

## Transient elevation of corticosterone alters begging behavior and growth of white-crowned sparrow nestlings

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### SUMMARY

**Developing animals may face a cost–benefit tradeoff during growth mediated through hormones such as glucocorticoids, as the hormone is essential for development but can have detrimental consequences. To investigate potential tradeoffs caused by brief, moderate elevations of corticosterone in avian young, we artificially elevated the hormone levels in two ways: feeding corticosterone-containing worms and applying corticosterone dermal patches. The former experiment tested the effects of an acute corticosterone elevation (25 min) on begging behavior, whereas the latter explored the effects of artificially elevated corticosterone for 24 to 48 h on growth. Corticosterone altered both begging behavior and growth of white-crowned sparrow nestlings. It increased latency to beg immediately after the treatment and suppressed growth as early as 24 h after the patch application. These experiments also showed that the effects depended on the age or types of development (e.g. gaining mass or growing feathers) that the nestlings were going through.**

Key words: corticosterone, glucocorticoids, begging behavior, growth, altricial, nestling.

### INTRODUCTION

Actions of glucocorticoids present a cost–benefit tradeoff for vertebrate young. Glucocorticoids are essential during development; for example, they play critical roles in fetal organ maturation (reviewed by Liggins, 1994) and many life history stage transitions such as metamorphosis and fledging (Krug et al., 1983; de Jesus et al., 1990; Galton, 1990; Brown and Kim, 1995; Heath, 1997; Schwabl, 1999; Kern et al., 2001; Sockman and Schwabl, 2001; Seabury Sprague and Breuner, 2005). Yet at the same time, glucocorticoids can be detrimental for development. Prolonged exposure to glucocorticoids can cause increased mortality (Mashaly, 1991; Saino et al., 2005; Eriksen et al., 2006; Janczak et al., 2006) (but see Meylan and Clobert, 2005), reduced growth and/or body condition (Hayward and Wingfield, 2004; Meylan and Clobert, 2005; Eriksen et al., 2006), and may result in a hypersensitive hypothalamic–pituitary–adrenal (HPA) axis as adults (Hayward and Wingfield, 2004). Recent studies also suggest corticosterone (CORT) hinders feather growth in adult European starlings (*Sturnus vulgaris*) (Romero et al., 2005) and barn swallow nestling (*Hirundo rustica*) (Saino et al., 2005) which can delay fledging. Thus, the duration, timing, and intensity of CORT exposure may be key factors determining the balance of cost–benefit tradeoffs during development.

Studies to date have utilized numerous methods for glucocorticoid administration during pre- and postnatal development. Researchers have used injections of CORT into eggs or mothers for transient, ‘acute’ CORT elevation (Dean and Matthews, 1999; Rubolini et al., 2005; Saino et al., 2005; Freire et al., 2006; Janczak et al., 2006; Uller and Olsson, 2006). This prenatal exposure presumably mimics a maternal transfer of CORT to her offspring, especially when performed very early in development (Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006). More prolonged, ‘chronic’ elevation of CORT is traditionally achieved by using subcutaneous

implants; these often elevate the hormone for weeks, and sometimes months (Morici et al., 1997; Catalani et al., 2000; Glennemeier and Denver, 2002; Spencer et al., 2003). Although some species may naturally elevate CORT for such extended periods of time, it is probably not biologically relevant for most. Thus it will be valuable to investigate the effect of short, moderate exposure to CORT, especially in species with shorter developmental periods (i.e. short-lived organisms). It is important to note that many studies investigate classical actions of glucocorticoids; however rapid actions are often overlooked, especially in young.

During the early postnatal development in birds, a possible conflict exists between diverse effects of CORT; it can retard growth (Spencer et al., 2003; Saino et al., 2005) but can also facilitate begging (Kitaysky et al., 2001b; Kitaysky et al., 2003) (but see Rubolini et al., 2005). To investigate potential tradeoffs resulting from brief, moderate (physiologically relevant) elevations of CORT, we evaluated the effects of CORT on growth and begging through the nestling phase in Nuttall’s white-crowned sparrows (*Zonotrichia leucophrys nuttalli* Forster 1722). In the first experiment, we tested the effects of an acute CORT elevation (25 min) on begging behavior, by feeding nestlings CORT- or oil-containing wax moth (*Achroia grisella*) worms. In the second experiment, we artificially elevated CORT between 24 and 48 h using a non-invasive dermal patch, and observed changes in growth.

### MATERIALS AND METHODS

#### Animals

Nuttall’s white-crowned sparrow *Zonotrichia leucophrys nuttalli* Forster 1772 nestlings were captured from a free-living population on Bodega Marine Reserve, University of California, Davis, CA, USA. Nestlings in this species develop from a body mass of ~3 g to over 20 g and fledge within ~10 days (Banks, 1959). For the purpose of the study, the ~10-day nestling period was divided into

three age groups: days 1–3, 4–6 and 7–9 post-hatching (D1–3, 4–6, 7–9, respectively). In D1–3 nestlings, eyes are closed or have just opened. During this period, nestlings gain mass in near-logarithmic fashion and pin feather break occurs in 2.5 days (Banks, 1959). Eyes are fully open by D4–6. Nestlings of this age switch from ectothermy to endothermy, complete body mass gain and increase alertness and coordination of movements (Morton and Carey, 1971; Morton, 2002). D7–9 nestlings switch from gaining mass to developing feathers. They are alert, show a fear reaction to an observer, and may fledge if disturbed. Ages of the nestlings are estimated in two ways: (1) by monitoring hatch date or (2) by comparing growth characteristics of nestlings with known ages. Experiment 1 (acute elevation of CORT) was conducted in the spring of 2005 and experiment 2 (extended elevation of CORT) was conducted in the spring of 2006. Experimental protocols were approved by the Institutional Animal Care and Use Committees (the University of Texas at Austin, #06022301; University of California, Davis, #12200).

**Corticosterone manipulation in nestlings**

Each nest was randomly assigned to one of the three age groups for the experiment. On the day of the experiment, two nestlings (non-run) from each nest were randomly selected for two treatments, control and experimental groups. Each nest and each individual was treated and observed only for one age group.

**Experiment 1: acute elevation and begging behavior**

To deliver a transient increase of CORT non-invasively, we fed nestlings wax moth worms containing either CORT dissolved in peanut oil or peanut oil alone. This method was modified after Breuner et al. (Breuner et al., 1998). The sample size for this experiment was 10, 13, 6 for the control and 12, 13, 9 for the CORT in D1–3, 4–6 and 7–9, respectively.

