Dexamethasone treatment supports age-related maturation of the stress response in altricial nestling birds

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Abstract

In birds, the magnitude of the adrenocortical stress response can be down-regulated during specific life-history stages. Such modulation likely occurs when the effects of mounting robust corticosterone (Cort) elevations interfere with the normal progression of critical lifecycle activities (e.g. development, molt, migration, reproduction). The Developmental Hypothesis posits that altricial birds should display a “Stress Hyporesponsive Period” during the early post-natal life stages, characterized by reduced adrenocortical stress responses compared to adult birds and a gradual age-related increase. Such modulation would allow avoiding the potential deleterious effects that long-term elevations of circulating Cort might exert on growth and development, when the physiological and behavioral abilities to cope with disturbance are limited. Two proximate hypotheses have been proposed to explain this age-dependent pattern of Cort secretion. The *Maturation Hypothesis* proposes a progressive age-related growth, maturation and enhanced sensitivity to sensory input of the Hypothalamic-Pituitary-Adrenal (HPA) axis tissues, whereas the *Negative Feedback Attenuation Hypothesis* proposes a gradual attenuation in the intensity of the negative feedback in the HPA axis. Here we tested these hypotheses by experimentally inducing negative feedback on the HPA axis via dexamethasone (DEX) treatment in nestling white storks *Ciconia ciconia*. Nestling age positively affected stress-induced plasma Cort (STRESS-Cort) levels during experimental handling and restraint, thus supporting the Developmental Hypothesis. DEX treatment significantly reduced STRESS-Cort levels compared to saline (SAL) treatment, thus eliciting the expected negative feedback on the HPA axis. However, inter- and intra-individual comparisons indicated no age effects on the intensity of the negative feedback exerted by DEX. Our results do not support the *Negative Feedback Attenuation Hypothesis* and suggest that progressive *Maturation* of the HPA axis tissues is the proximate mechanism responsible for age-related changes in the stress response during avian post-natal development. We encourage further tests of the proposed proximate mechanisms during migration, breeding and molt.

Key-words: adrenocortical modulation, Developmental Hypothesis, dexamethasone, Maturation Hypothesis, Negative Feedback Attenuation Hypothesis, Stress Hyporesponsive Period.

Introduction

In birds, exposure to environmental disturbance (e.g., inclement weather, famine, predation, experimental capture and restraint) typically leads to the activation of the adrenocortical response to stress (Blas 2015).

This response involves the Hypothalamic-Pituitary-Adrenal (HPA) axis, and results in a pronounced elevation of circulating corticosterone (Cort), the main avian glucocorticoid. Short-term elevations of blood Cort levels (i.e., during minutes to a few hours) are considered to be beneficial for the individual, as they facilitate rapid physiological (e.g., mobilization of stored energy reserves) and behavioral (e.g., escape, fight) responses aimed to cope with stress and overcome disturbance (Wingfield et al. 1998).

However, repeated or longer-term exposure to disturbance (i.e., modifying factors; Wingfield 2013) may cause an energy imbalance mediated through an over-activation of the HPA axis (i.e., allostatic overload type 2; McEwen and Wingfield 2003), resulting in chronic Cort elevations that potentially exert deleterious effects on critical traits such as growth, reproduction and neuronal function (e.g. Müllner et al. 2004, Angelier et al. 2009, Müller et al. 2009, Newman et al. 2010).

Numerous studies show that the magnitude of the adrenocortical response to stress can be down-regulated. Such modulation likely occurs when the effects of robust Cort elevations may interfere with the expression of activities and traits that are crucial for the development of specific life cycle stages. For example, the secretion of Cort may be down-regulated during molt, thus avoiding the inhibitory effect of this hormone on the deposition of the proteins that make up the feathers (Romero et al. 2005). Similarly, the secretion of Cort in response to disturbance can be down-regulated during reproduction (e.g., Parental Care Hypothesis (Wingfield et al. 1995); Brood Value Hypothesis (Wingfield and Sapolsky 2003, Bókony et al. 2009)); during migration (i.e., Migration-Modulation Hypothesis; Holberton et al. 1996, Wilson et al. 2017) or during development (Sims and Holberton 2000, Blas et al. 2006). In this sense, the negative feedback, a self-inhibitory mechanism of Cort secretion acting on the HPA axis tissues and hippocampus (Jacobson and Sapolsky 1991, Romero 2004), allows modulating the adrenocortical response to stress, as demonstrated in studies explaining personality or sexual differences in HPA axis reactivity (Astheimer et al. 1994, Baugh et al. 2017). This regulatory mechanism may reduce the deleterious effects associated to elevated Cort levels. For example, stronger negative feedback has been linked to lower levels of infection in song sparrows *Melospiza melodia* (Schmidt et al. 2012) and lower probabilities of abandoning reproduction in response to experimental challenges (e.g., reduced flight efficiency and increased predation risk) in tree swallows *Tachycineta bicolor* (Zimmer et al. 2019).

