Intra-Clutch Ratio of Yolk Progesterone Level Changes with Laying Date in Rockhopper Penguins: A Strategy to Influence Brood Reduction?

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Abstract

Hatching asynchrony in avian species generally leads to a size hierarchy among siblings, favouring the first-hatched chicks. Maternally deposited hormones affect the embryo and chick’s physiology and behaviour. It has been observed that progesterone, a hormone present at higher levels than other steroid hormones in egg yolks, is negatively related to body mass in embryos, chicks and adults. A differential within-clutch progesterone deposition could therefore be linked to the size hierarchy between siblings and to the resulting brood reduction. We tested whether yolk progesterone levels differed between eggs according to future parental ability to feed the entire clutch in wild rockhopper penguins Eudyptes chrysolome. This species presents a unique reversed egg-size dimorphism and hatching asynchrony, with the larger second-laid egg (B-egg) hatching before the smaller first-laid egg (A-egg). Yolk progesterone levels increased only slightly with female body mass at laying. However, intra-clutch ratios were not related to female body mass. On the other hand, yolk progesterone levels increased significantly with the date of laying onset for A-eggs while they decreased for B-eggs. Early clutches therefore had proportionally more progesterone in the B-egg compared to the A-egg while late clutches had proportionally less progesterone in the B-egg. We propose that females could strategically regulate yolk progesterone deposition within clutches according to the expected food availability during chick growth, an adaptive strategy to adjust brood reduction to conditions. We also discuss these results, relating to yolk progesterone, in the broader context of other yolk steroids.

Introduction

An individual’s experiences of the environment often lead to variation in its growth, condition and physiological state [1–3]. Similarly, environmental conditions that organisms experience during early life influence their development and can also have permanent effects in adulthood [4–7]. Consequently, maternal state can affect offspring via cytoplasmic factors (e.g. nutrients, hormones and carotenoids) that may influence offspring development [8,9]. Therefore, prenatal maternal effects may provide a powerful mechanism for adaptive phenotypic plasticity, in which the environment experienced by the mother is translated into phenotypic variation in the offspring [8,9].

In altricial and semi-altricial birds, asynchronous hatching generally leads to a size hierarchy among siblings, and to a competitive disadvantage for the youngest siblings compared to older ones [10–12]. This size hierarchy is thought to optimize the fitness of the parents by facilitating brood reduction when resource availability does not enable parents to feed the entire clutch properly [13–15]. In this context, females should adjust prenatal maternal effects over the laying sequence, and thus increase or offset the chick’s disadvantage in hatching later, according to breeding conditions such as the parents’ future ability to feed the chicks. Females unable to bear the cost of feeding a large clutch should give an advantage to the first egg to hatch in order to accelerate brood reduction. For instance, avian mothers deposit hormones differentially among their eggs, and these hormones have been shown consequently to affect the embryo and chick’s physiology and behaviour (see reviews in Groothuis & von Engelhardt [16], Gil [17] and Groothuis & Schwabl [18]). It has therefore been proposed that elevated yolk hormone levels may modulate sibling competition and facilitate brood reduction under unfavourable conditions in birds (see review in [19]). Several studies have suggested that intra-clutch variation in yolk testosterone and yolk corticosterone can act as an adaptive maternal effect in altricial species where modulation of competition between siblings would benefit mothers (see for example [20,21]). However, in precocial species where mothers would not benefit from a modulation of sibling quality, intra-clutch variation in yolk hormones may play little or no adaptive role (see for example [20,22]).

According to Lipar et al. [23], the patterns of hormone deposition (progesterone, testosterone and estradiol) in terms of timing and quantity, in yolk of altricial species (dark-eyed junco Junco hyemalis and red-winged blackbird Agelaius phoeniceus) matched those predicted by previous studies of the temporal patterns of...
steroidogenesis in the follicle of the domestic hen *Gallus gallus*, a precocial species [24]. We can therefore assume that the pattern of yolk hormone deposition is relatively similar between altricial and precocial species. Variations in yolk steroid levels among and within clutches are then not simply by-products of the steroid levels in the plasma of the female. Moreover, as steroid levels can vary independently between the follicle and the plasma, it has been suggested that the follicle is the primary influence on the pattern of steroid deposition within an individual yolk (see [23]).