The concentration used in this study was 0.4 mg CORT ml<sup>-1</sup> peanut oil (Sigma, St Louis, MO, USA). Peanut oil with or without CORT was injected into the worm using a 30-gauge needle mounted on a Hamilton syringe. The amount of solution injected into the moth worm was determined depending on the average mass of the two nestlings (Table 1A).

Nestlings were captured from nests one at a time. Immediately after capture, the body mass was recorded (Fig. 1A). The nestling was then transported to the laboratory in a transportable nest box (a natural nest in a small cardboard box) covered by an opaque cloth and moved into the observation box upon arrival. The observation box consisted of a nest in a small box taped onto a larger box (Fig. 1B). The outer box had a ~3 cm slit where the experimenter could tap the small nest box inside with a finger without being seen by the nestling. A video camera was placed on a tripod just outside of the observation box and aimed at the nest. The observation box plus the video camera were covered, for the entire duration of the behavioral observation, by a black plastic cover with an eye hole.

The room was kept dark except for inside the observation box. An electric body warmer was placed underneath the observation box to keep nestlings warm. Upon transfer, nestlings were fed wax moth

worms, the amount supplied was dependent on the nestlings body mass in order to bring all the nestlings to a similar fed state (17% of nestlings refused this first worm; we assumed those nestlings were fed and continued without the first feeding). The nest box within the observation box was then covered with an opaque cloth and nestlings were left undisturbed for approximately 35 min. After the quiescence period, the nest box was uncovered to feed the nestling with a CORT- or oil-containing wax moth worm. The nestling’s behavior was observed for 25 min (see below) immediately following the worm ingestion. Blood sample was collected after the behavioral observation to ensure the hormone manipulation was successful. Nestlings were then returned to their nest.

Begging behaviors of the nestlings were videotaped for 25 min. During the observation, the nest box was tapped for 3 sec every 5 min after an initial 5 min acclimation period. Tapping mimics a signal of parents’ return from their feeding trips and reliably elicited nestlings’ begging behavior in a preliminary study (H.W., unpublished). Videotapes were later analyzed for four parameters of begging behavior: latency to beg [time (s) for nestlings to beg after the start of each tapping], duration of begging (s), number of head lifts regardless of whether they resulted in actual begging, and number of peeping noises. The experimenter did not observe the behavior of the nestlings during the recording and the experimenter and the scorer did not know the treatment group of the subjects during the experiment.

**Experiment 2: extended elevation and growth parameters**

In the second experiment, CORT levels were artificially elevated for 24 to 48 h using a dermal patch containing either CORT dissolved in peanut oil or peanut oil alone. This method was modified after Knapp and Moore (Knapp and Moore, 1997). The sample size in this experiment was 9, 10, 10 for both treatment groups in D1–3, 4–6 and 7–9, respectively.

The concentration used in the dermal patches was 12.5 mg CORT ml<sup>-1</sup> peanut oil (Sigma). The amount of oil and the size of a patch were adjusted according to the nestlings’ mass (see Table 1B). The patch consisted of Johnson & Johnson clear Band-Aid, black vinyl electrical tape and 3M Nexcare transparent dressing. Patches were assembled the night before or the morning of the application to avoid drying up. The peanut oil with or without CORT was loaded on the band-aid portion of the patch in the morning of the application using 20-gauge needles.

Table 1. (A) Volume of peanut oil ± corticosterone injected into moth worms in experiment 1 and (B) size of dermal patch used in experiment 2

(A) Mass of chick (g)	Volume (μl)			
6–8	10.5			
8–10	13.5			
10–12	16.5			
12–14	19.5			
14–16	22.5			
16–18	25.5			
18–20	28.5			
20–22	31.5			
22–24	34.5			
(B) Mass of chick (g)	Volume (μl)	Band-Aid (mm)	Electrical tape (mm)	Dressing (mm)
0–10	5	2×4	6.5×8	11.5×25
10–15	10	4×4	7.5×8	13.5×25
15–20	15	6×4	7.5×9	13×30
>20	20	8×4	7.5×10	13×30

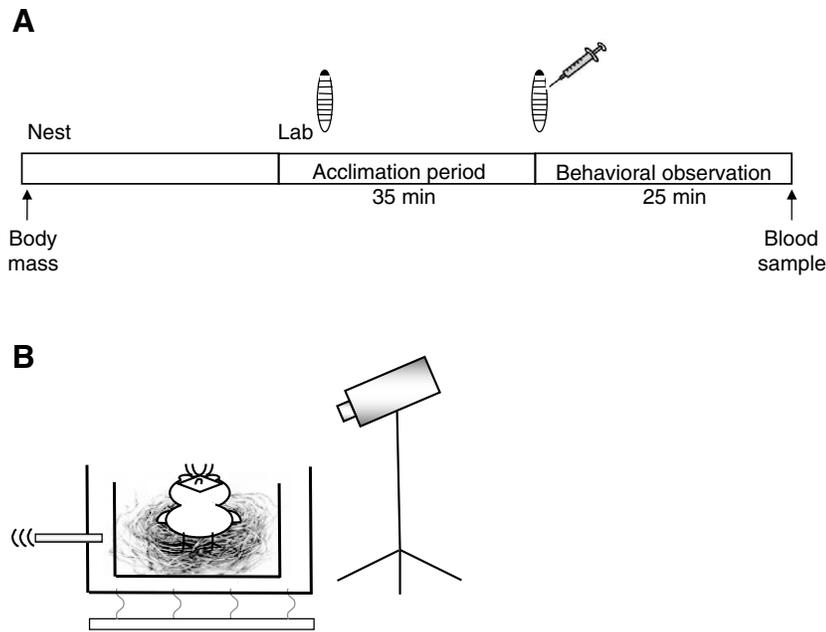


Fig. 1. Timeline (A) and diagram (B) for behavioral observations in experiment 1. (A) Immediately after nestlings were captured from their nest was body mass recorded. Upon arrival at the lab, nestlings were fed wax moth worms (total worm weight scaled to chick body mass) and left undisturbed for ~35 min. After the quiescence period, nestlings were fed with a worm injected with peanut oil with or without corticosterone. Behavior was observed for the following 25 min. After collecting a blood sample and taking measurements of growth, nestlings were returned to their nest. (B) Nestlings were placed in a natural nest within a small box taped onto a larger observation box. A small slit in the observation box allowed the experimenter to tap the nest box in place without being seen by the subjects. A video camera placed next to the observation box was aimed at the nest box. An electric body warmer was placed underneath the observation box to keep the nestlings warm.

