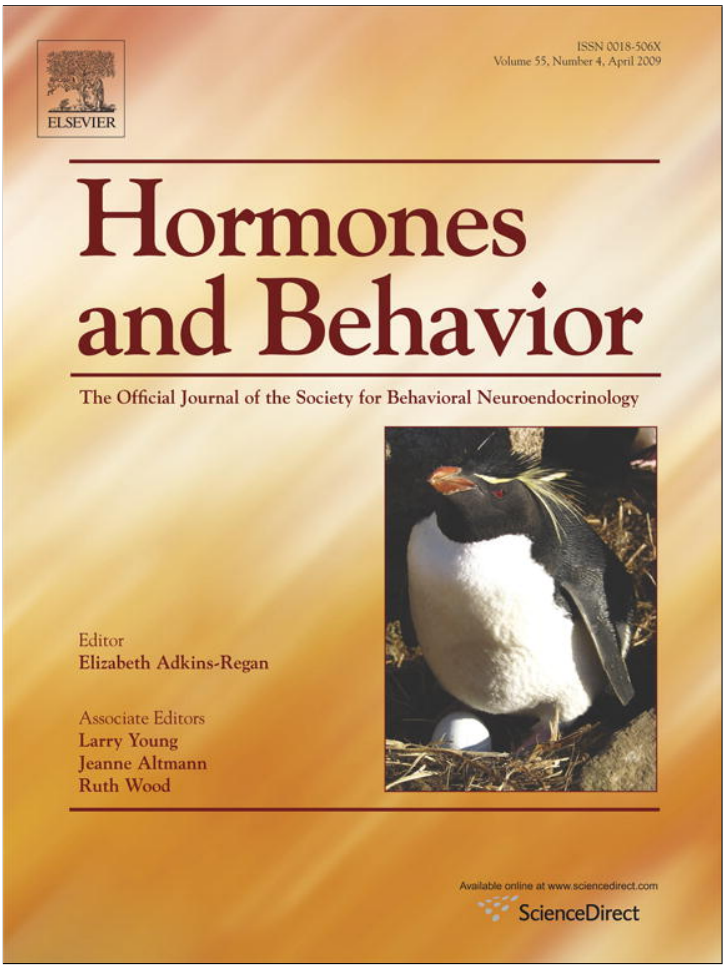

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# Maternal deposition of yolk corticosterone in clutches of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*)

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ABSTRACT

High corticosterone levels can have deleterious effects in developing avian embryos and chicks. Therefore, it may be adaptive for avian mothers to reduce corticosterone transfer to their eggs. However, until now, data about the active or/and passive role of mothers in corticosterone transfer to eggs are inconclusive. Here, we study maternal investment into A- and B-eggs of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*). This species exhibits reversed hatching asynchrony and provides a unique model to test whether there is a strategic investment in corticosterone favoring the B-chick, which is most likely to survive. We found that rockhopper penguins had the highest yolk concentrations of any wild bird species studied so far. Contrary to our expectations, B-eggs had more yolk corticosterone both in concentration and in quantity than A-eggs, independently of the laying period and the level of human disturbance. Additionally, females deposited more yolk corticosterone in their eggs when they were disturbed. However, this disturbance effect was particularly strong for A-eggs and for late-laid eggs. The present data support neither the predictions for an active regulation nor for a passive deposition, and hormone deposition mechanisms still need to be explored. The adaptive value, if any, of high yolk corticosterone is presently unknown.



## Introduction

In birds, stressors typically activate the hypothalamic–pituitary–adrenal (HPA) axis to release corticosterone (Sapolsky, 1992; Wingfield and Farner, 1993; Wingfield, 1997). Poor environmental and individual conditions lead to a chronic stress response, which affects basal plasma levels of corticosterone. Additionally, acute stressors such as severe bad weather conditions or predatory attacks cause a short-term acute stress response with a rapid increase of plasma corticosterone. Both chronic and acute stress may negatively affect reproduction through the elevated plasma corticosterone level (Greenberg and Wingfield, 1987). For example, only 56% of female zebra finches (*Taeniopygia guttata*) implanted with corticosterone initiated laying (compared with 100% of sham-implanted females, Salvante and Williams, 2003). In those females that did breed, corticosterone administration delayed the onset of egg laying. Additionally, wild female European starlings (*Sturnus vulgaris*) with higher corticosterone levels deserted their nests and abandoned reproduction significantly more than females with low levels (Love et al., 2004).