Progesterone is mainly produced during yolk formation by granulosa cells of the prevovulary follicle [25]. This hormone is present in yolks of freshly laid eggs at higher levels than the other steroids [23,26,27]. Progesterone receptors are found very early in developing embryos (for examples, in the urogenital tract [28] and in the brain [29] of domestic hen embryos). Although we are not aware of any studies that looked for progesterone receptors and enzymes at the embryonic stage in altricial birds, it has been shown that androgen and oestrogen receptors are abundantly expressed in the brains of altricial species’ embryos [18]. We can assume that it is probably the same for progesterone receptors. Progesterone is therefore likely to be a key hormone for avian embryos during early development. Progesterone has been implicated in the regulation of social interactions, parental behaviours and body mass in birds [30–33]. However, until now progesterone has received very little attention in the context of ecological and evolutionary studies.

Injection of high levels of exogenous progesterone into yolk caused significant reductions in body and shank mass and length in chick embryos [32]. Plasma progesterone levels and body mass gain have also been observed to be inversely correlated in altricial chicks [Nazca boobies *Sula granti* [33]]. Moreover, plasma progesterone is at its highest level when body mass is lowest, and at its lowest level when body mass is highest, in female king penguins *Aptenodytes patagonicus* [30]. Even if the effects of progesterone change with developmental phase and differ between altricial and precocial species, the relationship between progesterone and body mass appears to be consistently negative. Given the positive influence of body mass on the outcome of interactions among nestling birds [34], we hypothesized that females deposit higher progesterone levels in the last egg(s) to hatch compared to the first one(s) when the future parental ability to feed the clutch is likely to be poor. This differential investment could give an advantage to the first chick(s) and accelerate brood reduction. Conversely, females should deposit lower progesterone levels in the last egg(s) to hatch compared to the older sibling(s) when future parental ability is good and brood reduction is not necessary.

We tested this hypothesis in rockhopper penguins *Eudyptes chrysocome chrysocome*, a species exhibiting both reversed hatching asynchrony and brood reduction [35,36]. In this species, the larger second-laid egg (B-egg) hatches before the smaller first-laid egg (A-egg) although incubation starts only at clutch completion. Moreover, although both eggs commonly hatch, the chick hatched from the A-egg generally dies of starvation within days of hatching. However, in the Falkland Islands, parents can sometimes fledge both chicks of the clutch [37,38], although this phenomenon has not been quantified in detail and remains poorly understood (but see [38]). In this population, we predicted that the difference in yolk progesterone levels between A- and B-eggs should change with the female’s future ability to feed chicks (measured as her body mass at laying and as the date of laying onset).

Since in rockhopper penguins, females are the only parents feeding chicks during the first few weeks of life [39], we did not expect males to have a strong impact on this mechanism. Lightweight females should benefit from early brood reduction via an enhancement of the size hierarchy within the clutch, while heavy females could attempt to fledge two chicks. Heavy females were therefore expected to deposit proportionally more progesterone in the B-egg than in the A-egg while light females should deposit less progesterone in the B-egg than in the A-egg.

For many marine birds including penguins, prey availability varies according to the life cycle of the prey itself and to predators’ activity around the colony [40–43]. Such factors, which vary with the season, drive the breeding phenology of the species [44] and probably the highly synchronised laying and hatching periods of colonial seabirds such as rockhopper penguins [45,46]. Since this synchrony is an important component of the breeding success of colonial birds, we also examined whether the date of laying onset (i.e. the day the first egg of the clutch was laid) influenced yolk progesterone levels. We hypothesized that early breeders, which benefit from better food availability during chick feeding, would deposit proportionally more progesterone in the B-egg than in the A-egg compared to late breeders.

**Materials and Methods**

**Ethical statement**

The study was performed under proper legislation of the Belgian and Flemish law and was approved by the ethical committee on animal experimentation (ECD, ID numbers: 2011/44 and 2011/45). All work was conducted under a research license granted by the Environmental Planning Department of the Falkland Islands Government (Research Licence No: R06/2009). This license covered animal welfare in addition to collection of the egg samples. The methods that we used (nest check, female capture, egg collection) did not cause any desertion from nestling activity or mortality. Collected eggs were replaced with eggs found outside their own nest that we considered as lost by their original parents in order to avoid affecting the breeding success of the colony.

