



Reversed hatching order, body condition and corticosterone levels in chicks of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*)

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ABSTRACT

In altricial and semi-altricial species, asynchronous hatching gives the first chicks to hatch an initial advantage over other siblings and often leads to the elimination of the smallest chicks. Both baseline corticosterone and acute stress-induced corticosterone levels have been shown to be higher in food deprived chicks than in chicks fed ad libitum. However, first-hatched chicks have also been shown to exhibit higher corticosterone levels than last-hatched chicks, suggesting an influence of the initial differences between eggs on corticosterone levels. We subjected single-chicks of southern rockhopper penguins *Eudyptes chrysocome chrysocome* to a standardised capture-stress protocol. In this species having very dimorphic two-egg clutches, we examined whether corticosterone levels were different between the two chick categories and tested for the effect of body condition controlled by the chick category. Neither body sizes, nor corticosterone levels differed between A- and B-chicks at 18 days. In contrast to baseline corticosterone levels, acute stress-induced levels of corticosterone were negatively correlated to body condition: chicks with a good body condition had lower acute stress-induced levels of corticosterone than chicks with a poor condition, whatever the chick category. Our results do not support the idea that initial differences in egg characteristics could drive the difference in corticosterone levels between siblings. On the contrary, they show that the A-egg of rockhopper penguins has, when reared alone, the same intrinsic potential to develop into a fledged chick as the B-egg. Later differences in body condition appear to lead to variation in the acute stress-induced levels of corticosterone.

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1. Introduction

The hormones of the hypothalamic–pituitary–adrenal (HPA) axis play an important role in maintaining homeostasis and energetic balance in vertebrates under stressful and even non-stressful situations (Sapolsky et al., 2000; DeVries, 2002; DeVries et al., 2003). In birds, corticosterone is the primary steroid hormone secreted by the adrenal cortex. Indeed, plasma corticosterone levels rapidly rise when a bird is subject to unpredictable and sudden environmental changes such as a storm, food shortage or predation risk which are seen as acute stressful conditions (Sapolsky, 1992; Wingfield and Farner, 1993; Wingfield, 1997). This corticosterone stress response, facilitating the mobilisation of the energy necessary for the behavioural response, acts to reduce the impact of the stressor. After the perturbation has passed, corticosterone levels return to baseline levels and normal activities can be resumed.

However, a bird unable to escape this stressor will suffer the deleterious effects of sustained high corticosterone levels (i.e. chronic stress). These negative effects may include memory and immune system suppressions, wasting of muscle tissue and neuronal cell damage (Sapolsky et al., 1986; Sapolsky, 1992; Kitaysky et al., 2001a; and see Wingfield, 1994).

Altricial chicks which are completely dependent on their parents for survival cannot escape a stressor while precocial chicks are able to move away from perturbations (Starck, 1993). Chicks have been shown to exhibit age-related variation in the development of their corticosterone stress responses during growth. Precocial chicks typically exhibit a well-developed corticosterone stress response at hatching while altricial chicks have a small corticosterone stress response at hatching that increases until adult age (see Schwabl, 1999; Love et al., 2003a; Walker et al., 2005b; Quillfeldt et al., 2009).

In altricial and semi-altricial species, asynchronous hatching gives the first chicks to hatch a head start at vigorously begging for food and successfully attracting parental attention (Magrath, 1990). Therefore, elimination of the smallest chicks usually occurs by starvation as result of competition with their larger siblings for

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parental feeding, from overt parental neglect, or psychological and physical intimidation by their larger siblings (Magrath, 1990). Food deprivation experiments on chicks individually kept in cages also showed that both baseline corticosterone level and acute stress-induced corticosterone level were higher in food deprived chicks than in chicks fed *ad libitum* (Kitaysky et al., 1999a, 2001a; Pravosudov and Kitaysky, 2006; but see Kitaysky et al., 2005). The same results have been obtained on single-chicks and two-chick broods during food deprivation experiments in wild conditions (Nuñez-De La Mora et al., 1996). Body condition per se has also been directly and negatively linked to corticosterone levels in chicks (Kitaysky et al., 1999a, 2001a; Sockman and Schwabl, 2001).

