

# Age-related variation in the adrenocortical response to stress in nestling white storks (*Ciconia ciconia*) supports the developmental hypothesis

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## Abstract

The post-natal development of the adrenocortical response to stress was investigated in European white storks. Sixty wild nestlings aged 24–59 days old were subjected to a standardized capture and restraint protocol, and the time-course pattern of the response to stress was assessed through determination of circulating corticosterone in blood samples collected at five fixed times during the 45-min period following capture. The time course of the response was best fit to a third-order function of handling time, and showed a strong effect of age. Although age did not affect baseline titers and all birds showed a positive post-capture increase in circulating corticosterone, age had a positive effect on the relative increase from baseline titer, the recorded time to reach maximum level, and the acute concentration after 10 min following capture and restraint. While young nestlings displayed very little response to capture, the response near fledging resembled the typical adrenocortical pattern widely reported in fully developed birds. Our results concur with those found in altricial and semi-altricial species, and suggest that non-precocial birds follow a similar mode of development of the hypothalamic–pituitary–adrenal (HPA) axis. The fact that HPA sensitivity to stress is functional suggests that young storks gradually develop emergency responses of adaptive value and are able to overcome acute perturbations in spite of their parental dependence, at least during the last two-thirds of post-natal development. According to the Developmental Hypothesis, such gradual changes would allow nestlings to respond to perturbations as a function of the specific behavioral and physiological abilities of their age. The potential sources of stress that nestlings have to face during development (i.e., weather conditions, dietary restrictions, and social competition) are discussed according to developmental changes in behavioral and physiological abilities.

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## 1. Introduction

In birds, short-term activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to a suite of perturbations (e.g., harsh weather, decreased food resources, and predators; Wingfield and Silverin, 2002) results in the release of corticosterone (cort) into the blood. Elevated cort triggers emergency responses such as changes in locomotor activity (Astheimer et al., 1992; Breuner et al., 1998), decreased nocturnal oxygen consumption (Astheimer et al.,

1992), lipogenesis (Gray et al., 1990; Holberton, 1999; Jenni et al., 2000; Piersma et al., 2000), increased foraging (Bray, 1993; Koch et al., 2002, 2004; Wingfield et al., 1990), and mobilization of body energy resources (Jenni et al., 2000; Wingfield et al., 1995). These responses redirect animals to a life-saving state (“emergency life-history stage” Wingfield et al., 1998) allowing them to overcome the source of stress and recover homeostasis in the best possible physical condition. Moreover, the inability of individuals to adjust their physiology and behavior to unpredictable perturbations may elevate their circulating cort to chronic levels, with detrimental consequences to cognitive ability, growth, immune defenses, body condition, reproduction, and survival (reviewed in Kitaysky et al., 2003; Sapolsky et al., 2000).

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Birds modulate baseline and stress-induced glucocorticoid levels to meet the specific physiological and behavioral requirements associated with different life-history stages. For example, according to the Migration Modulation hypothesis, migrating birds elevate baseline cort to facilitate migratory fattening while corticosterone stress response is reduced to avoid catabolism of skeletal muscle (Holberton, 1999; Holberton et al., 2000; Jenni et al., 2000; Long and Holberton, 2004; Piersma et al., 2000). During breeding, baseline and stress-induced cort levels are also modulated as a function of the different reproductive stages (e.g., mating, laying, and brooding, Lormée et al., 2003; O'Reily and Wingfield, 2003; Blas et al., 2006), and specific adaptations such as the parental hyperphagia exhibited by brooding doves are facilitated by elevated baseline cort (Koch et al., 2002, 2004).