**Study site and birds**

The study was carried out in the “Settlement colony” on New Island, Falkland Islands (51°43’S, 61°17’W) from late October to early November 2009. This colony has approximately 5000 pairs of breeding southern rockhopper penguins. Their breeding biology at this colony has been described by Poisbleau et al. [37]. Briefly, males arrive at the colony first (early October) and establish nest sites. Females arrive a few days later, for pairing and copulation. Laying intervals are highly standardized in this species. Within clutches, the B-egg is generally laid four days after the A-egg.

After the arrival of the first males, we visited study sites daily; initially to mark active nests and subsequently to follow the egg laying.

**Egg collection**

When a new A-egg was detected in a study nest, we collected it. We replaced this egg with an egg found outside its own nest that we considered as lost by its original parents. Afterwards, we checked the nest daily until the laying of the B-egg. We collected the B-egg as soon as it was detected in the study nest and replaced it with a lost egg. As incubation in rockhopper penguins typically does not start before clutch completion [39], the A-eggs were not incubated and the B-eggs were not incubated for longer than 24 h at collection. We therefore assumed that embryo development and (potential) change in progesterone concentrations (see [27]) had not yet begun and that the eventual difference between A- and B-egg progesterone concentrations was not due to the procedure of
egg collection. No embryo development was observed during the preparation of any of the collected eggs.

In total, we collected 60 whole clutches. After collection, we weighed the eggs to the nearest 0.1 g using a digital balance and froze them whole at −20°C.

**Egg preparation**

The same method was used to prepare all frozen eggs for subsequent hormone analysis [47,48]. 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 [23,49]. We recorded the mass of the yolk to the nearest 0.1 g using a digital balance. Since progesterone concentrations are not homogeneous within the yolk, being the highest in the external layers [23,50,51], we carefully homogenized the yolk by swirling it with a mini-spatula. We therefore obtained a yolk sample representative of the whole yolk. A small quantity of each homogenized yolk was transferred to a 1.5-ml Eppendorf tube and stored at -20°C until hormone analysis.

**Hormone analysis**

Yolk concentrations of progesterone were determined by radioimmunoassay at the Centre d’Études Biologiques de Chizé (CEBC-CNRS) according Mauget et al. [52] adapted for yolk.

Briefly, 200 mg of each sample (weighed to the nearest 0.01 mg) were homogenised in 1 ml of distilled water. Steroids were extracted by adding 3 ml of diethyl-ether to 100 μl of the resulting emulsion, vortexing and centrifuging. The diethyl-ether phase was decanted and poured off after snap freezing the tube in an alcohol bath at −40°C. This was done twice for each sample and the resultant was then evaporated. Extraction efficiency was estimated by adding 1,000 counts per minute (CPM) of 3H-progesterone to samples. Recoveries were higher than 90%. The dried extract was re-dissolved in 200 μl of phosphate buffer and incubated overnight at 4°C with 7000 CPM of 3H-progesterone to samples. Results were then related to the date of laying onset and female body mass at laying using GLMs. All GLMs were initiated with full-factorial interactions, and subjected to backwards stepwise model simplification. We used Pearson’s correlations to explore the correlation in yolk progesterone concentration and total yolk progesterone in each clutch. These ratios followed normal distributions (Kolmogorov-Smirnov tests, P>0.05). They were then related to the date of laying onset and female body mass at laying using GLMs. All GLMs were performed in SPSS 16.0. Yolk progesterone concentrations and total yolk progesterone followed a normal distribution (Kolmogorov-Smirnov tests, P>0.05). We first tested for variation in yolk progesterone concentration and total yolk progesterone using General Linear Models (GLMs) with egg category (A- or B-eggs) as a fixed factor and date of laying onset and female body mass at laying as covariates. Secondly, we calculated intra-clutch ratios (A-egg:B-egg) for yolk progesterone concentration and for total yolk progesterone in each clutch. These ratios followed normal distributions (Kolmogorov-Smirnov tests, P>0.05). They were then related to the date of laying onset and female body mass at laying using GLMs. All GLMs were initiated with full-factorial interactions, and subjected to backwards stepwise model simplification. We used Pearson’s correlations to explore the correlation in yolk progesterone concentration and total yolk progesterone between A- and B-eggs of the same clutch and also to explore the association between the date of laying onset and female body mass at this date. We used paired t-tests to test whether yolk progesterone concentration and total yolk progesterone were significantly different between A- and B-eggs of the same clutch, and univariate linear regressions to explore the relationships of yolk progesterone concentration and total yolk progesterone with date of laying onset and with female body mass. Values are presented as means ± standard deviation (SD).

