



Migratory constraints on yolk precursors limit yolk androgen deposition and underlie a brood reduction strategy in rockhopper penguins

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Hormonally mediated maternal effects link maternal phenotype and environmental conditions to offspring phenotype. The production of lipid-rich maternal yolk precursors may provide a mechanism by which lipophilic steroid hormones can be transported to developing yolks, thus predicting a positive correlation between yolk precursors in mothers and androgen levels in eggs. Using rockhopper penguins (*Eudyptes chrysocome*), which produce a two-egg clutch characterized by extreme egg-size dimorphism, reversed hatching asynchrony and brood-reduction, we examined correlations between circulating concentrations of the primary yolk-precursor vitellogenin (VTG) and levels of yolk androgens. Previous work in *Eudyptes* penguins has shown that egg-size dimorphism is the product of migratory constraints on yolk precursor production. We predicted that if yolk precursors are constrained, androgen transport to developing yolks would be similarly constrained. We reveal positive linear relationships between maternal VTG and androgens in small A-eggs but not larger B-eggs, which is consistent with a migratory constraint operating on the A-egg. Results suggest that intra-clutch variation in total yolk androgen levels depends on the production and uptake of yolk precursors. The brood reduction strategy common to *Eudyptes* might thus be best described as the result of a migratory constraint.

Keywords: androstenedione; testosterone; physiological epiphenomenon hypothesis; vitellogenin; maternal effects; carryover effects

1. INTRODUCTION

Maternal effects are developmental modifications to offspring phenotype that reflect maternal phenotype

and environmental conditions, and affect traits such as offspring size, quality and sex. Steroid hormones have been identified as potentially important physiological mediators of maternal effects, but we still know relatively little about the underlying mechanisms through which hormones are transferred to eggs [1–3]. The lipophilic nature of steroids provides a parsimonious mechanism for the maternal transfer of steroids, and may explain the positive correlations between maternal plasma and yolk steroids—a phenomenon termed the ‘physiological epiphenomenon hypothesis’ by Groothuis & Schwabl [4]. This model suggests that the developing lipid-rich yolk forms a ‘sink’ for lipophilic steroid hormones [2], and predicts that the synthesis, transport and uptake of yolk precursors (e.g. vitellogenin, VTG) might provide a key mechanism for the deposition of maternally derived steroids to yolk. As VTG is transported from maternal circulation and released from capillaries directly into follicular thecal cells where sex-steroids are produced [1,4], the amount of VTG deposited there might influence the final size of follicular yolk-sinks and the amount of steroids deposited. This would predict (i) a direct, positive relationship between maternal VTG and yolk steroids, and (ii) that factors perturbing VTG synthesis and/or yolk formation (e.g. a migration–reproduction overlap or carryover effect, [5]) would have correlated effects on yolk steroids. However, to our knowledge, no studies have examined the inter-relationships between yolk precursors and yolk steroids during egg production. This is important to resolve, especially in the light of counter-examples wherein inverse relationships, or indeed no relationships, between maternal and yolk hormone levels have been identified [1].

Here, we test the role of VTG in mediating the deposition of maternal androgens to yolk by examining relationships between these variables in gravid female rockhopper penguins (*Eudyptes chrysocome*) and their eggs. Crested penguins (*Eudyptes* spp.) are good models for exploring patterns of yolk and androgen deposition as females produce extremely dimorphic clutches characterized by reversed hatching asynchrony (the small first-laid A-egg is approx. 65–90% of the size of the larger second-laid B-egg) and brood reduction. The larger B-eggs hatch first, and contain higher androgen levels, which may facilitate brood reduction via competitive asymmetry [6,7]. Furthermore, extreme egg-size dimorphism has been linked to a migratory carryover effect that constrains the production of VTG, and thus egg-size [5] and viability [8]. If constraints on VTG also constrain the transport of androgens to developing yolks, this would explain the lower androgen levels observed in smaller A-eggs.

