



## What ecological factors can affect albumen corticosterone levels in the clutches of seabirds? Timing of breeding, disturbance and laying order in rockhopper penguins (*Eudyptes chrysocome chrysocome*)

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### ARTICLE INFO

#### Article history:

Received 13 December 2008

Revised 15 March 2009

Accepted 23 March 2009

Available online 31 March 2009

#### Keywords:

Albumen corticosterone

Maternal investment

Disturbance

Reversed hatching asynchrony

Laying sequence

Laying date

Southern rockhopper penguins (*Eudyptes chrysocome chrysocome*)

### ABSTRACT

Female birds deposit corticosterone into their eggs. Elevated concentrations of this hormone may interfere with the development of their offspring, and mothers should thus regulate corticosterone levels deposited into the eggs adaptively. However, if females are unable to regulate deposition, then the corticosterone concentration in eggs should reflect that in female plasma and should be influenced by stressors to the females. We measured corticosterone levels in the albumen of rockhopper penguins, and assessed their relationship with hatching order, human disturbance and laying date. Rockhopper penguins (*Eudyptes chrysocome chrysocome*) lay two eggs, of which the second egg (B-egg) is larger and hatches faster than the first egg (A-egg). The chick hatching from the B-egg is also much more likely to survive than its sibling. Albumen corticosterone concentrations were lower in B-eggs. However, as B-eggs contained more albumen than A-eggs, the total corticosterone deposited in the albumen was not significantly different between the two eggs. Daily disturbance by human observers during albumen production did not influence albumen corticosterone levels. Laying date had an effect on total albumen corticosterone through a higher albumen mass. However, we observed a high individual component in the composition of eggs from the same clutch. Thus, more work is required to explore the hypotheses of passive versus active transfer to eggs and to understand the adaptive value of contrary effects on the amount and concentration of corticosterone.

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### 1. Introduction

The interest in studies on the effects of maternal hormones on embryos has considerably increased during the last decade (see Groothuis and Schwabl (2008) for a recent review). Fitness consequences of an early exposure of the embryo to different levels of corticosterone levels have been widely documented in bird species (Rubolini et al., 2005; Love et al., 2005; Janczak et al., 2006, 2007; Hayward et al., 2006; Love and Williams, 2008; Martin and Schwabl, 2008). Elevated egg corticosterone levels can have a negative impact on the development of the embryo, but also on the fitness of the offspring (see Groothuis and Schwabl (2008) for a review). For example, corticosterone injections in eggs of hens *Gallus gallus domesticus* resulted in greater embryonic mortality, earlier termination of foetal development, reduced developmental stability and reduced growth (Eriksen et al., 2003). Similarly, eggs

of barn swallows *Hirundo rustica* injected with corticosterone had lower hatchability and produced fledglings with smaller body size and slower plumage development than did control eggs (Saino et al., 2005).

Corticosterone seems to be transferred from the female organism to the egg during the egg's formation (see Groothuis and Schwabl, 2008). Indeed, an increase in maternal plasma corticosterone can lead to elevated levels of egg corticosterone in several bird species (Downing and Bryden, 2002; Hayward and Wingfield, 2004; Hayward et al., 2005; Love et al., 2005). Similarly, stressed females have generally been found to lay eggs with more corticosterone (Downing and Bryden, 2002; Saino et al., 2005; Hayward et al., 2005). These results indicate that corticosterone is passively transferred from the mother to the eggs during egg formation without active female control, and that this transfer is an inevitable cost associated with poor maternal condition or deleterious environmental conditions at the time of egg formation (Love et al., 2005; Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006; Love and Williams, 2008). Indeed, since corticosterone is produced by the adrenal gland which is distant from the egg formation site and requires transport in the female bloodstream to reach the egg, independent regulation of the cortico-

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sterone levels between maternal circulation and egg seems unlikely (Groothuis and Schwabl, 2008).

It has also been proposed that maternal corticosterone deposition in eggs could be regulated at a certain level. We could suppose that different levels of egg hormones may represent a way for mothers to affect offspring phenotype and hence to influence the development of offspring behavioural and life-history traits adaptively (Groothuis et al., 2005; Müller et al., 2007; Carere and Balthazart, 2007). Accordingly, maternal corticosterone influenced primary offspring sex ratio in different bird species (Pike and Petrie, 2005; Bonier et al., 2007) and females and males did not respond similarly to the same level of maternal corticosterone (Love et al., 2005; Hayward et al., 2006; Janczak et al., 2007; Love and Williams, 2008). The fact that females may communicate environmental conditions to their offspring through hormonal mechanisms that modify offspring traits, and thereby optimise their reproduction, suggests some positive effects of maternal corticosterone (Love and Williams, 2008). In this context, it is now important to investigate the different factors influencing maternal corticosterone deposition in eggs leading to natural variation within and between clutches, in order to understand by which mechanisms maternal corticosterone accumulates in the egg, and what is the degree of regulation over this process (Groothuis and Schwabl, 2008).

