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ScienceDirect
Hormones and Behavior 53 (2008) 295–305
Hormones and Behavior
www.elsevier.com/locate/yhbeh

Corticosterone metabolites in blue tit and pied flycatcher droppings: Effects of brood size, ectoparasites and temperature

Elisa Lobato [a,*], Santiago Merino [a], Juan Moreno [a], Judith Morales [a,c], Gustavo Tomás [b], Josué Martínez- de la Puente [a], José Luis Osorno [c,†], Alexandra Kuchar [d], Erich Möstl [d]

[a] Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, C/ José Gutiérrez Abascal 2, 28006, Madrid, Spain
[b] Netherlands Institute of Ecology (NIOO-KNAW), Centre for Terrestrial Ecology, PO Box 40, 6666ZG Heteren, The Netherlands
[c] Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, AP 70-275, México D.F.
[d] Institute of Biochemistry, Department of Natural Sciences, University of Veterinary Medicine, Vienna, Veterinärplatz 1, A-1210 Vienna, Austria

Received 22 March 2007; revised 8 October 2007; accepted 19 October 2007
Available online 7 November 2007

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis of birds induces the secretion of corticosterone (CORT) as a response to different ecological variables. In this study we tested experimentally if manipulations of brood size or ectoparasitism led to subsequent differences in the concentration of excreted CORT metabolites of adult and nestling blue tits (*Cyanistes caeruleus*). No significant effect of the manipulation of brood size was detected in adults or nestlings. No significant effect of ectoparasitism was detected in males or nestlings, although females from uninfested nests showed lower concentrations of excreted CORT metabolites. In addition, we analysed if weather conditions had an influence on the concentration of excreted CORT metabolites of blue tits and pied flycatchers (*Ficedula hypoleuca*) breeding in the same forest. We detected no effect of weather conditions on adults, but nestlings of both species showed a negative correlation between their excreted CORT metabolites and the average mean temperatures they were subjected to during their growth. This effect was not found in blue tits in a colder year, suggesting that the sensitivity of the HPA axis to ambient temperature may be subjected to interannual variation. Moreover, we found a positive effect of the maximum temperature on the day of sampling on the concentration of CORT metabolites of blue tit nestlings in one of the years. These results suggest that weather conditions may act as environmental stressors to which the HPA axis of blue tit and pied flycatcher nestlings may be sensitive.

Keywords: Corticosterone; Droppings; Ectoparasites; Brood size; Weather conditions; Cyanistes caeruleus; Parus caeruleus; Ficedula hypoleuca

Introduction

The endocrine response to stress includes the secretion of glucocorticoids as a modulator mechanism of physiological processes directed to overcome the stressful events. The hypothalamic-pituitary-adrenal (HPA) axis is known to respond to different ecological variables which can generate stressful situations. Thus, under stress, the corticotropin-releasing hormone (CRH) and vasopresin (AVP) are secreted by the hypothalamus and stimulate the secretion of adrenocorticotropic hormone (ACTH), which regulates the synthesis of glucocorticoids. In birds, the main steroid secreted under this situation is corticosterone (CORT). Among the actions modulated by glucocorticoids we can mention their cardiovascular effects, their suppressive actions on immune and inflammatory reactions, their effect on mobilization of lipids and proteins or their action stimulating appetite or their inhibitory effect on reproduction (see Sapolsky et al., 2000 for a review). Basal concentrations of glucocorticoids, secreted constitutively, can also exert some of these actions and are involved in the maintenance of basic life processes (Landys et al., 2006).

Feeding behavior constitutes a daily activity necessary for survival that seems to be affected by glucocorticoids. Food availability and internal energy stores are factors that can influence feeding behavior and may determine the level to

* Corresponding author.
E-mail address: elobato@mncn.csic.es (E. Lobato).
† José Luis Osorno died on 2 April 2004. We mourn the loss of a bright behavioural ecologist and excellent colleague.

0018-506X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2007.10.010

which glucocorticoids vary (Landys et al., 2006). In adult birds, it has been reported that corticosterone facilitates foraging behavior under nutritional stress (Astheimer et al., 1992) and increases under uncertain food availability (Reneerkens et al., 2002). For nestling birds, food availability may depend on food shortages and on competition between nest mates for the food brought by the parents. The size of the brood may be determinant in the availability of food as is demonstrated by a number of studies in which the body mass of the nestlings is negatively associated to the brood size (Martin, 1987; Merilä and Wiggins, 1995; Moreno et al., 1997; Sanz and Tinbergen, 1999; Saino et al., 2001). Thus, it could be expected that the HPA axis may be responsive to manipulations of brood size, given that the secretion of glucocorticoids may help to mobilize energy to overcome food shortages (Kitaysky et al., 1999; Sapolsky et al., 2000) and also increase begging frequencies and parental provisioning rates (Kitaysky et al., 2001a). Accordingly, corticosterone may be secreted as a response to the stress imposed by enlarged broods, as it is shown in the study of Saino et al. (2003) in nestlings of barn swallows (*Hirundo rustica*). Enlarged broods may also result in an increase on corticosterone levels on parents as a reflect of increased parental effort (see Ilmonen et al., 2003). Thus, a first objective of the present study was to evaluate whether a brood size manipulation experiment performed in a population of blue tits (*Cyanistes caeruleus*) in 2003 (see Merino et al., 2006) had an effect on the corticosterone levels of adults and nestlings.

Parasitism constitutes a stressor that may have consequences for life-history traits in wild birds (Loye and Zuk, 1991). The susceptibility to parasite infections may be associated to the immunosupression ocurred during chronic stress. The glucocorticoids secreted under prolonged stressful situations may exert immunosupressive and anti-inflammatory actions that may result in impaired disease resistance (Sapolsky et al., 2000; Sapolsky, 2002). As a consequence, under non-manipulated conditions, a positive correlation between parasitism and glucocorticoid levels might be reflecting the higher susceptibility to infection induced by potential chronic stress. On the other hand, release of glucocorticoids under parasitism may help the organism to face this pressure by increasing energy uptake through increased feeding behavior or mobilization of lipids (Raouf et al., 2006). In fact, a recent experiment by Raouf et al. (2006) has shown that free living cliff swallows *Petrochelidon pyrrhonota* are responsive to ectoparasite loads and colony size, with higher levels of corticosterone in individuals from parasitized nests and large colonies. Also, a positive correlation between the degree of infestation with ticks and baseline CORT levels of red-legged kittiwakes was detected by Kitaysky et al. (2001b). Other consequences of parasites have been evaluated in relation to modifications of behavior in order to compensate the deleterious effect of parasitism (Loye and Zuk, 1991; Simon et al., 2005). Increased parental provisioning seems to be a compensatory strategy in parasitized bird nests (Hurtrez-Boussès et al., 1998; Bouslama et al., 2002; Merino et al., 1998a,b), although evidences exist for variation in this behavior (Møller, 1994; Tripet et al., 2002). Thus, a second objective of the present study was to evaluate the effects of ectoparasitism on corticosterone levels and compensatory behavioral responses. Thus, we manipulated ectoparasitic load in nests of a free-living population of blue tits and measured if parasitism affected corticosterone levels of birds and parental provisioning behavior.

As a third objective, we evaluated if weather conditions function as ecological variables to which the HPA axis of birds may respond. Physiological changes orchestrated by glucocorticosteroids may be a useful mechanism to cope with perturbations of the environment, such as climate changes (Wingfield, 2003). As corticosterone may favour energy mobilization, via its stimulatory effects on glycogenolysis, lipolysis and proteolysis (Sapolsky et al., 2000) an increased adrenal activity under cold weather may have a role in the thermoregulation of birds. This possibility is suggested by the negative covariation of corticosterone levels and ambient temperature found by Frigerio et al. (2004) in Greylag geese (*Anser anser*). Moreover, several studies on birds have detected an increase in corticosterone levels as a response to severe weather episodes (Wingfield, 1984; Astheimer et al., 1995; Romero et al., 2000). Therefore, climatic variables (the maximum temperature registered on the day of sampling, the average mean temperature of the nestling period and the number of days of precipitation) were evaluated in this study as possible predictors of corticosterone levels of adult and nestling blue tits and pied flycatchers (*Ficedula hypoleuca*).

Methods

All procedures conform to the requirements of animal welfare and conservation of Spanish laws.