Using rockhopper penguins, we predicted (i) that VTG would increase from pre-laying concentrations at colony arrival (post-migration) to a peak at A-egg laying, and (ii) there would be a positive relationship between pre-laying VTG and egg-size dimorphism. We also predicted (iii) that pre-laying VTG would be directly proportional to androgens levels in first laid A-eggs, but (iv) VTG prior to laying of B-eggs (which are produced 4 days behind A-eggs; [9]) may not be as strongly related to B-egg androgens. In

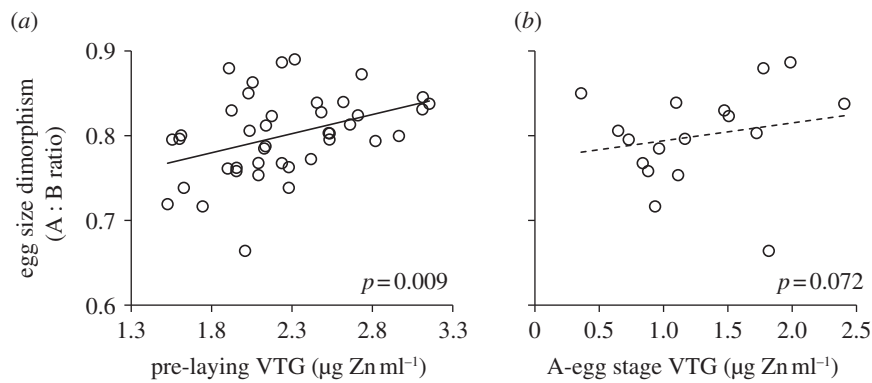


Figure 1. Relationships between intra-clutch egg-size dimorphism (A : B ratio) and circulating VTG concentrations in female rockhopper penguins (a) during the pre-laying period shortly after arrival at the breeding colony, and (b) at the time of egg laying.

other words, owing to a temporal separation between A- and B-egg development, migratory constraints on VTG production would affect androgen deposition to a greater extent in A- versus B-eggs.

2. MATERIAL AND METHODS

Sampling occurred at October–November 2008 at New Island, Falkland Islands [7]. Males return from sea in early October, with females days to weeks later. After arrival, females from 60 active nests were weighed and measured at three different times: after nest-establishment ($n = 42$), day of A-egg laying ($n = 43$) and B-egg laying (i.e. clutch completion, $n = 39$). Blood samples were collected from individuals serially: once ($n = 15$ individuals), twice ($n = 26$) and thrice ($n = 19$). We collected both eggs from each of the 60 nests as each was laid. One replacement A-egg was added to each nest from non-experimental nests to minimize breeding disturbance. Pre-laying samples were not necessarily collected on the day of arrival and nest-establishment; pre-laying blood was collected opportunistically, with some on the day of nest-establishment shortly after colony arrival, while others were collected some days later. Therefore, we could not calculate the interval between arrival and laying with confidence. However, laying dates were carefully recorded; pre-laying samples were obtained 7.9 ± 2.1 days s.e.m. prior to A-egg laying (range 1–12 days).

We regard 'pre-laying VTG' samples as those that reflect post-migratory VTG concentrations, collected sometime during the interval between colony arrival and laying. 'A-egg stage VTG' reflects concentrations within 24 h of A-egg laying (unequivocally 3–4 days before B-egg laying). 'B-egg stage VTG' reflects concentrations within 24 h of B-egg laying.

Both eggs from the 60 nests were collected, weighed (± 0.1 g) and frozen whole until analysis (see methods in Poisbleau *et al.* [10]). Plasma samples were assayed in duplicate for vitellogenic zinc (Zn; zinc kit, Wako Chemicals) as an index for VTG following methods in Crossin *et al.* [1]. All assays were measured on a Biotek 340i microplate reader. Intra-assay coefficient of variation for VTG, from a laying hen (*Gallus domesticus*) plasma pool, ranged from 5.7 to 7.3 per cent. Inter-assay coefficient of variation was 5.8 per cent. Radioimmunological analysis of yolk androstenedione (A4) and testosterone (T) are described in Poisbleau *et al.* [9].