At the beginning of the experiment, an initial blood sample was drawn and growth parameters were measured as a baseline for the individual. After the growth measurement (see below), patches were applied between two ventral sternal/abdominal tracts. The skin was first cleaned using 70% ethanol. Patches were applied to the skin after dabbing peanut oil on the skin area to aid the transfer of oil from patch to skin. Nestlings were then returned to their nest. The subsequent blood and growth samples were collected approximately 1, 3, 6 and 24 h (30 and 48 h when possible) after the patch application. New patches were applied after the 24 h sample was taken.

In this experiment, five growth parameters were measured: body mass (g), tarsus (mm), first primary (P1; mm) and wing (mm) length, and developmental scores. The wing length measurement here is slightly different from that used for adults, which is traditionally the length between the wrist joint and the tip of the longest primaries. Since bones are not yet defined in young birds, wing length was measured from the leading edge of the wing to the longest part of the primaries and secondaries. The developmental scores are the systematic scores of feather development on five parts of the body (wing, head, back, abdomen, and tail) on the scale of 0–5 (0=no pin, 2=pin and 4=sheath). As in experiment 1, nestling treatment was concealed from the experimenter for the duration of the study.

#### Blood sampling

All blood samples in experiment 1 and 2 were obtained within 4 min of capture, by puncturing the alar vein with a 26-gauge needle to measure baseline levels of CORT (Wada et al., 2007). The blood samples were kept on ice until they were spun for 8 min in the centrifuge at 13 460 *g* (11 500 r.p.m.) at the end of the day. Plasma and red blood cell samples were stored at  $-20^{\circ}\text{C}$  or below, until assay.

#### Corticosterone assays

Plasma CORT levels were determined using Enzyme Immunoassay (EIA) kits (cat # 901-097, Assay Designs). Plasma dilution and steroid displacement buffer (SDB) values were optimized previously

for this species (Wada et al., 2007). Samples were run in duplicate, and standard curves and standards were run in triplicate.

In 0.5 ml Eppendorf tubes, 7  $\mu\text{l}$  1% SDB was added to the equal volume of raw plasma. After a 5 min incubation, 266  $\mu\text{l}$  of assay buffer was added to the plasma (1:40 dilution). All plasma samples, standard curve, total binding, non-specific binding, and 500  $\text{pg ml}^{-1}$  standards were placed into a 96-well plate; conjugated CORT and secondary antibody were added to each well, except for non-specific binding wells, which received only antibody. The plate was incubated for 2 h on a shaker at  $26^{\circ}\text{C}$ . After the first incubation, the wells were rinsed three times with wash buffer. The plate was then incubated with substrate solution for 1 h at  $26^{\circ}\text{C}$  (without shaking). After the second incubation, stop solution was added to each well and the plate was read at 405 nm, with correction at 595 nm (Multiskan Ascent microplate reader).

Samples from experiment 1 and 2 were run in two separate EIA assays. Samples from experiment 1 were completely randomized within the assay, whereas samples from the same nest were analyzed on the same plate for experiment 2. All the nests were, however, randomized within the assay. Detection limits for the first and the second experiment were 0.64  $\text{ng ml}^{-1}$  and 0.87  $\text{ng ml}^{-1}$ , respectively (detectability=% bound of total binding – 2 standard deviations, i.e. CORT values that were significantly different from blank wells). The detection limit of the plate was used when the levels of a sample fell under the limit. Inter-plate and intra-plate variations for the first and the second experiment were 3.6%, 6.6%, 5.4% and 6.6%, respectively.

#### Corticosteroid binding globulin assays

Plasma corticosteroid binding globulin (CBG) levels were determined using a ligand-binding assay with tritiated CORT [described in Breuner et al. (Breuner et al., 2003)]. Optimal assay parameters in white-crowned sparrows (WCS) have been characterized previously (Lynn et al., 2003) and were validated for WCS nestlings (Wada et al., 2007). CBG levels of individual samples were measured in a point sample assay with 50  $\mu\text{l}$  1:300 diluted plasma, 50  $\mu\text{l}$  [ $^3\text{H}$ ]CORT, and either 50  $\mu\text{l}$  1  $\mu\text{mol l}^{-1}$  unlabelled CORT (non-specific binding) or 50  $\text{mmol l}^{-1}$  (pH 7.40)

Tris assay buffer (total binding); tubes were then incubated for 2 h at 4°C. After the incubation period, bound hormones were separated from free hormones by running through a rapid vacuum filtration, followed by three 3 ml rinse with 25 mmol l<sup>-1</sup> Tris buffer (pH 7.40). The glass fiber filters were soaked with 25 mmol l<sup>-1</sup> Tris buffer with 3% polyethylenimine for 1 h before harvesting. Intra-assay variation for the point sample assay was 22.1%.

Free hormone levels were estimated using an equation by Barsano and Baumann (Barsano and Baumann, 1989):

$$H_{\text{free}} = \frac{0.5[H_{\text{total}} - B_{\text{max}} - 1/K_a \pm \sqrt{(B_{\text{max}} - H_{\text{total}} + 1/K_a)^2 + 4(H_{\text{total}}/K_a)}]}{2}$$

where  $K_a$  is  $1/K_d$  (nmol l<sup>-1</sup>),  $K_d$  is affinity of CORT for CBG,  $B_{\text{max}}$  is total CBG capacity, and  $H_{\text{total}}$  is total plasma hormone concentration.  $K_d$  values were previously determined in equilibrium binding analyses using pooled plasma: day 2–3, 4–6 and 7–9 nestlings had  $K_d$  values of  $3.13 \pm 0.60$  nmol l<sup>-1</sup>,  $3.12 \pm 0.33$  nmol l<sup>-1</sup> and  $4.19 \pm 0.67$  nmol l<sup>-1</sup>, respectively (Wada et al., 2007).