Species and study area

The blue tit (*Cyanistes caeruleus*) is a small (10–11 g) hole-nesting passerine of European woodlands. It is a resident bird, which adapts readily to breeding in nest boxes. Egg laying in central Spain typically begins in late April, clutch sizes range from 4 to 14 eggs with a mean of 9 eggs and the number of fledglings averages 7. Females incubate and brood the chicks alone and both sexes feed the young (Potti et al., 1988; Moreno et al., 1996; Fargallo and Johnston, 1997). Similarly, the pied flycatcher (*Ficedula hypoleuca*) is a small (12–13 g) hole-nesting passerine of European woodlands. It is a summer visitor, which adapts readily to breeding in artificial nest-boxes. Egg laying in the study population typically begins in late May and clutch sizes range from 2 to 7 eggs with a mode of 6 eggs (mean 5.73) (Sanz and Moreno, 1995). Nest-boxes are periodically inspected and the dates of clutch initiation, clutch sizes and hatching dates (1 = 1 April) are determined.

The study was conducted in 2003 and 2005 in a deciduous forest of Pyrenean oak *Quercus pyrenaica* at 1200 m a.s.l. in the vicinity of La Granja, Segovia province, central Spain (40° 53′ N, 4° 01′ W.). Nest-boxes (125 × 17 mm bottom area) are cleaned every year after the breeding season.

In 2005 we visited nests on two consecutive days and we collected fresh droppings of nestlings in both visits. An effect of the visit of the previous day could be expected in the concentration of excreted CORT metabolites of nestlings the following day, thus, age of nestlings was considered in the analyses. In addition, previous studies have detected age-related changes in baseline CORT in nestlings of altricial and semi-altricial species (Schwabl, 1999; Kern et al., 2001; Love et al., 2003). Also, adrenocortical responses to stress have been shown to increase with age in nestlings of white storks (*Ciconia ciconia*) (Blas et al., 2006) and American kestrels (*Falco sparverius*) (Love et al., 2003). Adults of blue tits and pied flycatchers were captured at the nest boxes using traps activated when parents got inside the nest-box to feed the nestlings. They were held in cotton bags before ringing and measurement. Some birds defecated during handling. The whole manipulation did not take longer

than 10–15 min. In 2005 and in order to increase the number of droppings of adults collected, individuals that did not defecate during the process of ringing were held in a wooden box for aproximately two min. In some cases, birds defecated before release and allowed the collection of faeces from aluminium foil placed on the bottom of the box In pied flycatchers, adults were weighed to the nearest 0.1 g and their tarsus length measured to the nearest 0.01 mm (apparatus precision) with a digital calliper according to Svensson (1984), when nestlings were 11 days old. Similarly, nestlings were weighed when they were 11 days old and they were weighed and measured when they were 12 days old. The same number of visits to the nests were done in all nests. In blue tits, measurements of adults were taken when their nestlings were 13 days old. In 2003, nestlings were weighed and measured when they were 13 days old, while in 2005 nestlings were weighed when they were 12 days old and, again, weighed and measured when they were 13 days old. Residuals of the simple regression of body mass on tarsus length were used as an estimate of body condition. Age of adults, yearlings or older, was estimated according to Svensson (1984) or previous ringing.

Brood size manipulation experiment (Blue tits)

In 2003, a brood size manipulation experiment was performed during the breeding season to evaluate the effects of parental effort on physiological stress of female blue tits (Merino et al., 2006). Three days after hatching, two nestlings were transferred from one nest (reduced nests) to another (enlarged nests), while a third nest of the same hatching date and a similar brood size was maintained as a control. After the manipulation, mean (±SE) brood sizes of reduced, control and enlarged nests were 6.0 (0.3) (N=29), 7.7 (0.3) (N=29) and 9.9 (0.3) (N=29) respectively. Details of the experiment are described in Merino et al. (2006). Females were captured twice, before the manipulation on day three after hatching, and 10 days later. A blood sample was obtained in each capture from the brachial vein. Therefore, in this brood manipulation experiment we visited all the nests twice (day 3 and day 13 of nestling period). Physiological stress of females was analysed by means of the measurement of stress proteins (heat shock protein HSP60) and immunoglobulin Y (IgY) (details of the procedures are described in Merino et al., 2006). Heat shock proteins play an important role in the repair of damaged proteins in injured cells exposed to stressful environments (Morimoto, 1991). It has been reported that heat shock proteins are synthesized under a variety of stressors (see Sørensen et al., 2003 for a review) including high or low temperatures (Sonna et al., 2002; Martinez et al., 2001) parasites (Merino et al., 1998a,b, 2002; Martinez et al., 1999; Feder and Hofmann, 1999; Tomás et al., 2005), pollution (Eeva et al., 2000) or poor nutrional conditions (Moreno et al., 2002). Thus, HSPs are also called stress proteins and considered as a valid measure of physiological stress (Buchanan, 2000; Sørensen et al., 2003). Immunoglobulin levels show a negative association with HSP levels in peripheral blood (Tomás et al., 2005) and appear therefore to respond to physiological stress at cell level. The results of the effect of the experiment on maternal effort and physiological stress of females and nestlings are published elsewhere (Merino et al., 2006). In the present study, we measured the concentration of excreted corticosterone metabolites of adults and nestlings of the experimental broods. Fresh excreta of adults and nestlings were collected on day 13 of the nestling period. No excreta were collected on day 3 of nestling period.

Fumigation experiment (Blue tits)

During the breeding season of 2005, a fumigation treatment was performed in nests of blue tits. Nests were paired according to laying date (±1 day) and brood size (± one chick) and randomly assigned to one of the two experimental groups of the treatment. On days 3, 7 and 11 after hatching we sprayed the nest material with either an insecticide solution (Stockade ©, Fort Dodge Veterinaria, S.A., Vall de Bianya,132 Girona, Spain) comprising 0.5% Permethrin and 1% Piperonil butoxide (hereafter fumigated nests) or water (hereafter control nests). Nestlings were removed from the nests before the application of the treatment and replaced after the spraying of the insecticide or the water. This insecticide has been previously used in our population without negative effects on birds (Tomás et al., 2007). On day 20 after hatching, when all nestlings had already fledged, nests were collected for ectoparasite quantification. The intensity of infection by mites (*Dermanyssus gallinoides*) and fleas (*Ceratophyllus gallinae*) was determined by means of a Berlese funnel and a magnifying glass under which the number of individuals of each type of arthropod was estimated as described in Merino and Potti (1995). Later, the nest material was dismantled over a white piece of paper in order to count the total number of pupae of blowflies (*Protocalliphora azurea*) (Merino and Potti, 1995). Adults were captured when nestlings were 3 days of age (hatching day = 0) and both members of the pair were equipped with a uniquely identified transponder glued to two colour bands. In sum, 59 males and 68 females of blue tits were fitted with transponders. On day 12, a datalogger (Trovan, EID Iberica, Madrid, Spain) connected to an antenna was attached to the nest box entrance during an average (±SD) of 2.84±1.66 h (N=42), not including the first half hour to allow birds to get used to the datalogger. We used this recording system to register parental visits to the nest box and estimate parental provisioning rates per chick per hour. Adults were recaptured on day 13 and the transponders were unglued and the colour bands taken off. Therefore we visited all the nests on day 3, 7 and 11 (to spray the nests with insecticide or water solution) and on day 12 (to connect the data logger on the nest box entrance) and on day 13 (to capture adults). On this day, fresh excreta of adults were collected. Fresh excreta of nestlings were collected on days 12 and 13.

Weather conditions (Blue tits and pied flycatchers)

Weather data were available from the nearest meteorological station at Embalse del Pontón Alto, which is approximately 3 km from the study area. As a measure of the temperature on the day of sampling, we used the maximum temperature (T) provided by the station, given that the maximum T is a more representative measure of diurnal temperatures than the minimum or the mean daily temperatures. We used the average of the daily mean temperatures of the nestling period (the previous 10 days before the samples were collected) as a measure of the mean environmental temperature during the growth of the nestlings. We used 10 days in order to homogenize the data base because pied flycatcher nestlings sampled on day 11 only experienced 10 days of life before the samples were taken and the blue tit nestlings experienced at least this period of time. To evaluate a possible effect of rainfall on the concentration of excreted CORT metabolites we used the number of days of precipitation during the nestling period of blue tits and pied flycatchers per nest. To evaluate differences in weather conditions in the blue tit nestling periods of 2003 and 2005, mean values of maximum T, average mean T and days of precipitation for each nest were used.