Analyses were run with the JMP v. 9.0 or SAS v. 9.0 software packages. Shapiro–Wilk tests showed that all variables were normally distributed. Pearson's correlations were examined among sampling date, pre-laying body mass, VTG at all three sampling dates, yolk androgens and egg-size dimorphism (e.g. A : B ratio = A-egg mass/B-egg mass), and Bonferroni corrections were applied. A repeated-measures mixed linear model (SAS PROC MIXED) with *post hoc* Tukey's tests was used to compare temporal, intra-individual changes in VTG between the pre-laying, A-egg and B-egg stages. A similar model was used to compare intra-clutch changes in yolk androgen levels between A- and B-eggs. Backwards stepwise regressions were used to explore relationships between maternal VTG and yolk androgen concentrations while accounting for variation in date and body mass. The relevant comparisons are: pre-laying VTG versus A-egg yolk androgen levels; and A-egg stage VTG versus B-egg yolk androgen levels. Similar models were used to explore relationships between A : B ratio with both VTG and yolk androgens.

3. RESULTS

Sampling date was not correlated with body mass at any time (all $p > 0.120$). Neither pre-laying sampling date nor pre-laying body mass was significantly correlated with VTG or yolk androgen levels at any stage, or with A : B ratio (all $p > 0.110$).

Consistent with our first prediction, maternal VTG concentrations increased significantly from pre-laying to the A-egg stage, and then decreased at the B-egg stage (repeated measures, VTG, exact $F_{2,13} = 44.6$, $p < 0.001$). Pre-laying VTG was 2.08 ± 0.06 $\mu\text{g Zn ml}^{-1}$, which increased (Tukey's test, $p < 0.001$) to 2.58 ± 0.12 $\mu\text{g Zn ml}^{-1}$ at the A-egg stage, then decreased ($p < 0.001$) to 1.26 ± 0.13 $\mu\text{g Zn ml}^{-1}$ at the B-egg stage. Supporting our second prediction, relationships were found between A : B ratio and pre-laying VTG ($F_{1,39} = 9.822$, $p = 0.012$; figure 1), independent of date or mass effects (both $p > 0.202$). There was no significant relationship between A : B ratio and A-egg stage VTG ($p = 0.303$). For both eggs, total egg mass and yolk mass were significantly correlated (both $p < 0.001$).

Intra-clutch yolk androgen levels increased from the A-egg to B-egg stage, which supports our third prediction (repeated measures, A4: $F_{1,41} = 259.2$, $p < 0.001$; T: $F_{1,41} = 311.1$, $p < 0.001$). The mean yolk A4 in A-eggs was 3347.3 pg yolk^{-1} (or 177.27 ± 9.25 pg mg yolk^{-1}), and in B-eggs was 7143.7 pg yolk^{-1} (or 330.10 ± 9.97 pg mg yolk^{-1}). Mean yolk T in A-eggs was 119.0 pg yolk^{-1} (or 6.38 ± 0.27 pg mg yolk^{-1}), and in B-eggs was 270.7 pg yolk^{-1} (or 12.51 ± 0.42 pg mg yolk^{-1}).

Backwards stepwise regressions revealed a significant positive relationship between pre-laying VTG and A-egg yolk A4 ($F_{1,39} = 10.795$, $p = 0.002$), independent of body mass and date effects (both $p > 0.01$). Pre-laying VTG was also related to A-egg yolk T ($F_{1,39} = 7.805$, $p = 0.008$; figure 2), without date or body mass effects (both $p > 0.10$). Both observations support our third prediction. VTG at the pre-laying and A-egg stages however were unrelated to B-egg androgens (all $p > 0.292$; figure 2), which supports our fourth prediction.

4. DISCUSSION

When female *Eudyptes* penguins initiate egg production during the final days of migration back to a breeding