Although there is a growing body of literature on the role of cort in adult birds, the function of the HPA axis and associated responses to stress during development has received little attention until recently. Adrenocortical responsiveness to standardized stressors such as human handling seems highly variable among bird species during ontogeny. While mallards (*Anas platyrhynchos*) can rapidly elevate plasma cort to acute levels in response to capture and handling as soon as they hatch (Holmes et al., 1990), northern mockingbirds (*Mimus polyglottos*) show little, if any, cort response to handling during life as nestlings (Sims and Holberton, 2000). Intermediate stages of responsiveness during development as nestlings, with age-related increases in stress-induced cort elevations, have been reported in American kestrels (*Falco sparverius*, Love et al., 2003a) and Magellanic penguins (*Spheniscus magellanicus*, Walker et al., 2005). Such inter-specific variability has been postulated to reflect the species-specific life-history strategy along the precocial–altricial spectrum (Starck and Ricklefs, 1998), leading to the formulation of the Developmental Hypothesis (e.g., Kitaysky et al., 2003; Schwabl, 1999; Sims and Holberton, 2000). The Developmental Hypothesis is based on the following arguments: (1) because the physiological and behavioral capacity of a developing bird to overcome a perturbation depends on the degree of post-hatching parental dependence, the physical bond to a nest, and the capacity to thermoregulate, locomote, and forage, young birds may be limited in their abilities to perform many of the adult-like responses to overcome stressful situations (see Starck and Ricklefs, 1998). As a consequence (2) an adult-like adrenocortical response to stress may expose young to chronic cort elevations, with potentially deleterious consequences for development such as reduced growth, inadequate thyroid function, and reduced cognitive capabilities (Hayward and Wingfield, 2004; Kitaysky et al., 2003; Kühn et al., 1998). According to these arguments, the Developmental Hypothesis predicts that inter-specific variation in the adrenocortical response to stress of hatchlings reflects the species-specific degree of altricial development. Therefore, true altricial species such as northern mockingbirds that hatch almost naked, blind, unable to locomote or

thermoregulate, and depend on their parents for food and protection are expected to show little or no response to stress as nestlings (i.e., hyporesponsive period). At the other extreme of the developmental spectrum, precocial species hatch with sight, covered with down and are able to thermoregulate, locomote, and feed independently of their parents, and therefore the hypothesis predicts full adrenocortical function in response to stressors such as human handling during early post-hatching stages, as occurs in 1-day-old hatchling mallards (Holmes et al., 1990). A second prediction that derives from the Developmental Hypothesis at the intra-specific level is a positive association between the adrenocortical response to stress and nestling age. Although during early stages post-hatching non-precocial species are expected to show little response to stress, nestlings develop physiological and behavioral abilities useful in dealing with a perturbation, which may be modulated by the elevation of cort to acute levels. For example, studies performed on nestling seabirds show that cort elevation facilitates begging and aggression, two forms of adaptive responses to cope with food stress in early life (Kitaysky et al., 2001b, 2003). Therefore, at least in non-precocial species, the adrenocortical response to stress of nestlings is expected to increase with age, eventually reaching adult-like responses near fledging, when the young are able to show many of the adult-like behavioral and physiological responses in emergency situations. This prediction is supported by studies performed on nestlings of semi-altricial species such as the American kestrel (Love et al., 2003a) and Magellanic penguin (Walker et al., 2005).

To date, studies on developmental changes in HPA stress response of nestlings are limited to only three non-precocial species (see references above and Love et al., 2003b; Sockman and Schwabl, 2001). Additional data related to the Developmental Hypothesis during nestling stages come from studies showing age-related changes in baseline cort (Kern et al., 2001; Schwabl, 1999), or studies focused on other topics (e.g., anthropogenic disturbances, sibling competition, body condition, nutritional state, and begging behavior) that report age effects in the HPA stress response with developmental covariates: boobies *Sula* spp. (Nuñez de la Mora et al., 1996; Ramos-Fernández et al., 2000; Tarlow et al., 2001), hoatzins *Opisthocomus hoazin* (Mullner et al., 2004), and kittiwakes *Rissa* spp. (Kitaysky et al., 1999, 2001a,b, 2003). In addition, a number of studies report age effects on baseline cort titers during the periods, of fledging and natal dispersal (Belthoff and Dufty, 1995, 1998; Dufty and Belthoff, 1997; Heath, 1997; Kern et al., 2001; Love et al., 2003a,b). Considering that over 7600 avian species across 148 families have non-precocial modes of development (del Hoyo et al., 1994; Starck, 1993), and the wealth of variability in life-history strategies within the precocial–altricial spectrum (Starck and Ricklefs, 1998), the topic calls for additional research from a wider range of species.

Sex is another endogenous trait that could potentially explain intra-specific variation in the development of the

HPA response to stress. Sex differences in glucocorticosteroid levels have been reported in adult birds, and are thought to be the result of the different roles of males and females during breeding (Lormée et al., 2003; O'Reily and Wingfield, 2003). Although nestlings are unable to reproduce, and so potential sex differences in adrenocortical response to stress must be attributed to other factors, pre- and post-natal exposure to glucocorticosteroids can interfere with the sexual differentiation of behavior (Ward, 1972), and result in the permanent modification of offspring traits (Montano et al., 1991; Roberts et al., 1997; Sachser and Kaiser, 1996; Shapiro and Insel, 1990). Furthermore, neonatal sex steroids can have organizational effects on the function of the HPA axis in male rats (McCormick et al., 1998), suggesting that sexual differences in adrenocortical function may occur during early development, although this has only been tested in two avian species (American kestrel: Love et al., 2003a; Sockman and Schwabl, 2001; canary: Schwabl, 1999).