Recent studies have indicated that corticosterone is transferred from the mother to the eggs during egg formation. Accordingly, stressed females generally lay eggs with more albumen and/or yolk corticosterone (Saino et al., 2005; Hayward et al., 2005) and an increase in maternal plasma corticosterone generally leads to an elevated level of yolk corticosterone (Hayward and Wingfield, 2004; Hayward et al., 2005; Love et al., 2005). For European starling, Love et al. (2005) found a positive correlation between plasma corticosterone levels in mothers at the first egg-stage and yolk corticosterone levels in first eggs. However, a negative correlation between female plasma and yolk corticosterone was reported for the same bird species blood sampled at clutch completion (Love et al., 2008). This "stress effect" was also not found in eastern bluebird (*Sialia sialis*) eggs (Navara et al., 2006).

Manipulative studies have also demonstrated that elevated levels of corticosterone have mainly negative impacts on embryo and future chick survival and development. In barn swallows (*Hirundo rustica*), eggs 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). Also in other bird species, high corticosterone levels in eggs decreased hatching success (Eriksen et al., 2003; Love et al., 2005). High egg corticosterone level also had more long-term effects on chicks. It decreased their hatching mass and size, their growth speed or their cognitive abilities while it increased their body asymmetry (Eriksen et al., 2003; Hayward and Wingfield, 2004; Love et al., 2005; Janczak et al., 2006; Hayward et al., 2006; Janczak et al., 2007). Some of these detrimental effects have been observed, even after injection of only

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physiological doses of corticosterone corresponding to one standard deviation of the amount of corticosterone for the entire albumen or yolk (Saino et al., 2005) or after an experimental elevation of maternal baseline plasma corticosterone in turn increasing egg corticosterone (Hayward and Wingfield, 2004; Love et al., 2005).

In this context, the interest of females appears to be to avoid massive corticosterone transfer to their eggs. However, for the moment, we do not know whether females play an active or/and passive role in this maternal transfer (see review in Groothuis and Schwabl (2008)). Some authors have suggested that yolk hormones represent a way for mothers to affect offspring phenotype and hence influence the development of offspring behavioral and life-history traits adaptively (Groothuis et al., 2005; Müller et al., 2007; Carere and Balthazart, 2007). This idea has mainly been explored in the light of the hatching asynchrony adjustment hypothesis (Groothuis et al., 2005) for testosterone and of the sex ratio adjustment hypothesis for corticosterone (Pike and Petrie, 2005; Bonier et al., 2007). On the other hand, the maternal transfer of corticosterone to eggs could be an inevitable cost associated with poor maternal condition at the time of egg formation (Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006).

In this study, we examined the variability of yolk corticosterone levels within the breeding population and within clutches of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*). As do all other crested penguins (genus *Eudyptes*), rockhopper penguins exhibit brood reduction: two eggs are laid but only one chick usually fledges (Warham, 1975). They also present a unique reversed hatching asynchrony among birds (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 after hatching (Gwynn, 1953; Lamey, 1990). If corticosterone deposition in yolk is at least partially under the control of females and if elevated yolk corticosterone levels have deleterious effects for the embryo, we expect that females deposit less yolk corticosterone in the B-egg, which is the one most likely to produce a fledging chick. We moreover tested whether laying date (as an index of declining breeding environment quality caused by increasing nest density and social interactions) and human disturbance affect yolk corticosterone deposition. If females are not able to avoid or reduce corticosterone deposition in yolks, we expect that eggs from late nests and in disturbed areas have more yolk corticosterone than eggs from early nests and in undisturbed places.

## Methods

### *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. 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 two weeks later, for pairing and copulation. The laying period was similar between both breeding seasons, ranging from 27 October to 10 November, with less than 5% of new A-eggs found after 5 November (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. This study was conducted under a research license granted by the Environmental Planning Department of the Falkland Islands Government. This research license also covered animal welfare in addition to collection of the egg samples.

### *Egg collection*

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 replaced it 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 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.

In 2007, we additionally collected eggs at two dates (early: 03 November 2007 and late: 08 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 daily for at least 3 h from the 6 October (prior to the arrival of the females) to the 30 November. During the first visit, we labeled all eggs at the undisturbed site. The following day, we collected both A- and B-eggs from ten nests for which the A-egg was labeled whereas the B-egg was not labeled. We considered that the laying dates were the collection date for B-eggs and four days prior the collection date for A-egg (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 that we had considered as lost for their original parents.