Measurement of faecal corticosterone metabolite

We used non-invasive measures of corticosterone metabolites in excreta of adult and nestling blue tits and pied flycatchers. Steroid metabolites measured in excreta constitute an integrated measure of steroid levels over a long period of time (Goymann, 2005) and may provide an accurate measure of long-term glucocorticoid levels (see Millspaugh and Washburn, 2004). This measure constitutes a very useful technique for hormone studies in wild birds because it avoids problems induced by a rapid increase of corticosterone after handling of birds (Romero and Romero, 2002). In small birds, the study of droppings as a source of information of hormonal status is particularly useful due to the small volume of blood that can be obtained and the analytical disadvantages of small volumes of plasma. We have previously validated the measurement of corticosterone metabolites in blue tits and pied flycatchers droppings as required (see methods and Möstl et al., 2005; Goymann, 2005; Touma and Palme, 2005).

To validate the measurement of excreted CORT metabolites in these species, stimulation of adrenocortical activity was performed by adrenocorticotropic hormone (ACTH) administration. The use of ACTH to stimulate the secretion of steroids by the adrenal gland is a method widely used to validate the non-invasive technique of measurement of steroids metabolites in mammals and birds (reviewed in Touma and Palme, 2005). Injected ACTH induces the secretion of adrenal steroids and can mimic the action of stressful events that induce the release of stress hormones. In birds, the main glucocorticoid present in plasma is corticosterone and it is expected that an ACTH injection would increase to a large extent the concentration of corticosterone in plasma. Initial

excreta were collected immediately after the capture and preceding the ACTH administration. Two male and four female blue tits and three males and one female pied flycatchers were injected subcutaneously with 0.34 pmol of synthetic ACTH (1–24; Calbiochem) in 100 μl saline serum. The data of these individuals were only used for the validation of faecal corticosterone metabolite measurement. The dose of ACTH injected was selected according to previous studies with birds of similar size (Goymann et al., 2002). After the injection, birds were placed in the dark inside a wooden box with an aluminium foil on the base. Every 15 min we checked for new droppings and registered the time interval in which the birds defecated. The aluminium foil was changed every time a dropping was found. We expected to detect a peak of glucocorticoid increase within 1.5 h after the ACTH administration given that gut passages reported for small passerines do not seem to exceed this time (Goymann et al., 2002). Thus, we kept each bird for a maximum of 1.5 h in the box. Excreta were collected in 1.5 ml eppendorf tubes and stored in cool bags with cold-blocks until freezing. The challenge was carried out when nestlings were at least 7 days old and only one member of the pair attending nestlings was tested so the other could raise the chicks alone in case of desertion of the injected bird. All nests were checked after the experiment and in every case nestlings fledged normally. Steroids were extracted with 60% methanol into double-distilled water. Samples were vortexed for 15 min, centrifuged (11.000g, 1 min) and the supernatant collected. The determination of the recovery rate requires the immunoreactive metabolites in their radioactive form in order to estimate the lost of radioactivity after the extraction. However, the immunoreactive metabolites are not available in labeled (or unlabeled) forms and reporting the recovery rate of radiolabelled corticosterone may not reflect the actual recovery of corticosterone metabolites as they may have different polarity (Möstl et al., 2005). A possibility to obtain the radiolabeled metabolites would be to infuse a radiolabeled quantity of corticosterone to the birds and obtain naturally metabolized steroids in their droppings and then obtain their recovery rate afterwards. However, we are not authorized to inject radioactive substances into individuals of wild protected species. Therefore, when the exact extraction efficency cannot be calculated, it is recommended to use simple extraction procedures such as the dilution of samples in methanol–water mixtures (Touma and Palme, 2005; Palme, 2005), because when the steps in the extraction procedure are reduced, the variability among samples caused by extraction losses is minimized (Goymann, 2005). Thus, we have used an extraction that covers a broad polarity range with methanol–water 60% to extract conjugated and unconjugated steroids and remove undissoluble substances from droppings. In addition, because the same storage conditions, homogeneization processes and analyses procedures where conducted for all samples, we are confident that variability among samples due to field and laboratory procedures is minimized in our study as much as possible. Glucocorticoids are extensively metabolized and can be excreted via the gut or via the urine in nonconjugated form or conjugated in more polar substances as sulfates or glucuronides. Thus, enzymatic hydrolysis of a fraction of the samples with a mixture of β-glucuronidase-arylsulfatase was performed to cleave conjugated steroids. 100 μl of the total volume were transfered to new tubes and evaporated under a stream of nitrogen. After evaporation, 100 μl of enzymatic mixture (1:250) in sodium acetate buffer (0.1M, pH 4.8) were added and the tubes were sealed and incubated at 40 °C overnight. Five enzyme immunoassays successully used in previous bird studies were tested with and without hydrolysis of the samples: corticosterone, described by Palme and Möstl (1997) and previously used in geese (Kotrschal et al., 1998; Hirschenhauser et al., 2000), 11-oxoetiocholanolone (described by Möstl et al., 2002 and successfully used in great tits by Carere et al., 2003), 11β-hydroxyetiocholanolone (detailed in Frigerio et al., 2004), tetrahydrocorticosterone (established by Quillfeldt and Möstl (2003) and used by Nakagawa et al. (2003) in Adélie penguins) and cortisone, described by Rettenbacher et al. (2004) and recently used in capercailles (Thiel et al., 2005). In nestlings of blue tits, higher amounts of immunoreactive substances were obtained after hydrolysis of the samples, so in both adults and nestlings, samples were subjected to enzymatic hydrolysis. In pied flycatchers no significant differences in steroid concentrations measured with and without hydrolysis were found, so samples were not subjected to enzymatic hydrolysis. The cortisone assay was selected as it detected a higher baseline-to-peak ratio after ACTH administration as it is shown in Figs. 1 A and B (see Möstl et al., 2005). Two peaks of immunoreactivity were detected after the ACTH challenge. The first peak may correspond to the faster increase in corticosterone produced in urine with respect to the slowest increase in

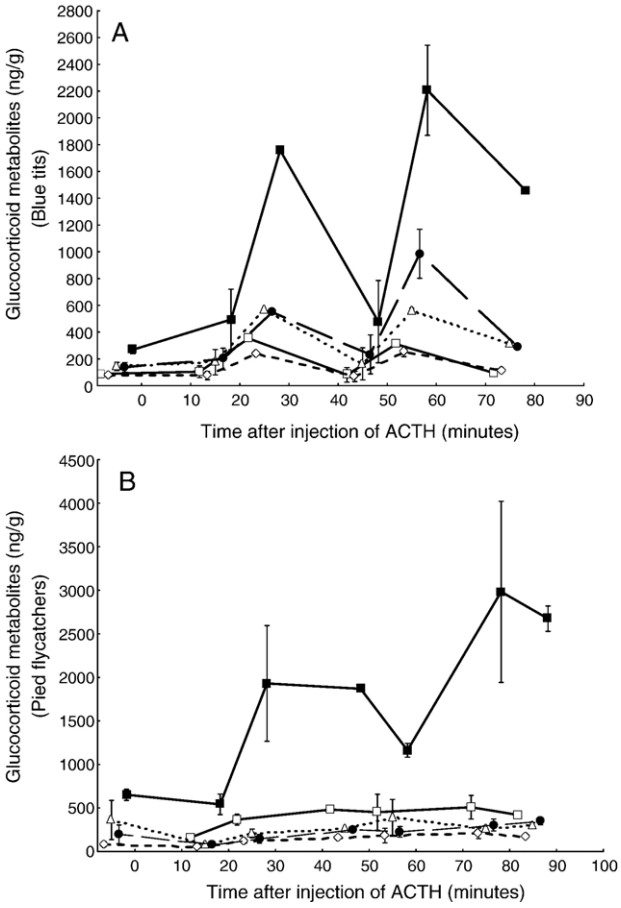

Fig. 1. Profile of excreted corticosterone metabolites in A) four female and two male blue tits and B) three males and one female pied fycatchers injected with ACTH (mean ±SE) measured with five enzyme immunoassays (corticosterone □, 11-oxoetiocholanolone ●, 11β-hydroxyetiocholanolone ◇, tetrahydrocorticosterone △and cortisone ■). The cortisone assay detected a higher baseline-to-peak ratio after ACTH administration. Number of droppings collected: A) N = 17 and B) N = 18. Note that two peaks are detected.