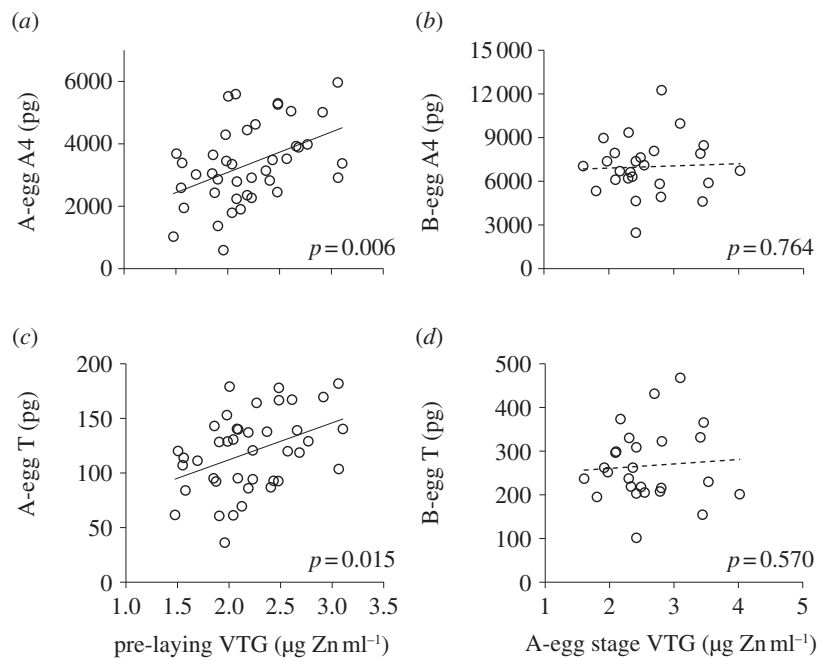


Figure 2. Relationships of circulating VTG concentrations in female rockhopper penguins with absolute yolk androstenedione (A4) and yolk testosterone (T) levels in their eggs (*a,c*) during the pre-laying period shortly after arrival at the breeding colony, and (*b,d*) at the time off egg laying.

colony, the demands of migration place constraints on VTG production. This migratory carryover effect leads to high variation in pre-laying VTG, which predicts egg-size (intra-clutch egg-size dimorphism; [5]). Because androgen hormones are highly lipophilic, we hypothesized that constraints on VTG production would constrain androgen deposition to developing yolks, to a greater extent in small A-eggs (developed mostly at sea) than in larger B-eggs (developed mostly on land). Consistent with previous work in *Eudyptes*, we found that VTG concentrations in newly arrived rockhopper penguins were directly related to egg-size dimorphism, thus indicating a potential migratory constraint, and that deposition of yolk androgens to A- and B-eggs was consequently asymmetric. The results suggest that the synthesis and transport of yolk precursors to the site of androgen synthesis in follicular thecal cells, and the receptor-mediated endocytotic uptake of yolk precursors to developing oocytes, are key mechanisms for the maternal deposition of androgens to yolk.

In *Eudyptes*, the degree of overlap between migration and egg production (measured as the time-interval between colony arrival and laying) forms the basis for this constraint on VTG, which then determines egg-size dimorphism [5]. Although we could not precisely determine the time-interval between colony arrival and laying in rockhopper penguins (and thus the relative degree of overlap between migration and egg production), pre-laying samples were nevertheless collected shortly after arrival, 1–12 days before laying. As yolk deposition takes 16 days in penguins, with a 4-day lag between sequential follicles [9], this means that *ca* 25–94% of A-eggs were developed at sea during migration, whereas only 0–69% of B-eggs were developed at sea. We had therefore predicted A-egg androgen levels to correlate with pre-laying VTG

concentrations, which our data support. Conversely, androgens in B-eggs did not correlate with either pre-laying VTG or VTG just after A-egg laying, which suggests that VTG was no longer constrained at the B-egg stage as the bulk of B-egg development occurs on land. Individual variation in B-egg androgens is thus probably related to other factors, including direct maternal effects [6]. Our results are novel because they suggest that intra-clutch allocation of maternal androgens depends on the production of yolk precursors. Furthermore, because *Eudyptes* penguins have evolved a breeding system in which egg production overlaps with migration, our results lend an insight into an evolutionary maladaptation [11] characterized by extreme egg-size dimorphism and reversed hatching asynchrony, and provide a mechanism that facilitates brood reduction [6]. On the basis of our results, the brood reduction strategy common to *Eudyptes* might therefore be best described as the result of a migratory constraint that yields a competitive disadvantage to A-eggs.

Research was conducted under a licence from the Falkland Islands Government.

We thank the New Island Conservation Trust, and Colette Trouvé, Stéphanie Dano and André Lacroix. Funding was provided by the Deutsche Forschungsgemeinschaft DFG (Qu 148/1-ff), the Falkland Islands Environmental Studies Budget, the University of Antwerp, the FWO Flanders and NSERC (Canada).

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