The Developmental Hypothesis (Sims and Holberton 2000; reviewed in Blas and Baos 2008) proposes that the adrenocortical stress responses displayed by birds during post-natal development should be down-regulated. Such modulation would be adaptive when the physiological and behavioral abilities that allow coping with disturbance (e.g., thermoregulation, flight and aggression responses, foraging, etc.) are limited, thus avoiding the prolonged or repeated elevations of Cort which may jeopardize growth and development. The Developmental Hypothesis predicts that the magnitude of the stress response in nestling birds will depend on: (i) the degree of parental dependence at hatching (the responses will be less robust in altricial than in precocial birds), (ii) the physical link to a nest (the responses will be less robust in nidicolous than in nidifugous birds) and (iii) age (the responses will be less robust in younger nestlings). In general, altricial, nidicolous and young nestlings typically display a "Stress Hyporesponsive Period" (SHRP; Sapolsky and Meaney 1986) characterized by a reduced secretion of Cort in response to disturbance during the first days of life, and a gradual increase with age. At the end of the nestling period, altricial birds have typically acquired some of the physiological and behavioral abilities that allow coping with disturbance, thus showing more robust adrenocortical responses (e.g., Love et al. 2003, Blas and Baos 2008, López-Jiménez et al. 2016).

Positive age-related effects on stress-induced plasma Cort levels (STRESS-Cort) have been shown in numerous avian species (e.g., Blas et al. 2005, Wada et al. 2007, Müller et al. 2010), providing broad support for the Developmental Hypothesis in birds (Blas and Baos 2008). From a proximate perspective, age-related increased Cort secretion can be explained by two hypotheses (based on Sapolsky and Meaney 1986). On the one hand, (1) the *Maturation Hypothesis* proposes a gradual growth, maturation and enhanced sensitivity to sensory input of the HPA axis tissues as individuals age. This maturation would increase the abilities to secrete Cort in response to stress, thus generating a pattern consistent with the Developmental Hypothesis. On the other hand, (2) the *Negative Feedback Attenuation Hypothesis* proposes a gradual reduction (i.e., age-related attenuation) in the intensity of the adrenocortical negative feedback, which would generate a gradual increase in the secretion of Cort. At young ages the feedback would be stronger, thus reducing the secretion of Cort in response to stress. Conversely, the efficacy of the negative feedback would become weaker in older birds (i.e. attenuation), thus increasing the secretion of Cort in response to stress. Such mechanism (also described as "enhanced negative feedback", when referring to younger birds; see Wada and Breuner 2010) could potentially generate the widely reported

age-related elevations of the stress responses even if the maturation of the HPA axis tissues was complete from the earliest developmental stages.

The main goal of this study is testing the Negative Feedback Attenuation Hypothesis in birds through experimentally inducing negative feedback in the HPA axis via dexamethasone (DEX) treatment. DEX is a synthetic glucocorticoid that competes with circulating endogenous Cort for the same receptors, typically inducing negative feedback in the HPA axis and generating a decrease in endogenous Cort secretion that can be quantified by measuring circulating levels of this hormone (Romero and Wikelski 2010). Figure 1 illustrates some of the predictions associated to the hypotheses proposed above, involving (i) STRESS-Cort levels (Fig. 1a) and (ii) changes in circulating Cort (Fig. 1b). The Developmental Hypothesis predicts an age-related increase in STRESS-Cort levels, illustrated as line *A* in Fig. 1a. DEX treatment should produce an overall decrease in plasma Cort levels, represented by arrow *B* in Fig. 1a. If the ability to perform negative feedback is independent of age, the decreased plasma Cort levels displayed by DEX-treated birds would still show a positive age-related pattern that should be identical to control birds, generating the pattern illustrated by line *C* (which is lower than, and parallel to, line *A* depicting control birds; Fig. 1a). At the intra-individual level (Fig. 1b), the change (the decrease) in circulating Cort levels after DEX treatment (arrow *B* in Fig. 1b) would be constant throughout the developmental period, generating line *C* in Fig. 1b. Such a result would be incompatible with the Negative Feedback Attenuation Hypothesis, thus supporting the progressive maturation of the HPA axis. Conversely, the Attenuation Hypothesis predicts a decline in the negative feedback intensity related to age. As a consequence, the expected decrease in plasma Cort levels after DEX treatment would be maximal in younger nestlings and minimal in older birds, resulting in the pattern illustrated by line *D* in Fig. 1a (which should not be parallel to the pattern of control birds, illustrated as line *A* within the same figure). At the intra-individual level, the change (the decrease) in plasma Cort levels after DEX treatment (Fig. 1b), would be age-dependent, generating line *D* within this figure.