corticosterone in the faeces that may correspond to the second peak. The cross reactions of the cortisone assay are described in Rettenbacher et al. (2004) and they include: 4-pregnene-17α,21-diol-3,11,20-trione (cortisone) 100%; 4-androstene-3,11,17-trione, 30%; 5α-androstane-3,11,17-trione, 20%; 4-pregnene-11β,17α,20α,21-tetrol-3-one, 9%; 4-pregnene-3,20-dione, 2.3%; 5β-androstane-3,11,17-trione, 2.2%, 4-pregnene-11β,21-diol-3,20-dione, 1.8%; 4-androstane-3,17-dione, 0.9%; 5α-androstane-3,17-dione, 0.5%; 5α-androstane-3α-ol-11,17-dione; 5α-androstane-3β-ol-11,17-dione; 5β-androstane-3α-ol-11,17-dione has cross reactions below 0.1%. Thus, the cortisone assay is able to detect steroids with 3,11-dioxo structures. In the corticosterone metabolism of birds a oxidization at position C_{11} may take place (Kučka et al., 2006; Mazancová et al., 2005) leading to metabolites with 3,11-dioxo structures. The fact that other steroids that may increase after an ACTH administration such as progesterone (Möstl et al., 2005) are not susceptible to be oxidized at the position C_{11} (they do not have an oxygen function in that position), makes the cortisone assay a suitable immunoassay to measure corticosterone metabolites non-invasively in avian droppings. Moreover, corticosterone is the main steroid in the avian plasma (see references in Mazancová et al., 2005; Kučka et al., 2006) thus we are confident that the increase in 3–11 dioxo metabolites after the ACTH injection detected by the cortisone assay correspond to corticosterone metabolites. Enzyme immunoassays were performed on anti-rabbit-IgG-coated microtitre plates using the double antibody technique and biotinylated steroids as labels. Details of the procedure of the assay are described in Möstl et al. (2002). The parallelism tests confirmed that the change in absorbance with the dilution factor of pooled samples of blue tits and

pied flycatchers was not statistically different to the change in absorbance of the linear range of standard curve of cortisone ($P > 0.05$, test of homogeneity of slopes). The number and relative proportions of immunoreactive corticosterone metabolites were determined by means of HPLC. Two methanol extracted samples of two different individuals of blue tits and pied flycatchers collected after ACTH administration and, consequently, showing a high immunoreactivity were run in high performance liquid chromatography (HPLC) (Nova-Pak C-18 column). Details of the procedure are described elsewhere (Goymann et al., 2002). Ninety five fractions were collected and corticosterone metabolites of each fraction were quantified with the cortisone assay. Immunoreactive substances with different cromatographic pattern than cortisone were measured (Figs. 2 A and B).

Fresh excreta of adult and nestling blue tits and pied flycatchers were collected in 1.5 ml plastic vials, stored in cool bags with cold-blocks (not exceeding 15 °C) and frozen at −20 °mC (1–5 h later) until laboratory analyses. Droppings were collected when adults and nestlings defecated spontaneously during the process of ringing so fresh excreta could be individually identified. The samples were weighed in the laboratory just before analyses. In birds, voiding of faecal and urinary excreta is simultaneous and it is not possible to separate both parts in the droppings. However, in nestlings, droppings are excreted inside a well formed sac in which it is possible to distinguish a brown part (faeces), a white part (uric acid) and a liquid part, the last two corresponding to urine. A homogenised sample of each nestling dropping was prepared. We separated a small amount of the brown part of randomly chosen droppings of 62 and 86 samples of pied flycatcher and blue tit nestlings respectively, and compared the levels of CORT metabolites with those of their homogenised samples. In both species, CORT metabolites of faecal component of droppings and homogenised samples were highly positively correlated ($P < 0.0001$). Excreta from nestlings included in the study were analysed as a homogenised sample, given that separation of droppings in parts is not practicable for a large number of samples and mixed samples may be more representative of the amount of excreted CORT metabolites (Millspaugh and Washburn, 2004). Excreta from adults were all homogenised as well. Steroids were extracted as described above and the concentration (ng/g) of excreted CORT metabolites was determined in each sample using the cortisone enzyme immunoassays. All samples were analysed in duplicates and if the coefficient of variation between the individual results was higher than 8%, the sample was reanalysed. Interassay coefficient of variation was 14%.

We evaluated the effects of time of day, body condition, sex and date on the concentration of CORT metabolites given that these variables may have an influence on the measurement of the excreted glucocorticoid metabolites (see review in Millspaugh and Washburn, 2004).

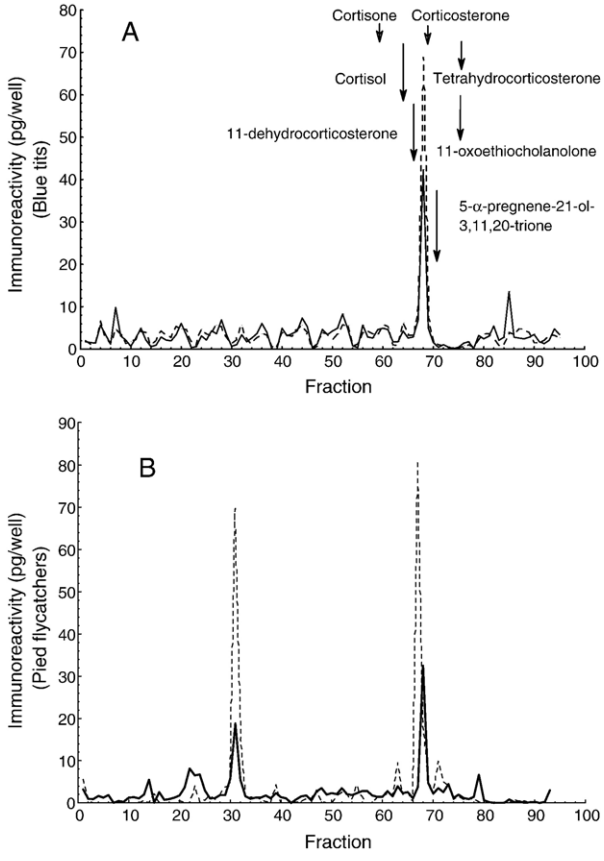

Fig. 2. HPLC profile of immunoreactive glucocorticoid metabolites measured with the cortisone assay in samples of two individuals (represented by a dotted and a solid line) of blue tits A) and pied flycatchers B) collected after the ACTH treatment. Note that immunoreactive substances with different cromatographic pattern than cortisone are measured.

Statistics

Statistical analyses were performed with STATISTICA 6.0 (StatSoft Inc.). Effects of the experiments were analysed by means of the General Lineal Models module (GLM). The brood manipulation treatment on blue tits in 2003 was included as a categorical factor (enlarged, reduced and control broods) and the CORT metabolites of adult blue tits were included as dependent variables. In order to avoid pseudoreplication, effects of the experiment on the nestling CORT metabolites were analysed with a generalized linear mixed model (GLMM) including the nest as a random factor and the brood manipulation experiment as a fixed factor.

In 2005, age effects on the concentration of excreted CORT metabolites of nestlings were evaluated using repeated measures ANOVA analyses when samples of the same individuals were available for the two categories of age (12 and 13 days in blue tits, and 11 and 12 days in pied flycatchers). As a significant effect of age was detected (see Results), we corrected further analyses by nestling age (categorical factor). For individuals with samples in the two categories of age, one of the two data points was eliminated randomly in order to avoid pseudoreplication.

The fumigation treatment on blue tit nests in 2005 was included as a categorical factor (fumigated or not fumigated) and ectoparasites, body condition, provisioning rates and immunoreactive CORT metabolites of adults as continuous dependent variables in respective analyses. When analysing these effects in nestlings, brood was included as a random factor in GLMM.

Effects of weather on excreted CORT metabolites were evaluated by introducing simultaneously in GLM as explanatory variables the maximum temperatures registered on the day of sampling, the average mean temperatures during the nestling period and the number of days of precipitation in the nestling period with CORT concentrations as dependent variables for blue tits and pied flycatchers. Because a significant effect of the treatment reducing ectoparasites was detected in female blue tits in 2005, we used as dependent variable the residual of excreted CORT metabolites on fumigation treatment. In this last analysis, we used precipitation as a categorical variable (raining and not-raining) as not enough variance for the number of days of precipitation was observed. Sex was included as a categorical factor in the models when we analysed weather effects on CORT of adult blue tits and pied flycatchers. To study the effects of temperature on nestling excreted CORT metabolites we used GLMM and included brood as a random factor and nestling age as a fixed factor. In all GLMM degrees of freedom of the error term have been computed using the Satterthwaite method.

