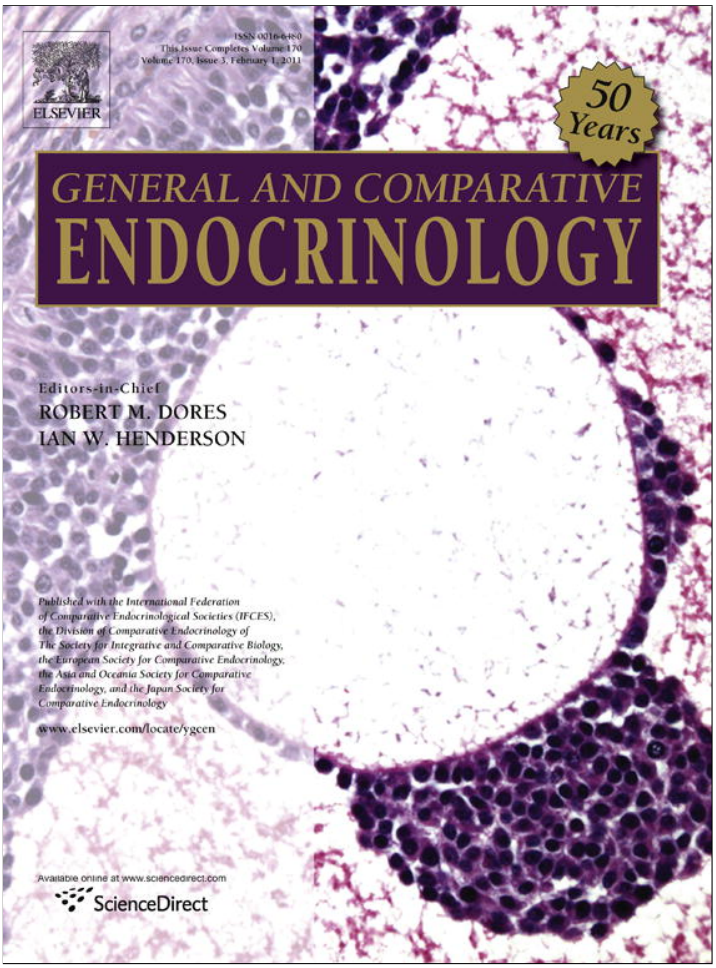

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Yolk androgen deposition in rockhopper penguins, a species with reversed hatching asynchrony

M. Poisbleau [a,b,*], L. Demongin [a,b], O. Chastel [c], M. Eens [b], P. Quillfeldt [a]

[a] Max Planck Institute for Ornithology, Vogelwarte Radolfzell, Schlossallee 2, 78315 Radolfzell, Germany
[b] University of Antwerp, Campus Drie Eiken, Department Biology – Ethology, Universiteitsplein 1, 2610 Antwerp (Wilrijk), Belgium
[c] Centre d'Études Biologiques de Chizé, CNRS UPR 1934, 79360 Beauvoir-sur-Niort, France

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ABSTRACT

To maximize fitness, females should invest optimally in the siblings within a litter or brood and adapt this investment to environmental conditions. Chick mass and yolk androgens have been shown to influence the outcome of sibling competition. In birds, asynchronous hatching plays a major role in this process and often leads to brood reduction. We studied maternal deposition of yolk androgens in eggs of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*). Contrary to other avian models, laying and hatching sequences do not coincide in this species, which exhibits reversed hatching asynchrony. This provides a unique model to test whether the first egg to hatch (B-egg), which is the most likely to survive, differs in composition from the second egg to hatch (A-egg). We found that B-eggs had higher egg masses, yolk masses, yolk androgen concentrations and total yolk androgen amounts than A-eggs. This was observed consistently for the three androgens analyzed (testosterone, androstenedione and 5α-dihydrotestosterone). Laying date affected androgen deposition into A- and B-eggs differently. Interestingly, late clutches had proportionally higher androgen levels in the B-egg compared to the A-egg than early clutches. We discuss these results in relation to the chronology of egg formation and the potential effect of the observed differences on embryo development and brood reduction.

1. Introduction

In animals, competition among siblings affects their growth and can even lead to the elimination of part of the litter or brood [28]. Brood size and differences in size and development between siblings have been shown to influence sibling competition [28]. In birds, asynchronous hatching of the eggs within a single clutch plays a major role in this process. Together potentially with variation in egg size within clutches, hatching asynchrony generally leads to a size hierarchy among siblings, and to a competitive disadvantage for younger siblings compared to older ones [60,44,51]. In this context, hatching asynchrony and the resulting brood reduction has been proposed to serve different functions (see review in [50]). It might be (1) an adaptation to unpredictable food availability, (2) a means of saving time, ultimately to increase lifetime reproductive success, or (3) a not-necessarily-adaptive by-product of constraints on reproduction.