To test these predictions, we used nestling white storks *Ciconia ciconia*, Linnaeus 1758, an altricial bird species showing a long period of growth and development in the nest (i.e., 60-90 days according to Redondo et al. 1995). Previous studies have shown that storks' adrenocortical responses follow an age-related pattern consistent with the Developmental Hypothesis (Blas et al. 2005, Blas et al. 2006). However, it remains unknown whether this pattern is proximately the consequence of a gradual attenuation of the negative feedback intensity. Here we tested the Negative Feedback Attenuation

Hypothesis via DEX treatment in 77 stork nestlings. To the best of our knowledge, this hypothesis has not yet been tested experimentally in any bird species.

Materials and methods

Fieldwork

From June 2014 to July 2016, we performed 93 stress series in 77 white stork nestlings from a colony near Doñana National Park (Dehesa de Abajo, Puebla del Río, Seville, Spain). Nestlings age, estimated through morphometric measurements (following Chozas 1983) ranged between 34 and 68 days. Each stress series involved the collection of four sequential blood samples (except for 2 individuals whose first blood sample could not be obtained) from the brachial vein. Each sample involved the extraction of 0.5 mL of blood, which together accounted for less than 0.05% body mass per stress series. The first blood sample was taken within the first 3.25 min post-capture (97 ± 4 s, mean $\pm$ s.e.m.), allowing us the determination of baseline Cort levels (BAS-Cort). BAS-Cort levels were independent of the time elapsed between bird capture and blood sampling ($P > 0.05$). A second blood sample was obtained 30 min post-capture (1815 ± 5 s, mean $\pm$ s.e.m.), allowing the determination of stress-induced plasma Cort levels (STRESS-Cort-30) corresponding to the phase of increase of the adrenocortical response to stress, prior to the experimental treatment with DEX. At 35 min post-capture, nestlings randomly received an intramuscular injection (in the pectoral muscle) of either DEX (Fortecortín, Merck S.L., Madrid, Spain; dose: 1 mg kg^{-1} body mass; $N = 54$ experimental birds) or the same volume of saline (only vehicle) solution (SAL; Vitulia, Laboratorios ERN, S.A., Barcelona, Spain; dose: 0.25 mL kg^{-1} body mass; $N = 39$ birds) as control treatment. The dose of DEX was selected after verifying that previous studies obtained a significant reduction in circulating Cort levels in birds (Dickens et al. 2009, Lattin et al. 2012). After DEX or SAL injection, two additional blood samples were taken at 65 min (3901 ± 9 s; mean $\pm$ s.e.m.) and 95 min (5702 ± 8 s; mean $\pm$ s.e.m.) post-capture (i.e., 30 and 60 min after injection). Each individual was randomly assigned to a single treatment of either DEX or SAL except for a sub-sample of 16 birds which were sequentially subjected to both treatments on different days in random order (to further assess negative feedback intra-individually). For this purpose, two complete stress series were performed in each individual, allowing 4.5 ± 0.7 days between treatments. All birds were held individually inside cloth bags and in the shade between sequential blood sampling (i.e., during a stress series). Before returning birds to their nests, each nestling was banded, weighed and the wing chord was measured. All blood samples were

kept in portable coolers until centrifuged (1252 g, 10 min) the same day, storing the resulting plasma at -80°C until laboratory analyses.