We collected either the A-egg ($n=33$), or the B-egg ($n=42$) from 75 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 therefore collected a total of 53 A-eggs and 62 B-eggs from 95 different nests. After collection, we weighed the eggs and froze them whole at −20 °C for at least four days.

### *Egg preparation*

The same method was used in both years to prepare the frozen eggs for subsequent hormone analysis. We first removed the shell while the egg was still frozen. Then, we separated the yolk from the albumen by taking advantage of the fact that albumen thaws more quickly than yolk (Lipar et al., 1999a,b). After recording the mass of the yolk (to the nearest 0.1 g using a digital balance), we carefully homogenized it by swirling with a mini-spatula (Lipar et al., 1999a). A small quantity of each homogenized yolk was transferred to a 1.5-ml Eppendorf and stored at −20 °C until hormone analysis.

### *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 homogenize it and took 100 µl 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 °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 °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 redissolved in 0.01 M phosphate-buffered saline (pH 7.4) containing 0.1% bovine albumin serum (PBS-BSA) and incubated overnight at 4 °C with ca. 9000 cpm of the appropriate $^{3}$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 5.97% ($n=3$ duplicates) for 2006 and 11.20% ($n=6$ duplicates) for 2007. The inter-assay coefficient of variation between years was 9.25% ($n=9$ duplicates). The lowest detectable concentration was 0.55 pg/mg while the lowest measurement was 34.17 pg/mg.

### *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 for the former. We therefore calculated the total yolk corticosterone (in ng) by multiplying yolk mass (in g) and yolk corticosterone concentration (in pg/mg). As the 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 standardize dates between the two breeding seasons.

Both yolk corticosterone concentration and total yolk corticosterone followed normal distributions (Kolmogorov–Smirnov tests: $Z=0.80$, $P=0.54$ for yolk corticosterone concentration and $Z=0.64$, $P=0.81$ for total yolk corticosterone). We first tested whether A- and B-eggs differed in their hormonal characteristics between the breeding seasons of sampling (2006 and 2007) within disturbed sites by using Student's tests (*t*-tests). Secondly, we performed two General Linear Models (GLM) based on Type III sum of squares with egg category (A- or B-eggs) and disturbance level (undisturbed or disturbed nests) as fixed factors and laying date as covariate to test for the impact of these parameters on yolk corticosterone concentration and total yolk corticosterone. As we collected both eggs for 20 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 examining the correlations of these hormonal parameters between A- and B-eggs of the same clutch with Pearson's correlations, we used paired *t*-tests to test whether they were significantly different between A- and B-eggs of the same clutch. We additionally examined changes in corticosterone levels with egg category according to laying period (early nests: clutches collected the 03 November 2007 and late nests: clutches collected the 08 November 2007) with two-way repeated measures ANCOVAs. Statistical tests were performed in SPSS 15.0. Although *t*-tests were conducted, data are presented as medians in Figs. 1 and 2.