Yolk is the primary source of energy in eggs and an important source of proteins for the embryo [2]. It is also the main source of maternal androgens for developing birds [41,15]. Yolk androgens have been shown to 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 [9]). However, these effects are largely inconsistent, being either positive, negative or nonexistent according to the species, the environmental conditions or the sex of the individuals. We also do not yet know whether females play an active and/or passive role in the maternal transfer of yolk androgens (see review in [18]), but it appears that yolk androgen concentrations vary within and between clutches according to various intrinsic and extrinsic factors which could have an indirect effect on later brood reduction (see review in [9]).

One of the first sources of variation in yolk androgen that was considered was within-brood variation with the laying sequence [41]. Since Schwabl [41] showed that the testosterone content of canary (*Serinus canaria*) eggs increased with laying sequence within clutches, numerous studies have also demonstrated that laying sequence strongly and positively correlates with androgen concentration [23,22,45,4,37,16,32,26,56,53,13,12,39,54]. However, several studies have highlighted a negative relationship [42,10,36,11] and others failed to find any trend [8,58,38]. So far, a comparative study of how these differences in within-clutch distribution relate to patterns of hatching asynchrony or brood reduction has not been conducted [9].

* Corresponding author at. Max Planck Institute for Ornithology, Vogelwarte Radolfzell, Schlossallee 2, 78315 Radolfzell, Germany.
E-mail address: maud.poisbleau@ua.ac.be (M. Poisbleau).

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Laying date could also be a factor affecting yolk androgen deposition and thus affecting brood reduction. Yolk androgen levels have generally been found to be negatively associated with laying date [32,14,55]. Additionally, within-clutch variation in yolk androgens may also change with time. For example, the increase in androgen levels over the laying sequence was more pronounced in clutches of black-headed gull (*Larus ridibundus*) produced later in the season [30]. However, the opposite trend was observed for consecutive clutches of domestic canaries: third and fourth eggs had significantly higher testosterone levels than first eggs in first clutches while such an increase was absent in third or fourth clutches [40].

Crested penguins (genus *Eudyptes*) exhibit brood reduction: two eggs are laid but only one chick usually fledges [57]. They also present a reversed hatching asynchrony unique among birds [49,48]: the incubation starts only at clutch completion and the larger second-laid egg (B-egg) hatches before the smaller first-laid egg (A-egg). As a result, although both eggs commonly hatch, the chick hatching from the A-egg generally dies of starvation within days of hatching [19,21]. Collectively, these characteristics provide the circumstances to test whether females deposit different yolk androgen concentrations and/or total yolk androgens in the first egg to be laid or in the first egg to hatch as, contrary to other study models, laying and hatching sequences do not match in crested penguins. Here, we collected freshly laid clutches (A- and B-eggs) of rockhopper penguins (*Eudyptes chrysocome chrysocome*) and described variation in maternal androgen deposition.

The first aim of this paper was to provide baseline data for yolk androgen concentrations and total yolk androgens, as this is the first such study for any penguin. Consistently with previous studies, we measured testosterone (T), androstenedione (A4) and 5α-dihydrotestosterone (DHT). In addition, we examined whether these yolk androgen concentrations and total yolk androgens were correlated with egg and yolk masses and tested for within-clutch covariation in these parameters. We further tested whether yolk androgen concentrations and amounts differ with laying order, and whether laying date affects yolk androgen concentrations and amounts.

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) in November 2007. This colony has approximately 5000 pairs of breeding southern rockhopper penguins. Their breeding biology at this colony has been described by Strange [52], and more recently by Poisbleau et al. [34]. Briefly, males arrive at the colony first (early October) and establish nest sites. Females arrive two weeks 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, but rarely three days (8%) or five days (16%) after the A-egg (n = 432 clutches, unpublished data and Poisbleau et al. [34]). Similarly, hatching is highly synchronized, 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, unpublished data and Poisbleau et al. [34]). The laying period is short and very synchronised between females, usually ranging from 27 October to 10 November each year, with less than 5% of new A-eggs found after 5 November (see [34]).

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 with regard to the collection of the egg samples.

2.2. Egg collection

We collected eggs on two dates (early: 3 November 2007 and late: 8 November 2007) at an undisturbed colony not visited by humans during this breeding season prior to our first visit (see [35]). These two egg collections took place at two distinct but similar sites within the colony in order to ensure that eggs collected on the second date came from birds that had not seen us during the first collection. The two collection sites were approximately 20 m apart and visually separated by tussock (*Parodiochloa flabellata*) bushes that constituted some vegetation cover for the colony. The breeding environment was similar between the sites: they did not differ in nest density, vegetation community and density or distance from the ocean.