Laboratory plasma Cort analysis

We performed plasma Cort extractions using diethyl ether (Fisher Chemical, Fair Lawn, New Jersey, USA) and following the protocol described in Blas et al. 2005. We extracted all samples in 3 separate batches and more than 93% of the radioactivity was recovered in each batch. We determined plasma Cort levels by radioimmunoassay (RIA) on reconstituted extracts (300 µl of phosphate-buffered saline, 0.05 M and pH 7.6). Plasma extracts were frozen at -20°C until Cort was measured. We purchased antiserum (C8784; lot 090M47520) and purified Cort (C2505, Lot 22K1439) for standards from Sigma-Aldrich Chemicals (Saint Louis, Missouri, USA). We measured samples in duplicate and we found a parallel relationship between serial dilutions of the plasma extracts and the standard curve. Assay variability was calculated as the coefficient of variation (CV) resulting from repeated measurement of six samples of a known amount of Cort in each assay. We measured all samples in 15 RIAs, with intra- and inter-assay CV being 5.4% and 13.1% respectively. The average limit of detection ($ED_{80} \pm s.d.$) was 14.44 ± 2.33 pg per assay tube.

Statistical analyses

The intensity of the negative feedback induced by DEX treatment was estimated in two ways (respectively illustrated as panels (a) and (b) in Fig. 1). We first used plasma Cort concentrations (N=370) and established inter-individual comparisons between DEX and SAL groups. Then, we calculated intra-individual changes in circulating Cort levels (i.e., Cort-DEX minus Cort-SAL titres) at each sampling time post-treatment (i.e., ΔCort_{65} and ΔCort_{95} ; N=32). We used an information-theoretic approach to assess the support provided by our data to several a priori defined competing hypotheses. Model selection was performed using Akaike Information Criterion corrected for small sample sizes, AICc (Burnham and Anderson 2002). Within a given set of models we first calculated ΔAICc_i as the difference between the AICc of model i and that of the best model (i.e. the model with the lowest AICc). As a rule of thumb, a $\Delta\text{AICc}_i < 2$ suggests substantial evidence for the model. Following our main hypotheses, inter-individual

differences in plasma Cort levels were tested through General Mixed Models GLMMs, which allowed us assessing the effect of age on the intensity of negative feedback (Models a1 and a2). Using plasma Cort levels (ng mL^{-1}) as the response variable, the following potential effects were considered as fixed, independent terms: sampling time (factor: 0 vs 30 vs 65 vs 95 minutes), treatment (factor: DEX-treated vs SAL-treated) and age (days; continuous variable). Model a1 also considered the interactions "sampling time X treatment" (the effects of treatment should occur at times 65 and 95 minutes but not before; i.e., only after experimental injection) and "sampling time X age" (age effects should increase with handling time; Blas et al. 2006). Model a2 included all the terms described above, plus the three-way interaction "sampling time X treatment X age". The latter interaction in our Model a2 would provide support to the Negative Feedback Attenuation Hypothesis, as we would expect significant age effects on the intensity of negative feedback (i.e., age effects on treatment; see line D in Fig. 1a) occurring at sampling times after (and not before) actual experimental treatment. According to the Negative Feedback Attenuation Hypothesis, Model a2 should be better than Model a1. Conversely, the Maturation Hypothesis does not predict age effects on the intensity of negative feedback (line C in Fig. 1a), and therefore we would expect Model a1 to be better than Model a2. To avoid potential pseudoreplication, Models a1 and a2 also included the following random terms nested in descending hierarchy: year (the same colony was sampled in different years), nest (the same nests were repeatedly sampled in different years), brood identity (several nestlings composed a given brood during the same year) and individual identity (the same birds were repeatedly sampled within a stress series).

Intra-individual differences in the intensity of negative feedback were tested in a second set of GLMMs that used ΔCort as the response variable (Models b1-b5). According to the Maturation Hypothesis the intensity of negative feedback should be independent of age (line C, Fig. 1b), thus predicting a constant response (i.e., a Model b1 containing only the intercept). Alternatively, the intensity of negative feedback could be affected by age (in days; continuous variable; Model b2), sampling time (factor: 65 vs. 95 min; Model b3), both variables additively (Model b4) or interacting (Model b5). According to the Negative Feedback Attenuation Hypothesis, the best model should involve age effects (line D, Fig. 1b). Since both SAL and DEX treatments were administered in random order to the same individuals on different days, GLMMs considered the average age between both stress series. The fact that birds "aged" (albeit minimally) between sequential treatments was ethically and logistically unavoidable, because the nestlings needed to fully recover between one experimental treatment (e.g., from DEX injection) and the