**Sex determination**

The extraction and PCR procedure were modified after Freeman-Gallant et al. (Freeman-Gallant et al., 2001). Red blood cells (10 µl), 150 µl Tris-EDTA (TE) buffer, 3 µl 20% SDS, and 2 µl proteinase K were incubated at 65°C for 2 h on a shaker. DNA was extracted in three steps: with phenol, phenol–chloroform mixture, then with chloroform, all in 1:1 ratio with samples. At each step of the extraction, a reagent–red blood cell mixture was spun down in a centrifuge for 10 min at 16 060 g. At the end of the extraction, 20 µl ammonium acetate and 0.5 ml 100% ethanol were added to the supernatant. After purifying the DNA using 0.5 ml 70% ethanol, 50 µl TE buffer was added to re-suspend DNA. DNA samples (1 µl each) were then run in PCR machine with 1 µl forward (gagaactgtgcaaacag) and 1 µl reverse primers (tcagaatctctctgctcc) (Integrated DNA Technologies, Coralville, IA, USA). Post-PCR samples (10 µl) were run in an agarose gel stained with ethidium bromide and read with a UV light. Adult samples with known sex were run together to confirm the sexing results.

**Data analysis**

All data analyses were performed using SPSS 15.0. For experiment 1, the effects of treatment and age on CORT levels were determined using two-way ANOVA. The four parameters of begging behavior were reduced to three after principle axis factoring. Duration and number of head lift had factor loadings higher than 0.6; therefore they were combined by taking an average. The effects of treatment and age on three parameters of begging behavior were determined using MANOVA.

For the second experiment, the effect of treatment on CORT and CBG levels was analyzed using repeated measures ANOVA. The CORT levels and growth parameters were regressed using hierarchical multiple regression analysis. Five growth parameters were regressed separately to determine the effects of CORT on different types of growth. Prior to analyses, areas under the curve for both variables were calculated for each individual. Since CORT and growth parameters did not always increase with time, this approach allowed us to incorporate both rates and direction of the change into one variable. In the multiple regression analysis, age was coded as the following: Age1 denotes for D1–3 nestlings (D1–3=1, D4–6 and 7–9=0), Age2 codes for D4–6 nestlings (D1–3 and 7–9=0, D4–6=1), and the oldest age group was a reference. Since sex and treatment did not have a significant effect on the

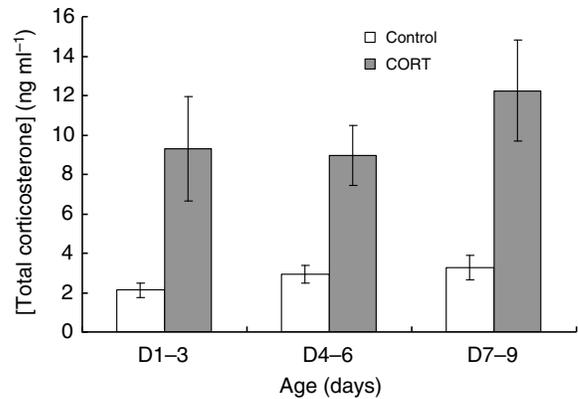


Fig. 2. Total (unbound and bound to corticosteroid binding globulin) corticosterone (CORT) levels at the end of the behavioral observation in control and CORT-treated nestlings in experiment 1. There was no effect of age but there was a significant effect of treatment on the hormone levels ( $P < 0.001$ ).  $N = 9, 13, 8$  for the controls and  $10, 14, 13$  for the CORT-treated groups D1–3, 4–6 and 7–9, respectively (D, days post-hatching).

CORT–growth regression in experiment 2, they were excluded from the further analyses.

Homogeneity of variance was tested using Levene’s test. When results were  $P \leq 0.05$ , the data were log transformed (begging behavior). Data were considered to be significant when  $P \leq 0.05$  after Bonferroni corrections when appropriate. Data are presented as mean  $\pm$  s.e.m.

**RESULTS**

**Experiment 1: acute elevation**

**CORT levels in nestlings**

There was no significant effect of age ( $F = 0.418, P = 0.66$ ) but a significant effect of treatment ( $F = 26.54, P < 0.001$ ) on nestlings’ CORT levels at the end of the behavioral observation (Fig. 2). CORT-treated nestlings had a significantly higher CORT than control nestlings. No significant interaction was observed between age and treatment ( $F = 0.329, P = 0.721$ ).

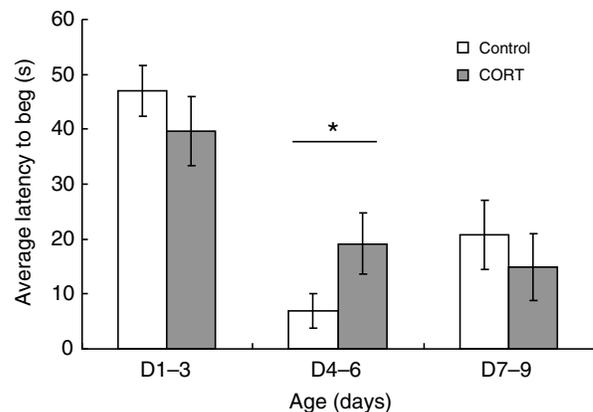


Fig. 3. Latency to beg in the three age groups. Latency was measured as the time it took for nestlings to beg after the start of tapping.  $N = 10, 13, 6$  for the control and  $12, 13, 9$  for the corticosterone (CORT)-treated groups D1–3, 4–6, and 7–9, respectively (D, days post-hatching). \* $P < 0.05$ .

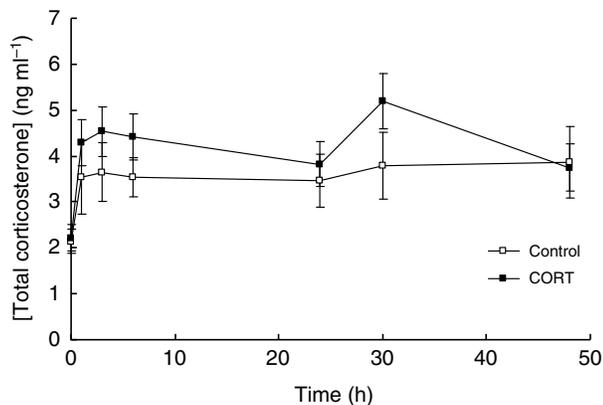


Fig. 4. Changes in total corticosterone (CORT) levels over 48 h of treatment with dermal patches containing CORT and patches with vehicle only (control; experiment 2). Minimum blood samples were collected prior to and 1, 3, 6 and 24 h (30 and 48 h when possible) after the patch application. After the 24 h sample, a new patch was applied. There was a marginal effect of treatment ( $P=0.094$ ) on CORT levels.