As not all data were available for every variable, degrees of freedom may differ across analyses. Log transformation was used to normalize variables when necessary. In the variables where log transformation was needed, descriptive data show median and interquartile ranges. When original variables were normally distributed, descriptive data show means and standard errors.

Results

Time of day of sampling, laying date, body condition and age

In blue tits sampled in 2005, 12 days old nestlings had significantly lower concentration of excreted CORT metabolites

Table 1
Effects of the fumigation treatment on ectoparasites, provisioning rates, body condition and concentration of immunoreactive CORT metabolites using GLM procedures in adults and mixed models (GLMM) in nestlings

	Treatment (Fumigation)		Descriptive statistics	
	F	*P*	Fumigated nests	Non-fumigated nests
Ectoparasites				
Blowflies	13.25 [1,62]	**0.0006**	3.3 (3.2) (*N*=31)	20.2 (3.3) (*N*=33)
Mites	13.08 [1,59]	**0.0006**	119.2 (27.3) (*N*=31)	373.2 (65.6) (*N*=30)
Fleas	7.67 [1,59]	**0.007**	3.4 (1.5) (*N*=31)	76.2 (26.7) (*N*=30)
Provisioning rates per chick per hour				
Male	2.10 [1,20]	0.16	2.18 (0.53) (*N*= 10)	1.40 (0.21) (*N*=12)
Female	0.47 [1,26]	0.50	2.06 (0.52) (N=12)	1.69(0.24) (N=16)
Body condition				
Male	13.2 [1,51]	0.26	−0.04 (0.11) (*N*=25)	−0.20 (0.08) (*N*=28)
Female	0.19 [1,54]	0.66	−0.15 (0.07) (*N*=27)	−0.20 (0.09) (*N*=29)
Nestlings	0.02 [1,35.8]	0.88	0.04 (0.07) (*N*=95)	0.01 (0.12) (*N*=106)
Corticosterone metabolites (log)				
Male	0.673 [1,17]	0.42	267 (109.8) (*N*=9)	427 (304.6.) (*N*=10)
Female	5.224 [1,23]	**0.032**	501 (370) (*N*=10)	954 (995.2) (*N*=14)
Nestlings	1.08 [1,39.3]	0.30	1236 (64) (*N*=84)	1367 (63) (*N*=97)

Degrees of freedom of the error term in GLMM have been computed using the Satterthwaite method.
Descriptive statistics show mean (SE) except for adult corticosterone metabolites which was not normally distributed and median (interquartile range) is shown.

than the same nestlings with 13 days of age (repeated measures ANOVA: $F_{2,10}$=41.38, P<0.001, N=12). Mean (±SE) CORT metabolites of 12 and 13 days old blue tit nestlings were 1152 (183) and 1375 (168) respectively. Similarly, pied flycatcher nestlings of 11 days of age showed significantly lower levels of excreted CORT metabolites than the same nestlings with 12 days of age (repeated measures ANOVA: $F_{2,\ 38}$=117.56, P<0.001, N=40). Mean (±SE) CORT metabolites of 11 and 12 days old pied flycatcher nestlings were 232 (19) and 292 (29) respectively. In order to avoid pseudoreplication, we randomly eliminated one datum for nestlings from which we collected droppings in 2 days. Thus, in further analyses, sample size available for excreted CORT metabolites of nestlings was N=145 and N=181 for blue tits in 2003 and 2005 respectively, and N=197 for nestling pied flycatchers in 2005. In adults, sample sizes were N=17 and N=31 for male and female blue tits in 2003, N=20 and N=26 for male and female blue tits in 2005 and N=46 and N=51 for male and female pied flycatchers in 2005.

No effect of time of the day of sampling, laying date or body condition on excreted CORT metabolites was detected in adults or nestlings of blue tits in 2003 or 2005 or in pied flycatcher adults or

Table 2
Effects on the excreted CORT metabolites of the maximum temperature (T) on the day of sampling, the average mean temperature (T) and precipitation during the nestling period of blue tits in 2003 and 2005 and in pied flycatchers in 2005

A)			Explanatory variables									
Year	Species (Adults)	Dependent variable	Maximum T (day of sampling)		Average mean T (nestling period)		Precipitation		Sex		Precipitation x Sex	
			F [df]	*P*	*F* [df]	*P*	*F* [df]	*P*	*F* [df]	*P*	*F* [df]	*P*
2003	Blue tit	(log) CORT metabolites	0.00 [1,24]	0.99	0.09 [1,24]	0.77	1.05 [3,24]	0.339	0.25 [1,24]	0.62	0.67 [2,24]	0.52
2005	Blue tit	Residuals (log) CORT on Fumigation	0.10 [1,27]	0.94	0.07 [1,27]	0.80	0.12 [1,27]	0.73	1.44 [1,27]	0.24	1.16 [1,27]	0.29
2005	Pied flycatcher	(log) CORT metabolites	2.52 [1.57]	0.12	0.95 [1,57]	0.33	0.20 [4,57]	0.20	0.10 [1,57]	0.75	0.20 [4,57]	0.94

B)			Explanatory variables							
Year	Species (Nestlings)	Dependent variable	Maximum T (day of sampling)		Average mean T (nestling period)		Precipitation		Age	
			F [df]	*P*	*F* [df]	*P*	*F* [df]	*P*	*F* [df]	*P*
2003	Blue tit	CORT metabolites	1.42[1,35.7]	0.24	3.12[1,35.0]	0.09	0.36[3,33.4]	0.78		
2005	Blue tit	CORT metabolites	6.25 [1,34.1]	**0.02**	9.94[1,48.6]	**0.003**	2.38 [2,26.8]	0.11	0.76 [1,26.7]	0.39
2005	Pied flycatcher	CORT metabolites	0.68 [1,36.1]	0.41	7.49 [1,40.5]	**0.009**	1.05 [4,41.4]	0.39	0.001 [1,31.2]	0.97

Significant statistics are shown in bold. A) Shows results for adult blue tits and pied flycatchers (GLM).
Sex is included in the models. B) Shows results for nestlings blue tits and pied flycatchers (GLMM). Nestling age is included in the models. Degrees of freedom of the error term in GLMM have been computed using the Satterthwaite method.

nestlings in 2005 ($P>0.05$ in all cases). No effect of the age of adult blue tits and pied flycatchers was detected (all $P>0.05$).

Brood size manipulation experiment in blue tits (2003)

The results of this experiment concerning parental effort are described in Merino et al. (2006).

No significant correlation was found between the levels of heat shock proteins or the levels of total immunoglobulins with the concentration of excreted CORT metabolites of adults or nestlings ($P>0.09$). The brood size manipulation did not affect the concentration of excreted CORT metabolites of adults (males: $F_{2,14}=0.33$, $P=0.73$, females: $F_{2,28}=0.06$, $P=0.94$) or nestlings ($F_{2,51.7}=0.104$, $P=0.90$). Median (interquartile range) excreted CORT metabolites (ng/g) of adults was: males 538.0 (473.8) and females 533.5 (558.7). Mean (±SE) excreted CORT metabolites (ng/g) of nestlings was 868.2 (34.3).

Fumigation experiment in blue tits (2005)

The fumigation treatment significantly reduced the ectoparasite intensities for blowflies, mites and fleas in blue tit nests (Table 1). No effect of treatment on the body condition of adults or nestlings was detected (Table 1). No differences in provisioning rates were detected between treated and control nests (Table 1). Females from treated nests showed significantly lower concentration of CORT metabolites in their droppings (Table 1). Nestlings did not show significant differences between experimental groups in their excreted CORT metabolites (Table 1). Correcting by nestling age did not change the results.

Weather

Weather conditions were significantly different in 2003 and 2005 during the nestling period of blue tits. The maximum temperatures registered on the day of sampling was significantly higher in 2005 with respect to 2003 (Kolmogorov Smirnov test, $P<0.001$: median Tmax (interquartile range) being 25.0 (6.2) ($N=56$) and 29.5 (0.7)(($N=43$) in 2003 and 2005 respectively. Similarly, the average mean temperature during the nestling period of blue tits were significantly higher in 2005 with respect to 2003 (Kolmogorov Smirnov test, $P<0.001$: average mean T (interquartile range) being 15.4 (1.7)($N=86$) and 18.5 (0.8) ($N=54$) in 2003 and 2005 respectively. The distribution of the number of days of precipitation was significantly different during the nestling period of blue tits of 2003 and 2005 ($\chi^2=16.0$, $P<0.005$, $N=140$). The mode of the number of days of precipitation during the nestling period in 2003 was 2 days, while in 2005 the mode was 3 days. Mean (±SE) hatching dates were earlier in 2003 (hatching date: 54.0±0.5) with respect to 2005 (57.0±0.3) ($F_{1,145}=22.86$, $P<0.001$).