alternative treatment (e.g., SAL injection). However, the number of days elapsed between treatments was rather short (4.5 days on average), specially compared to the range of ages among the different individuals included in our experiment (i.e. 18 days). In fact, the age-related increase in STRESS-Cort levels previously reported for nestling white storks (Blas et al. 2006) predicted a slight, c.a. 6% increase in Cort levels for 4.5 days of growth whereas 18 days of growth would imply an increase over 30%. For these reasons we expected minimal age effects intra-individually, justifying the use of the average age value for each individual between sequential stress series, as well as a test for age-related changes in STRESS-Cort levels. In order to avoid potential pseudoreplication, Models b1-b5 also included brood identity and individual identity as hierarchically nested random terms. As an additional procedure for assessing negative feedback intra-individually, we also calculated additional variables that took into consideration changes in STRESS-Cort levels within and between stress series (see Supplementary material Appendix 1) and tested age effects in a third set of GLMMs (c1-c5). All GLMMs were build following Zuur et al. (2009) using the library 'nlme' (Pinheiro et al. 2017) in R 3.2.3 (R Development Core Team 2015). Statistically significant interactions ($P \leq 0.05$) were evaluated through subgroups analyses, either subsetting data or performing Tukey *post-hoc* tests with Bonferroni correction. We checked the normality and homocedasticity assumptions of the model residuals.

Results

At the inter-individual level, the best model based on AICc indicated no age effects on the intensity of the negative feedback (Table 1, Model a1). This model, revealed two statistically significant interactions involving “sampling time X treatment” and “sampling time X age” (Table 2). Plasma Cort levels were independent of experimental group before applying DEX or SAL treatment (see Fig. 2 and Supplementary material Appendix 2, Table A1). However, after experimental injections, DEX treatment significantly reduced Cort levels by -8.01 ng mL^{-1} and $-15.65 \text{ ng mL}^{-1}$ compared to the control situation at 65 and 95 minutes post-capture respectively, implying a decrease of 17.83% and 37.99% respectively (Fig. 2; Supplementary material Appendix 2, Table A1). BAS-Cort levels were independent of age (Fig. 3; Supplementary material Appendix 2, Table A2, Model a1.1), but age had a positive and statistically significant effect on STRESS-Cort levels at all sampled times (Fig.3; Supplementary material Appendix 2, Table A2, Models a1.2-a1.4).

At the intra-individual level, the best model (Table 1, Model b1) revealed a negative change (i.e., a significant decrease) in plasma titers associated to DEX treatment (Intercept $\pm$ s.e.m: -12.35 ± 4.21 ; $\square^2$

$t_1 = 8.61$; $P < 0.01$), but age and sampling time post-treatment had no effects on such decrease (Table 1, Models b1-b5; Fig. 4; see also Supplementary material Appendix 2, Table A3). STRESS-Cort-30 levels (i.e. pre-injection samples) did not change between the first and the second stress series (GLMM: STRESS-Cort-30 as a function of order of sampling (individual as random) $\chi^2_1 = 1.33$; $P = 0.25$; $N = 32$). On the contrary, STRESS-Cort-65 levels and STRESS-Cort-95 levels (i.e. post-injection samples) decreased between SAL and DEX treatments at both sampling times (GLMM: STRESS-Cort-65 as a function of treatment (regardless of order of sampling and keeping individual as random) $\chi^2_1 = 11.51$; $P < 0.001$; $N = 32$; STRESS-Cort-95: $\chi^2_1 = 5.00$; $P = 0.025$; $N = 32$).