#### Effects on begging behavior

There was no direct effect of treatment on any of the behaviors observed ( $P>0.05$ ). Significant effects of age were observed for all parameters: latency ( $F=15.88$ ,  $P<0.001$ ), duration–head lifts ( $F=8.169$ ,  $P=0.001$ ), and peeping ( $F=3.55$ ,  $P=0.035$ ). No significant interactions were observed between age and treatment in duration–head lift or peeping ( $P>0.05$ ), however, significant interaction was detected in latency to beg ( $F=4.27$ ,  $P=0.019$ ;

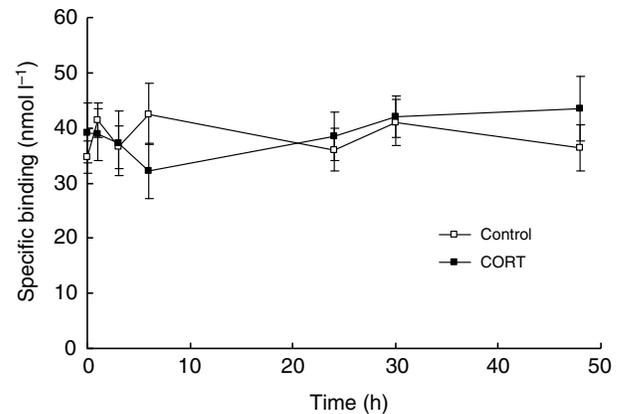


Fig. 5. Changes in corticosteroid binding globulin (CBG) levels over 48 h of treatment with dermal patches containing CORT and patches with vehicle only (control; experiment 2). Treatment had no effect on plasma CBG levels ( $P=0.976$ ).

Fig. 3). A pairwise comparison showed that in D4–6 nestlings, CORT-treated nestlings had longer latency to beg than controls.

#### Experiment 2: extended elevation

##### CORT and CBG levels in nestlings

Repeated measures ANOVA showed that there was a marginal effect of treatment on CORT levels ( $F=2.91$ ,  $P=0.094$ ; Fig. 4) and no effect of treatment on CBG levels ( $F=0.001$ ,  $P=0.976$ ; Fig. 5).

Table 2. Hierarchical multiple regression analysis outputs for corticosterone and five developmental measures in experiment 2

Developmental measures	Model summary				Coefficients		
	Predictors	Adjusted $R^2$	$R^2$ change	$F$ change	Predictors	Std Beta	$P$ value
Mass	CORT	-0.006	0.012	0.418	CORT	-0.013	0.841
	Age	0.881	0.875	<0.001 <sup>†</sup>	Age1	-0.916	<0.001 <sup>†</sup>
					Age2	-0.307	0.015*
	Age×CORT	0.89	0.013	0.044*	Age1×CORT	-0.194	0.047*
Tarsus	CORT	0.001	0.018	0.311	Age2×CORT	-0.239	0.038*
	Age	0.922	0.908	<0.001 <sup>†</sup>	CORT	0.001	0.982
					Age1	-0.924	<0.001 <sup>†</sup>
	Age×CORT	0.931	0.011	0.014*	Age2	-0.396	<0.001 <sup>†</sup>
P1 length	CORT	0.048	0.065	0.054	Age1×CORT	-0.211	0.007*
	Age	0.939	0.878	<0.001 <sup>†</sup>	Age2×CORT	-0.179	0.05*
					CORT	0.048	0.295
	Age×CORT	0.943	0.006	0.068	Age1	-0.994	<0.001 <sup>†</sup>
Wing length	CORT	0.031	0.048	0.1	Age2	-0.614	<0.001 <sup>†</sup>
	Age	0.941	0.897	<0.001 <sup>†</sup>	Age1×CORT	-0.07	0.314
					Age2×CORT	-0.191	0.022*
	Age×CORT	0.947	0.007	0.025*	CORT	0.032	0.468
Developmental scores	CORT	0.05	0.066	0.051	Age1	-0.983	<0.001 <sup>†</sup>
	Age	0.946	0.883	<0.001 <sup>†</sup>	Age2	-0.554	<0.001 <sup>†</sup>
					Age1×CORT	-0.111	0.099
	Age×CORT	0.949	0.004	0.114	Age2×CORT	-0.209	0.01*

CORT, corticosterone. Age1 denotes day 1–3 (D1–3=1, D4–6 and 7–9=0); Age2 denotes D4–6 nestlings (D1–3 and 7–9=0, D4–6=1), and the oldest age group was a reference. The relationship was considered significant when \* $P\leq 0.05$ , <sup>†</sup> $P\leq 0.001$ .

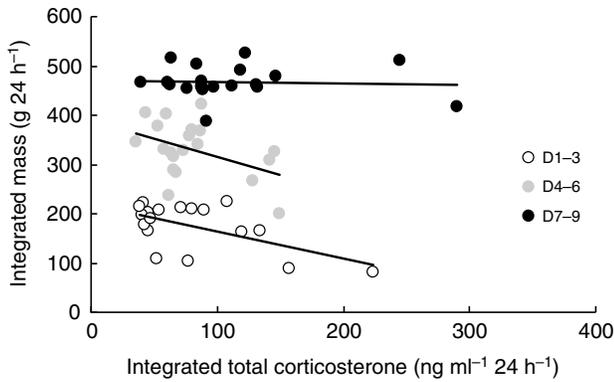


Fig. 6. Integrated mass vs integrated total corticosterone (CORT) for the first 24 h of treatment with dermal patches containing CORT and patches with vehicle only. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatment and control groups are plotted together.  $N=18, 20, 20$  for D1–3, 4–6 and 7–9, respectively (D, days post-hatching). Trend lines were added for visualization.

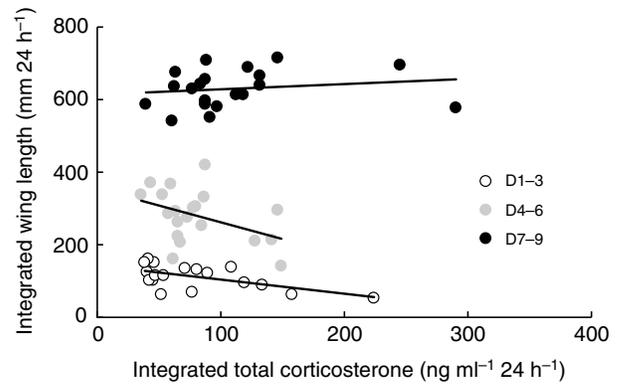


Fig. 8. Integrated wing length vs integrated total corticosterone (CORT) for the first 24 h of treatment with dermal patches containing CORT and patches with vehicle only. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatment and control groups are plotted together.  $N=18, 20, 20$  for D1–3, 4–6 and 7–9, respectively (D, days post-hatching). Trend lines were added for visualization.