Under the above scenario, we thus expect the last-hatched chicks (i.e. food deprived and poor condition chicks) to have lower baseline and acute stress-induced levels of corticosterone than their older siblings (i.e. well-fed chicks). Hatching order has indeed been shown to influence corticosterone levels in altricial bird species. Baseline corticosterone levels of canary *Serinus canaria* chicks vary with hatching order (Schwabl, 1999). In American kestrel *Falco sparverius* chicks, both hatching order and hatching span (difference in days between first- and last-hatched chicks) have a significant effect on both baseline corticosterone levels and acute stress-induced corticosterone levels between siblings at ages 10, 16, 22, and 28 days post-hatching (Love et al., 2003b). However, in both of these studies, first-hatched chicks exhibited higher baseline and acute stress-induced levels of corticosterone than last-hatched chicks. This observation suggests that initial egg/chick characteristics directly linked to its position within the brood could impact baseline corticosterone levels and acute stress-induced corticosterone levels more than the consequent chick condition does.

Penguins have semi-altricial chicks that depend on their parents for food and defence, although they have well developed down and can thermoregulate soon after hatching (Williams, 1995). Similar to altricial species, penguin chicks show little corticosterone stress response at hatching, a robust corticosterone stress response near fledging and an intermediate corticosterone stress response at half time (Walker et al., 2005b). However, in response to an adrenocorticotropic hormone (ACTH) challenge, hatchlings are capable of secreting corticosterone at adult-like levels (Walker et al., 2005b). Crested penguins (genus *Eudyptes*), exhibit brood reduction: two eggs are laid but only one chick usually fledges (Warham, 1975). They also present, uniquely among birds, a reversed hatching asynchrony (St Clair, 1995, 1998): the larger second-laid egg (B-egg) hatches before the smaller first-laid egg (A-egg). As a result, the chick from the A-egg generally dies of starvation within days of hatching (Gwynn, 1953; Lamey, 1990). Both eggs of southern rockhopper penguins *Eudyptes chrysocome chrysocome* are very different in mass, size (Poisbleau et al., 2008; Demongin et al., 2010a) and composition (Poisbleau et al., 2009a,b, 2011, in preparation). Chicks from A-eggs (A-chicks) have, therefore, a lower initial mass and size than chicks from B-eggs (B-chicks) at hatching (Poisbleau et al., 2008). Therefore, we expected both chicks to have a different organisation of the HPA axis directly linked and fixed to their position within the brood.

We subjected chicks of rockhopper penguins to a standardised capture-stress protocol (Wingfield, 1994). To exclude any impact of age, sibling presence and hatching asynchrony, we sampled chicks at the same age and only used chicks when the sibling had disappeared before hatching. We then examined whether baseline corticosterone levels and acute stress-induced corticosterone levels were different between A- and B-chicks. We also tested for the effect of body condition. If initial egg/chick characteristics directly linked to their position within the brood leads to different organisations of the HPA axis between chicks, we expected a higher baseline and acute stress-induced levels of corticosterone in

B-chicks than in A-chicks. On the contrary, if body condition is the main driver of corticosterone levels in chicks, we should observe no difference in these levels between A- and B-chicks and decreasing baseline and acute stress-induced levels of corticosterone as chick body condition increases.

2. Methods

2.1. Study site and birds

This study and experiment were carried out at the “Settlement colony” on New Island, Falkland Islands (51°43'S, 61°17'W) in December 2007. This colony has approximately 5000 pairs of breeding southern rockhopper penguins. Their breeding biology at this colony has been described by Strange (1982), and more recently by Poisbleau et al. (2008). Briefly, males arrive first at the colony (early October) and establish nest sites. Females arrive a few days later, for pairing and copulation. The laying and then hatching are very synchronised within this population, both taking place in less than two weeks from the laying (or hatching) of the first egg to the laying (or hatching) of the last egg (see Poisbleau et al., 2008).