Discussion

The age of stork nestlings had no effect on BAS-Cort levels, but it exerted a significant effect on STRESS-Cort titers (Fig. 3). Older nestlings displayed more robust stress responses, characterized by higher elevations of STRESS-Cort levels that were in agreement with previously published studies and consistent with the Developmental Hypothesis (Blas et al. 2006). The early life of white storks is characterized by a long period of stay in the nest (60 days at least), when physiological and behavioral abilities are progressively developed (Bernis 1975, Redondo et al. 1995, Jovani and Tella 2004, Blas et al. 2006). A parallel development of the stress response would allow the younger nestlings (who depend almost completely on their parents for food, thermoregulation and protection) to avoid the negative consequences that prolonged elevations of Cort might exert on their ontogenetic development. In contrast, when nestlings grow older and approach fledging age, they acquire physiological and behavioral abilities that allow coping with possible sources of stress (e.g. they can respond to thermal stress through thermoregulation and locomotion). These arguments are compatible with the positive age-related pattern on the stress response we found in white stork nestlings, and agree with our initial predictions for the Developmental Hypothesis (i.e., line A in Fig. 1a), with previous studies in this species (Blas et al. 2006) and with studies in other species of altricial birds (Love et al. 2003, Walker et al. 2005, López-Jiménez et al. 2016).

The selected dose of DEX we applied to white stork nestlings produced a significant reduction of plasma Cort levels compared to control, SAL-treated birds (Fig. 2). This result was also consistent with our initial predictions (i.e., arrows B in Fig. 1), thus justifying the experimental use of DEX to evaluate the effects of nestling age on the intensity of the negative feedback inter- and intra-individually. At the inter-individual level, the magnitude of the decrease in plasma Cort titers induced by DEX treatment was independent of

age. In other words, the effect of age in control birds (i.e., the slopes of line A in Fig. 1a and dashed lines in Fig. 3) was similar to the effect of age in DEX-treated birds (i.e., the slopes of line C in Fig. 1a and solid lines in Fig. 3), indicating that the intensity of the negative feedback was the same across ages. This result does not support the predictions associated to the Attenuation Hypothesis (line D in Fig. 1a), and thus suggests that the Maturation Hypothesis (line C in Fig. 1a) is the proximate mechanism explaining age-related development of stress responses. At the intra-individual level, there was a negative change in plasma Cort levels associated with DEX treatment (i.e., plasma Cort levels decreased in response to DEX treatment), but more important, such a decrease was also independent of age (Fig. 4). This result is contrary to the effect predicted by the Attenuation Hypothesis (line D in Fig. 1b), and also supports the Maturation Hypothesis (line C in Fig. 1b). Individual birds showed similar STRESS-Cort-30 levels (pre-injection) between sequential stress series, thus supporting the idea that the starting conditions were similar in both treatments. Therefore the difference in STRESS-Cort levels between SAL and DEX treatments at time 65 (i.e. 30 minutes post-injection) and also at time 95 (i.e. 60 minutes post-injection) were due to the treatment *per se*, providing accurate information about the negative feedback intra-individually. To the best of our knowledge, this is the first study that has experimentally induced a negative feedback on the HPA axis in birds to test the potential mechanisms explaining age-related development of stress responses. Two studies have previously contributed to test the hypotheses presented here in altricial nestlings of white-crowned sparrows *Zonotrichia leucophrys*. In a first study, Wada and Breuner (2010) measured the ability of Cort to bind to its receptors in the hypothalamus and hippocampus (thus estimating the ability to induce negative feedback) and analyzed the effect of nestlings age. In the latter work, the intensity of negative feedback was independent of age, allowing rejection of the Attenuation Hypothesis (that was presented as “enhanced negative feedback” in younger nestlings; Wada and Breuner 2010). In a second study, Wada et al. 2007 used an experimental treatment with adrenocorticotrophic hormone (ACTH) to study the effect of age on the expected increases in circulating Cort. Plasma Cort levels in response to capture and handling increased with age, and the age effect was also reported in ACTH-treated birds, supporting in both cases the Developmental Hypothesis (Wada et al. 2007). However, the increase in circulating Cort caused by the injection of ACTH was similar across nestling ages, indicating that the effect of age reflected a gradual increase in the secretory capacity of Cort by the adrenal tissues, as suggested by our results on white storks and in agreement with the Maturation Hypothesis. Despite the youngest white-crowned sparrow nestlings were almost unable to elevate plasma