We investigated post-natal development of the adrenocortical response to stress in white storks (*Ciconia ciconia*), a large ciconiform (3100–3600 g) widely distributed throughout Africa and Europe (del Hoyo et al., 1994). Storks show an altricial type of development (Skutch, 1976), as hatchlings are unable to thermoregulate or locomote, depend completely on their parents for food and shelter, and stay in an open nest during the 60–90 days of nestling growth and development (Redondo et al., 1995; Tortosa and Castro, 2003). However, as hatchlings show a loose downy plumage and open eyes, they may, depending on the classification, be considered as semi-altricial (Nice, 1962), or semi-altricial-1 (Starck, 1993). Previous studies indicate that nestling storks respond to environmental perturbations by modulating their adrenocortical levels, and also that age has a positive effect on acute cort levels (Blas et al., 2005). However, to make the age-specific pattern of HPA response of storks comparable with existing literature, and to discuss ontogenic variation in light of the Developmental Hypothesis, additional information and a higher resolution in the course of a time response to experimental handling is required. In this sense, the determination of circulating cort levels at several time-intervals following exposure to capture allows a better assessment of HPA responsiveness of young than a single measurement taken at a fixed time, as specific parameters shaping the time course of the response (e.g., relative increase from baseline titer, time to reach maximum level, and absolute acute concentration) may depend on age. Our objectives were (1) to establish when during post-natal development cort levels elevate in response to a stressor, and (2) to investigate qualitative and quantitative changes in the response with age. These aims were accomplished by evaluating the ability of nestlings of different ages to release cort in response to a standardized “capture-stress protocol” (Wingfield, 1994). Based on the Developmental Hypothesis, we predicted that corticosterone levels would increase above initial baseline levels more rapidly and/or more robustly with age, to

eventually reach maximal adrenocortical responsiveness to capture and handling close to the age of fledging.

## 2. Materials and methods

### 2.1. Field sampling

Fieldwork was conducted in June 2000 at two breeding sites in southern and mid-western Spain (provinces of Sevilla and Caceres). Selected nests had either two or three nestlings, but blood sampling was always performed on two chicks per nest, which were the oldest and youngest. After accessing a nest with a ladder, storks were captured by hand and immediately brought down to the ground. The first blood sample was taken within the first minute post-capture, and the synchronized work of several workers allowed simultaneous sampling of two birds per nest. Nestlings were held in the shade in open plastic boxes covered with opaque cloth and additional blood samples were collected from the brachial vein at 2, 10, 30, and 45 min after capture. After blood sampling, nestlings ( $N = 60$ ) were banded and their wing chord and body mass were measured before returning them to the nest. All field activities were performed between 8:00 and 11:00 a.m. to minimize potential diel effects. Blood samples were kept in ice coolers until centrifuged (3000 rpm for 10 min) the same day of capture, and plasma was frozen and stored at  $-80^{\circ}\text{C}$ . Nestling age, which ranged from 24 to 59 days, was estimated according to a regression equation of age on wing chord ( $\text{age} = 5.068 + 0.117 \times \text{wing chord}$ ,  $r = 0.99$ ,  $N = 12$ ,  $P < 0.01$ ) calculated with data from nestlings of the Spanish population whose hatching date was known (Chozas, 1983).

### 2.2. Laboratory analyses

Plasma cort was determined through radioimmunoassay following standard methods described elsewhere (Wayland et al., 2002). Antiserum and purified cort for the standards were purchased from Sigma Chemicals; [ $^3\text{H}$ ]cort was purchased from New England Nuclear. Corticosterone measurements were performed on reconstituted organic ethyl ether extracts of the plasma samples. Extraction efficiency was estimated to be consistently greater than 90%, and each extract was measured in duplicate tubes. Average assay precision (assessed by including an internal control with  $0.080 \text{ ng cort ml}^{-1}$ ) was  $0.084 \text{ ng ml}^{-1}$ . The minimum detection limit of the assay was  $0.10 \text{ ng ml}^{-1}$ . Samples were measured over several separate assays, with intra- and inter-assay coefficients of variation of 7.08 and 7.99%, respectively. In the white stork both sexes look alike, and we resorted to molecular sexing of the birds by using the cellular fraction of the blood as a source of DNA. For this analysis, primers 945F, cfR, and 3224R were used, following the method of Ellegren (1996).

