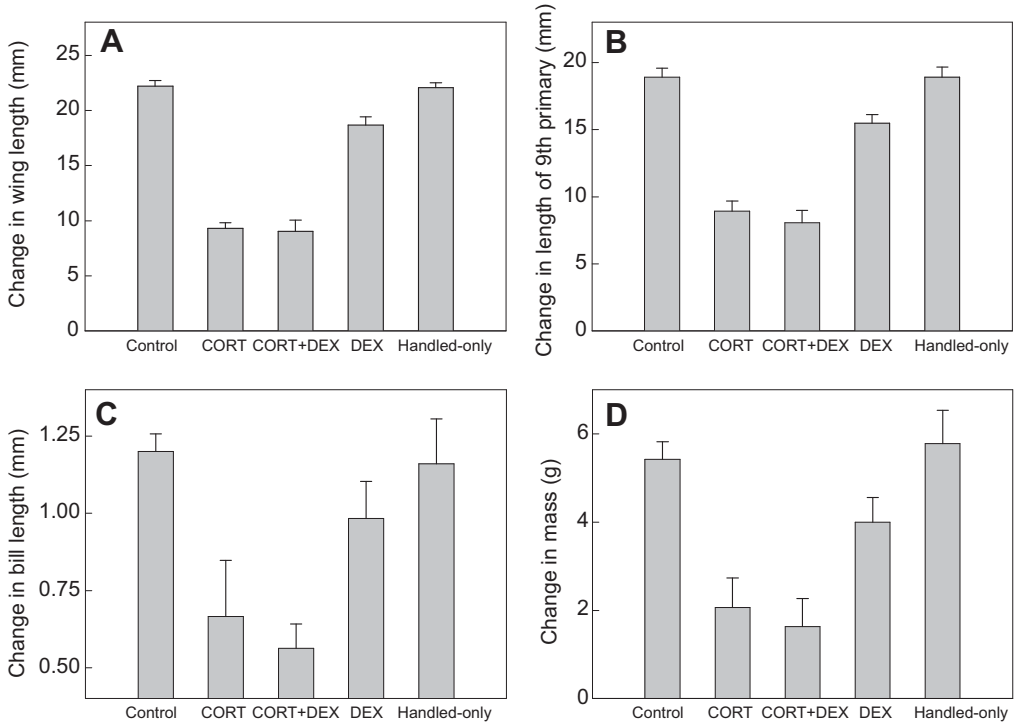

Fig. 2. Changes from day 7 to day 11 post-hatch in wing length (A), length of the ninth primary (B), bill length (C) and mass (D) of nestling tree swallows (means+s.e.m.). Birds were implanted on day 7 with time-release pellets containing CORT (*N*=17), DEX (*N*=20) or both hormones (CORT+DEX; *N*=20). Control birds had no hormones but were bled (Control; *N*=39) or no hormones and were not bled (Handled-only; *N*=16). See Table 1 for further description of treatments.

also evident in the size, mass and growth of birds, as all were significantly reduced relative to controls. As predicted, there was a significant positive relationship between $CORT_f$ and both baseline and handling-induced plasma levels of CORT, though model results suggested a stronger influence of baseline values. This was not a seasonal effect because date of sampling was not significant in any model of CORT. However, contrary to our prediction, $CORT_f$ correlated significantly with plasma levels of CORT only at day 9 and not at the end of the implantation period (day 11).

$CORT_f$ only correlated with plasma levels of CORT when the latter were highest and most variable across treatments (day 9). We reason that only at this point in the experiment were exogenous CORT levels sustained long enough that instantaneous plasma samples were representative of the total amount of CORT in the feather. CORT is deposited incrementally as the feather grows (Bortolotti et al., 2008), and the amount of feather grown during the time frame of an instantaneous plasma sample (i.e. seconds) is extremely small. $CORT_f$ values represent the summation of numerous sections of feather, each reflecting plasma CORT levels at the instant of feather growth. Plasma CORT levels during the short period of feather growth likely fluctuate within the normal reactive scope (Cockrem et al., 2009; Romero et al., 2009; Ouyang et al., 2011), so it is unlikely that any single instantaneous sample of CORT would significantly reflect the total CORT in the feather. This was likely the case prior to hormone implantation (day 7) in our experiment. However, CORT implants significantly elevated and sustained plasma CORT levels for a period long enough to alter $CORT_f$. Thus, instantaneous plasma levels of CORT measured during this period (day 9) better represented the total amount of CORT in the feather.

