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Immunoreactive cortisone in droppings reflect stress levels, diet and growth rate of gull-billed tern chicks

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ABSTRACT

Blood levels of corticosterone have been traditionally analyzed to assess stress levels in birds; however, measuring steroid hormone metabolites in feces and droppings has gained much interest as a noninvasive technique successfully used for such purposed in vertebrates. Diet may affect these fecal metabolite levels (e.g., due to nutritional stress), however, this variable has not been taken into account in studies with chicks despite the great dietary flexibility of many avian species. In this study, we addressed for the first time this key issue and validated the technique in wild gull-billed tern chicks (*Gelochelidon nilotica*). Several enzyme immunoassays were used to determine the most appropriate test to measure the stress response. Subsequently, we performed an experiment in captivity to assess adrenocortical activity in gull-billed tern chicks fed with two diets: piscivorous vs. insectivorous. Finally, the relation between the chicks' growth rate and excreted immunoreactive glucocorticoid metabolites (EGMs) was also evaluated. We found the immunoreactive cortisone metabolites to be a good index of stress (as being an index of adrenocortical reactivity) in chicks of this species. Fish-fed chicks had higher levels of cortisone metabolites when comparing both concentration and total daily excreted metabolites. Within each treatment diet, cortisone metabolite levels and growth rates were negatively correlated. These findings suggest that the diet should be considered when using this technique for comparative purposes and highlight the trade-off between stress levels and chicks growth rates.
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1. Introduction

The study of stress hormones may shed light on understanding how animals face threats to health, reproductive success and survival (von Holst, 1998; Sapolsky et al., 2000). Stress hormones play an important role in increasing the amount of energy available for vital functions, because they suppress nonessential physiological functions to promote immediate survival (von Holst, 1998; Romero and Wikelski, 2001; Sapolsky, 2002). However, chronically elevated stress hormones may suppress growth, body condition, immune function, reproduction and survival (Sapolsky, 2002; Kitaysky et al., 2003; Romero, 2004).

Glucocorticoids (GCs) are released into the blood after activation of the hypothalamo–pituitary–adrenocortical (HPA) axis when an animal faces a stressor (Sapolsky et al., 2000; Möstl and Palme, 2002). Such stressors can be either natural events, like food limitation, adverse weather conditions or predators, or disturbances resulting from human activities, like hunting, tourist visits or scientific research itself (Wasser et al., 1997; Kitaysky et al., 1999; Romero et al., 2000; Müller et al., 2006; Thiel et al., 2008; Creel et al., 2009). Since the level of activation of the adrenocortical response often correlates with the animal's condition (Heath and Dufty, 1998; Raja-aho et al., 2010; Müller et al., 2010), measuring GCs has been widely used as a biomarker that suitably reflects the combined effects of health, physiological constraints, allocation of energy resources and anthropogenic disturbances on individuals (e.g., Breuner and Hahn, 2003; Romero, 2004); therefore, measurements of stress hormone profiles may reflect their habitat quality (reviews by Homyack, 2010; Albano, 2012).

Glucocorticoids have traditionally been measured in blood after capture, and therefore an accurate assessment of stress in this way could be compromised by the effects of the manipulation itself (Le Maho et al., 1992; Walker et al., 2005; Sheriff et al., 2011). Plasma levels of GCs can be rapidly increased by capture, handling, and bleeding within time periods as short as 3 min, and bleeding wild animals within this time interval is not possible in most cases (Romero and Romero, 2002; Sheriff et al., 2010). In recent years, measurements of steroid hormone metabolites in feces have attracted much interest as a noninvasive technique to study the