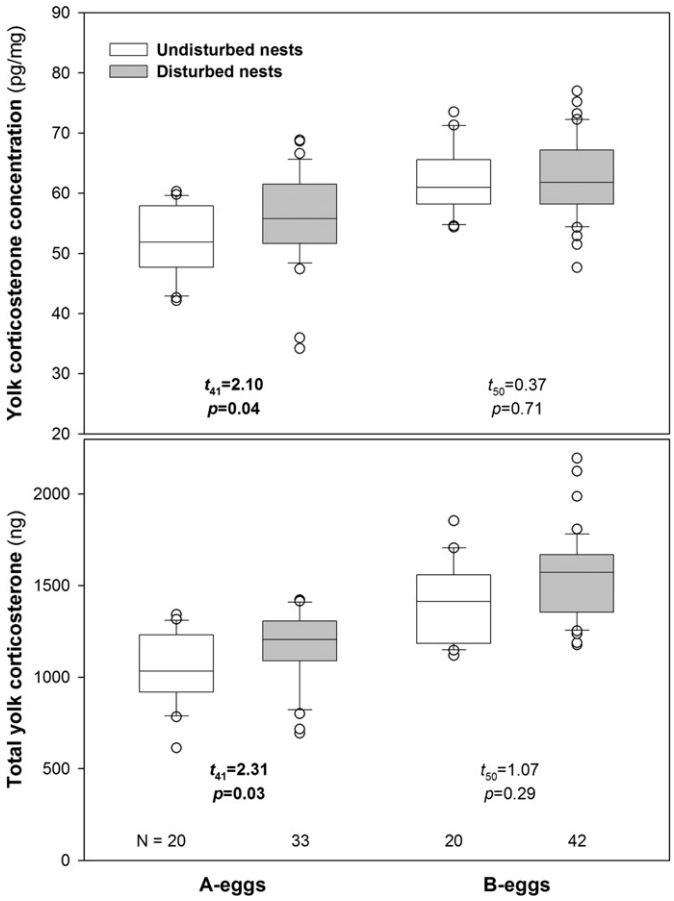


**Fig. 1.** Differences in yolk corticosterone concentration (upper frames, in pg/mg) and total yolk corticosterone (bottom frames, in ng) according to egg category (left boxes: A-eggs; right boxes: B-eggs) and disturbance level (white boxes: undisturbed nests; gray boxes: disturbed nests). Boxes show 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 under the boxes. Results of *t*-tests with disturbance level (undisturbed or disturbed nests) as the grouping variable are presented under respective boxes within each egg category. Significant *P*-values are marked bold.

## Results

### *Variations in yolk corticosterone levels*

Yolk corticosterone concentration was not significantly different between the 2006 and 2007 breeding seasons for either A- or B-eggs from disturbed nests (*t*-test: $t_{31}=1.43$, $P=0.16$ for A-eggs and $t_{40}=0.68$, $P=0.50$ for B-eggs). The same result was observed for total yolk corticosterone (*t*-test: $t_{31}=-0.89$, $P=0.38$ for A-eggs and $t_{40}=0.29$, $P=0.59$ for B-eggs). Therefore, we assumed that mechanisms of yolk corticosterone deposition did not differ between 2006 and 2007 and we combined both breeding seasons in the following analyses.

Both yolk corticosterone concentration and total yolk corticosterone were different between egg categories and disturbance levels but did not vary with laying dates (Table 1). Indeed, B-eggs and eggs from disturbed nests had more yolk corticosterone than A-eggs and eggs from undisturbed nests (Fig. 1). For both these parameters, there were also significant interactions between egg category and disturbance level, and between disturbance level and laying date (Table 1). Specifically, A-eggs from disturbed nests had significantly more yolk corticosterone than A-eggs from undisturbed nests, while in contrast, B-eggs from disturbed nests did not have significantly more yolk corticosterone than B-eggs from undisturbed nests (Fig. 1). Moreover, we observed a significant increase of yolk corticosterone levels with laying date in disturbed nests ($F_{1,72}=6.01$, $P=0.02$ for yolk corticosterone concentration and $F_{1,72}=6.68$, $P=0.01$ for total yolk corticosterone in GLM procedures with egg category as fixed factor and laying date as covariate, Fig. 2). This effect was not significant for undisturbed nests for which we had only two different laying dates per egg category ($F_{1,17}=1.78$, $P=0.20$ for yolk corticosterone concentration and $F_{1,17}=1.07$, $P=0.32$ for total yolk corticosterone, Fig. 2).

### *Within-clutch variations*

Within undisturbed clutches in 2007, the yolk corticosterone concentration and total yolk corticosterone were not correlated between A- and B-eggs ($R=0.09$, $P=0.72$ for yolk corticosterone concentration and $R=0.28$, $P=0.23$ for total yolk corticosterone). The B-eggs generally had a higher yolk corticosterone concentration and total yolk corticosterone (Table 2). However, we observed that for two early nests both yolk corticosterone concentration and total yolk corticosterone were slightly lower in the B-egg than in the A-egg (Fig.