### 2.3. Statistical analyses

To test whether age and sex affect the response to stress, and also to study how these factors shape the time-course patterns of cort secretion, we performed repeated measures analyses as well as specific tests on selected parameters of the curves. First, we tested whether nestlings show any adrenocortical responsiveness to our capture and handling. Individual responses were qualitatively classified as either positive or negative depending upon a bird's ability to elevate cort post-capture by 10% above its recorded baseline concentration (i.e., positive response). Lower increases (<10%) or absence of changes were classified as negative responses. The threshold value was decided a priori based on the intra- and inter-assay coefficients of variation of the cort RIAs, which were always below 8%. The percent increase was also analyzed quantitatively, as a function of age, sex, and the interaction age  $\times$  sex, while controlling for potential sources of pseudoreplication (see below).

We then considered the whole response to capture and handling in a repeated measures analysis. Our data matrix comprised 300 corticosterone values, corresponding to 60 birds each subjected to a single stress series (i.e., five blood samples collected at fixed times following capture). Because several observations came from a given individual, corticosterone values

were not totally independent. In addition, the observations of a given individual were not independent from the measures taken from a sibling, or from the other individuals sharing a given colony. Although the potential effects of the specific colony, nest, and individual were not the focus of our study (but see Blas et al., 2005), all these factors had to be controlled to avoid pseudoreplication. To solve this problem, we analyzed our data by means of Generalized Linear Mixed Models GLMMs, using the macro GLIMMIX for SAS/STATS (for details see Breslow and Clayton, 1993; Wolfinger and O'Connell, 1993). Compared to a classical repeated measures ANOVA, data analysis through GLMM allows one to incorporate random, categorical, and continuum effects as independent variables, and thus control for potential pseudoreplication due to the individual, nest, and colony (random variables) while studying the significance and estimating the magnitude of the categorical (sex) and continuous (time, age) variables of interest, as well as their interactions. Because corticosterone observations were normally distributed ( $K-S = 0.037$ ,  $P = 0.2$ ), we used a normal distribution of errors and the default identity link function (see Crawley, 1993; SAS, 1997). The models were constructed following a backward procedure, where the least significant terms were sequentially removed until obtaining a minimum adequate model that only retained significant effects at  $>5\%$  rejection probability (Crawley, 1993; McCullagh and Nelder, 1989). Handling time was also included as a squared term, allowing us to test whether maximum cort values occur in intermediate positions along the 45-min response, through quadratic ( $\text{time}^2$ ) and cubic (interaction  $\text{time} \times \text{time}^2$ ) data fitting. Because in a previous study we already showed that body condition, brood hierarchies and brood size do not affect plasma cort in nestling white storks from two- and three-chick broods (Blas et al., 2005), and in order to focus our analyses on the ontogenic, endogenous (i.e., non-environmental) factors modulating the stress response, the former variables were not included in the models. For statistical purposes, rather than grouping birds in different age categories, age was considered a continuous variable, allowing us to increase the degrees of freedom in the analyses. We also did so because we could not select a priori the age of the chicks in the nests that were accessible for sampling, and because the Developmental Hypothesis predicts gradual age-related changes in cort. Therefore, both in the analyses and in the resulting figures, the effect of age corresponds to a continuum, although we also grouped this variable in discrete categories for illustrative purposes. The significant interactions were always analyzed post hoc, applying the Bonferroni correction with the aim of avoiding a false discovery rate. Finally, we tested age, sex, and interaction effects on maximum cort titers while controlling for other sources of pseudoreplication (i.e., nest and colony) in the GLMMs.