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stress response, both in mammals (e.g., Möstl et al., 2002; Ayres et al., 2012; Murray et al., 2013; Stetz et al., 2013) and birds (e.g., Goymann, 2005; Klasing, 2005; Möstl et al., 2005; Legagneux et al., 2011). Metabolites excreted in the urine and feces can be independently measured in mammals, but in birds two peaks of concentration in droppings collected over time would be expected, corresponding to the urinary and the fecal contributions. The first one would be in early samples, as a result of metabolites excreted in the urine, and the second peak as a result of metabolites excreted in the feces (Möstl et al., 2005). The measurement of excreted immunoreactive glucocorticoid metabolites (EGMs) reflects plasma GC levels (Sheriff et al., 2010) and avoids samples containing undesirable increases in circulating GCs induced by capture and manipulation of individuals (Harper and Austad, 2001; Millspaugh et al., 2001), since the EGMs integrate information on hormone levels over a long time period (Harper and Austad, 2000; Millspaugh and Washburn, 2004; Sheriff et al., 2010).

Several factors such as sex, age, or life history stage (e.g., moult, reproductive status, etc.) can affect the HPA axis response causing intra-specific variations (Millspaugh and Washburn, 2004; Touma and Palme, 2005; Goymann, 2012). In addition, it has been suggested that nutritional status may be inferred by EGMs in vertebrates (Ayres et al., 2012; Stetz et al., 2013), and the varying quality of the diets may lead to differences in nutritional conditions, that may also involve nutritional stress in the case of low-quality diets (Kitaysky et al., 2001; Quillfeldt et al., 2007; Jenni-Eiermann et al., 2008). However, although many birds (especially long-distance migratory species) show remarkable dietary flexibility to meet their energy and nutrient requirements (reviewed by McWilliams and Karasov, 2001), potential dietary effects on EGMs have rarely been analyzed in adult birds (but see Goymann, 2005; Klasing, 2005), necessitating additional studies that include this variable (Dantzer et al., 2011).

Moreover, the detrimental effects of the chronic stress response may be particularly relevant in chicks since their growth may be compromised in the short term (Korte et al., 2004), and long term physiological effects have been documents later in life (Lindström, 1999; Lendvai et al., 2009). Such short and longer term consequences might even affect population dynamics (Kitaysky et al., 2006; Monaghan et al., 2012). However, very few studies have validated the measurement of EGMs to assess stress in wild chicks (but see Lobato et al., 2008; Stöwe et al., 2008, 2010 with passerine chicks). Because there are clear species-specific differences in metabolism and excretion of GMs, experiments dealing with the validity of EGM analyses are essential and must be performed before applying the technique in a given species (Palme, 2005; Touma and Palme, 2005). However, validation that ensures a reliable monitoring of adrenocortical activity has rarely been done in adults or chicks of wild waterbirds (but see e.g., studies in adult birds by Nakagawa et al., 2003; Frigerio et al., 2004; Ninnes et al., 2010).

The first objective of this study was to validate the use of EGMs to measure stress in wild gull-billed tern chicks (*Gelochelidon nilotica*), by monitoring changes in EGM levels after inducing an increase in circulating GCs in free-living chicks. The second objective, taking advantage of an experiment conducted in captivity, was to address for the first time the effect of diet on adrenocortical activity in gull-billed tern chicks by measuring EGMs in chicks fed on two different diets, and assess if those levels may reflect their growth rates. Given the wide variety of diets documented for this species and the diversity of its foraging habitats (Sánchez et al., 1991; Sánchez and Fasola, 2002), the gull-billed tern represents a good model species for our purposes.

2. Materials and methods

2.1. Validation study in free-living gull-billed tern chicks

The measurement of EGMs was validated by ensuring that elevations in their levels were detectable. The experiment for validation of the use of EGM analysis to evaluate stress in gull-billed tern chicks was conducted in July 2009 in an approximately 300-pair breeding colony located on an island in the Alange reservoir in Extremadura, Spain (38°45′N 6°15′W). Twelve chicks, from 12 different nests, with an estimated age of 14 days were captured immediately upon arrival on the island. They were taken away from the colony to a hidden part of the island at a distance far enough (~150 m) from the colony, thus minimizing disturbances to the rest of the colony. Six chicks received an injection of adrenocorticotropic hormone (ACTH, porcine hormone, 20IU/animal, Sigma) in the pectoral muscle to induce increases in circulating GC levels. The administration of ACTH to stimulate the secretion of GCs by the adrenal gland is a widely used method to physiologically validate the non-invasive technique, since it verifies that the increased levels of circulating GCs is reflected in the levels of EGMs (see review by Touma and Palme, 2005). To control for the stress of capture, handling, injection and isolation per se (since they are know to cause an increase in GCs) the biological validation of the technique is required to ensure that the assay system can detect biologically meaningful alterations in the endocrine status (Touma and Palme, 2005). This was performed by injecting the rest of the chicks ($n = 6$) with a saline solution (0.9% NaCl), acting as a control group.