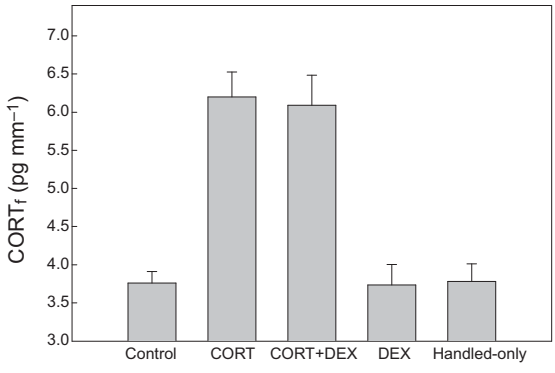

Fig. 3. Mean (+s.e.m.) values of corticosterone from feathers ($CORT_f$) collected from nestling tree swallows on day 15. Birds were implanted on day 7 post-hatch with time-release pellets containing CORT (*N*=18), DEX (*N*=20) or both hormones (CORT+DEX; *N*=20). Control birds had no hormones but were bled (Control; *N*=39) or no hormones and were not bled (Handled-only; *N*=16). See Table 1 for further description of treatments.

Considering we used 5 day time-release implants, why did plasma CORT not correlate with $CORT_f$ when nestlings were 11 days old? We presumed plasma CORT levels would remain high to day 11 but clearly they did not. It is possible that by day 11 the endogenous system was downregulated to avoid the deleterious effects of sustained elevated CORT (Romero, 2004), or that the kinetics of the degradable implants differed from the manufacturer's specifications (Müller et al., 2009a). Additionally, increases in levels of CORT binding globulin (CBG), alone or in concert with increased clearance of CORT from the general circulation, could have reduced levels of free CORT by day 11. How CBGs respond to stressors (or exogenous CORT) is context and species specific (Breuner et al., 2013), and CORT pellets like the ones we used can increase CBG capacity (Müller et al., 2009a). Therefore, it is possible that such an increase in CBG capacity decreased the free CORT available for deposition into feathers. Regardless, by day 11, plasma CORT values in the CORT-treated birds no longer reflected the sustained elevated levels that influenced total CORT in the feather,

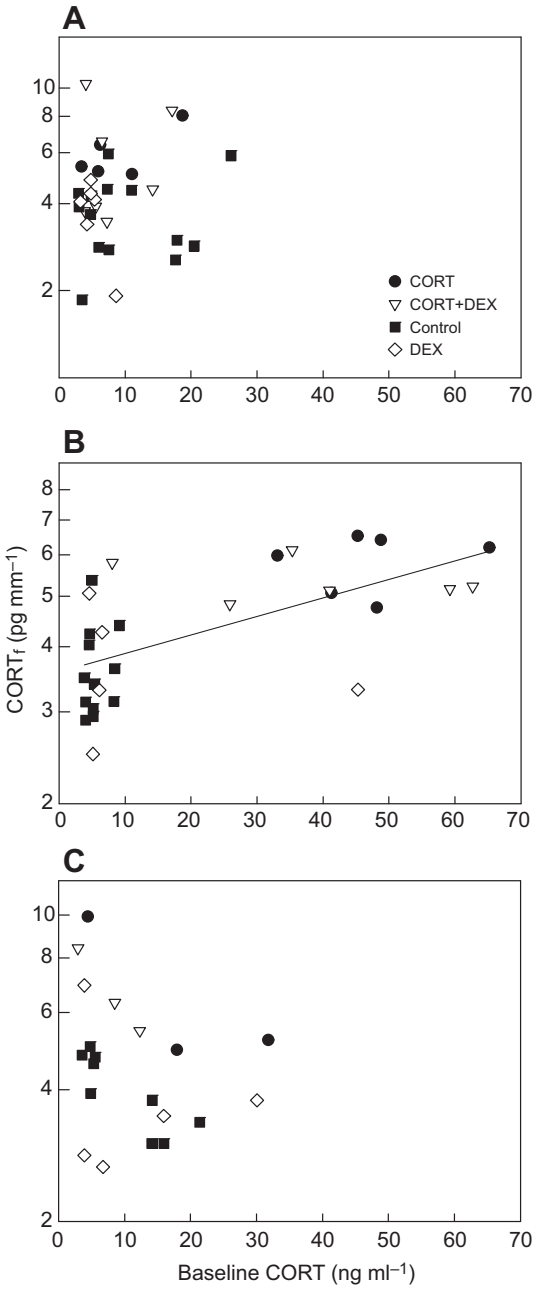

Fig. 4. Relationship between $CORT_f$ from 15 day old nestling tree swallows and baseline levels of plasma CORT sampled before (A), during (B) and at the end of (C) the hormone implantation period, corresponding to days 7, 9 and 11 post-hatch. On day 7 post-hatch, birds were blood sampled prior to implantation with time-release pellets containing CORT, DEX or both hormones (CORT+DEX); controls received no hormones. See Table 1 for further description of treatments. Note log scale of *y*-axes.

so a correlation between plasma CORT and $CORT_f$ was not observed.