### 3. Results

All birds but one showed a positive adrenocortical response to capture and handling, elevating their cort levels above 10% of the recorded baseline. The percent increase was nonetheless highly variable, ranging from 12 to 980% depending on age (GLMM:  $F_{1,28} = 5.84$ ,  $P = 0.022$ ,  $N = 60$ ), but not sex (sex:  $F_{1,27} = 0.28$ ,  $P = 0.599$ , sex  $\times$  age:  $F_{1,26} = 0.01$ ,  $P = 0.993$ ,  $N = 60$ ). In the population as a whole, maximum cort values for the sampling times taken corresponded to the sample at 45 min post-capture, which was significantly higher than baseline (ANOVA  $F_{1,118} = 121.5$ ,  $P < 0.001$ ) although statistically similar to the sample taken after 30 min of restraint (ANOVA  $F_{1,118} = 107.3$ ,  $P = 0.970$ ). The repeated measures analysis along the full capture and restraint series ( $N = 300$  samples) also revealed a strong effect of age on the time course of the response, which was best fitted to a third-order function of handling time. The final model showed that the time at maximum cort secretion and the absolute maximum titers was dependent on the

Table 1

Results from the GLMM analyzing ontogenic effects (i.e., age and sex) on baseline and stress-induced corticosterone ( $N = 300$  samples), along the 45 min of experimental handling and repeated sampling of 60 birds

	Estimate	St. Error	Test value	<i>P</i>
<i>Random effects</i>				
Bird ( $n = 60$ )	16.7848	6.7169	$Z = 2.50$	0.0062
Nest ( $n = 30$ )	9.4873	7.1781	$Z = 1.32$	0.0931
Colony ( $n = 2$ )	21.9258	33.8683	$Z = 0.65$	0.2587
Residual	41.5586	3.8339	$Z = 10.84$	$<0.0001$
<i>Fixed effects</i>				
Intercept	24.3948	5.9700	—	—
Age	-0.1252	0.1096	$F_{1,234} = 1.30$	0.2547
Sex	—	—	$F_{1,235} = 2.63$	0.1064
Time	0.3203	0.4944	$F_{1,234} = 0.42$	0.5177
Time <sup>2</sup>	-0.05787	0.0160	$F_{1,234} = 13.15$	0.0004
Age $\times$ Time	0.04185	0.0098	$F_{1,234} = 18.18$	$<0.0001$
Age $\times$ Time <sup>2</sup>	-0.00051	0.0002	$F_{1,234} = 5.72$	0.0176
Age $\times$ Sex	—	—	$F_{1,233} = 0.44$	0.5076
Sex $\times$ Time	—	—	$F_{1,234} = 0.22$	0.6373
Sex $\times$ Time <sup>2</sup>	—	—	$F_{1,233} = 0.73$	0.3925
Time $\times$ Time <sup>2</sup>	0.00094	0.0002	$F_{1,234} = 27.00$	$<0.0001$

Test values and associated probabilities are shown for the random effects, the fixed terms that were retained by the final model, and the non-significant fixed effects when excluded during the backwards procedure. The model was constructed using a normal distribution of errors and identity link function.

individual's age (Table 1, Fig. 1B). The youngest nestlings tended to increase baseline levels shortly after capture but slightly decrease thereafter. As they grew older, cort secretion continued for longer times, delaying the time when maximum titers are attained and showing higher concentrations. The model explained a high proportion of the recorded variability in plasma cort (i.e., 75% deviance). Neither sex alone nor sex interacting with age or handling time showed any significant effect on circulating cort (Table 1).

When the analysis was performed separately on each sampling time ( $\alpha = 0.01$  after applying Bonferroni correction), age and sex affected neither baseline cort concentration nor cort values recorded 2 or 10 min post-capture (Table 2). However, after this time age had a positive effect on plasma titers (Table 2, Fig. 2). Maximum cort titers were, regardless of sampling time, higher in older birds (GLMM: age  $F_{1,28} = 13.67$ ,  $P < 0.001$ ) and there was a non-significant trend for sex differences (sex:  $F_{1,28} = 4.18$ ,  $P = 0.0503$ ; age  $\times$  sex:  $F_{1,27} = 0.78$ ,  $P = 0.384$ ).

### 4. Discussion

Stork nestlings responded to our standardized protocol by showing a quantitative change in circulating cort following capture. However, the magnitude of the adrenocortical response to handling was highly variable among individuals, and age had a positive effect on different parameters defining the 45-min time-course pattern of cort secretion, including the relative increase from baseline titers, the time to reach maximum levels, and the absolute acute concentrations after 10 min of handling and restraint. Although our