**Results**

Neither yolk progesterone concentrations nor total yolk progesterone were correlated between A- and B-eggs of the same clutch (Pearson correlation: r = −0.114, P=0.387 and r = −0.015, P=0.910, respectively). B-eggs had significantly higher yolk progesterone concentrations than A-eggs (mean 263.2±59.2 pg/mg and 227.5±49.6 pg/mg, respectively; table 1; paired t-tests: t9g = 3.590, P = 0.001). This difference remained for total yolk progesterone (5891.7±1359.0 ng and 4528.7±1295.6 ng, respectively; table 1; paired t-tests: t9g = 5.581, P<0.001).

Female body mass at laying explained a very small part of the variation in both these outcomes (table 1), with both yolk progesterone concentrations and total yolk progesterone slightly increasing with female body mass at laying (univariate linear regression: t= 1.807, P=0.073 and t= 1.583, P=0.062, respectively; figure 1). However, the intra-clutch ratios of yolk progesterone concentrations and total yolk progesterone between A- and B-eggs were not related to female body mass at laying (F,57 = 0.229, P=0.634, ηp2 = 0.004 for yolk progesterone con-
centrations and $t_{1.57} = 0.642, P = 0.426, \eta^2_p = 0.011$ for total yolk progesterone when this covariate was removed from the model).

Both yolk progesterone concentrations and total yolk progesterone changed significantly with the date of laying onset in interaction with egg category (table 1). Yolk progesterone concentrations and total yolk progesterone increased with time for A-eggs (univariate linear regression: $t = 3.881, P < 0.001$ and $t = 2.909, P = 0.005$, respectively; figure 2), while they decreased with time for B-eggs (univariate linear regression: $t = -5.008, P < 0.001$ and $t = -4.955, P < 0.001$, respectively; figure 2). Consequently, the proportion of clutches with lower yolk progesterone concentrations and total yolk progesterone in the B-egg than in the A-egg increased with the date of laying onset. The intra-clutch ratios were significantly related to the date of laying onset ($F_{1.58} = 48.468, P < 0.001, \eta^2_p = 0.455$ for yolk progesterone concentration and $F_{1.58} = 37.311, P < 0.001, \eta^2_p = 0.391$ for total yolk progesterone): early clutches had proportionally more progesterone in the B-egg compared to the A-egg while late clutches had proportionally less progesterone in the B-egg (figure 3).

The date of laying onset was not related to female body mass at laying (Pearson correlation: $r = 0.018, P = 0.894$).

**Discussion**

We tested whether yolk progesterone levels differed between eggs according to the future parental ability to feed the entire clutch in wild rockhopper penguins. Overall, we observed that B-eggs had significantly higher yolk progesterone levels (both in concentrations and total amounts) than A-eggs in rockhopper penguins. However, this general result was reversed in many clutches. This variation in the intra-clutch pattern of yolk hormone levels seems less common for the other hormones measured in this species [48,59,60]. Among 20 clutches collected in 2007, only one had slightly higher testosterone and dihydrotestosterone (DHT) levels in the A-egg than in the B-egg and none had higher androstenedione (A4) levels [59]. Whatever the positive or negative effects of measured yolk hormone (other than progesterone) levels on embryos and chicks, the constancy of their intra-clutch ratios showed that one chick category was always favoured over the other one. This is clearly not the case for progesterone, yet it is a precursor of testosterone. Since a recent study showed that the corticosterone measurements reported on rockhopper penguin yolks [48] in reality also reflect high concentrations of yolk progesterone and its precursors [61], it is not relevant to further compare our present findings with corticosterone levels measured on the same species [48].