The chicks were individually held in cardboard boxes provided with a mesh floor over an aluminum foil to collect droppings. To obtain control (pre-treatment phase) data and establish the initial concentration of naturally occurring EGMs, the first dropping defecated by each chick (at 13.83 ± 2.68 min) was immediately taken after capture (and just before ACTH or saline injection), since EGMs reveal individual physiology over a long period of time before collection (Harper and Austad, 2000; Millspaugh and Washburn, 2004; Sheriff et al., 2010). All droppings excreted spontaneously during the course of 3 h after injection [time period chosen to minimize disturbances on chicks, but enough to detect gut passage time in tern chick droppings (e.g., Dahdul and Horn, 2003)] were collected one by one, changing the foil after each successive sample. The time from the injection to the deposition was noted. Dropping samples were kept on ice during field work and transport and then were frozen at −80 °C until laboratory analysis, at which point they were also weighed.

2.2. Determining the concentrations of EGMs

Glucocorticoid metabolites were extracted from the droppings (0.1 g from each fecal sample) with 60% methanol in double-distilled water. This percentage of methanol has been optimal for obtaining the highest levels of recovery based on the polarity of the EGMs present in bird droppings (Möstl et al., 2005). Samples were shaken in a vortex for 1–2 min, centrifuged (2500*g* for 15 min), and the supernatants were collected. An aliquot of each supernatant (1 ml) was used in each of the subsequent tests. Six enzyme immunoassays (EIAs) were used to test for EGMs in the chick droppings (Table 1), previously used in other bird species (e.g., Nakagawa et al., 2003; Quillfeldt and Möstl, 2003; Rettenbacher et al., 2004; Stöwe et al., 2008, 2010) and taking into account the different group specificity of the EIAs in detecting GC metabolites (Möstl et al., 2005).

2.3. Evaluating the effect of diet and growth rate on EGM levels

For this purpose, we used data derived from Albano et al. (2011), which included a detailed study on growth rates

Table 1
Six different enzyme immunoassays (EIA) used to test for immunoreactive glucocorticoids metabolites in droppings of gull-billed tern chicks, following the procedures performed by author cited in the last column. Its main specific antigens and other similar structures involved in its specificity are indicated.

EIA	Standard used	Antigen	Reacting also with	Described by
1	11β-Hydroxyetiocholanolone	11β-Hydroxy-etiocholanolone-17-CMO:BSA	5β, 3α-ol, 11β-diol structure	Frigerio et al. (2004)
2	Corticosterone	Corticosterone-3-CMO:BSA	5α-Pregnane metabolites with a 11β, 21-diol-20-one structure	Palme and Möstl (1997)
3	11-Oxoetiocholanolone	11-Oxoetiocholanolone-17-CMO:BSA	5β-Androstane and 5β-pregnane metabolites with 3α-ol, 11-one structure	Möstl et al. (2002)
4	Tetrahydrocortisone	Tetrahydrocortisone-20-CMO:BSA	5β-Pregnanes with 3α-ol, 11β-diol structure	Quillfeldt and Möstl (2003)
5	Cortisone	Cortisone 20-CMO:BSA	5α-Pregnanes with 3,11-dioxo structure	Rettenbacher et al. (2004)
6	5α-Pregnane-3β, 11β, 21-triol-20-one	5α-Pregnane-3β, 11β, 21-triol-20-one-20-CMO:BSA	5α-Pregnanes with a 3β, 11β-structure	Touma et al. (2003)