Corticosterone could accumulate in yolk during follicle proliferation and also in albumen during its deposition in the oviduct after ovulation (see Groothuis and Schwabl, 2008). Until now, studies exploring maternal corticosterone deposition in eggs were largely focussed on the yolk egg component rather than in the albumen egg component (see Groothuis and Schwabl (2008) for a recent review). However, stress is known to have an effect on corticosterone concentration in the albumen (Downing and Bryden, 2002; Saino et al., 2005) and albumen corticosterone was also observed to affect offspring quality (Rubolini et al., 2005; Saino et al., 2005). As albumen deposition in the female oviduct occurs after yolk formation in the female ovary (see Groothuis and Schwabl, 2008), we can suppose that factors influencing maternal corticosterone deposition in eggs differ between yolk and albumen. Additionally, maternal corticosterone probably differs in availability and use for the developing embryo according to its location, in the yolk or in the albumen. The general aim of this study was to examine the influence of ecological factors on albumen corticosterone levels in a bird species.

Southern rockhopper penguins *Eudyptes chrysocome chrysocome*, as all other crested penguins (genus *Eudyptes*), exhibit brood reduction: two eggs are laid but only one chick usually fledges (Warham, 1975). Crested penguins 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, even though both eggs usually hatch, the chick hatched from the A-egg generally dies of starvation within days of hatching (Gwynn, 1953; Lamey, 1990). This species provides a unique model to test whether there is strategic maternal investment in corticosterone favouring the B-chick, which is most likely to survive. Moreover, studies on penguins have demonstrated that breeding adults react to human presence at their nest site with a significant increase in plasma corticosterone (Fowler, 1999; Walker et al., 2006).

In the present study, we examined the variability of corticosterone levels (in terms of concentration and total amount) within the breeding population and within clutches of southern rockhopper penguins during two consecutive breeding seasons. If corticosterone deposition in albumen is, at least partially, under the control of females and if elevated albumen corticosterone levels have deleterious effects for the embryo, we would expect females to deposit less albumen corticosterone in the B-egg, which the one most

likely to produce a fledging chick. We moreover tested whether human disturbance and laying date (as an index of declining breeding environment quality caused by increasing nest density and social interactions) affect albumen corticosterone deposition. If females are unable to avoid or reduce corticosterone deposition in albumens, we expect that eggs in disturbed areas and from late nests have more albumen corticosterone than eggs in undisturbed places and from early nests.

## 2. Materials and methods

### 2.1. Study site and birds

The study was carried out at the “Settlement colony” on New Island, Falkland Islands (51°43'S, 61°17'W) during two consecutive breeding seasons (from late October to early November in 2006 and 2007). This colony has around 5000 pairs of breeding southern rockhopper penguins. After the arrival of the first males (early October), we visited study sites daily, initially to mark active nests and subsequently to monitor laying dates. The laying period was similar between both breeding seasons, ranging between 27 October and 10 November, with less than 5% of new A-eggs found after 5 November (see Poisbleau et al., 2008). The overall laying period was 15 days, but almost all the eggs were laid within only 10 days.

### 2.2. Egg collection and preparation

Eggs were collected under licence from the Falkland Islands government. When a new A-egg was detected in a study nest, we recorded its laying date, marked it and weighed it to the nearest 0.1 g using a digital balance. Afterwards, we placed it back in its nest and checked its presence daily until the laying of its B-egg sibling. We collected eggs as soon as the B-egg was detected in a nest randomly selected to be part of the study. As incubation in rockhopper penguins typically does not start before clutch completion (Williams, 1995), neither A- nor B-eggs were incubated for longer than about 24 h at collection. We therefore assumed that embryo development had not yet begun and we indeed observed no growth of the germinal disk when we prepared the eggs for hormone analyses.

In 2007, we additionally collected eggs at two dates (early: 3 November 2007 and late: 8 November 2007) at two distinct undisturbed sites. The breeding environment was similar between sites: undisturbed sites did not differ from study sites (disturbed) in nest density, vegetation community and density or distance to the ocean. Undisturbed sites were never visited by humans prior to our first visit, while at least two researchers visited disturbed sites for at least 3 h daily from 6 October (prior to females' arrival) to 30 November. According to previous results conducted on penguin species (Fowler, 1999; Walker et al., 2006), we are therefore very confident that females in disturbed and undisturbed sites were subject to different stress regimes.