Nestlings with higher $CORT_f$ were smaller, lighter and less likely to fledge. Of those individuals that did fledge, birds that fledged at an older age had significantly higher $CORT_f$. Measures of temporal variation were not significant in any of these models, indicating that these relationships were not due to seasonal effects. Instead, effects were likely due to CORT treatment affecting nestling development and subsequent timing of departure from the nest. Interestingly, DEX

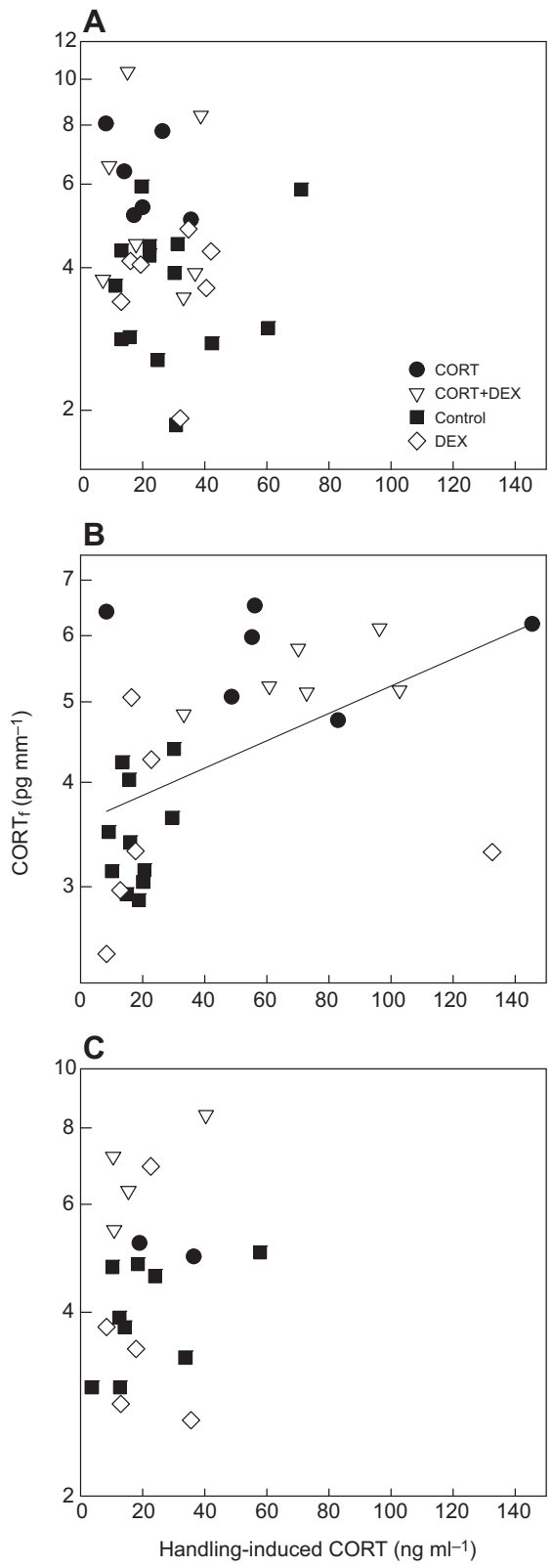

Fig. 5. Relationship between $CORT_f$ from 15 day old nestling tree swallows and handling-induced levels of plasma CORT sampled before (A), during (B) and at the end of (C) the hormone implantation period, corresponding to days 7, 9 and 11 post-hatch. Birds were implanted with time-release pellets containing CORT, DEX or both hormones (CORT+DEX); controls received no hormones. See Table 1 for further description of treatments. Note log scale of *y*-axes.