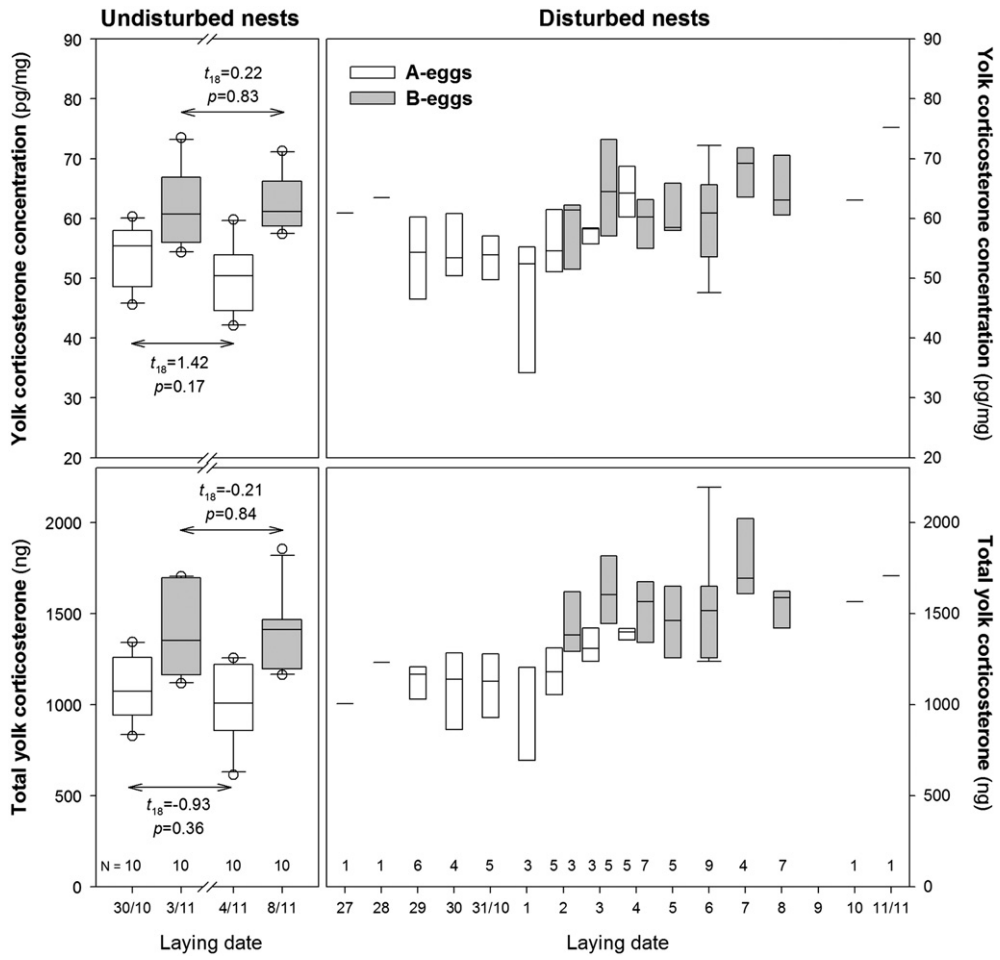


**Fig. 2.** Differences in yolk corticosterone concentration (upper frames, in pg/mg) and total yolk corticosterone (bottom frames, in ng) according to disturbance level (left frames: undisturbed nests; right frames: disturbed nests), egg category (white boxes: A-eggs; gray boxes: B-eggs) and laying dates. 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 under the boxes. Results of *t*-tests between both laying dates within each egg category are presented above or under respective boxes for undisturbed nests. Significant *P*-values are marked bold.

3). For all other early nests and all of the late nests, B-eggs had both a higher corticosterone level and a total yolk corticosterone than A-eggs (Fig. 3). The laying period did not significantly affect yolk corticosterone concentrations (*t*-test: $t_{18} = -1.42$, $P = 0.17$ for A-eggs and $t_{18} = 0.22$, $P = 0.83$ for B-eggs, Fig. 3) or total yolk corticosterone (*t*-test: $t_{18} = -0.93$, $P = 0.36$ for A-eggs and $t_{18} = -0.21$, $P = 0.84$ for B-eggs, Fig. 3). Additionally, the increases of both yolk corticosterone concentration and total yolk corticosterone from the A-egg to the B-egg were not affected by the laying period itself (repeated measures GLM between A- and B-eggs, $F_{1,18} = 1.48$, $P = 0.24$ for yolk corticosterone concentration and $F_{1,18} = 0.26$, $P = 0.62$ for total yolk corticosterone, Fig. 3).