We proposed that females could adjust the levels of yolk progesterone (as of other yolk hormones, see review in [19]) over the laying sequence to reduce or enhance the effects of the size hierarchy between siblings according to the breeding conditions they experience during egg formation and/or they expect to experience during chick rearing. Breeding conditions here were assessed through both female body mass at laying and date of laying onset.

We detected a slight general increase of yolk progesterone levels with female body mass at laying. However, the intra-clutch ratios of yolk progesterone levels did not vary with female body mass, implying that the increase in yolk progesterone levels with female

| Table 1. Test of the variation in yolk progesterone (in pg/mg) concentrations and total yolk progesterone (in ng). |
|---------------------------------------------------------|------------------|-----------------|------------------|
| Parameter                                              | df   | $F$  | $P$  | $\eta^2_p$ |
|---------------------------------------------------------|------|------|------|------------|
| Yolk progesterone concentration                         |      |      |      |            |
| Egg category                                            | 1,115| 59.652| <0.001| 0.342      |
| Female body mass                                        | 1,115| 5.004 | 0.027 | 0.042      |
| Laying onset                                            | 1,115| 1.469 | 0.228 | 0.013      |
| Egg category * Laying onset                             | 1,115| 43.377| <0.001| 0.264      |
| Total yolk progesterone                                |      |      |      |            |
| Egg category                                            | 1,115| 61.877| <0.001| 0.350      |
| Female body mass                                        | 1,115| 5.827 | 0.017 | 0.048      |
| Laying onset                                            | 1,115| 1.812 | 0.181 | 0.016      |
| Egg category * Laying onset                             | 1,115| 31.666| <0.001| 0.216      |

Results of GLMs with egg category (A- or B-eggs) and as a fixed factor and female body mass and date of laying onset as covariates. $n = 120$ for both dependent variables. Only significant interactions are shown in these models, while other non-significant ones were removed from the model during the backwards-stepwise procedure. As a measure of effect sizes we used partial Eta-Square values ($\eta^2_p$; i.e. the proportion of the effect + error variance that is attributable to the effect) for the factors and covariates tested with a GLM.

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![Figure 1. Relationship between female body mass and (a) yolk progesterone concentration and (b) total yolk progesterone. doi:10.1371/journal.pone.0027765.g001](image-url)
body mass increase was similar for A- and B-eggs. Female body mass is therefore not likely to affect the size hierarchy through progesterone levels. These findings do not follow those obtained on testosterone and A4 levels for the same species [60]: androgen levels were significantly negatively related to female body mass in B-eggs while these measurements varied independently for A-eggs [60]. These results suggest that, if female body mass acts as a driver of brood reduction in rockhopper penguins, maternal yolk progesterone is not mediating this relationship, while maternal yolk androgen deposition could be. Given that yolk androgens positively influence the outcome of sibling competition through potential effects on embryonic developmental time and on chick begging behaviour, food competitiveness and growth rates (see review in [17]), this pattern of yolk androgen deposition between eggs favours B-eggs and probably ensures a quicker brood reduction in females with low body mass.

Yolk progesterone levels increased with the date of laying onset for A-eggs while they decreased for B-eggs. We therefore observed that the within-clutch difference in yolk progesterone levels between A- and B-eggs gradually changed with time. At the beginning of the laying period, all clutches had higher yolk progesterone in the B-egg than in the A-egg, but the trend was reversed for clutches laid after the middle of the laying period, with some clutches having more yolk progesterone in the A-egg than in the B-egg. For androgens, it was observed that late clutches had proportionally higher levels in the B-egg compared to the A-egg than early clutches [59]. Interestingly, we note that yolk androgen deposition is modulated only in B-eggs, while yolk progesterone is modulated simultaneously in both egg categories.