Cort in response to capture and handling, Cort levels readily increased following ACTH treatment (Wada et al. 2007). Such a reduced response to handling in young birds may indicate that there is an upstream control in the HPA axis on the production of Cort, for example, a lower sensitivity of the hypothalamus to sensory input or reduced secretory capacity of the pituitary and hypothalamus, related to their small size. The latter is, in fact, the mechanism postulated to explain the post-natal development of the stress response in altricial rodents, whose cytological and morphological development of the HPA axis, provides favorable evidence for the Maturation Hypothesis at three levels: adrenal tissues, pituitary and hypothalamus (Sapolsky and Meaney 1986). At birth, the adrenal cortex is able to respond to ACTH but due to its small size, the synthesis and release of Cort is significantly lower compared to older ages, and the adrenal cortex develops progressively during the growth of the individual until reaching adult size. In addition, the amount of corticotropin-releasing hormone (CRH) in the hypothalamus is considerably lower at early ages of post-natal growth, and the cellular organization of the pituitary continues after birth and throughout the growth of the individual, thus showing an age-related increase of its ACTH secretory capacity (Sapolsky and Meaney 1986). This process of maturation of HPA axis tissues during postnatal development would act as a quantitative protection mechanism against exaggerated stress responses (i.e., adult-like Cort elevations) during a life history stage when the physiological and behavioral abilities to deal with disturbances are limited. In other words, the growth and maturation of the HPA axis tissues seem to limit the secretory capacity of Cort in response to stress, thus avoiding over-exposure to Cort and its potential negative effects on the organism.

In summary, our results provide experimental evidence that the age-related pattern of response to stress in wild storks is not the consequence of a gradual attenuation in the intensity of the negative feedback, and it likely reflects a progressive maturation of the HPA axis. Although a gradual maturation may be the underlying mechanism explaining the stress hyporesponsive period widely reported in birds during post-natal development, there is a marked lack of studies testing the hypotheses and predictions we presented here, and we encourage further experimental tests in other bird species. Since down-regulation of the stress response is typically observed in other stages of the avian life cycle (e.g., during migration, molt or reproduction) we also encourage further tests on the proximate mechanisms and the associated predictions we proposed here.

Declarations

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Permits- The experimental procedures were approved by the CSIC Ethical Committee and the Andalusian Committee of Animal Experimentation (refs. CEBA-EBD-11-24/12-39) to comply with Spanish and European legislation on the protection of animals used for scientific purposes.

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Author contributions- F. T-M, J.B, S.C and T.A.M conceived the ideas and designed methodology; F. T-M, J.B and S.C collected the data; F. T-M and J.B analyzed the data; F. T-M, J.B and S.C led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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- Supplementary material (Appendix JAV-02091 at <www.oikosoffice.lu.se/appendix>). Appendix 1 and Appendix 2.

Figure legends

Figure 1. Summary of hypotheses and predictions explaining the development of avian stress

responses. Panel (a) represents STRESS-Cort levels in relation to nestlings' age. The *Developmental Hypothesis* (line A) predicts an age-related increase in STRESS-Cort levels in unmanipulated or saline (SAL)-treated altricial nestlings (older nestlings should display more robust stress responses).

Experimental treatment with dexamethasone (DEX; arrow B) typically induces a negative feedback on the HPA axis, resulting in overall decreased STRESS-Cort levels. According to the *Maturation Hypothesis* (line C) the intensity of negative feedback would be independent of age: STRESS-Cort levels in DEX-treated birds will be lower compared to SAL-treated individuals, and both groups should display similar age-related patterns (resulting in line A and line C being parallel). Alternatively, the *Negative Feedback Attenuation Hypothesis* (line D) predicts an age-related decrease in the intensity of negative feedback.

Therefore, STRESS-Cort levels in DEX-treated birds should be lower than in SAL-treated individuals, and both groups will display different age-related patterns (i.e., line A and line D should not be parallel).

The reduction in STRESS-Cort levels will be maximal in the youngest birds and minimal in the oldest individuals (i.e. young and old birds will display enhanced and reduced feedback intensity, respectively).

Panel (b) represents intra-individual changes in STRESS-Cort levels following DEX-treatment (i.e. DEX-treated minus SAL-treated, STRESS-Cort levels; arrow B) and in relation to nestlings' age. The *Maturation Hypothesis* (line C) predicts no age effects (i.e. constant intensity of the negative feedback across nestlings' age), whereas the *Negative Feedback Attenuation Hypothesis* (line D) predicts an age-related decrease in the intensity of negative feedback, and therefore a positive effect of age on STRESS-Cort change.

Figure 2. Time-related changes in plasma Cort levels in nestling white storks in relation to experimental treatment. Plasma Cort levels (ng mL^{-1}) at different times post-capture (X-axis) in birds treated with dexamethasone (DEX; grey circles) or saline (SAL; open circles). Error bars represent the mean $\pm$ s.e.m. both before (0-30 min) and after (65-95 min) experimental injection. Groups sharing the same lowercase letter showed statistically indistinguishable Cort levels.