**Table 1**
Test of the differences in yolk corticosterone concentration and total yolk corticosterone

| Parameter | Yolk corticosterone concentration | | | Total yolk corticosterone | | |
|---|---|---|---|---|---|---|
| | $F_{1,89}$ | $P$ | $\eta_p^2$ | $F_{1,89}$ | $P$ | $\eta_p^2$ |
| Egg category | **16.00** | **<0.001** | **0.15** | **30.53** | **<0.001** | **0.25** |
| Disturbance level | **4.99** | **0.03** | **0.05** | **4.63** | **0.03** | **0.05** |
| Laying date | 0.01 | 0.92 | <0.001 | 0.05 | 0.83 | <0.01 |
| Egg category × Disturbance level | **9.33** | **<0.01** | **0.10** | **4.62** | **0.03** | **0.05** |
| Disturbance level × Laying date | **5.04** | **0.03** | **0.05** | **4.71** | **0.03** | **0.05** |

Results of GLM procedures with egg category (A- or B-eggs) and disturbance level (undisturbed or disturbed nests) as fixed factors and laying date as covariate. $n = 95$ for both variables.
Only significant interactions are shown in these models, other non-significant ones 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.

## Discussion

Female southern rockhopper penguins deposited a high concentration and quantity of corticosterone in yolk. Comparisons with other published studies for birds (see Love et al. (2008) for a review) show that rockhopper penguins have the highest yolk concentrations of any species studied so far. Indeed, data reported for other wild species range from 2.5 pg/mg for eastern bluebirds (Navara et al., 2006) to 15.34 pg/mg for European starlings (Love et al., 2008) while we measured 54.45 ± 7.29 pg/mg and 62.38 ± 6.30 pg/mg for A- and B-eggs of rockhopper penguins, respectively. Because disturbance slightly increased these corticosterone levels in yolk, we cannot

**Table 2**
Mean ± SD values of yolk corticosterone concentration (pg/mg) and total yolk corticosterone (ng) of 20 pairs of A- and B-eggs collected from the same clutch in undisturbed nests in 2007

| Parameter | A-eggs | B-eggs | $t_{19}$ | $P$ |
|---|---|---|---|---|
| Yolk corticosterone concentration | 51.93 ± 5.75 | 62.04 ± 5.56 | −5.90 | <0.001 |
| Total yolk corticosterone | 1043.5 ± 191.1 | 1393.3 ± 223.2 | −6.31 | <0.001 |

Within-clutch differences between A- and B-eggs were tested with paired *t*-tests.

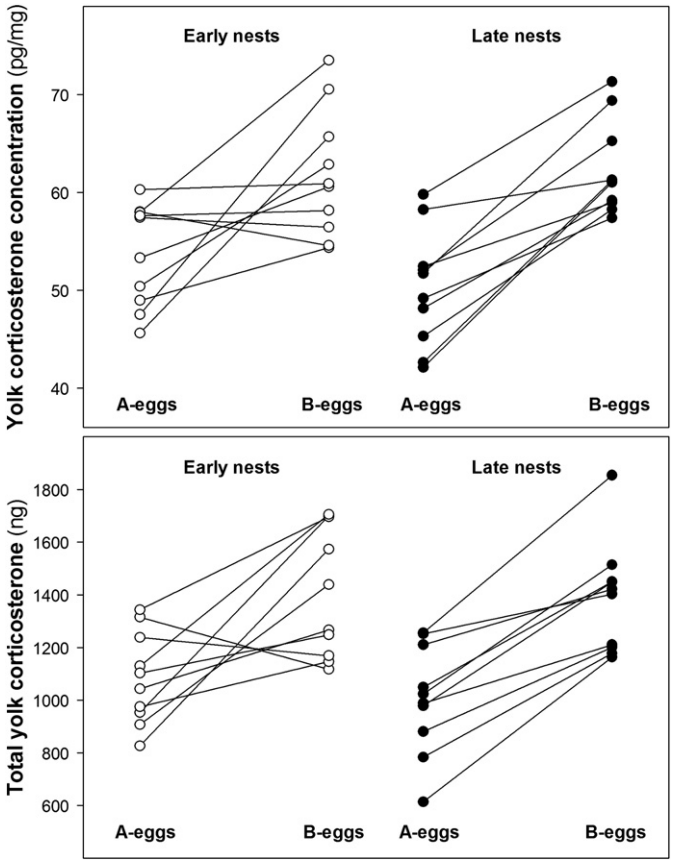


**Fig. 3.** Within-clutch variation of yolk corticosterone concentration (upper frames, in pg/mg) and total yolk corticosterone (bottom frames, in ng) according to laying period (left side: early nests for clutches collected the 03 November 2007; right side: late nests for clutches collected the 08 November 2007) for the 20 undisturbed nests in 2007.