After the arrival of the first males, we visited study sites daily, initially to mark active nests and subsequently to follow the egg laying and future breeding developments. During and after the hatching period, we visited study sites daily in order to record hatching dates and chick survival in each study nests. Chicks in nests with siblings less than 5 days of age were marked on one nail with non-toxic paint marker. Study chicks older than 5 days of age were all marked with a numbered and coloured Velcro band on each flipper (see Poisbleau et al., 2008 for more details about the nest survey). This study was conducted under a research licence granted by the Environmental Planning Department of the Falkland Islands Government.

2.2. Blood sampling

We selected 25 study chicks (11 A- and 14 B-chicks) from which the sibling had disappeared before hatching. We, therefore, could assume that they all had, more or less, the same rearing conditions.

We blood sampled all of the chicks at 18 days of age, when they were still brooded permanently by males. All birds were bled according to the following standardised technique (Wingfield, 1994): immediately after capture, an initial blood sample (500 µl) was collected from the brachial vein with a 1 ml heparinised syringe and a 25 gauge needle, and additional samples (300 µl) were taken 20 and 30 min later. None of the chicks sampled during the experiment had seen us on the day of capture previous to our first approach to catch it. For each individual, we measured the time elapsed between the onset of trapping (i.e. when the manipulator was visible from the nest) and the end of initial blood sampling to control for possible effect of handling time during initial bleeding on hormone levels. Mean handling time was 107 ± 27 s (SD), maximum: 163 s. Only one chick at a time was captured in an area of approximately 100 m² to avoid the possibility that subsequently sampled chicks may have already witnessed the capture process (Walker et al., 2005a). Because the study area was uneven, and interspersed with, and fringed by, tussock grass *Poa flabellata*, it was possible to approach close to the nests without being seen by sampled birds.

After each blood sample, the bird was kept in an individual box. Blood samples were put into ice and centrifuged as soon as possible. Subsequently, plasma (for corticosterone assay) was stored at –20 °C. All chicks were bled at the same period of the day (between 10:00 and 13:00 the first time) to minimise the effect

of diurnal fluctuations on plasma corticosterone levels (Breuner et al., 1999; Romero and Remage-Healey, 2000; Tarlow et al., 2003).

2.3. Hormone analysis

Corticosterone levels were determined at the CEBC following the procedure detailed in Lormée et al. (2003). All samples were run in one assay. The intra-assay coefficient of variation assessed using three reference plasmas was 4.91% ($n = 9$ duplicates). The lowest detectable concentration was 0.2 ng/ml while the lowest measurement was 0.31 ng/ml.

There was no significant relationship between handling time and corticosterone levels measured during the initial bleeding (Spearman's correlation, $r_s = -0.002$, $P = 0.991$). Thus, initial blood samples were considered to reflect baseline corticosterone levels (Wingfield et al., 1982; Kitaysky et al., 1999b). Corticosterone levels (baseline, 20 and 30 min) were not correlated with time of day (Spearman's correlation, $r_s = -0.202$, $P = 0.334$, $r_s = 0.223$, $P = 0.284$ and $r_s = 0.329$, $P = 0.108$, respectively). This suggests that our protocol was effective in minimising this source of variation.

2.4. Morphometric measurements and body condition

The chicks were weighed to the nearest gram after blood collection using a spring balance. We measured flipper length, extended from axilla, to the nearest millimetre with a ruler (Poisbleau et al., 2010). All measurements were made by the same observer to minimise observer biases. We calculated an index of body condition for each 18-day chick using the ratio of body mass (g) to flipper length (mm) (Robinson et al., 2005; Poisbleau et al., 2008). Birds with a high value were in a relatively better body condition for their size than birds with low values.

2.5. Statistical analysis

Plasma corticosterone levels (baseline, 20 and 30 min) and body condition followed a normal distribution (Kolmogorov–Smirnov tests: $Z = 1.069$, $P = 0.203$, $Z = 0.450$, $P = 0.987$, $Z = 0.526$, $P = 0.945$ and $Z = 0.492$, $P = 0.969$, respectively). We used a General Linear Model (GLM) based on Type III sum of squares with chick category (from A- or from B-eggs) as fixed factor to test for the difference in body condition between both chick categories. We also performed a GLM with chick category as fixed factor and body condition index as covariate to test for the impact of these parameters on baseline corticosterone levels. We examined changes in corticosterone levels with time after capture with two-way repeated GLMs, where chick category was included as a factor, body condition index as a covariate and serial bleeds of the same bird during the stress protocol as repeated measures. All statistical analyses were performed using SYSTAT 7.0 (release 9 for Windows; SPSS Inc., Chicago, USA, Wilkinson, 1997). Values are presented as means \pm standard error.