Figure 3. Effects of age on plasma Cort levels across time and in relation to experimental group.

Plasma Cort levels (ng mL^{-1}) as a function of nestlings age (X-axis) at different times post-capture (i.e., 0, 30, 65 and 95 minutes) for birds treated with dexamethasone (DEX; grey circles) or saline (SAL; open circles). Lines represent Cort values predicted by the best model for SAL-treated (dashed lines) and DEX-treated birds (solid lines), both before (0-30 min) and after (65-95 min) experimental injection.

Figure 4. Intra-individual changes in plasma Cort levels following DEX treatment, in relation to nestlings age. Intra-individual changes in STRESS-Cort levels after injecting DEX (i.e., DEX-treated minus SAL-treated, STRESS-Cort levels; all times post-treatment combined) as a function of nestlings age (X-axis). The line represents predicted values from the best model (which only contained the intercept), selected through AICc .

Table Legends

Table 1. Comparison of models aimed at testing whether developmental changes in Cort secretion in birds occur as a consequence of an age-related attenuation of negative feedback mechanisms (Negative Feedback Attenuation Hypothesis) *versus* growth and maturation of the HPA tissues (Maturation Hypothesis). The hypotheses were tested in nestling white storks using inter-individual (a) as well as intra-individual (b) approaches. Model selection was based on second-order Akaike Information Criterion (AICc).

Approach	Response (N)	Model	Tested effects	K	AICc	$\Delta AICc$
a (inter-individual*)	Plasma Cort, ng ml ⁻¹ (N=370)	a1	Sampling time	17	2953.17	0.0
			Treatment			
			Age			
			Sampling time X treatment			
			Sampling time X age			
		a2	Sampling time	21	2958.99	5.82
			Treatment			
			Age			
			Sampling time X treatment			
			Sampling time X age			
b (intra-individual**)	Plasma Cort, ng ml ⁻¹ (N=32)	b1	Null model (only intercept)	4	277.74	0.00
		b2	Age	5	279.92	2.18
		b3	Sampling time	5	280.57	2.83
		b4	Age	6	282.97	5.23
			Sampling time			
		b5	Age	7	285.67	7.93
			Sampling time			
			Age X Sampling time			

*General Linear Mixed Models always considered year, nest, brood and individual as random terms.

** General Linear Mixed Model always considered brood and individual as random terms.

Table 2. Summary of the best model (Model a1 in Table 1) explaining age-related changes in plasma Cort levels (ng mL⁻¹; N=370) during a stress series in storks subjected to either dexamethasone or saline treatment. The GLMM was constructed using a normal distribution of errors and identity link function, with year, nest, brood and individual as random terms.

Parameter	Estimate ($\pm$ s.e.m.)	<i>Chi</i> ² (df)	<i>P</i>
Intercept*	11.20 ($\pm$ 14.77)	0.58 (1)	0.45
Sampling time (30 γ)	2.76 ($\pm$ 17.86)	16.18 (3)	<0.01
Sampling time (65 γ)	-32.55 ($\pm$ 17.86)		
Sampling time (95 γ)	-58.64 ($\pm$ 17.86)		
Treatment $\blacklozenge$	-1.42 ($\pm$ 2.59)	0.30 (1)	0.58
Age $\clubsuit$	-0.07 ($\pm$ 0.25)	0.09 (1)	0.77
Sampling time (30 γ) X treatment $\blacklozenge$	2.94 ($\pm$ 3.47)	26.11 (3)	<0.01
Sampling time (65 γ) X treatment $\blacklozenge$	-6.16 ($\pm$ 3.47)		
Sampling time (95 γ) X treatment $\blacklozenge$	-13.37 ($\pm$ 3.47)		
Sampling time (30 γ) X age $\clubsuit$	0.51 ($\pm$ 0.31)	33.58 (3)	<0.01
Sampling time (65 γ) X age $\clubsuit$	1.24 ($\pm$ 0.31)		
Sampling time (95 γ) X age $\clubsuit$	1.63 ($\pm$ 0.31)		

* Estimates for baseline samples and saline treatment included in the intercept.

$\blacklozenge$ Estimate for DEX-treated group.

$\clubsuit$ Days post-hatch