Effects on growth

In the hierarchical multiple regression analyses, independent variables were added to the regression model in the following order: CORT, age, and interaction between age and CORT. Developmental measures were not explained by CORT levels alone ( $P>0.05$ , Table 2), although some developmental measures showed a marginal correlation with CORT (P1 length and developmental scores,  $P=0.054, 0.051$ , respectively). When age (Age1 and Age2) was added to the regression model,  $R^2$  increased significantly for all five developmental measures ( $P<0.001$ ). When age  $\times$  CORT interaction was added to the regression model,  $R^2$  again increased significantly for mass, tarsus and wing length ( $P<0.05$ ; Figs 6–8). This significant and negative correlation between growth parameters and CORT was observed after 24 h of CORT treatment in D1–6 nestlings.

DISCUSSION

Our study demonstrated that moderate, transient CORT elevation can alter behavior and growth of white-crowned sparrow nestlings. In the first experiment, a stress-response-like elevation in CORT over 25 min increased latency to beg in the middle-staged nestlings. This contrasts the previous findings where CORT promotes begging behavior in nestlings (Kitaysky et al., 2001b; Kitaysky et al., 2003). In the second experiment, mass, tarsus length, and wing length were negatively correlated with CORT levels of the nestlings. The effect of CORT was apparent as early as 24 h after the treatment. These results suggest that even a moderate increase in CORT is detrimental for early postnatal development in white-crowned sparrows. Moreover, within the observed measures, CORT appears more costly for nestlings.

Glucocorticoids and behavior

Both adult and developmental studies suggest that the effect of CORT on behaviors is condition or context dependent. In rodents, postnatal handling (brief separation) and maternal separation (three hours or more) have opposite effects on young's HPA reactivity in adulthood (for a review, see Anisman et al., 1998). Similarly, acute vs chronic elevation of CORT may have opposite effects on begging behavior in avian young. Our study showed that transient increases in CORT suppress subsequent begging in middle-staged nestlings. Acute prenatal elevation of CORT levels also have a similar effect in yellow-legged gulls (Rubolini et al., 2005), where begging rate is reduced in freshly hatched nestlings. By contrast, chronically elevated CORT (for 1–3 days) increases begging behavior in black-legged kittiwakes (Kitaysky et al., 2001b). When conditions are unfavorable for a brief period of time, it may be beneficial for the young to conserve energy by reducing body movements. However, it may be more beneficial for young to increase begging when the body goes into a negative energy balance. Distinct effects of CORT for acute and chronic elevation may be a mechanism for avian young to adjust energy balance during diverse types of challenges. It is also plausible that the receptor types may be responsible for the difference in effects of CORT; the suppression of begging seen in this study may be mediated through membrane receptors ('rapid'

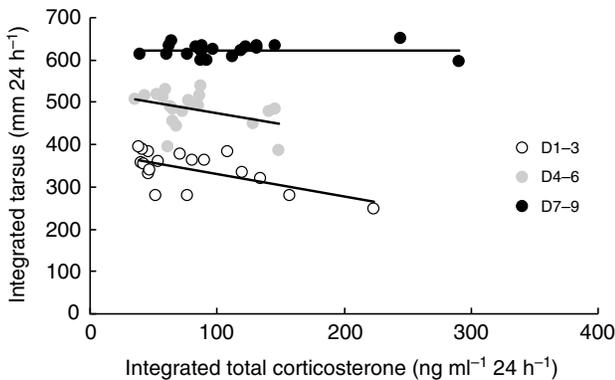


Fig. 7. Integrated tarsus vs integrated total corticosterone (CORT) for the first 24 h of treatment with dermal patches containing CORT and patches with vehicle only. Integrated measures were used to incorporate both rate and direction of changes. Individuals from both treatment and control groups are plotted together.  $N=18, 20, 20$  for D1–3, 4–6 and 7–9, respectively (D, days post-hatching). Trend lines were added for visualization.

actions), while the enhancement of begging in kittiwakes is likely mediated by classical actions of CORT through intracellular receptors.

Context dependency may also reflect nestling age. In our study, acute CORT elevations had an effect only on the middle-staged nestlings; this may reflect the physical and physiological stage of development. A similar phenomenon is seen in young domestic chickens. CORT has an effect on anti-predatory and anxiety behaviors only when administered prenatally (day 18 of incubation) and not postnatally (day 1 post-hatch) (Freire et al., 2006). Across vertebrates, CORT is known to act in a highly context-dependent manner (Orchinik, 1998), and developmental stages may be one of those determinants for actions of CORT. It is also possible that nestlings are highly sensitive to dose. CORT levels in the experimental group reached between 9 and 12 ng ml<sup>-1</sup> in experiment 1 which are well within the physiological range and equivalent to or less than those reached after a handling stress during a nestling period in the species (Wada et al., 2007). However, effects of CORT are highly dose dependent (Diamond et al., 1992; Breuner and Wingfield, 2000), and the 'effective dose' may change with age. (Early-staged nestlings have peak levels of ~11.5 ng ml<sup>-1</sup> after capture and handling stress, whereas late-staged nestlings reach ~37 ng ml<sup>-1</sup>.) Hence, it is possible that the current dose was relatively low for the late-staged nestlings, and a higher dose of CORT would be necessary to stimulate changes in begging behavior.

Many developmental studies acutely elevate CORT by giving a single injection into eggs or mothers (Dean and Matthews, 1999; Rubolini et al., 2005; Saino et al., 2005; Freire et al., 2006; Janczak et al., 2006; Uller and Olsson, 2006). It is important to note that this acute, prenatal exposure of CORT differs from our study in terms of timing of the treatment. In the former case, behaviors are observed days after the administration. This may reveal an organizational effect rather than an activational action of CORT.