During the first visit, we labelled all eggs at the undisturbed site. The following day, we collected both A- and B-eggs from 10 nests for which the A-egg was labelled whereas the B-egg was not labelled. We considered that the laying dates were the collection date for B-eggs and 4 days prior to the collection date for A-eggs (i.e. the average laying interval between A- and B-eggs from a same clutch, see Poisbleau et al., 2008). To avoid impact on the breeding success of the colony, we replaced the eggs of these nests with one or two eggs found outside their own nest and which were lost for their original parents.

We collected either the A-egg ( $n = 32$ ), or the B-egg ( $n = 41$ ) from 73 different study nests included in a brood reduction study (see Poisbleau et al. (2008) for more information) and 20 whole

clutches from two undisturbed sites. We collected a total of 52 A-eggs and 61 B-eggs from 93 different nests.

After collection, we weighed the eggs and froze them whole at  $-20^{\circ}\text{C}$  for at least 4 days. Afterwards, the same method was used in both years to prepare the frozen eggs for subsequent hormonal analysis. We first removed the shell while the egg was still frozen. We cut the extremity of its acute pole and collected a small amount of albumen that we transferred to a 1.5-ml Eppendorf and stored at  $-20^{\circ}\text{C}$  until hormone analysis. We then separated the albumen from the yolk by taking advantage of the fact that albumen thaws more quickly than yolk (Lipar et al., 1999a,b). After recording the mass of the shell and the yolk (to the nearest 0.1 g using a digital balance), we calculated the albumen mass (in g) subtracting shell and yolk masses to egg mass.

### 2.3. Albumen sampling

In 2007, we additionally collected albumen samples non-destructively at the different sites. At the study site, we sampled either the A-egg ( $n = 19$ ), or the B-egg ( $n = 20$ ) from 39 different nests. Collections were homogeneously spread out over the laying period and, as for egg collection, they were done the day the B-egg was detected in the nest both for A- and B-eggs. We also sampled albumen in the same two undisturbed sites, during the same two periods of sampling as egg collection. We sampled both eggs from 10 different nests during the first period and from 11 more nests during the second period.

We used exactly the same protocol for each sampling (Schwabl, 1993; Ferrari et al., 2006). The acute pole was carefully disinfected with alcohol. A 22-gauge sterile needle connected to a 1-ml sterile syringe was inserted close to the acute pole of the egg, approximately 6 mm into the albumen and in the direction of the egg's centre. We gently removed 0.5 g of the albumen (i.e. less than 1% of the albumen). Eggshells were patched with a small square of Op-Site transparent and breathable wound dressing (Smith & Nephew Medical Limited, Hull, England).

### 2.4. Hormone analysis

We added 1 ml of distilled water and eight glass beads to 200 mg of each sample (weighed to the nearest 0.01 mg). We vortexed this mixture repeatedly to homogenise it and took 100  $\mu\text{l}$  of the resulting emulsion for further corticosterone analysis.

Steroid extraction and corticosterone analyses by radioimmunoassay were carried out at the CEBC laboratory according to Lormée et al. (2003). Briefly, steroids were extracted from the aqueous phase by adding 3 ml of a diethyl-ether solution, vortexing for 1 min and centrifuging for 5 min ( $4^{\circ}\text{C}$  at 2000 rpm). The diethyl-ether phase containing the steroids was decanted and poured off after snap freezing the tube in an alcohol bath at  $-30^{\circ}\text{C}$ . This was done twice for each sample and the resultant was then evaporated under a stream of nitrogen. The recovery rate of this double extraction was above 95%.

The dried extracts were re-dissolved in 0.01 M phosphate-buffered saline (pH 7.4) containing 0.1% bovine albumin serum (PBS-BSA) and incubated overnight at  $4^{\circ}\text{C}$  with ca. 9000 cpm of the appropriate  $^3\text{H}$ -corticosterone (GE Healthcare, F-91898-Orsay) and a rabbit corticosterone antiserum (Sigma-Aldrich, F-18297-St. Quentin-Fallavier). The bound corticosterone fraction was separated by addition of dextran-coated charcoal and counted in a Packard scintillation spectrometer.

Two assays were performed. Intra-assay coefficients of variation were 4.25% ( $n = 3$  duplicates) for 2006 and 5.49% ( $n = 9$  duplicates) for 2007. The inter-assay coefficient of variation between both years was 5.02% ( $n = 12$  duplicates). The lowest detectable concentration was 1.48 pg/mg while the lowest measurement was

16.76 pg/mg. Albumen corticosterone concentrations were not significantly different between the 2006 and 2007 breeding seasons for disturbed nests ( $t$ -test:  $t_{49} = -1.95$ ,  $P = 0.06$  for A-eggs and  $t_{59} = -0.90$ ,  $P = 0.37$  for B-eggs). Therefore, we assumed that mechanisms of albumen corticosterone deposition did not differ between 2006 and 2007 and we combined both breeding seasons in the following analyses.