3. Results

3.1. Body condition and baseline corticosterone levels

At 18 days of age, A- and B-chicks did not differ in body mass (GLM, $F_{1,23} = 0.003$, $P = 0.951$), flipper length (GLM, $F_{1,23} = 3.072$, $P = 0.093$) and body condition (GLM, $F_{1,23} = 0.899$, $P = 0.353$). Baseline corticosterone levels were not different between A- and B-chicks (GLM, $F_{1,21} = 0.019$, $P = 0.891$), were not related to body condition (GLM, $F_{1,21} = 0.455$, $P = 0.507$, Fig 1) and were also not dependent on the interaction between these parameters (GLM, $F_{1,21} = 0.057$, $P = 0.814$).

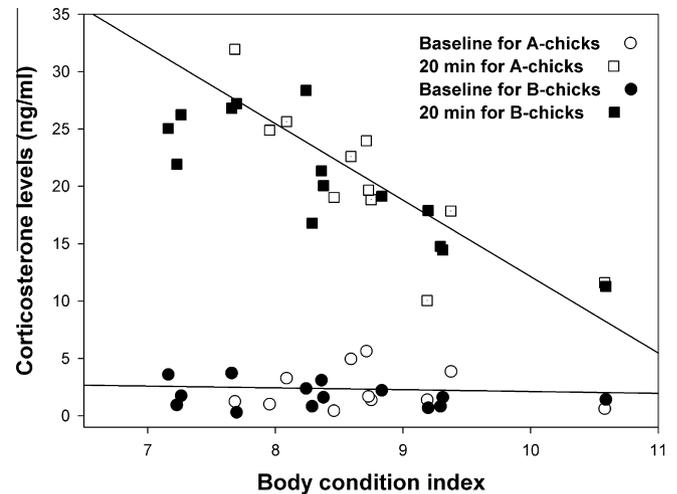


Fig. 1. Relationships between body condition index, baseline corticosterone levels (0 min, circles) and acute stress-induced levels of corticosterone at 20 min after handling (squares) for chicks from A-eggs (open symbols) and chicks from B-eggs (closed symbols).

3.2. Acute stress-induced levels of corticosterone

Rockhopper penguin chicks responded to the stress of being captured and held by a rapid and significant response of the adrenocortical system (repeated measures GLM between 0, 20 and 30 min, $F_{2,48} = 134.020$, $P < 0.001$, Fig. 2). Corticosterone levels rapidly increased between 0 and 20 min after capture and restraint (repeated measures GLM between 0 and 20 min, $F_{1,24} = 296.874$, $P < 0.001$, Fig. 2). Therefore, maximum corticosterone levels were almost reached after 20 min of handling and restraint and did not significantly increase between 20 and 30 min (repeated measures GLM between 20 and 30 min, $F_{1,24} = 1.860$, $P = 0.185$, Fig. 2).

The acute stress-induced levels of corticosterone were quantitatively similar between A- and B-chicks (repeated measures GLM between 0, 20 and 30 min, $F_{2,42} = 1.383$, $P = 0.262$), which reflected no significant difference in plasma corticosterone levels between both chick categories at each time step (Fig. 2). The magnitude of the corticosterone increases was significantly and negatively affected by body condition (repeated measures GLM between 0, 20 and 30 min, $F_{2,42} = 8.909$, $P = 0.001$, Fig. 1). There was no significant