totally reject the idea that these high levels could be partially the result of an artificially stressful situation, as could be the case also for captive birds such as peafowl (*Pavo cristatus*, Pike and Petrie, 2005). However, yolk corticosterone levels of eggs from undisturbed nests are likely to be in the natural range for this species, and, they are much higher than in previous other studies (see Table 2).

*Yolk corticosterone differences between A- and B-eggs*

Overall, yolk corticosterone has been found to have a negative effect on the embryo and future chick (Eriksen et al., 2003; Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2005; Janczak et al., 2006; Hayward et al., 2006; Janczak et al., 2007). Thus, we were expecting higher yolk corticosterone levels in A-eggs than in B-eggs. However, females of rockhopper penguins deposited more yolk corticosterone in B-eggs than in A-eggs on average, whatever the laying period and the disturbance level. This finding was confirmed for 90% of the undisturbed clutches. Only two of 20 clutches had a lower total yolk corticosterone in the B-egg than in the A-egg and these two clutches were laid during the early-laying period. Similarly, both Saino et al. (2005) and Love et al. (2008) found that last-laid eggs exhibited higher yolk corticosterone levels than their first-laid sibling eggs. In contrast, Navara et al. (2006) found no intra-clutch patterns in yolk corticosterone.

All crested penguin species lay two eggs and, depending on the species, the first A-egg is 15 to 45% smaller than the second B-egg and rarely produces a fledging chick (Warham, 1975; Williams, 1995). However, yellow-eyed penguins (*Megadyptes antipodes*), which are the closest relative of crested penguins, lay two similarly sized eggs that both hatch and both chicks are often reared (see Williams (1995)). As such, crested penguins could appear to be evolving away from a two-egg clutch (Lack, 1968). If this is the case, it is therefore difficult to determine which maternal effect (egg size, hatching order or yolk corticosterone) plays a greater adaptive role (if any) in shaping fitness for mothers. As proposed for passerines by Love et al. (2008), it is then possible that intra-clutch yolk corticosterone patterns in the close ancestor do in fact act as an adaptive maternal effect by which to spread competition across the brood. If so, this raises the possibility that the increase in yolk corticosterone in rockhopper penguins has been carried over from its ancestor, and it may no longer be adaptive since the chick from the A-egg nearly never survives.

Love et al. (2008) also recently proposed that increasing yolk corticosterone across the clutch might simply be due to an increased accumulation of physiological stress across the laying sequence in the mother, which is then passively passed onto the eggs. Accordingly, the transfer of large amounts of corticosterone to eggs may most plausibly be explained as passive deposition by mothers who are unable to avoid or control it (Groothuis et al., 2005; Müller et al., 2007; Carere and Balthazart, 2007; and see Groothuis and Schwabl (2008) for a review of the debate on maternal control). This idea may also explain the positive correlation between plasma corticosterone levels in mothers at the first egg-stage, and yolk corticosterone levels in first eggs but not in the next ones for European starling (Love et al., 2005). In this context, we expected, and we have observed, that disturbance also results in an increase in yolk corticosterone levels.

*Disturbance effects*

Female rockhopper penguins arrive at the colony only from ten to fifteen days before laying the A-egg (Strange, 1982 and personal observations) while egg formation lasts around 25 days for A-eggs and a little less for B-eggs (see Fig. 4, data obtained 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)). Therefore, most of the A-egg yolk formation occurs at sea while the B-egg yolk is mainly formed at the colony. As female arrival is even more synchronized than laying (Strange, 1982 and personal observations), we could assume that females laying early may almost have finished forming the yolk of the A-egg when they arrive at the colony while females laying later may have to build a higher proportion of the A-egg's yolk at the colony. This chronology would also explain why we observed only a slight (if any)