determined by nonlinear regression using logistic models. In that study, 13 gull-billed tern chicks were hand-reared in captivity from hatching to flight after having been randomly assigned either to an insect- or a fish-based diet. This is an opportunistic species with a wide spectrum of preys, including fishes and insects during the breeding period (Sánchez et al., 1991, 2004; Dies et al., 2005). The feeding protocol, explained in detail by Albano et al. (2011), mainly consisted of hand-feeding the chicks *ad libitum* 8–9 times per day at regular time intervals. At each feeding, they were fed until they refused to eat more, and water was provided with a syringe until satiation. The insect diet consisted mainly of common crickets (*Acheta domestica*), and the fish diet consisted primarily of bleak (*Alburnus alburnus*). Total daily amounts of excreta for each chick were collected at three different ages (10, 15 and 22 days old). We analyzed excreted immunoreactive cortisone metabolites (CMs; the best EGM found to use as a stress index, see Results, Section 3.1) contained in those droppings by EIA as described in Section 2.2, corrected assuming a moisture content in the droppings of 70% (Koch et al., 2009), since they had been dried before analysis. Droppings excreted over 24 h were collected and weighed, allowing for the calculation of the amount of CMs produced in one day (ng day^{-1}) by multiplying the measured CM concentration (ng g^{-1}) per total daily mass of excreta (g day^{-1}).

2.4. Statistical analysis

Since droppings for the experimental validation protocol were collected after spontaneous excretion, the times and frequencies of the samplings differed among individuals. As we aimed to find the peak response time (i.e., the peak concentration of CMs in droppings), the samples were assigned to regular time intervals of 45 min, allowing for a comparison between EIAs. For each EIA, we define the range of EGMs as the difference between the minimum and maximum concentration of EGMs over three hours of the experiment (see Möstl et al., 2005). To compare these ranges between EIAs, we performed a repeated measures ANOVA (rmANOVA; with EIA type representing the repeated measure), including treatment (two levels: ACTH- and saline-injected chicks) as fixed factor for the biological validation.

Concentrations and total daily amounts of CMs were repeatedly assessed in droppings of captive chicks at different ages. Therefore, a rmANOVA was performed to evaluate the effects of diet (two levels: fishes and insects), age (three levels: 10, 15 and 22 days old), and interaction between both fixed factors.

Immunoreactive cortisone metabolites measured in 15-days-old chicks were used to study the influence of diet and body mass growth rate of chicks (*k*, growth constant of logistic growth model) on CMs, as they were taken coinciding with the linear phase of growth (i.e., maximum growth rate; see Albano et al., 2011). For this purpose, we used a general linear mixed model (GLMM), with CMs as dependent variable, diet as fixed factor (two levels) and growth rate (derived from the work by Albano et al. (2011) using a logistic growth equation) as covariate, as well as the interaction diet × growth rate. We used Tukey's test where post-hoc comparisons were necessary. All tests were performed using Statistica (StatSoft, version 7.0), after checking the variables to meet parametric assumptions. Means ± SE are shown.

3. Results

3.1. Physiological and biological validation

There were highly significant differences in the ranges between basal and peak EGMs between the six EIAs (rmANOVA: $F_{5,50} = 59.46$, $P < 0.0001$) regardless of the treatment (rmANOVA: $F_{1,10} = 0.62$, $P = 0.45$). The EIA designed to measure CMs was the most useful to detect an increase in EGMs, showing the highest range both in ACTH- and saline-injected chicks (Tukey's test, $P < 0.001$ in all cases; Fig. 1). The peak response (i.e., the peak concentration of CMs in droppings), occurred around 90 min after the ACTH injection (2007.03 ± 332.57 ng g^{-1}; Fig. 2A) and around 180 min after the saline injection (1554.07 ± 377.48 ng g^{-1}; Fig. 2B), significantly differing from basal levels, while differences in the rest of EGMs were unappreciable (Fig. 2A and B).