#### Glucocorticoids and growth

It is generally accepted that chronically elevated CORT retards growth of young (Morici et al., 1997; Glennemeier and Denver, 2002; Spencer et al., 2003; Hayward and Wingfield, 2004). However, CORT may alter growth rates more rapidly than previous studies have suggested. In studies demonstrating deleterious effects of CORT on growth, young are often exposed to CORT for extended period of time, ranging from 7 days to three months (Morici et al., 1997; Leonhardt et al., 2002). In others, embryos are exposed to CORT by a prenatal injection into eggs or mothers. Results from latter studies are mixed; some show significantly slower growth (Saino et al., 2005; Janczak et al., 2006), whereas others indicate no effect of CORT (Preest et al., 2005; Rubolini et al., 2005; Uller and Olsson, 2006). The current study showed that negative relationships between CORT and mass, tarsus, and wing length were apparent after 24 h of patch application. The greatest effects were seen in D1–3 and D4–6 nestlings. This is the time when nestlings of this species grow rapidly both in terms of body mass and structural size (i.e. skeleton) (Banks, 1959). During days 7–10, as they reach fledging, the development switches from mass gain to feather growth. When the length of the first primary was regressed against CORT levels, we only observed a marginal interaction between age and CORT. This suggests that CORT may have a stronger effect on mass and structural development than feather growth in this species.

However, feather growth is also important for young birds, especially for the transitions between nestling, fledgling and independence. In adult European starlings, CORT is shown to

inhibit feather growth (Romero et al., 2005). In young barn swallows, an acute prenatal exposure to CORT (single injection within two days of laying) slows the wing feather and rectrix growth (Saino et al., 2005). In our study, we observed a negative relationship between wing length and CORT but not between P1 and CORT. Wing length in our study included carpometacarpus, patagium and flight feathers. Hence, the significant effect of CORT on wing length may be a result of reduction in development rate for both bones and feathers.

CORT may serve as a mechanism to adjust to current body condition, as suggested above and by other researchers (Breuner and Hahn, 2003; McEwen and Wingfield, 2003). Food restriction is known to elevate CORT (Kitaysky et al., 2001a). When nestlings respond to CORT by slowing growth, there may be a shift in energy allocation from growth to maintenance, until conditions improve. If they do, the energy allocation may shift back to growth and there may be no permanent alteration in body size, cognition or HPA reactivity. However if conditions do not improve, there may be irreversible changes, such as reduced body size/condition or song quality (e.g. Spencer et al., 2003). Such consequences of CORT elevation in this species are still not well understood.

CORT levels observed in response to the patch application were moderate in experiment 2. The highest level observed was 19 ng ml<sup>-1</sup> of a middle-staged nestling. Virtually all individuals had CORT levels below the age-specific stress-induced levels for the whole duration of the study. We observed higher variation around the mean in experimental plasma CORT levels than expected. In addition, preliminary studies using adult white-crowned sparrows showed an extensive effect of CORT patches on plasma hormone levels (data not shown), whereas levels in nestlings changed little. We do not know the exact cause of this variation or the disparity between adults and young, however, it may be due to a greater leakage, a differential skin diffusion rate, clearance rate, or magnitude of negative feedback, and physical interactions between siblings and parents in the field.

#### Conclusion

The current study demonstrated that brief and moderate increases of CORT can affect begging and growth in white-crowned sparrow nestlings. To our knowledge this is the first study to demonstrate (1) the rapid and negative effects of CORT on begging behavior and (2) the negative relationship between CORT and growth as early as 24 h after treatment. These results together indicate that both transient and extended CORT elevations are costly in this species. Then again, effects of CORT appear to be highly context dependent. Future studies are needed to determine the effects of more prolonged CORT elevations as well as effects on begging behaviors when a nestling's energy balance falls negative. These studies will help us understand whether CORT is more costly or poses more balanced cost–benefit tradeoffs to sparrow nestlings during development.