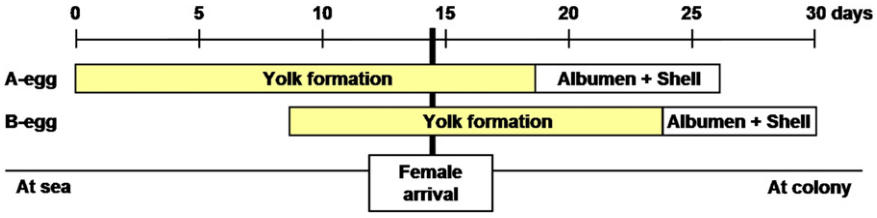


**Fig. 4.** General chronology of egg formation for A- and B-eggs. Day 0 corresponds to the beginning of the formation of the A-egg yolk. Day 30 corresponds to the laying date of the B-egg. Modified from Grau (1982) for Fiordland crested penguins (*Eudyptes pachyrhynchus*).

impact of the disturbance caused at the early laying dates, but an increase in the yolk corticosterone levels with the laying date afterwards (see Fig. 2). However, this does not explain why B-eggs were not consistently affected by disturbance despite being formed mostly at the colony for both early and late breeders.

On the other hand, disturbance may not have impacted corticosterone levels in B-eggs because females were able, at least partially, to regulate their yolk corticosterone deposition (see Groothuis and Schwabl (2008) for a review of the debate on maternal control). It is possible that females regulated yolk corticosterone only for B-eggs, these being the privileged eggs and chicks, but not for A-eggs. A-eggs could have been used as a reservoir of corticosterone. This idea, first proposed for androgens (Navara et al., 2006), suggests that females depositing high hormone levels into yolks may be left with a temporary deficit of this hormone in the plasma after laying (Love et al., 2008). Indeed, across studies no consistent pattern has been found: even if disturbed females had increased plasma corticosterone levels, yolk corticosterone was not always affected, and a negative relationship between plasma and yolk corticosterone was even found when birds were blood sampled at clutch completion (Love et al., 2008). So, if females used the first-laid egg as corticosterone reservoir, this mechanism might allow females to deposit an optimal hormone level in the following egg. This idea is consistent with the fact that disturbed females may have deposited their overdose of plasma corticosterone in A-eggs to allow an optimal corticosterone level in B-eggs. The question is then why the optimal corticosterone level may be so high.

The role, if any, of the higher yolk corticosterone level for B-eggs in their reduced incubation period is currently unknown. However, gull eggs injected with corticosterone had similar hatching success to controls, but hatched later (Rubolini et al., 2005). Yolk corticosterone injection also tended to increase the willingness to feed in a competitive environment for newly hatched chicken males but not for females (Janczak et al., 2007). However, chicks from B-eggs are generally not in a competitive situation when they hatch. As high plasma and yolk corticosterone levels seem to be associated with a female-biased sex ratio (Pike and Petrie, 2005; Love et al., 2005; Bonier et al., 2007), we could alternatively propose that females actively influence offspring sex ratio through their corticosterone deposition (see Groothuis and Schwabl (2008)). However, we collected eggs before the beginning of incubation because hormone levels have been shown to change through incubation (Groothuis et al., 2005). Therefore, the present study unfortunately did not allow determination of the sex of the embryos from collected eggs because determination of an embryo's sex requires more tissue than was present before incubation began. Moreover, this hypothesis supposes that females would like to favor female offspring. Penguins being monogamous and obligatory biparental carers (Davis and Renner, 2003), it is not clear why females would bias the offspring sex ratio towards females. Ultimately, recent studies on European starling indicated that the transfer of stress hormones to eggs by mothers could be adaptive since corticosterone investment matched the quality of a mother to offspring demand (Love and Williams, 2008) and fledglings exposed to experimentally increased corticosterone in ovo performed better during flight than control fledglings (Chin et al., 2009).

In conclusion, despite its potentially negative impact on embryo and chick, females deposited on average more yolk corticosterone in the first egg to hatch (B-eggs) than in the egg to be lost (A-eggs) whatever the laying period and the disturbance level. Moreover, disturbance had a larger impact on yolk corticosterone levels in A-eggs than in B-eggs. These findings support neither the predictions for an active regulation nor for a passive deposition of the corticosterone in yolk (see Groothuis and Schwabl (2008)). The present results show that the regulation of yolk corticosterone deposition, at least for rockhopper penguins, is more complex than initially expected, with interactions between different factors. More experimental explorations of the mechanisms underlying hormone deposition in eggs and its potential positive and negative effects on embryos and chicks are necessary.

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