3.2. Effect of diet on CM levels in captive chicks

Both CM concentrations in droppings (Fig. 3A) and total daily CMs excreted (Fig. 3B) were significantly affected by diet

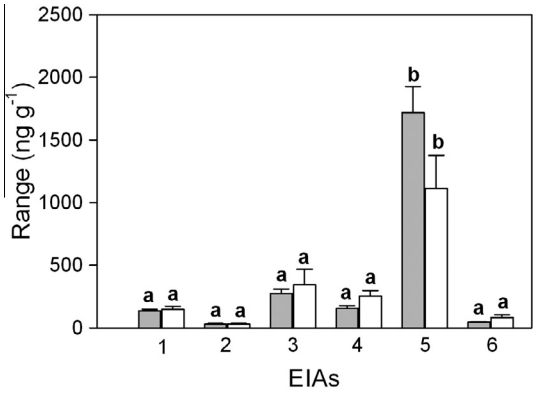

Fig. 1. Ranges between maximum and basal levels (mean ± SE) of excreted immunoreactive glucocorticoids metabolites (EGMs) in six gull-billed tern chicks injected with ACTH (gray bars) and six injected with saline solution (white bars), measured with six different enzyme immunoassays (EIAs), described in Table 1. Different letters indicate means significantly different (Tukey test).

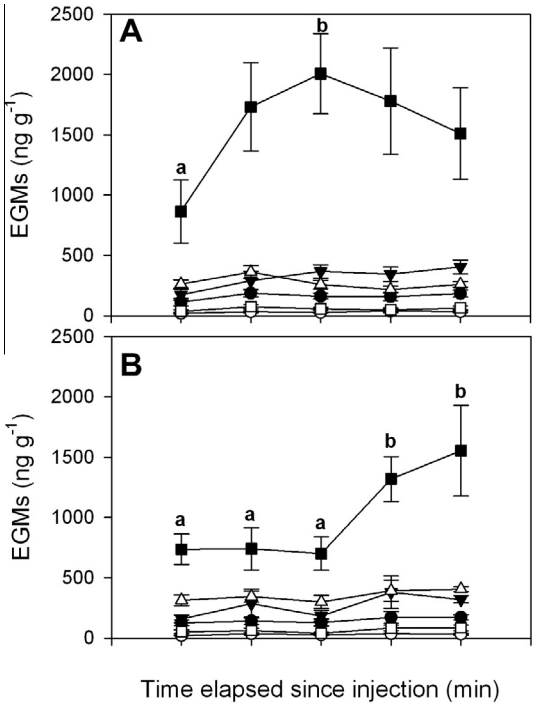

Fig. 2. Excreted immunoreactive glucocorticoids metabolites (EGMs) levels (mean ± SE) in (A) six gull-billed tern chicks injected with ACTH and (B) six injected with saline solution within 3 h after administration, measured with six different enzyme immunoassays, described in Table 1. Cortisone (EIA number 5) is represented by black squares. Different letters indicate means significantly different (Tukey test).

($F_{1,11}$ = 71.16, P < 0.0001 and $F_{1,11}$ = 27.01, P < 0.0003, respectively), being higher in fish-fed chicks than in those raised on an insect diet (Fig. 3A and B), whereas the total amount of excreta was overall higher in insect-fed chicks (Fig. 3C). There were no significant effects of age or age × diet in either CM concentrations or total daily CMs excreted (P > 0.05 in all cases).

3.3. Relationship between growth rate, diet and CM levels

The GLMM results showed that diet had no significant effect on the relationship between growth rate and CMs measured at 15 days of age ($F_{1,9}$ = 1.65, P = 0.230), therefore, the interaction of diet × growth rate was removed from the model. Consistent with results in Section 3.2, the effect of diet on CMs of 15-days-old chicks was highly significant ($F_{1,10}$ = 107.68, P < 0.0001), with fish-fed chicks showing higher CM levels (Fig. 4). Immunoreactive cortisone metabolites were also affected by growth rate ($F_{1,10}$ = 12.20, P < 0.01), being negatively correlated both in chicks fed fish ($F_{1,5}$ = 10.43, P < 0.05, R^2 = 0.70, b = −0.72) and insects ($F_{1,4}$ = 30.80, P < 0.01, R^2 = 0.88, b = −0.94) (Fig. 4). As explained in Albano et al. (2011), fish-fed chicks showed higher growth rates. Diet and growth rate together, the two retained variables in the model, explained 91% of the variance in CM levels in gull-billed tern chicks.