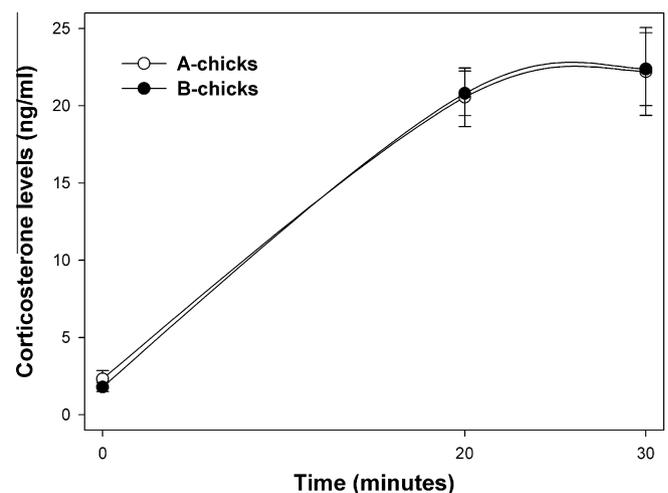


Fig. 2. Corticosterone levels (ng/ml) over 30 min of capture and handling for A-chicks (open circles) and B-chicks (closed circles). Time 0 min corresponds to the baseline corticosterone levels, i.e. from the blood samples taken immediately after capture.

interaction between chick category and body condition ($F_{1,42} = 1.336, P = 0.274$). Therefore, chicks with a poor body condition had higher acute stress-induced levels of corticosterone than chicks in good body condition independent of whether they came from A- or B-eggs (Fig. 1).

4. Discussion

4.1. Difference between A- and B-chicks in corticosterone levels

Neither body mass, nor flipper length or body condition differed between A- and B-chicks at 18 days of age. Similarly, baseline corticosterone levels and acute stress-induced corticosterone levels did not differ between A- and B-chicks at this age. These results suggest that chicks from A-eggs had a growth and a HPA axis development which was not different from chicks from B-eggs despite their initial differences. All the sampled chicks had lost their sibling before hatching thus they never endured sibling competition. Hatching asynchrony and its effects on growing chicks were, therefore, not a source of difference between the two chick categories. This also supports the idea that the A-egg of the southern rockhopper penguin has, when reared alone, theoretically the same intrinsic potential to lead to a fledged chick as the B-egg (Poisbleau et al., 2008). However, although the hatching success is not significantly different between A- and B-eggs and both eggs hatch in a large majority of the nests, A-chicks usually disappear immediately after hatching (Poisbleau et al., 2008).

Schwabl (1999) proposed that a differential maternal investment in yolk steroids could influence the neuroendocrine development and function of siblings and could lead to difference in corticosterone secretion between them. Accordingly, Sockman and Schwabl (2001) found that treatment of American kestrel eggs with maternal androgens produced chicks with higher corticosterone levels than control chicks. Yolk androgen levels are higher in late laid eggs (B-eggs) than in early laid eggs (A-eggs) of southern rockhopper penguins (Poisbleau et al., in preparation). If we assume that yolk androgens have the same effect in southern rockhopper penguins than in American kestrels, we should expect higher corticosterone levels in B-chicks than in A-chicks. However, corticosterone levels did not differ between the two chick categories in the present study. Our results, therefore, do not seem to support Schwabl's hypothesis (1999).

Schwabl (1999) and Love et al. (2003b) also believed that the effect of hatching order on corticosterone levels is largely due to the fact that all broods hatched asynchronously, resulting in competitive hierarchies. They then suggested that hierarchies in adrenocortical function among siblings should be one physiological mechanism by which the developmental competitive hierarchies are maintained, without the costs associated with chronic, acute stress-induced levels of corticosterone. This hatching asynchrony hypothesis still needs to be tested for rockhopper penguins with clutches in which both the A- and the B-chicks survive sufficiently after hatching. Although this occurrence is almost nil in crested penguins, the Falkland Island population of rockhopper penguins could enable this hypothesis to be tested. Indeed, in this population it appears possible for rockhopper penguins to raise two chicks (Strange, 1982; Pütz et al., 2001; Clausen and Pütz, 2002). In 2006/2007 for example, three of 114 study nests (Poisbleau et al., 2008) raised two chicks until fledging. In the same breeding population, more than 80% of pairs retained both eggs until at least one hatched successfully (Demongin et al., 2010a; Strange, 1982; Lamey, 1993). Because of the reversed hatching asynchrony exhibited by this species (St Clair, 1995, 1998) and the general decrease of corticosterone levels with hatching order observed in other studies (Schwabl, 1999; Love et al., 2003b), we expect, once more,

that first-hatched chicks (B-chicks) will have a higher baseline and acute stress-induced levels of corticosterone than last-hatched chicks (A-chicks).