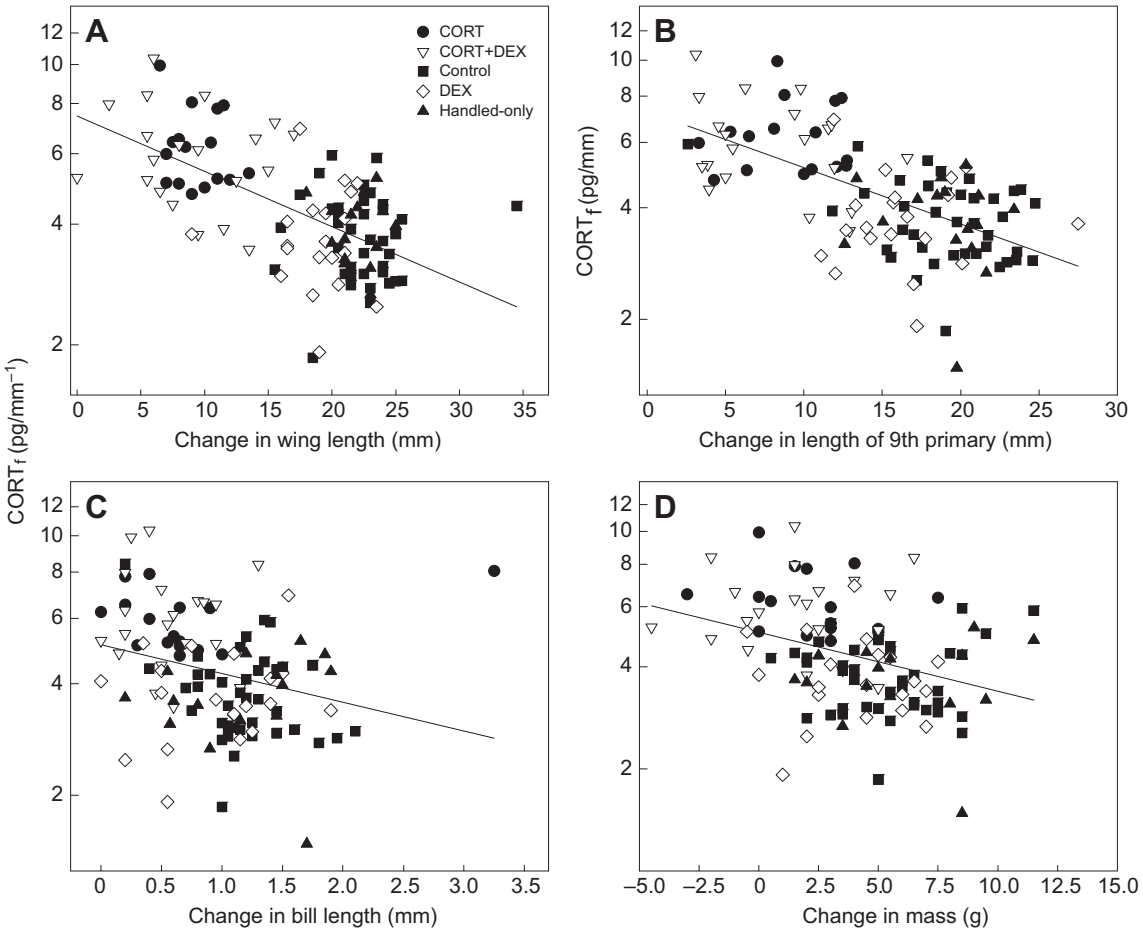

Fig. 6. Relationship between $CORT_f$ and changes in wing length (A), length of the ninth primary (B), bill length (C) and mass (D) from day 7 to day 11 post-hatch. On day 7 post-hatch, birds were implanted with time-release pellets containing CORT (*N*=16), DEX (*N*=20) or both hormones (CORT+DEX; *N*=20). Control birds had no hormones but were bled (Control; *N*=39) or no hormones and were not bled (Handled-only; *N*=16). See Table 1 for further description of treatments. Note log scale of *y*-axes.

treatment also influenced nestling development at day 9, but the effects were weaker than those of CORT. The effects of DEX were also more transient because changes in morphometrics from day 7 to day 11 of DEX-treated birds did not differ from those of placebo birds, suggesting compensatory growth (Müller et al., 2009b). We used the DEX treatment to induce negative feedback in the HPA (Westerhof et al., 1994) and expected decreased plasma CORT and $CORT_f$, but these variables did not differ significantly between DEX and control birds, and birds in the CORT+DEX group were statistically similar to birds in the CORT group. Thus, although the GC effects of DEX were temporarily evident in nestling growth, apparently the dosage we used was insufficient to induce negative feedback in the HPA axis or was only transiently effective. We were conservative when selecting a dose of DEX because we assumed nestlings would be particularly sensitive to the drug's effects. Our results suggest that a stronger dose of DEX may be necessary to significantly alter CORT levels in nestling swallows.