4. Discussion

4.1. Validation of the noninvasive technique to assess stress in gull-billed tern chicks

In this study we validated the analysis of excreted immunoreactive CMs to assess stress levels in gull-billed tern chicks. Acute stress produced by the administration of ACTH caused an increase

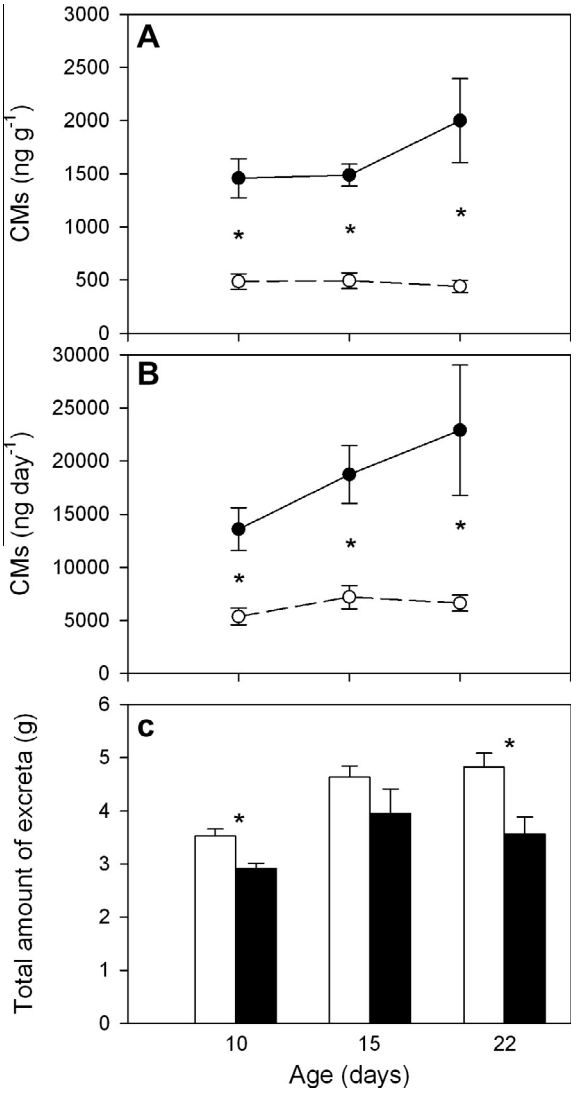

Fig. 3. Experiment with captive chicks showing (A) mean ± SE concentration of excreted immunoreactive cortisone metabolites (CMs) in droppings, (B) mean ± SE total daily amount of excreted CMs and (C) mean ± SE total amount of excreta in gull-billed tern chicks fed on a piscivorous (black) or an insectivorous (white) diet in three different developmental stages (10, 15 and 22 days old). Asterisks indicate means significantly different between diets.

in circulating GCs that was efficiently detected by the technique, since it reflected an increase of immunoreactive metabolites in droppings, while all the others EIAs failed to show any significant difference after the ACTH injection (Fig. 1). The cortisone EIA was also useful to reveal the physiological response of chicks after a stressful event (i.e., catching, handling and housing in our experiment) of biological relevance, contrarily to all the other ones, since it was the most useful to detect an increase in EGMs caused by increased adrenocortical activity in response to stressors, confirming its usefulness in measuring stress in chicks of this species (Fig. 1). Both the Saline and the ACTH injected groups followed a similar pattern of GMs excretion, matching the described two picked curve of EGMs detection in bird droppings (Fig. 2; minutes 45 and 90 in ACTH-injected chicks, and 135 and 180 in saline-injected chicks), according to urinary and fecal excretion (Möstl et al., 2005). In the experimental (ACTH-injected chicks) group these peaks appear earlier most probably due to the powerful stimulating action of the ACTH, linked to the fact that the stress of captivity may become stronger when chicks stay in the boxes for a prolonged time.