### 2.5. Statistical analysis

Because A- and B-eggs vary in size and mass in this species (Poisbleau et al., 2008), a higher corticosterone concentration in A-eggs than in B-eggs does not necessarily mean a higher quantity of corticosterone in the former. We therefore calculated the total albumen corticosterone (in ng) by multiplying albumen mass (in g) and albumen corticosterone concentration (in pg/mg). As laying was initiated at the same date (27 October) in both breeding seasons, we used dates as the number of days since the first of January of each year (Julian date) in order to standardise dates between the two breeding seasons.

Albumen corticosterone concentration, albumen mass and total albumen corticosterone followed normal distributions (Kolmogorov–Smirnov tests:  $Z = 0.41$ ,  $P = 1.00$ ,  $Z = 1.00$ ,  $P = 0.27$  and  $Z = 0.85$ ,  $P = 0.46$ , respectively). We performed General Linear Models (GLM) based on Type III sum of squares with egg category (A- or B-eggs) and disturbance level (disturbed or undisturbed nests) as fixed factors and laying date as a covariate to test for the impact of these parameters on corticosterone levels and albumen mass. As we collected both eggs for 20 clutches and additional albumen samples from both eggs for 21 more clutches, we randomly selected one of them for each of these nests and used only that one in the GLMs in order to avoid pseudo-replication.

After using Pearson's correlations to examine whether corticosterone levels and albumen mass of the A-egg were correlated with those of the B-egg within clutches, we used paired  $t$ -tests to test whether these variables significantly differed between A- and B-eggs of the same clutch. We additionally examined changes in corticosterone levels and albumen mass with egg category according to laying date (early nests: clutches collected on 3 November 2007 and late nests: clutches collected on 8 November 2007) with two-way repeated measures ANCOVAs. Statistical analyses were performed by using SPSS 15.0.

## 3. Results

### 3.1. Variations of corticosterone levels

Albumen corticosterone concentration was different according to egg category but not to disturbance level or laying date (Table 1): B-eggs had lower albumen corticosterone concentrations than A-eggs (Fig. 1a). Albumen mass was different according to egg category and laying date but not according to disturbance level (Table 1). Specifically, albumen mass was heavier for B-eggs than for A-eggs and increased with laying date (Fig. 1b). Finally, total albumen corticosterone was significantly different according to laying date only but not to disturbance level or egg category (Table 1): total albumen corticosterone increased with laying date (Fig. 1c).

### 3.2. Within-clutch variation of corticosterone levels

Within clutches, corticosterone levels and albumen mass were correlated between A- and B-eggs (Pearson's correlation,  $r = 0.62$ ,  $P < 0.001$ , Fig. 2a for albumen corticosterone concentration,  $r = 0.79$ ,  $P < 0.001$ , Fig. 2b for albumen mass and  $r = 0.51$ ,  $P = 0.02$ , Fig. 2c for total albumen corticosterone). Tests of the within-clutch differences between A- and B-eggs in 2007 also showed that B-eggs

**Table 1**

Differences in albumen corticosterone concentration (pg/mg), albumen mass (g), and total albumen corticosterone (ng). Results of GLMs with egg category (A- or B-eggs) and disturbance level (undisturbed or disturbed nests) as fixed factors and laying date as a covariate ( $n = 153$  for albumen corticosterone concentration and  $n = 93$  for albumen mass and amount of albumen corticosterone). None of the three- and two-way interactions were significant and they were removed from the model during the backwards-stepwise procedure. Significant  $P$ -values are marked bold. As a measure of effect sizes we used partial Eta-Square values ( $\eta_p^2$ ; i.e. the proportion of the effect + error variance that is attributable to the effect) in case of variables and covariates tested with a GLM.

Parameter	Albumen corticosterone concentration			Albumen mass			Total albumen corticosterone		
	$F_{1,149}$	$P$	$\eta_p^2$	$F_{1,89}$	$P$	$\eta_p^2$	$F_{1,89}$	$P$	$\eta_p^2$
Egg category	<b>10.33</b>	<b>&lt;0.01</b>	<b>0.07</b>	<b>51.34</b>	<b>&lt;0.001</b>	<b>0.37</b>	<0.01	0.96	<0.01
Disturbance level	1.80	0.18	0.01	1.44	0.23	0.02	0.32	0.57	<0.01
Laying date	0.26	0.61	<0.01	<b>4.40</b>	<b>0.04</b>	<b>0.05</b>	<b>6.81</b>	<b>0.01</b>	<b>0.07</b>

generally had a lower albumen corticosterone concentration (Table 2 and Fig. 3a), a higher albumen mass (Table 2 and Fig. 3b) and a higher total albumen corticosterone than their sibling A-eggs (Table 2 and Fig. 3c). However, 20% of the clutches (8 of 39) had an A-egg with lower albumen corticosterone concentration than the B-egg (Fig. 3a) and for 30% of the clutches (6 of 20), the total albumen corticosterone was higher in the A-egg than in the B-egg (Fig. 3c).