4.2. Body condition effect

Body condition had no significant effect on baseline corticosterone levels in southern rockhopper penguins. To our knowledge, only one other study has examined the relationship between corticosterone levels and nutritional status in penguin chicks which were not long-fasting (Walker et al., 2005b). They observed that magellanic penguins *Spheniscus magellanicus* chicks increased baseline corticosterone levels only when they were food deprived and that the size and mass asymmetries between the two siblings were well established. They noticed no differences in baseline corticosterone levels between well-fed chicks and food deprived chicks when the later were not yet food deprived or moribund (Walker et al., 2005b). In this study and in food deprivation studies (Kitaysky et al., 1999a, 2001a; Kitaysky et al., 2005; Pravosudov and Kitaysky, 2006), chicks were food deprived either experimentally or via intense sibling competition in low food availability conditions. We can, therefore, assume that the food restrictions were quite drastic. In the present study, rockhopper penguin chicks had been without a sibling since hatching, females were feeding them and food conditions were not particularly hard (see Demongin et al., 2010b). They have, therefore, never encountered competition or suffered from intense food restriction. Correlative studies under normal food conditions showed that body mass or condition were not correlated to baseline corticosterone levels (Schwabl, 1999; Love et al., 2003a; Pravosudov and Kitaysky, 2006; Adams et al., 2008; but see Sockman and Schwabl, 2001). These results are in line with our results. These higher baseline corticosterone levels in food-restricted chicks compared to non-restricted chicks suggest that chicks with an extremely low body condition due to food restriction may suffer chronic stress while small differences in body conditions between chicks when they are not food-restricted should not impact baseline levels of corticosterone.

Contrary to baseline corticosterone levels, acute stress-induced levels of corticosterone were negatively correlated to body condition implying that chicks in good body condition had smaller acute stress-induced levels of corticosterone than chicks with a poor body condition, whatever the chick category. This relationship between body condition and acute stress-induced levels of corticosterone in southern rockhopper penguin chicks is in accordance with previous studies in other species in which experimental food restriction caused a reduction in body mass or fat reserves and an elevation of acute stress-induced levels of corticosterone (Kitaysky et al., 1999a, 2001a; Pravosudov and Kitaysky, 2006; but see Kitaysky et al., 2005). Magellanic penguins chicks, however, showed no difference in acute stress-induced levels of corticosterone until near fledging (Walker et al., 2005b). At fledging, poor condition chicks had lower acute stress-induced levels of corticosterone than their siblings (Walker et al., 2005b). This difference is in contrast with what was predicted and to our results.

In the present study, we did not link variations in corticosterone levels to age differences as we sampled all chicks at the same age (i.e. 18 days old). At this stage, males were still permanently brooding their chicks and females were foraging during the day to feed chicks. However, males were quite quickly leaving the colony and leaving their chicks alone in crèches. At this age, chicks have to be ready to be unguarded, to protect themselves against aggression and bad weather. A good enough stress response is, therefore, required whatever their body mass. Why rockhopper penguin chicks with a poor body condition have a higher stress response than chicks in good body condition is unclear as it is not yet known

whether body condition influences acute stress-induced corticosterone levels or the other way around. One explanation could be that chicks with a poor body condition, possibly due to low parental quality, need to beg more to their parents. Indeed, corticosterone levels have been shown to facilitate begging and to affect resource allocation in birds (Kitaysky et al., 2001b; Loiseau et al., 2007; Quillfeldt et al., 2006). This hypothesis could also explain the dissimilarity between the two studies on penguins. Moribund magellanic penguin chicks, with a body condition too poor to enable them to beg for food, may have had no need for strong acute stress-induced corticosterone levels.

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