Hatching is highly standardized in rockhopper penguins, with the A-egg generally hatching one day later than the B-egg, but occasionally hatching on the same day (22%) or two days later (18%, n = 92 clutches, MP unpublished data and [37]). Since yolk progesterone levels have been observed to be negatively linked to nestling growth [32], lower yolk progesterone levels in A-eggs (the smallest and the second to hatch) than in B-eggs (the biggest and the first to hatch) for early clutches may be a way of compensating for the initial size hierarchy between A- and B-eggs. Because Nazca booby chicks with a sibling have lower plasma progesterone levels and higher mass increase than unchallenged chicks, Tarlow et al. [33] suggested that plasma progesterone could be regulated to promote exaggerated mass gain in socially challenged chicks.

**Figure 2.** Variation in (a) yolk progesterone concentration and (b) total yolk progesterone according to egg category (white boxes: A-eggs; grey boxes: B-eggs) and the date of laying onset. Boxes show medians, 25% and 75% quartiles for each day; whiskers indicate the range between the 10th and 90th percentiles. *: Data outside the 10th and 90th percentiles. Sample sizes are given under the boxes in the lower frame. Significance of paired t-tests with egg category (A- or B-eggs) as the grouping variable is presented above respective groups for each date of laying onset; NS: P>0.05, *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001. doi:10.1371/journal.pone.0027765.g002

**Figure 3.** Intra-clutch ratios of (a) yolk progesterone concentration and (b) total yolk progesterone between A- and B-eggs according to the date of laying onset. The solid lines are univariate regression models predicting ratios of yolk progesterone concentration or total yolk progesterone from the date of laying onset and dashed lines represent the ratio expected in the absence of any difference between A- and B-eggs. n = 60 clutches. doi:10.1371/journal.pone.0027765.g003
We therefore propose here that maternal yolk progesterone could also be regulated to influence mass gain of the embryos and chicks according to the parents’ future ability to feed the entire clutch. The fact that the pattern of deposition according to the date of laying onset is opposite between the two eggs of the clutch (increasing with time for A-eggs but decreasing for B-eggs) suggests that the mechanism governing deposition of yolk progesterone differs between egg categories. More specifically, maternal yolk progesterone deposition shows that the growth of embryos from A-eggs may be favoured at the beginning of the laying period, compared to embryos from B-eggs while neither egg category may be favoured in regard of yolk progesterone deposition at the end of the laying period (see figure 2). Since it does not seem adaptive to decrease the growth rates of either chick, even if it may enhance survival of the other, we suggest that yolk progesterone levels could be decreased in A-eggs that are laid early in the breeding season to give those chicks a better survival chance. The high yolk progesterone levels in their B-egg siblings might therefore only be a compensatory consequence because the female has to evacuate a certain amount of progesterone. However, the fact that yolk progesterone is produced in follicles, different for each yolk, and that its levels do not mirror female plasma levels (see [23]) do not support this hypothesis. Alternatively, the low yolk progesterone levels in A-eggs, compared to their B-egg siblings, might be an additional adaptive mechanism to increase the size hierarchy between siblings if females would be able to actively control hormone deposition (see discussion on the subject in [18]).

In conclusion, a differential investment with lower yolk progesterone levels in the second eggs to hatch (A-eggs) should be associated with a reduction in the advantage enjoyed by the first chick and with a reduced frequency of brood reduction when breeding conditions are expected to be good during chick rearing. Conversely, lower (or similar) progesterone levels in the first eggs to hatch (B-eggs) should allow the negative effects of hatching asynchrony to be expressed. Brood reduction would be accelerated in favour of B-eggs, the eggs with the highest expected value, when breeding conditions are expected to be bad during chick rearing. Therefore, the reported within-clutch difference in yolk progesterone supports the hypothesis that females can strategically deposit different progesterone levels within the clutch according to the future parental ability to feed the clutch. The present study stresses the importance of further investigation of role of progesterone in both altruical and precocial species and of exploring how the different hormones present in the yolk interact to affect brood reduction, chick development and behaviour in general.

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Author Contributions

Conceived and designed the experiments: MP LD ME. Performed the experiments: MP LD. Analyzed the data: MP LD. Wrote the paper: MP LD CP ME. Performed the hormone analyses: CP.

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