Eggs of early and later laying dates did not consistently differ in their albumen corticosterone concentration ( $t$ -test:  $t_{37} = -1.31$ ,  $P = 0.20$  for A-eggs and  $t_{37} = -2.09$ ,  $P = 0.04$  for B-eggs, Fig. 3a), albumen mass ( $t$ -test:  $t_{18} = 0.86$ ,  $P = 0.40$  for A-eggs and  $t_{18} = 1.08$ ,  $P = 0.29$  for B-eggs, Fig. 3b) and total albumen corticosterone ( $t$ -test:  $t_{18} = 1.22$ ,  $P = 0.24$  for A-eggs and  $t_{18} = 1.84$ ,  $P = 0.08$  for B-eggs, Fig. 3c). Additionally, the changes in albumen corticosterone concentration, albumen mass and total albumen corticosterone between A- and B-eggs were not impacted by the laying period itself (repeated measures GLM between A- and B-eggs,  $F_{1,37} = 0.21$ ,  $P = 0.65$ , Fig. 3a for albumen corticosterone concentration,  $F_{1,18} = 0.35$ ,  $P = 0.56$ , Fig. 3b for albumen mass and  $F_{1,18} = 1.45$ ,  $P = 0.24$ , Fig. 3c for total albumen corticosterone).

## 4. Discussion

### 4.1. Variations in corticosterone level

Rockhopper penguin females arrive at the colony only 10–15 days before laying the A-egg (Strange (1982) and personal observations) while egg formation lasts around 25 days for A-eggs (a little less for B-eggs) and albumen deposition occurs during less than the last 8 days (from Grau (1982) for Fiordland crested penguin (*Eudyptes pachyrhynchus*), a species genetically close to rockhopper penguin (see O'Hara, 1989) and very similar in mass (see Williams, 1995)). Contrary to the yolk which is partially formed at sea for A- and B-eggs, the entire albumen formation normally occurs at the colony for both eggs. Therefore, environmental breeding conditions at the colony, such as nest density, social interactions and disturbance, may have impacted corticosterone deposition in the albumen.

Previous studies on penguins have demonstrated that breeding adults react to human approaches to their nest site with a significant increase of plasma corticosterone (Fowler, 1999; Walker et al., 2006). However, these birds also showed some habituation or decreasing capability of the adrenocortical tissue to secrete corticosterone after a long history of disturbance (Fowler, 1999; Walker et al., 2006). In the present study, we disturbed females every day during the 10–15 days from their arrival to egg laying. Because habituation does not seem to occur within 10 days, we assumed that our presence impacted plasma levels of corticosterone during the entire period of albumen formation. If the transfer of this hormone from the plasma to the albumen was mainly passive (see Groothuis and Schwabl, 2008), disturbance should have then influenced albumen corticosterone levels for both A- and B-eggs. However, we detected no