Our study clearly demonstrates that an augmentation in circulating CORT can result in a substantial increase in the amount of CORT that can be extracted from feathers grown during the period of hormone elevation. Evidence strongly suggests that the CORT in feathers was of systemic origin. Although mammalian skin can synthesize and regulate local glucocorticoid production (e.g. Slominski, 2005; Slominski et al., 2007), to our knowledge no studies have provided evidence of local production of glucocorticoids in bird skin (Taves et al., 2011). Without such evidence, the extrapolation of findings in mammals to the situation in birds may not be warranted because hair and feathers evolved independently in lineages separated by over 300 million years of evolution (Dhouailly, 2009). All birds in our study were handled similarly, with the exception of treatments, and all treatments were applied within each nest box. Thus, local production of CORT by growing feather cells, if present at all, could not explain the patterns we observed across treatment groups. Similarly, skin secretions or external deposits on feathers cannot account for our results. Birds did not evolve a glandular skin as in mammals (Dhouailly, 2009), but it is possible that hormone manipulation in our experiment could have resulted in increased CORT in preen oil (waxes), which then would have been deposited on feathers and elevated $CORT_f$. However, previous experimental work has ruled out external deposits as the source of CORT in feathers [see appendix S1 in Bortolotti et al. (Bortolotti et al., 2008)], and preen oil from starlings did not contain detectable levels of CORT (Lattin et al., 2011). Although further work is needed to understand the precise mechanism of hormone deposition in feathers, the findings in our study and those of Lattin et al. (Lattin et al., 2011) provide support for the parsimonious explanation that the CORT found in bird feathers is of circulatory origin.

Our results from free-living birds generally agree with a previous study reporting plasma–feather relationships in captive birds (Lattin et al., 2011). Both studies found that exogenous CORT increased whole-feather levels of $CORT_f$, providing experimental evidence for the idea that the feather measure reflects changes in plasma CORT levels over the period of feather growth (Bortolotti et al., 2008). By analyzing the plasma–feather relationship on an individual basis, we found that plasma CORT at day 9 correlated positively with whole-feather $CORT_f$. Lattin and colleagues did not detect a relationship between plasma CORT and $CORT_f$ from feather sections at any point in their experiment (Lattin et al., 2011); these findings are in contrast to evidence that $CORT_f$ from feather sections reflects experimental manipulations occurring during the growth of those sections (Fairhurst et al., 2011). In Lattin and colleagues' study (Lattin et al., 2011), between-group differences in $CORT_f$ from sections of feather grown prior to hormone implants may have been influential. We implanted wild 7 day old nestlings with time-release dissolvable pellets at the beginning of their natural feather growth period; these pellets are known to deliver a sustained and better-controlled dosage of hormone than silastic implants (Meyer et al., 1979; Fusani, 2008). Lattin and colleagues worked with unknown age starlings that received silastic implants following an induced regrowth of 26 flight feathers in each bird (Lattin et al., 2011). As noted by Lattin et al., CORT can affect feather regrowth during molt (Lattin et al., 2011). It is possible that the high energetic demand of simultaneously regrowing numerous flight feathers may have influenced the deposition of CORT in their study.

Our results shed new light on the relationship between instantaneous and integrated measures of CORT in birds. Both the duration and amplitude of HPA activity are important determinants of $CORT_f$. Thus, direct correlations between plasma CORT and $CORT_f$ may not always be expected, especially if the elevation in plasma CORT is relatively modest and brief. Therefore, we suggest caution when using $CORT_f$ as a proxy for plasma measures. Our findings also provide a benchmark for interpreting when $CORT_f$ values reflect sustained activity of the HPA axis in nestling tree swallows. $CORT_f$ is a tool that provides a unique integrated measure of avian GC physiology that is best used to study activity of the HPA over relatively long time frames. By showing experimentally that $CORT_f$ from a free-living bird can reflect plasma levels of CORT, measures of individual quality and components of fitness, our findings strengthen the use of $CORT_f$ as a biomarker of individual CORT physiology in ecological studies. Future research should focus on better understanding the causes and consequences of variation in $CORT_f$. Given the growing body of literature indicating that $CORT_f$ provides important ecophysiological information, tests of direct relationships between $CORT_f$ and metabolism, reproductive strategies and survival may be particularly revealing.

LIST OF ABBREVIATIONS

CORT	corticosterone
$CORT_f$	feather corticosterone
DEX	dexamethasone
GC	glucocorticoid
HPA	hypothalamic-pituitary-adrenal

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AUTHOR CONTRIBUTIONS

The research was conceived and designed by all the authors; G.D.F. performed the field work, conducted the lab analyses and led the writing; G.D.F. and R.G.C. analyzed the data; other authors provided technical and editorial advice.

COMPETING INTERESTS

No competing interests declared.

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