Significant effects of temperature variables were only detected in 2005 in blue tits and pied flycatchers (Table 2 A and B). The maximum temperature on the day of sampling was positively associated with the nestling CORT metabolites only in blue tits. The average mean temperature during the nestling period was significantly and negatively associated with the

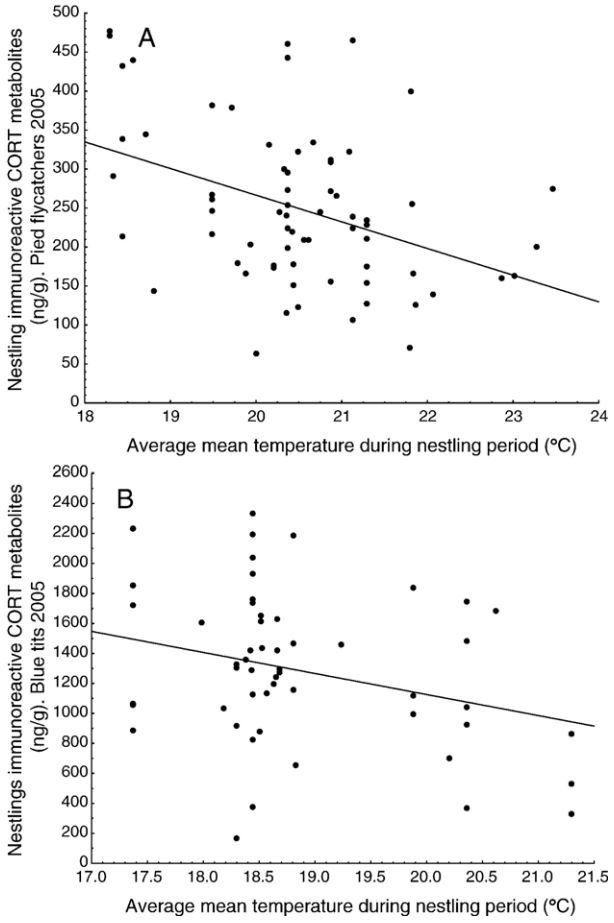

Fig. 3. Simple correlations between mean nestling excreted CORT metabolites in broods and the average mean temperature during the nestling period in A) pied flycatchers ($F_{1,66}=12.04$, $P=0.0009$, *adjusted* $R^2=0.14$) and B) blue tits ($F_{1,52}=4.80$, $P=0.033$, *adjusted* $R^2=0.07$).

excreted CORT metabolites of both blue tit and pied flycatcher nestlings. This association was significant on its own (Fig. 3) and including the maximum temperature, precipitation and nestling age (Table 2 B). No effect of the temperature variables were detected in adults of blue tits in 2003 or 2005 or in adults of pied flycatchers in 2005 (Table 2 A).

Discussion

In this study we have validated the measurement of corticosterone metabolites from droppings of wild blue tits and pied flycatchers. The acute stress produced by handling and ACTH administration resulted in an increase of immunoreactive corticosterone metabolites that could be detected in excreta after 1 h of the ACTH injection. This pattern of corticosterone secretion confirms the biological relevance of the assay (Touma and Palme, 2005), so the enzyme immunoassay validated in this study may be used in future studies on these species. Non-invasive measurements of corticosterone may be very useful in studies of small passerines given the small size of these birds and the limitation of plasma volume for hormone analyses. This is, to our knowledge, the first study of corticosterone variation in nestling birds that uses a non-invasive method of measurement.

In the present study, we report the measurement of the concentration of excreted CORT metabolites of individuals subjected to a brood size manipulation experiment. We show that the CORT levels did not differ in adults or nestlings between experimental treatments. In the manipulation of brood size performed by Saino et al. (2003) in barn swallows (*Hirundo rustica*), they found increased corticosterone levels in nestlings from enlarged broods, as a response to the stress imposed by the brood enlargement, and we had expected a similar response by blue tit nestlings. It is possible that the brood manipulation in this study may not represent such a stressful situation for nestlings as the brood manipulation experiment by Saino et al. (2003). Barn swallows lay clutches of 2–7 eggs, while blue tits lay clutches of 4–14 eggs. Thus, it could be that a brood manipulation of one nestling in barn swallows might reflect more clearly the effects of brood size than enlarging or decreasing blue tit broods with two nestlings. In our experiment, parental behaviour may have mediated the lack of effect on nestling CORT metabolites. Parents may modify their provisioning rates in manipulated broods (see Martin, 1987), which may counteract the negative effects on their nestlings of the stressful conditions of enlarged broods. In this study, provisioning rates could not be measured, but females attending enlarged brood showed higher levels of stress proteins possibly as a reflect of an increase in parental effort (see Merino et al., 2006). If this is the case, it could be possible that the increase in parental effort in enlarged broods compensated for the stressful conditions for nestlings as expressed by the lack of differences in CORT metabolites with respect to reduced or control nests. Similar conclusions were reported for growth and condition parameters in Merino et al. (2006). Although an effect of an increased parental effort in the CORT metabolites of parents could be expected (see Ilmonen et al., 2003), other physiological parameters such as stress proteins or plasma immunoglobulins seem to reflect more clearly the effects of the brood size manipulation on adult blue tits in our population (Merino et al., 2006).

The fumigation treatment performed in 2005 reduced the ectoparasite load in nests of blue tits for our estimates of abundance of mites, fleas and blowflies. This ectoparasite reduction only resulted in a decrease in CORT metabolite concentration in females. This may be in accordance with the study by Raouf et al. (2006) in which individuals of cliff swallows from fumigated colonies averaged significantly lower baseline levels of corticosterone. However, our data do not provide evidence in the same direction for males and nestlings as no effect of the ectoparasite load on their excreted CORT metabolites was detected in our population. It is possible that female blue tits suffer more the detrimental effects of nest parasites than males given that they brood the chicks alone (see Nur, 1984) and, consequently, spend more time in the nests than males. In our experimental design, nest were fumigated on day 11 but also on days 7 and 3 of nestling period, when females are still brooding the chicks. Thus, reducing the parasite load of nests on these days may have influenced the corticosterone concentration of the females, not having an influence on the males because of their lower prolonged contact with the nest material. Nestlings being the individuals more susceptible to ectoparasite impact because of their confinement to the nestbox at the stage of measurement, we had expected that corticosterone secretion would be increased in order to cope with the impact of parasitism. Parental compensatory behavior could have mediated this lack of effect of the treatment (Bouslama et al., 2002). Although our data do not show evidence of an influence of ectoparasites on the frequency of parental visits to the nest on day 12 (see Merino et al., 1998a,b, for a similar result), it could be possible that maternal compensatory effects during early phases of nestling period could be reflected in an absence of detrimental effect of parasites in nestlings. Thus, parasite effect on female CORT could have been mediated by an increased compensatory parental effort. On the other hand, ectoparasite loads in our population may not be high enough to produce a glucocorticoid response in nestlings. In the experiment by Raouf et al. (2006), nestlings in small colonies did not show differences in their corticosterone levels between fumigated and non-fumigated sites. Infestation is higher in large colonies, so it is possible that the parasite load of small colonies in that study may represent similar conditions to those of our population with regard to ectoparasite presence. Alternatively, if ectoparasitism constitutes a chronic stressor for nestlings, a downregulation of the adrenocortical response may have resulted in no changes or even lower CORT concentrations in the individuals exposed to chronic stress (see Rich and Romero, 2005) as a mechanism to avoid the pathogenic potential effect of constant elevated levels of glucocorticoids (Sapolsky, 2002), explaining also the absence of effect of the fumigation treatment. It could also be possible that other physiological variables not measured in the present study, such as heat shock proteins (HSP), may reflect more accurately the effects of ectoparasitism on nestlings, as it has been shown previously in house martin (*Delichon urbica*) nestlings (Merino et al., 1998a,b).