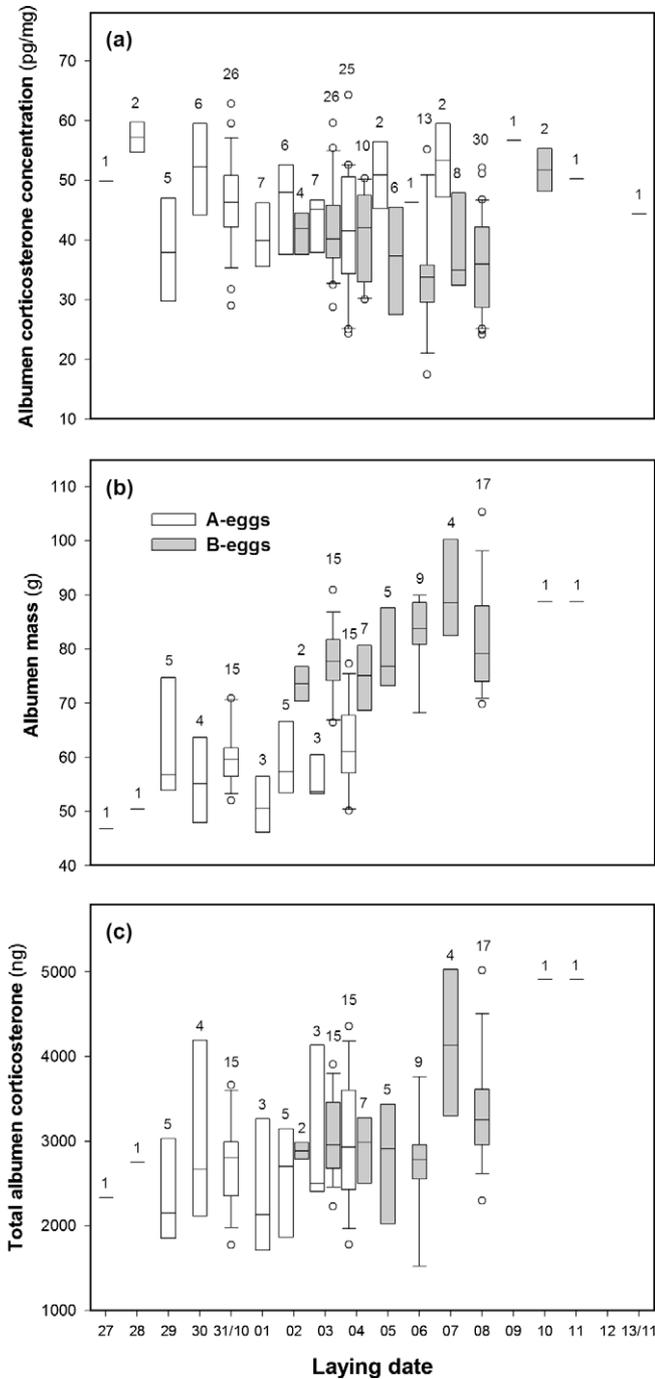
significant effect of disturbance on corticosterone levels both for A- and B-eggs. The observed lack of impact supports the idea that females may be able to play an active role in the regulation of the albumen corticosterone deposition. Nevertheless, a more powerful approach measuring both female plasma and albumen corticosterone concentrations in the same study would be necessary to confirm a stress effect on female plasma but not on albumen corticosterone.

We noticed no effect of laying date on albumen corticosterone concentration. However, laying date influenced albumen mass and then subsequently total albumen corticosterone, which both slightly increased with laying date. Female body condition at laying is known to influence egg mass for penguins (see, for example, Yorio et al., 2001). Our results suggest that late-laying females had a better body condition but that this condition had only a slight influence (if any) on their albumen corticosterone deposition. Investigation of female body masses and conditions before laying in relation to albumen masses and albumen corticosterone levels would be necessary to test this hypothesis.

We also observed that corticosterone levels and albumen mass were highly correlated between A- and B-egg from the same clutch. Interestingly, this observation shows that there exists a high individual component during the albumen formation. Females depositing higher albumen corticosterone levels or heavier albumen in their A-egg also do the same in their B-egg. As suggested by Reid and Boersma (1990) for magellanic penguins *Spheniscus magellanicus*, maternal quality may affect egg composition of the entire penguin clutch, and thus the female's breeding success. More importantly, this strong correlation indicates that corticosterone is incorporated into the egg at the same rate as albumen. This result represents evidence in favour of a passive deposition mechanism.

