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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.

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