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## REFERENCES

- Anisman, H., Zaharia, M. D., Meaney, M. J. and Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int. J. Dev. Neurosci.* **16**, 149-164.
- Banks, R. C. (1959). Development of nestling white-crowned sparrows in central coastal California. *Condor* **61**, 96-109.
- Barsano, C. P. and Baumann, G. (1989). Editorial: simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? *Endocrinol.* **124**, 1101-1106.
- Breuner, C. W. and Hahn, T. P. (2003). Integrating stress physiology, environmental change, and behavior in free-living sparrows. *Horm. Behav.* **43**, 115-123.
- Breuner, C. W. and Wingfield, J. C. (2000). Rapid behavioral response to corticosterone varies with photoperiod and dose. *Horm. Behav.* **37**, 23-30.
- Breuner, C. W., Greenberg, A. L. and Wingfield, J. C. (1998). Noninvasive corticosterone treatment rapidly increases activity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Gen. Comp. Endocrinol.* **111**, 386-394.
- Breuner, C. W., Orchinik, M., Hahn, T. P., Meddle, S. L., Moore, I. T., Owen-Ashley, N. T., Sperry, T. S. and Wingfield, J. C. (2003). Differential mechanisms for regulation of the stress response across latitudinal gradients. *Am. J. Physiol.* **285**, R594-R600.
- Brown, C. L. and Kim, B. G. (1995). Combined application of cortisol and triiodothyronine in the culture of larval marine finfish. *Aquaculture* **135**, 79-86.
- Catalani, A., Casolini, P., Scaccianoce, S., Patacchioli, F. R., Spinozzi, P. and Angelucci, L. (2000). Maternal corticosterone during lactation permanently affects brain corticosteroid receptors, stress response and behaviour in rat progeny. *Neuroscience* **100**, 319-325.
- de Jesus, E. G., Inui, Y. and Hirano, T. (1990). Cortisol enhances the stimulating action of thyroid hormones on dorsal fin-ray resorption of flounder larvae *in vitro*. *Gen. Comp. Endocrinol.* **79**, 167-173.
- Dean, F. and Matthews, S. G. (1999). Maternal dexamethasone treatment in late gestation alters glucocorticoid and mineralocorticoid receptor mRNA in the fetal guinea pig brain. *Brain Res.* **846**, 253-259.
- Diamond, D. M., Bennett, M. C., Fleschner, M. and Rose, G. M. (1992). Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. *Hippocampus* **2**, 421-430.
- Eriksen, M. S., Bakken, M., Espmark, A., Braastad, B. O. and Salte, R. (2006). Prespawning stress in farmed Atlantic salmon *Salmo salar*: maternal cortisol exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations. *J. Fish Biol.* **69**, 114-129.
- Freeman-Gallant, C. R., O'Connor, K. D. and Breuer, M. E. (2001). Sexual selection and the geography of Plasmodium infection in Savannah sparrows (*Passerculus sandwichensis*). *Oecologia* **127**, 517-521.
- Freire, R., van Dort, S. and Rogers, L. J. (2006). Pre- and post-hatching effects of corticosterone treatment on behavior of the domestic chick. *Horm. Behav.* **49**, 157-165.
- Galton, V. A. (1990). Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. *Endocrinology* **127**, 2997-3002.
- Glennemeier, K. A. and Denver, R. J. (2002). Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. *Gen. Comp. Endocrinol.* **127**, 16-25.
- Hayward, L. S. and Wingfield, J. C. (2004). Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *Gen. Comp. Endocrinol.* **135**, 365-371.
- Heath, J. (1997). Corticosterone levels during nest departure of juvenile American kestrels. *Condor* **99**, 806-811.
- Janczak, A. M., Braastad, B. O. and Bakken, M. (2006). Behavioural effects of embryonic exposure to corticosterone in chickens. *Appl. Anim. Behav. Sci.* **96**, 69-82.
- Kern, M., Bacon, W., Long, D. and Cowie, R. J. (2001). Possible roles for corticosterone and critical size in the fledging of nestling pied flycatchers. *Physiol. Biochem. Zool.* **74**, 651.
- Kitaysky, A. S., Kitaiskaia, E. V., Wingfield, J. C. and Piatt, J. F. (2001a). Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. *J. Comp. Physiol. B* **171**, 701-709.
- Kitaysky, A. S., Wingfield, J. C. and Piatt, J. F. (2001b). Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behav. Ecol.* **12**, 619-625.
- Kitaysky, A. S., Kitaiskaia, E. V., Piatt, J. F. and Wingfield, J. C. (2003). Benefits and costs of increased levels of corticosterone in seabird chicks. *Horm. Behav.* **43**, 140-149.
- Knapp, R. and Moore, M. C. (1997). A non-invasive method for sustained elevation of steroid hormone levels in reptiles. *Herpetol. Rev.* **28**, 33-36.
- Krug, E. C., Honn, K. V., Battista, J. and Nicoll, C. S. (1983). Corticosteroids in serum of *Rana catesbeiana* during development and metamorphosis. *Gen. Comp. Endocrinol.* **52**, 232-241.
- Leonhardt, M., Lesage, J., Dufourny, L., Dicks-Coopman, A., Montel, V. and Dupouy, J.-P. (2002). Perinatal maternal food restriction induces alterations in hypothalamo-pituitary-adrenal axis activity and in plasma corticosterone-binding globulin capacity of weaning rat pups. *Neuroendocrinology* **75**, 45-54.
- Liggins, G. C. (1994). The role of cortisol in preparing the fetus for birth. *Reprod. Fertil. Dev.* **6**, 141-150.
- Lynn, S. E., Breuner, C. W. and Wingfield, J. C. (2003). Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migrating songbird. *Horm. Behav.* **43**, 150-157.
- Mashaly, M. M. (1991). Effect of exogenous corticosterone on chicken embryonic development. *Poult. Sci.* **70**, 371-374.
- McEwen, B. S. and Wingfield, J. C. (2003). The concept of allostasis in biology and biomedicine. *Horm. Behav.* **43**, 2-15.
- Meylan, S. and Clobert, J. (2005). Is corticosterone-mediated phenotype development adaptive? Maternal corticosterone treatment enhances survival in male lizards. *Horm. Behav.* **48**, 44-52.
- Morici, L. A., Eisey, R. M. and Lance, V. A. (1997). Effect of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. *J. Exp. Zool.* **279**, 156-162.
- Morton, M. L. (2002). The mountain white-crowned sparrow: migration and reproduction at high altitude. *Stud. Avian Biol.* **24**, 1-236.
- Morton, M. L. and Carey, C. (1971). Growth and the development of endothermy in the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*). *Physiol. Zool.* **44**, 177-189.
- Orchinik, M. (1998). Glucocorticoids, stress, and behavior: shifting the timeframe. *Horm. Behav.* **34**, 320-327.
- Prest, M. R., Cree, A. and Tyrrell, C. L. (2005). ACTH-induced stress response during pregnancy in a viviparous gecko: no observed effect on offspring quality. *J. Exp. Zool. A* **303**, 823-835.
- Romero, L. M., Strohlic, D. and Wingfield, J. C. (2005). Corticosterone inhibits feather growth: potential mechanism explaining seasonal down regulation of corticosterone during molt. *Comp. Biochem. Physiol.* **142A**, 65-73.
- Rubolini, D., Romano, M., Boncoraglio, G., Ferrari, R. P., Martinelli, R., Galeotti, P., Fasola, M. and Saino, N. (2005). Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. *Horm. Behav.* **47**, 592-605.
- Saino, N., Romano, M., Ferrari, R. P., Martinelli, R. and Moller, A. P. (2005). Stressed mothers lay eggs with high corticosterone levels which produce low-quality offspring. *J. Exp. Zool. A* **303**, 998-1006.
- Schwabl, H. (1999). Developmental changes and among-sibling variation of corticosterone levels in an altricial avian species. *Gen. Comp. Endocrinol.* **116**, 403-408.
- Seabury Sprague, R. and Breuner, C. W. (2005). Timing of fledging, body condition, and corticosteroid binding globulin in Laysan albatross. *Integr. Comp. Biol.* **45**, 1070.
- Sockman, K. W. and Schwabl, H. (2001). Plasma corticosterone in nestling American kestrels: effects of age, handling stress, yolk androgens, and body condition. *Gen. Comp. Endocrinol.* **122**, 205-212.
- Spencer, K. A., Buchanan, K. L., Goldsmith, A. R. and Catchpole, C. K. (2003). Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). *Horm. Behav.* **44**, 132-139.
- Uller, T. and Olsson, M. (2006). Direct exposure to corticosterone during embryonic development influences behaviour in an ovoviparous lizard. *Ethology* **112**, 390-397.
- Wada, H., Hahn, T. P. and Breuner, C. W. (2007). Development of stress reactivity in white-crowned sparrow nestlings: total corticosterone response increases with age, while free corticosterone response remains low. *Gen. Comp. Endocrinol.* **150**, 405-413.