### 4.2. Difference between A- and B-eggs

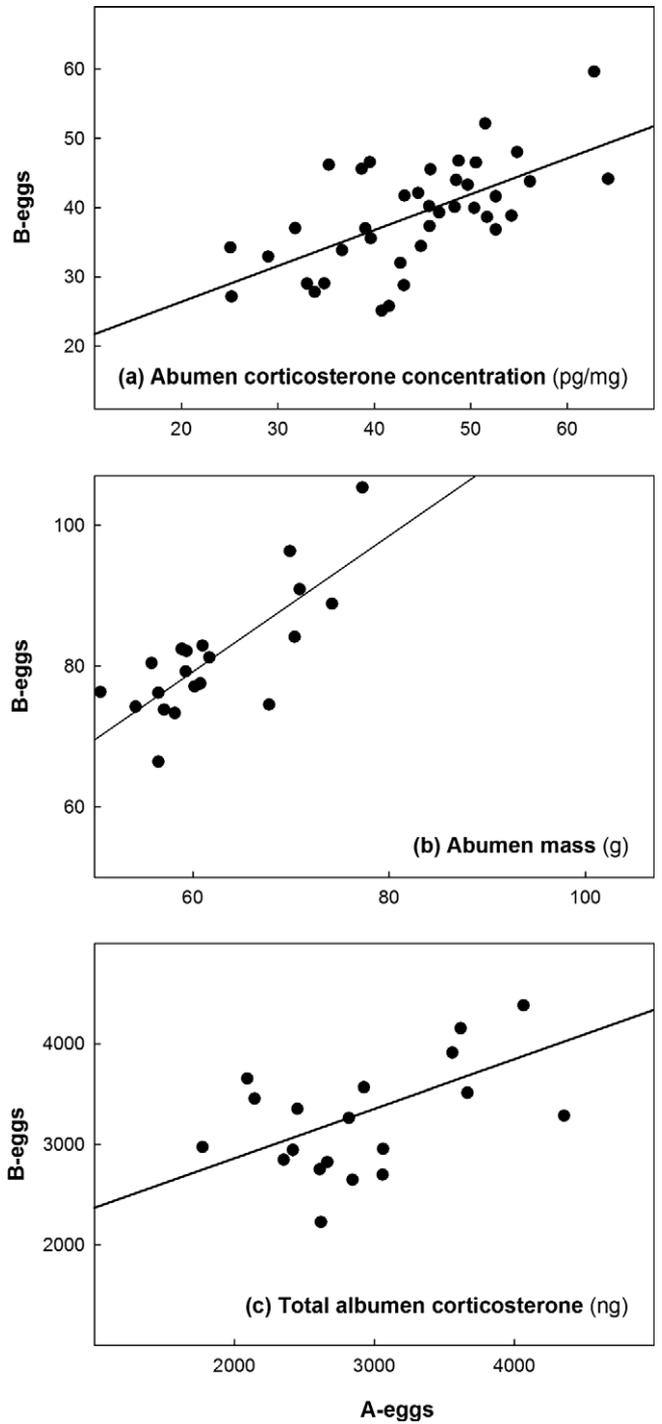
In the present study, a large part of the variation was explained by the egg category. The generally higher albumen corticosterone concentration in A-eggs than in B-eggs was also verified for 80% of the complete clutches collected (31 of 39). These results are in accordance with predictions for an active regulation of the deposition by females but do not support an obligate active regulation mechanism for all individuals. Females deposited lower concentrations in albumen corticosterone in the egg most likely to survive and produce a fledging chick. However, B-eggs had consistently higher albumen masses than A-eggs, and therefore the total albumen corticosterone was also higher in B-eggs than in A-eggs in most cases (70% of the complete broods collected). To sum up, embryos developing from A-eggs are generally exposed to a higher albumen corticosterone concentration but to a lower total albumen corticosterone than their B-eggs sibling. As embryos use the whole albumen before hatching, they are exposed to the entire amount of albumen corticosterone during the development process. We therefore expect that total albumen corticosterone may



**Fig. 1.** Differences in (a) albumen corticosterone concentration (pg/mg), (b) albumen mass (g) and (c) total albumen corticosterone (ng) according to egg category (white boxes: A-eggs; grey boxes: B-eggs) and laying date. Boxes show medians, 25% and 75% quartiles. Whiskers indicate the range between the 10th and 90th percentiles. ○: data outside the 10th and 90th percentiles. Sample sizes are given above the boxes. Significant *P*-values are marked bold.

have a more significant role for a developing embryo than albumen corticosterone concentration.

In all studies that used experimental injections of corticosterone, both the concentrations and the quantities of corticosterone in the egg were increased (Eriksen et al., 2003; Rubolini et al., 2005; Janczak et al., 2006, 2007). Moreover, all correlative studies examined only the effects of corticosterone concentrations in albumen but not the effects of the quantities of this hormone available for the embryo (Saino et al., 2005; Nordgreen et al., 2006 and see



**Fig. 2.** Within-clutch correlation between A- and B-eggs in (a) albumen corticosterone concentration (pg/mg, *n* = 39 pairs), (b) albumen mass (g, *n* = 20 pairs) and (c) total albumen corticosterone (ng, *n* = 20 pairs).