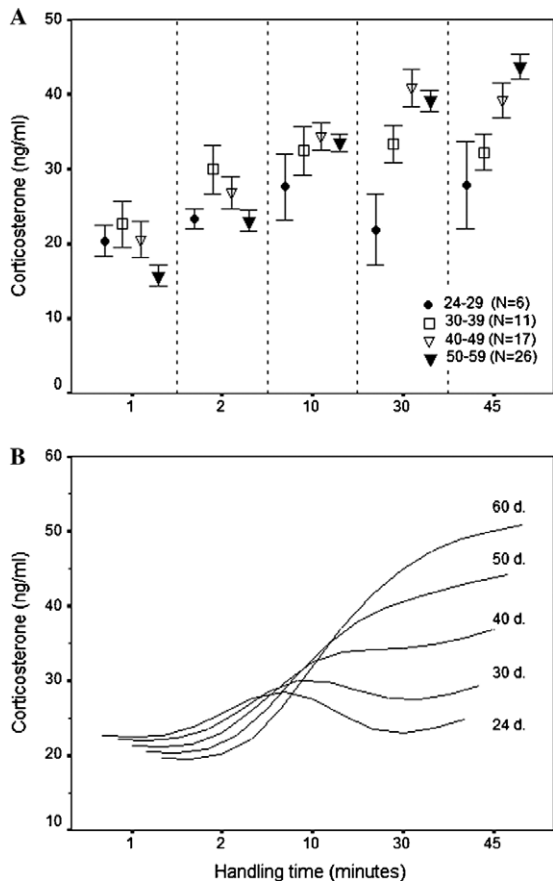


Fig. 1. Circulating corticosterone ( $\text{ng ml}^{-1}$ ) along the time course of the response to capture and handling. (A) Means  $\pm$  SE corticosterone at different time points (as indicated in the X-axis), after grouping the 60 individuals in several age-intervals of similar amplitude (total number of observations:  $N = 300$ ). Note that age (in days) was always considered a continuous variable in the analyses and the represented discrete categories (uncorrected for potential sources of pseudoreplication such as sampling site and nest) were created only for illustrative purposes. (B) Age-related changes in cort values predicted from the GLMM model. Numbers beside figure lines indicate the post-hatching age of the nestlings in days.

sample did not include birds from the earliest developmental stages (to avoid potential negative effects associated with sampling), the gradual age-related pattern we found (Figs. 1A and B) suggests that nestlings below 24 days may show little adrenocortical response to capture. Our results fitted initial predictions of the Developmental Hypothesis: the response to stress developed gradually as chicks aged resulting in a pattern near fledging that resembles typical adrenocortical responses of fully developed birds (i.e., progressive elevations along 45–60 min following capture, Wingfield, 1994).

With regard to the proximate basis for the age-pattern, two studies have provided evidence that the response to stress in altricial birds reflects changes within the hypothalamic–pituitary component of the HPA system, rather than a maturation of the adrenal glands (Sims and Holberton, 2000; Walker et al., 2005). Following intra-jugular injections of adrenocorticotrophic hormone (ACTH), nestling mockingbirds and penguins showed cort levels several fold

Table 2

Results from the GLMM models (A–E) analyzing circulating corticosterone as a function of age and sex, separately at the five different times post-capture ( $N = 60$  per sample time)

Model	Effect	df	F value	P
(A) 1 min post-capture	Age	1,28	0.18	0.6707
	Sex	1,29	0.97	0.3325
	Age * Sex	1,27	0.13	0.7216
(B) 2 min post-capture	Age	1,29	2.17	0.1512
	Sex	1,28	0.00	0.9467
	Age * Sex	1,27	1.18	0.2877
(C) 10 min post-capture	Age	1,29	4.88	0.0353
	Sex	1,28	3.48	0.0727
	Age * Sex	1,27	0.04	0.8440
(D) 30 min post-capture	Age	1,29	18.09	0.0002
	Sex	1,28	0.04	0.8510
	Age * Sex	1,27	2.15	0.1539
(E) 45 min post-capture	Age	1,29	18.48	0.0002
	Sex	1,28	2.12	0.1568
	Age * Sex	1,27	3.28	0.0813

Test values and associated probabilities are shown for the fixed terms that were retained by the final models and the non-significant terms when excluded during the backwards procedure. The models were constructed using a normal distribution of errors and identity link function.  $\alpha$  level was set to 0.01 after applying Bonferroni correction.

above the maximum elevations elicited by capture and restraint, indicating that the adrenal glands were functional and therefore that the maturation processes controlling the age-pattern of the response to stress take place within the hypothalamo–pituitary portion of the HPA axis. The fact that HPA sensitivity to stress is functional when the birds are still in the nest suggests that young storks may gradually show emergency responses of some adaptive value. According to the Developmental Hypothesis, gradual changes would allow nestlings to respond to perturbations as a function of the specific behavioral and physiological abilities that characterize post-hatching age. The questions now are (1) what kind of perturbations would nestlings have to face while in the nest, (2) what kind of responses, adequate to face such perturbations, are acquired gradually during development, and (3) what is the evidence for a corticosterone-mediated effect on the expression of the latter responses.