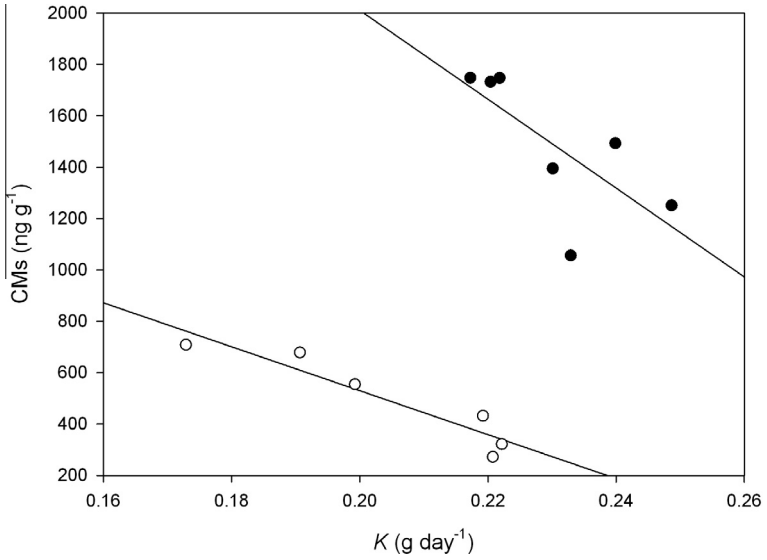

Fig. 4. Regressions between body mass growth rate (*k*, growth constant) and concentration of excreted immunoreactive cortisone metabolites (CMs) in 15-days-old captive gull-billed tern chicks fed on a piscivorous (black dots) or an insectivorous (white dots) diet.

Because steroid hormones are metabolized before excretion, for the detection of these metabolites in feces mainly group-specific antibodies are used, that react with a family of metabolites derived from an original steroid (Palme and Möstl, 1997). The cross-reactions of the cortisone EIA were described by Rettenbacher et al. (2004), including 3,11-dioxygen structures. The metabolism of corticosterone (the main steroid in the plasma of birds; Möstl and Palme, 2002; Palme et al., 2005) may cause an oxidation at position C11 (Mazancová et al., 2005; Kuĉka et al., 2006) originating those dioxo structures, which could explain the effectiveness of cortisone EIA in detecting EGMs found in droppings of gull-billed tern chicks. On this regard, it is necessary to highlight the importance of achieving a standardization of the procedures when measuring EGMs in droppings to properly compare results of different studies, a matter that still remains unsolved.

4.2. Effect of diet and growth rate on EGM measurements

The significant differences in EGMs in chicks fed different diets (Fig. 3A and B) suggest that this is a key factor to consider when evaluating stress using this noninvasive technique. Although all individuals were fed *ad libitum*, nutritional stress (i.e., suboptimal nutritional conditions) due to a low-quality diet may account for the higher levels of CMs in droppings, due to the relationships between nutritional state and adrenocortical activity (Kitaysky et al., 2001; Quillfeldt et al., 2007; Jenni-Eiermann et al., 2008). In that case, we would expect that increased CMs corresponded with a lower growth rate (Kitaysky et al., 2003), since it has been suggested that this glucocorticoid may be involved in the redistribution of energy from growth to maintenance (Landys et al., 2006; Müller et al., 2009). However, that was not the case because fish-fed chicks were more efficient at assimilating energy and macronutrients than chicks fed insects (Albano et al., 2011), showing also a higher growth rate (Fig. 4; Albano et al., 2011). Thus, higher levels of CMs in fish-fed chicks cannot be attributed to a lower-quality diet causing suboptimal conditions. Our results suggest, contrarily, an effect of diet *per se* in EGMs, not due to differences in the nutritional value of the diets.