Our data indicate that adults of blue tits and pied flycatchers do not seem to respond with variation in their CORT levels to the variation in ambient temperature. On the other hand, environmental temperature does have an influence on the concentration of excreted CORT metabolites of nestlings of these two passerine species. The adults may have reacted to the range of environmental temperatures reported in this study with modifications of their behavior (e.g. use of shade or sun exposed areas) which may avoid to detect an effect of temperature on their baseline CORT levels. However, nestlings are confined in the nest-boxes exposed to environmental changes and with limited possible behavioral modifications. In 2005, the average mean temperature during the nestling period was negatively and significantly associated to the CORT levels of nestlings of both species. This negative correlation may be coherent with the metabolic function exerted by corticosterone on the stimulation of lipolysis and proteolysis, as it was previously suggested by a similar negative correlation found by Frigerio et al. (2004) in Greylag geese (*Anser anser*). However, in 2003, no significant effect of the temperature on excreted CORT of blue tit nestlings was observed. Average mean temperatures in 2003 were significantly lower than in 2005, so it seems that in a cold year, the HPA axis of nestling

blue tits does not respond to lower average mean temperatures. This difference could be due to a possible habituation or downregulation of the HPA axis of nestlings (see Rich and Romero, 2005) when exposed to constant low temperatures in a colder year. In addition, weather conditions were different between 2003 and 2005 also in the number of days of precipitation during the nestling period of blue tits. Although this factor did not have an effect on nestling CORT metabolites within years, it is possible that the difference in the frequencies of precipitation between years had an influence on the climatic conditions that influenced nestling CORT metabolites in 2005. On the other hand, hatching dates of blue tits were significantly earlier in 2003 with respect to 2005 and this may have also had an influence on the availability of food during the nestling period leading to different nutritional condition of nestlings in both years. If the delay in the breeding season of 2005 was accompanied of a lower abundance of caterpillars, it could be possible that a lower nutritional condition of the nestlings in 2005 have made them more vulnerable to weather conditions than blue tit nestlings in 2003. Our study also detected a positive effect of the maximum temperature on the day of sampling on the concentration of excreted CORT metabolites of blue tit nestlings in 2005, suggesting that the high temperatures suffered in the same day of sampling may have been a stressor to which the HPA axis responded. Although this result may be in accordance with previous studies in mammals and birds in which either low or high temperatures have been shown to induce higher HPA response (Edens and Siegel, 1975; Djordjevic et al., 2003; Koko et al., 2004), we did not detect the same pattern in pied flycatcher nestlings in the same year, so this result should be considered with caution. In our study, we included age as a factor in the analyses and no significant change of the effects was detected, suggesting that the temperature effects are independent of age considered.

In sum, our results indicate that ectoparasitism may mediate changes in the concentration of excreted CORT metabolites of wild birds, suggesting a role of glucocorticoids in the mechanisms directed to overcome the impact of parasitism. Also, our results suggest that weather conditions may act as environmental stressors to which the HPA axis of blue tit and pied flycatcher nestlings may be sensitive. Previous studies have demonstrated that altricial nestlings perceive and respond to environmental stressors by changing the levels of circulating corticosterone (see references in Blas et al., 2005) and our data provide a new evidence of the adrenocortical responsiveness of nestlings of two passerine species. The fact that the corticosterone secretion of nestlings seems to be subjected to inter-annual variation in climatic conditions may be also considered when analysing potential effects of global climate change.

Acknowledgments

The study received financial support from projects CGL2004-00787/BOS to J. Moreno and BOS2003-05724 to SM (DGI-Ministerio de Educación y Ciencia). J. Morales and E.L. were supported by FPI and FPU grants MEC, G.T. by a CAM grant and J. Martínez-de la Puente by a 'El Ventorrillo' grant from CSIC. We thank T. Calvo and M. Gil for their help in the field. We also thank R. Palme, S. Rettenbacher, D. Gil and two anonymous reviewers for useful comments. We were authorised by J. Donés, Director of 'Centro Montes de Valsaín' (Ministerio de Medio Ambiente) to work in the study area. Dirección General del Medio Natural (Junta de Castilla y León) authorised the capture, ringing and sampling of birds in the study area. This paper is a contribution from the field station 'El Ventorrillo'.

References

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. Orn. Scand. 23 (3), 355–365.

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1995. Seasonal and acute changes in adrenocortical responsiveness in an artic-breeding bird. Horm. Behav. 29, 442–457.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T.A., Hiraldo, F., 2005. A multi-tier approach to identifying environmental stress in altricial nestling birds. Funct. Ecol. 19, 315–322.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T.A., Hiraldo, F., 2006. Age-related variation in the adrenocortical response to stress in nestling white storks (*Ciconia ciconia*) supports the developmental hypothesis. Gen. Comp. Endocrinol. 148, 172–180.

Bouslama, Z., Lambrechts, M.M., Ziane, N., Djenidi, R., Chabi, Y., 2002. The effect of nest ectoparasites on parental provisioning in a north-African population of the Blue Tit *Parus caeruleus*. Ibis 144 (2), E73–E78.

Buchanan, K.L., 2000. Stress and the evolution of condition-dependent signals. Trends Ecol. Evol. 15 (4), 156–160.

Carere, C., Groothuis, T.G.G., Möstl, E., Daan, S., Koolhas, J.M., 2003. Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. Horm. Behav. 43 (5), 540–548.

Djordjevic, J., Cvijic, G., Davidovic, V., 2003. Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiol. Res. 52 (1), 67–72.

Edens, F.W., Siegel, H.S., 1975. Adrenal responses in high and low ACTH response lines of chickens during acute heat stress. Gen. Comp. Endocrinol. 25 (1), 64–73.

Eeva, T., Tanhuanpää, S., Råbergh, C., Airaksinen, C., Nikinmaa, M., Lehikoinen, E., 2000. Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines. Funct. Ecol. 14, 235–243.

Fargallo, J.A., Johnston, R.D., 1997. Breeding biology of the Blue tit *Parus caeruleus* in a montane Mediterranean deciduous forest: the Interaction of Latitude and Altitude. J. Ornithol. 138, 83–92.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243–282.

Frigerio, D., Dittami, J., Möstl, E., Kotrschal, K., 2004. Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 137 (1), 29–36.

Goymann, W., 2005. Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences and the influence of diet on hormone metabolite levels. Ann. N.Y. Acad. Sci. 1046, 35–53.

Goymann, W., Möstl, E., Gwinner, E., 2002. Corticosterone metabolites can be measured noninvasively in excreta of European Stonechats (*Saxicola torquata rubicola*). Auk 119 (4), 1167–1173.

Hirschenhauser, K., Möstl, E., Wallner, B., Dittami, J., Kotrschal, K., 2000. Endocrine and behavioural responses of male greylag geese (*Anser anser*) to pairbond challenges during the reproductive season. Ethology 106 (1), 63–77.

Hurtrez-Boussès, S., Blondel, J., Perret, P., Fabreguettes, J., Renaud, F., 1998. Chick parasitism by blowflies affects feeding rates in a Mediterranean population of blue tits. Ecol. Lett. 1 (1), 17–20.

Ilmonen, P., Hasselquist, D., Langerfors, A., Wiehn, J., 2003. Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia 136, 148–154.

Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999. Stress response of black-legged kittiwake chicks in relation to dietary restrictions. J. Comp. Physiol., B. 169, 303–310.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001a. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behav. Ecol. 12 (5), 619–625.

Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001b. Dietary resctriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. J. Comp. Physiol., B. 171, 701–709.

Kern, M., Bacon, W., Long, D., Cowie, R.J., 2001. Possible roles for corticosterone and critical size in the fledging of nestling pied flycatchers. Physiol. Biochem. Zool. 74 (5), 651–659.

Koko, V., Djordjeviae, J., Cvijiae, G., Davidoviae, V., 2004. Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. J. Exp. Biol. 207 (24), 4225–4230.

Kotrschal, K., Hirschenhauser, K., Möstl, E., 1998. The relationship between social stress and dominance is seasonal in greylag geesee. Anim. Behav. 55, 171–176.

Kučka, M., Vagnerová, P., Klusoňova, Mikšik, I., Pácha, J., 2006. Corticosterone metabolism in chicken tissues: Evidence for tissue-specific distribution of steroid dehydrogenases. Gen. Comp. Endocrinol. 147, 377–383.

Landys, M.M., Ramenofsky, M., Wingfield, J.C., 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen. Comp. Endocrinol. 148, 132–149.

Love, O.P., Bird, D.M., Shutt, L.J., 2003. Corticosterone levels during post-natal development in captive American kestrels (*Falco sparverious*). Gen. Comp. Endocrinol. 130, 135–141.

Loye, J.E., Zuk, M. (Eds.), 1991. Bird-Parasite Interactions: Ecology, Evolution and Behaviour. Oxford Ornithology Series. Oxford University Press, Oxford.

Martin, T.E., 1987. Food as a limit on breeding birds: a life-history perspective. Ann. Rev. Ecolog. Syst. 18, 453–487.