Young white storks inhabit open nests, normally 1–2 m diameter platforms at the top of trees, poles or buildings, where there is little protection from the elements. Weather conditions represent a real environmental perturbation for young storks as they are known to have a strong effect on nestling mortality (Carrascal et al., 1993; Jovani and Tella, 2004). In cold weather, cort elevations may reduce oxygen consumption that lowers extended metabolic rate, and promote gluconeogenesis and mobilization of body energy stores as is known to occur in adults (Astheimer et al., 1992; Buttemer et al., 1991; Honey, 1990). Furthermore cort elevations modulate locomotor activity in adult birds (Astheimer et al., 1992; Breuner et al., 1998), and so increased

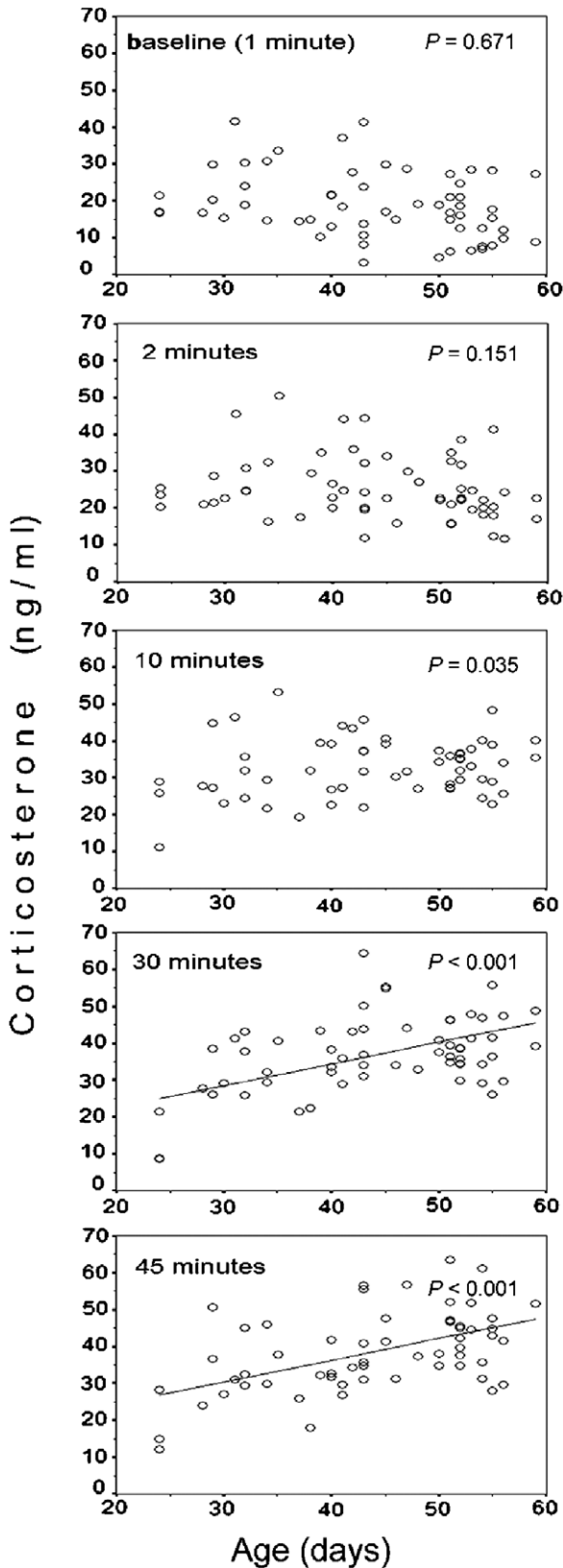


Fig. 2. Association between circulating corticosterone ( $\text{ng ml}^{-1}$ ) and nestling age (in days) for different handling and restraint times. Baseline values correspond to blood samples taken within 1 min following capture.  $P$  values into figures indicate the significance of the age effect and lines represent the predicted values according to the fitted GLMM models.