There are several not mutually exclusive hypotheses which could explain the significant differences in the CM concentrations in droppings (ng g^{-1}) and/or in total daily CMs excreted (ng day^{-1}) between diets: (1) The amount of indigestible material present in the food can affect the concentration of CMs by increasing the amount of droppings produced (Goldin et al., 1982; Wasser et al., 1993; Dantzer et al., 2011), thus leading to an underestimation of real EGM levels (Goymann, 2012). Since macronutrient assimilation efficiency of chicks fed insects is significantly lower than that for fish-fed chicks (Albano et al., 2011), the amount of droppings collected over a day was greater with the insect diet (Fig. 3C). Consequently, for a similar amount of EGMs, the final concentration measured in droppings of chicks with an insectivorous diet would be lower. Although in field studies it is generally not possible to measure the rate of excretion of GC metabolites over a defined time period, it has been suggested that it represents a better estimate of its production than its concentration in just one sample (Goymann, 2005; Goymann et al., 2006). However, the results of our study showed that although the differences between diets in total daily CMs were smaller than when considering the concentration, the amount of CMs occurring throughout the day was also significantly higher for fish-fed chicks. (2) Differences in dietary components may affect CMs in chick droppings, as suggested for EGMs in mammals (e.g., Goldin et al., 1981; van der Ohe et al., 2004; Dantzer et al., 2011). GCs are initially metabolized in various organs, primarily in the liver and then excreted in the bile to the intestine, where microbial enzymes play an additional role in the metabolic conversion (Möstl et al., 2005). It is well known that the activity of bacterial enzymes in the gut may change with diet (Martinez del Rio et al., 1995; Levey et al., 1999), which may affect the structure of the steroid metabolites formed (McDonald et al., 1983). Some of the metabolites present in the intestine are reabsorbed back (enterohepatic circulation), a process in which the enzyme activity is also crucial (Crowther et al., 1977; McDonald et al., 1983). The formation of EGMs in chicks could then be modified as a result of differences in the microbial metabolism in the intestine depending on the diet. (3) EGM levels could also be altered by direct ingestion of GCs. It has been speculated that the levels of circulating GCs may be increased by the consumption of large amounts of GCs in animal flesh (e.g., fish) or other dietary sources (van der Ohe and Servheen, 2002), and it has been recently shown that EGM levels may change even with slight differences in diet in mammals (Dantzer et al., 2011). This factor may also contribute, at least partially, to the highest concentration of EGMs

found in droppings of fish-fed chicks, but unfortunately we did not measure initial cortisone levels present in both diets of experimental chicks.

5. Conclusions

The determination of EGMs to assess stress in birds is often compromised by the limited variety of species in which the technique has been validated. Therefore, the mere validation of the cortisone EIA to assess stress in gull-billed tern chicks should be considered of great importance. This technique has emerged as a promising physiological tool to study many important ecological aspects, including assessing habitat quality (Homyack, 2010; Albano 2012), with a huge potential in conservation biology (Wielebnowski and Watters, 2007). Traditional measures of habitat quality generally provide information about how individuals are distributed according to available resources, while measures of stress may identify other habitat characteristics often overlooked, including negative stimuli (Walker et al., 2005). The adrenocortical response of individuals prepare them to deal with a stressor; however, taking into account the potential adverse effects of chronic stress, these studies are particularly relevant in endangered species (Millspaugh and Washburn, 2004).

Klasing (2005) suggested that the nutritional strategy could influence the noninvasive measurement of hormones in birds, since the amount and composition of feces and transit rate could vary with diet. To date, this potential effect of diet on EGM levels remains poorly tested in birds, in spite of the fact that one-third of species are omnivorous and consume a wide variety of foods. Our findings suggest that the effect of diet should be considered when using the measurement of EGMs with comparative purposes to avoid confusing interpretation of the results. Also, we have shown that EGM levels may be used to infer the status of growth rates in birds, at least in the period when chicks are growing faster, highlighting the importance of stress levels on their development.

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