Martinez, J., Pérez-Serrano, J., Bernadina, W.E., Rodriguez-Caabeiro, F., 1999. Using heat shock proteins as indicators of the immune function in wistar rats during a secondary *Trichinella spiralis* infection. Vet. Parasitol. 85, 269–275.

Martinez, J., Pérez-Serrano, J., Bernadina, W.E., Rodriguez-Caabeiro, F., 2001. Stress response to cold in Trichinella species. Cryobiology 43, 293–302.

Mazancová, K., Kučka, M., Mikšik, I., Pácha, J., 2005. Glucocorticoid metabolism and Na+ transport in chicken intestine. J. Exp. Zool. 303A, 113–122.

Merino, S., Potti, J., 1995. Mites and blowflies decrease growth and survival in nestling pied flycatchers. Oikos 73, 95–103.

Merino, S., Moreno, J., Potti, J., de León, A., Rodríguez, R., 1998a. Nest ectoparasites and maternal effort in pied flycatchers. Biol. Conserv. Fauna 102, 196–201.

Merino, S., Martínez, J., Barbosa, A., Møller, A.P., de Lope, F., Pérez, J., Rodríguez-Caabeiro, F., 1998b. Increase in a heat shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach. Oecologia 116, 343–347.

Merino, S., Martinez, J., Møller, A.P., Barbosa, A., de Lope, F., Rodríguez-Caabeiro, F., 2002. Blood stress protein levels in relation to sex and parasitism of barn swallows (*Hirundo rustica*). Écoscience 9, 300–305.

Merino, S., Moreno, J., Tomás, G., Martínez, J., Morales, J., Martínez-de la Puente, J., Osorno, J.L., 2006. Effects of parental effort on blood stress protein HSP60 and immunoglobulins in female blue tits: a brood size manipulation experiment. J. Anim. Ecol. 75, 1147–1153.

Merilä, J., Wiggins, D.A., 1995. Offspring number and quality in the blue tit: a quantitative genetic approach. J. Zool. 237, 615–623.

Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199.

Møller, A.P., 1994. Parasite infestation and parental care in the barn swallow *Hirundo rustica*. A test of the resource-provisioning model of parasite-mediated sexual selection. Ethology 97 (3), 215–225.

Moreno, J., Carrascal, L.M., Fargallo, J.A., Soto-Largo, E., 1996. Determination of clutch size in the Blue tit *Parus caeruleus* in central Spain: Field experiments. Ardeola 43, 9–17.

Moreno, J., Potti, J., Merino, S., 1997. Parental energy expenditure and offspring size in the pied flycatcher *Ficedula hypoleuca*. Oikos 79 (3), 559–567.

Moreno, J., Merino, S., Martinez, J.J., Sanz, J.J., Arriero, E., 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Écoscience 9 (4), 434–439.

Morimoto, R.I., 1991. Heat shock: The role of transient inducible responses in cell damage, transformation, and differentiation. Cancer Cells 3, 295–301.

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139.

Möstl, E., Rettenbacher, S., Palme, R., 2005. Measurement of corticosterone metabolites in birds' droppings: An analytical approach. Ann. N.Y. Acad. Sci. 1046, 17–34.

Nakagawa, S., Möstl, E., Wass, J.R., 2003. Validation of an enzyme immunoassay to measure faecal glucocorticoid metabolites from Adelie penguins (*Pygoscelis adeliae*): a non-invasive tool for estimating stress? Polar Biol. 26 (7), 491–493.

Nur, N., 1984. The consequences of brood size for breeding blue tits. 1. Adult survival, weight change and the cost of reproduction. J. Anim. Ecol. 53 (2), 479–496.

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N.Y. Acad. Sci. 1046, 75–80.

Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood Z. Saugetierkd. J. Mammal. Biol. 62 (Suppl.II), 192–197.

Potti, J., Montalvo, S., Sánchez-Aguado, F.J., Blanco, D., 1988. La reproducción del herrerillo común (*Parus caeruleus*) en un robledal del centro de España. Ardeola 35, 31–43.

Quillfeldt, P., Möstl, E., 2003. Resource allocation in Wilson's storm-petrels *Oceanites oceanicus* determined by measurement of glucocorticoid excretion. Acta Ethol. 5, 115–122.

Raouf, S.A., Smith, L.C., Brown, M.B., Wingfield, J.C., Brown, C.R., 2006. Glucocorticoid hormone levels increase with group size and parasite load in cliff swallows. Anim. Behav. 71, 39–48.

Reneerkens, J., Piersma, P., Ramenofsky, M., 2002. En experimental test of the relationship between temporal variability of feeding opportunities and baseline levels of corticosterone in a shorebird. J. Exp. Zool. 293, 81–88.

Rettenbacher, S., Möstl, E., Hackl, R., Ghareeb, K., Palme, R., 2004. Measurement of corticosterone metabolites in chicken droppings. Br. Poult. Sci. 45 (5), 704–711.

Rich, E.L., Romero, L.M., 2005. Exposure to chronic stress downregulates corticosterone response to acute stressors. Am. J. Physiol., Regul. Integr. Comp. Physiol. 288 (6), R1628–R1636.

Romero, L.M., Romero, R.C., 2002. Corticosterone responses in wild birds: The importance of rapid initial sampling. Condor 104 (1), 129–135.

Romero, L.M., Reed, J.M., Wingfield, J.C., 2000. Effects of weather on corticosterone responses in wild free-living passerine birds. Gen. Comp. Endocrinol. 118, 113–122.

Saino, N., Martinelli, R., Møller, A.P., 2001. Growth, immunity and begging behaviour of nestling barn swallows (*Hirundo rustica*) in relation to hatch order. J. Avian Biol. 32, 263–270.

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D., Møller, A.P., 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). Behav. Ecol. 14 (3), 318–325.

Sanz, J.J., Moreno, J., 1995. Mass loss in brooding female pied flycatchers *Ficedula hypoleuca*: No evidence for reproductive stress. J. Avian Biol. 26, 313–320.

Sanz, J.J., Tinbergen, J.M., 1999. Energy expenditure, nestling age and brood size: an experimental study of parental behaviour in the great tit *Parus major*. Behav. Ecol. 10, 598–606.

Sapolsky, R.M., 2002. Endocrinology of the stress response, In: Becker, J.B., Breedlove, S.M., Crews, D., McCarthy, M.M. (Eds.), Behavioral Endocrinology, 2nd edition. MIT Press, Cambridge, MA.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Schwabl, H., 1999. Developmental changes and among-sibling variation of corticosterone levels in altricial avian species. Gen. Comp. Endocrinol. 116, 403–408.

Simon, A., Thomas, D.W., Speakman, J.R., Blondel, J., Perret, P., Lambrechts, M.M., 2005. Impact of ectoparasitic blowfly larvae (*Protocalliphora* spp.) on the behaviour and energetics of nestlings Blue Tits. J. Field Ornithol. 76 (4), 402–410.

Sonna, L.A., Fujita, J., Gaffin, S.L., Lilly, C.M., 2002. Invited review: effects of heat and cold stress on mammalian gene expression. J. Appl. Physiol. 92, 1725–1742.

Sørensen, J.G., Kristensen, T.N., Loeschke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6, 1025–1037.

Svensson, L., 1984. Identification guide to European passerines. Ugga, Stockholm.

Thiel, D., Jenni-Eiermann, S., Palme, R., 2005. Measuring corticosterone metabolites in droppings of capercaillies (*Tetrao urogallus*). Ann. N.Y. Acad. Sci. 1046, 96–108.

Tomás, G., Merino, S., Martinez, J., Moreno, J., Sanz, J.J., 2005. Stress protein levels and blood parasite infection in blue tits (*Parus caeruleus*): a medication field experiment. Ann. Zool. Fenn. 42 (1), 45–56.

Tomás, G., Merino, S., Moreno, J., Morales, J., 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. Anim. Behav. doi:10.1016/j.anbehav.2006.06.016.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Ann. N.Y. Acad. Sci. 1046, 54–74.

Tripet, F., Glaser, M., Richner, H., 2002. Behavioural responses to ectoparasites: time-budget adjustments and what matters to blue tits *Parus caeruleus* infested by fleas. Ibis 144, 461–469.

Wingfield, J.C., 1984. Influence of weather on reproduction. J. Exp. Zool. 232 (3), 589–594.

Wingfield, J.C., 2003. Control of behavioural strategies for capricious environments. Anim. Behav. 66, 807–816.