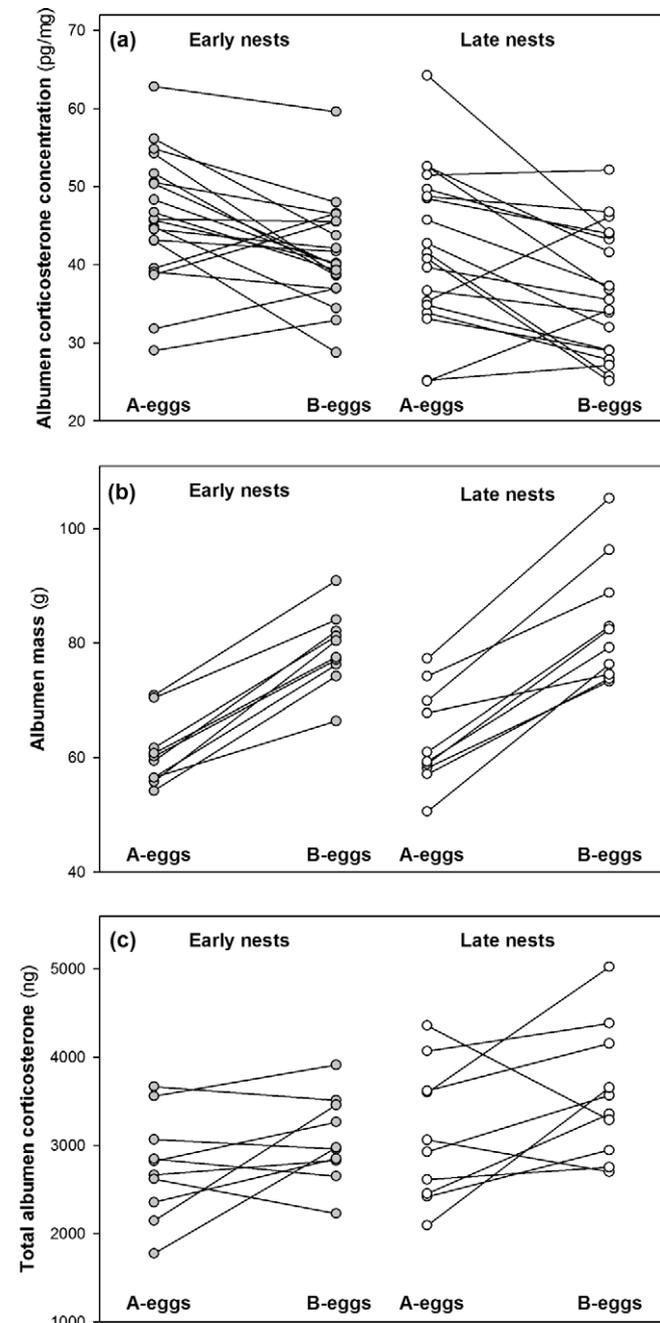
Love et al. (2008) for a review). With the present state of knowledge, it is therefore difficult to assess and discuss the potential differences in the physiological role of these two parameters. The present finding underlines the necessity to integrate total corticosterone levels as well as concentrations when possible into future studies.

In European starlings, a species with less size dimorphism between eggs and without reversed hatching asynchrony, yolk corticosterone concentrations increased with laying order and thus with hatching order (Love et al., 2008). For rockhopper penguins,

**Table 2**

Mean  $\pm$  SD values of albumen corticosterone concentration (pg/mg), albumen mass (g) and total albumen corticosterone (ng) in pairs of A- and B-eggs collected from the same clutch in 2007. Within-clutch differences between A- and B-eggs were tested with paired *t*-tests.

Parameter	A-eggs	B-eggs	<i>t</i>	df	<i>P</i>
Albumen corticosterone concentration	44.19 $\pm$ 9.21	38.92 $\pm$ 7.66	-4.41	38	<0.001
Albumen mass	62.04 $\pm$ 7.21	81.15 $\pm$ 8.85	15.61	19	<0.001
Total albumen corticosterone	2936 $\pm$ 687	3322 $\pm$ 669	2.56	19	0.02



**Fig. 3.** Within-clutch variation of (a) albumen corticosterone concentration (pg/mg), (b) albumen mass (g) and (c) total albumen corticosterone (ng) according to laying date (left side: early nests for clutches collected 3 November 2007; right sides: late nests for clutches collected 8 November 2007).

albumen corticosterone concentrations decreased with laying order but also increased with hatching order. Taking account of the general negative effect of corticosterone on survival and development of embryos and future chicks (Rubolini et al., 2005; Love et al., 2005; Janczak et al., 2006, 2007; Hayward et al., 2006; Love and Williams, 2008), a higher concentration for the second eggs to hatch might be a hormonal mechanism to facilitate brood reduction as proposed by Love et al. (2008).

## Acknowledgments

We are grateful to the New Island Conservation Trust for permission to work on the island. We wish to thank Ian, Maria and Georgina Strange and Dan Birch for their support during the field season. Charles-André Bost, Olivier Chastel, Léopold Denonfoux and Paulo Catry provided essential advice on methods. Thanks also to Helen Otley, Falkland Islands Government and British Antarctic Survey for their logistic help. We also highly appreciate the skilful assistance in corticosterone assays of Colette Trouvé and Adeline Berger. The manuscript benefited greatly from critical comments by David Carslake and two anonymous reviewers. This study was funded by a grant provided by the Deutsche Forschungsgemeinschaft DFG (Qu 148/1-ff) and conducted under a research licence granted by the Environmental Planning Department of the Falkland Islands Government.

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