locomotor activity of nestlings may allow them to select parts of the nest to avoid temperature extremes (Glasse et al., 2006). The ability to regulate body temperature and locomote changes gradually with age. Thermoregulatory capabilities are not fully attained until nestling storks are about 20 days old (Tortosa and Castro, 2003). This is the estimated age when HPA stress response starts to develop (Figs. 1A and B) and although chicks still have limited locomotor activity (Bernis, 1975), up to this age there is one adult permanently at the nest providing shelter. The lack or reduced ability of the younger age groups to show HPA stress response is hypothesized to be an adaptation (Sims and Holberton, 2000; Walker et al., 2005), because their reduced skills to evade the perturbation may lead to chronically elevated cort levels, with detrimental consequences to growth and development (Kitaysky et al., 2003). At 30–40 days of age storks are in the mid-point of development, they are able to stand up and walk around the nest, and their HPA stress response is also intermediate (Fig. 1A). By the end of the nestling period young storks show a strong adrenocortical response similar to adults of many bird species (Figs. 1A and B). At this age nestlings are almost totally covered by a definitive feather coat, fully able to move around the nest platform (Bernis, 1975), and show increased rates of flight exercises (Redondo et al., 1995). Adults leave nestlings unattended for long periods, and may limit the activity at the nest during the day to feeding bouts (Bernis, 1975), suggesting that nestlings are able to meet the challenge associated with weather as well as adults can.

The stress associated with food deprivation constitutes another potential source of perturbation for altricial young. Several studies indicate that circulating cort elevates in nestlings seabirds and penguins in response to food shortages (Kitaysky et al., 1999, 2001a; Walker et al., 2005). The probability of food stress is expected to increase gradually as nestlings age because their energy demands along development increase with body mass. The physiological ability of nestlings to overcome a period of food stress may also improve with age, as increased body mass involves larger muscles and body fat stores which constitute the main source of energy under conditions of limited food supply, and mobilization of energy stores is facilitated by the elevated cort levels triggered by emergency situations (Sapolsky et al., 2000). Nestling storks also display specific behaviors to elicit food delivery from parents (Cramp and Simmons, 1980). The intensity of begging behavior is likely to increase with age, as young improve their motor skills. Furthermore, experimental elevations of cort levels in seabird chicks have been shown to elicit begging behaviors (Kitaysky et al., 2001b, 2003). Therefore, the gradual maturation of the HPA responses to stress could parallel both increased exposure to food stress, and the capacity to accumulate and use body energy stores and perform begging behaviors.

Recent experimental work has also shown that cort plays a role in the expression of aggressive behaviors (Kitaysky et al., 2003), and this association is supported by

observational studies where social hierarchies within a brood correlate with between-sibling differences in circulating glucocorticoids (Love et al., 2003b; Ramos-Fernández et al., 2000; Schwabl, 1999; Tarlow et al., 2001). Although previous evidence suggests that cort does not play a role in the organization of within-brood social hierarchies in white storks (Blas et al., 2005), the strongest expression of nestling aggressive behavior is directed to unrelated, kleptoparasitic nestlings rather than to siblings (Redondo et al., 1995). If nestling HPA response to stress is associated with aggression to repel intruders, we may expect an increase in the intensity of this behavior during the last weeks of nestling development as reported in a previous study (Redondo et al., 1995).

Despite the strong effect of age on the HPA response to stress, baseline cort levels were independent on the degree of post-natal development. Age-related increases in baseline cort have been reported in nestlings of some non-precocial species (e.g., Schwabl, 1999; Love et al., 2003a) and this effect is often explained as a mechanism to facilitate the transition from nestling to fledging stages through the positive effects of elevated baseline cort on locomotor activity, food searching behaviors, and learning processes that may be critical in the preparation to independence (for studies on adrenocortical responses in birds during fledging stages, see Belthoff and Dufty, 1995, 1998; Dufty and Belthoff, 1997; Heath, 1997; Kern et al., 2001; Love et al., 2003b). However, the elevations of baseline cort to facilitate fledging may occur just a few days prior to independence rather than following a gradual age-pattern, and this may explain why we and some other studies (Mullner et al., 2004; Sims and Holberton, 2000; Walker et al., 2005) did not detect such an effect.

In summary, our study demonstrates that the ontogenic development of the HPA stress response in white storks is a gradual process. Although other factors such as the clearance rates of circulating cort and the concentration of binding globulins may play a role in the described patterns (Breuner and Orchinik, 2002; Breuner and Hahn, 2003), our results concur with those found in other altricial and semi-altricial birds and fit the predictions of the Developmental Hypothesis.

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