We followed exactly the same method for both egg collections. We labelled all eggs during the first visit to each site. The following day, we collected both A- and B-eggs from ten nests in which the A-egg was labelled, but the B-egg was not. We thus considered that the laying dates were the collection date for B-eggs and four days prior to the collection date for A-eggs (see [34]).

In total, we collected 20 whole clutches (10 early and 10 late). To avoid impact on the breeding success of the colony, we replaced the eggs from these nests with one or two eggs found outside their own nest that we considered as lost by their original parents. After collection, we weighed the eggs and froze them whole at −20 °C for at least four days.

2.3. Egg preparation

The same method was used to prepare all the frozen eggs for subsequent hormone analysis [33,35]. 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 [23,24]. 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 [23]. A small quantity of each homogenized yolk was transferred to a 1.5-ml Eppendorf tube and stored at −20 °C until hormone analysis.

2.4. Hormone analysis

Yolk concentrations of testosterone (T), androstenedione (A4) and 5α-dihydrotestosterone (DHT) were determined by radioimmunoassays at the Centre d'Études Biologiques de Chizé according to Mazuc et al. [27] and Gil et al. [13].

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 −30 °C. This was done twice for each sample and the resultant was then evaporated under a stream of nitrogen. Extraction recoveries were calculated on a random sample (n = 20) of these double yolk extractions by adding 1000 counts per minute (CPM) of tritiated hormones, with all the values above 90% for T, A4 and DHT (see [13]). The dried extract was re-dissolved in 1 ml of phosphate buffer and incubated overnight at 4 °C with approximately 9000 CPM of the appropriate ^{3}H-labeled hormone and a specific antibody (see [13] for their specificities). Bound and free fractions were separated by dextran-coated charcoal and centrifuged. A Packard 1600 liquid scintillation counter was used to count activity of the bound fractions. Cross-reactivity of T antiserum at 50% binding was 12% for DHT and <1% for the rest of steroids tested. Cross-reactivity of A4 antiserum was 0.9% for DHT, 0.3% for T, and <0.1% for other steroids. Cross-reactivity of DHT antiserum was 11% for T, 0.3% for A4, and <0.1% for other ste-

roids. As the cross-reactivity between T and DHT was >10%, we acknowledge that a significant proportion of measured DHT could have been T, given the higher concentration of T, while the impact would be minor in the T assay. However, as T and DHT showed different trends in the results section, we believe that we mainly underlined natural variations in each of these androgens.

One assay per hormone was performed. Intra-assay coefficients of variation were 6.9% ($n = 6$ duplicates), 15.7% ($n = 4$ duplicates) and 5.9% ($n = 9$ duplicates) respectively, for T, A4 and DHT.

2.5. Statistical analysis

Due to the fact that A- and B-eggs vary in size and mass in this species [34], a higher androgen concentration in A-eggs than in B-eggs does not necessarily mean a higher quantity of androgens for the former. We therefore calculated the total yolk androgens per yolk (in ng) by multiplying yolk mass (in g) and yolk androgen concentration (in pg/mg).

Statistical tests were performed in SPSS 16.0. Egg masses, yolk masses, yolk androgen concentrations and total yolk androgens followed normal distributions (tested with Kolmogorov–Smirnov tests). We first used Pearson's correlations to test for correlation among the three yolk androgen concentrations and among the three total yolk androgens, and then to explore the relation between these parameters and egg masses and yolk masses. After examining the correlations between A- and B-eggs of the same clutch in egg mass, yolk mass, yolk androgen concentrations and total yolk androgens with Pearson's correlations, we used paired *t*-tests to test whether there were significant differences between A- and B-eggs of the same clutch and between laying periods (early nests; clutches collected on 3 November 2007 and late nests; clutches collected on 8 November 2007) for each egg category. We finally examined changes in masses, yolk androgen concentrations and total yolk androgens with egg category according to laying period with repeated measures GLMs.

3. Results

3.1. Correlation between masses, yolk androgen concentrations and total yolk androgens

Concentrations of all three yolk androgens were highly positively correlated ($n = 40$, all $r > 0.742$ and $P < 0.001$). Similarly, total androgen amounts per yolk were also highly positively correlated ($n = 40$, all $r > 0.836$ and $P < 0.001$). Yolk androgen concentrations were positively correlated with both egg mass and yolk mass ($n = 40$, all $r > 0.487$ and $P < 0.001$), except that yolk DHT concentration was not significantly correlated with yolk mass ($n = 40$, $r = 0.275$ and $P = 0.086$). Moreover, all the total androgen amounts per yolk were positively correlated to egg mass and yolk mass ($n = 40$, all $r > 0.574$ and $P < 0.001$). These results indicate that larger eggs also had higher yolk androgen concentrations and total yolk androgens. Moreover, as yolk androgen concentrations increased with egg and yolk masses, this relation was disproportionate.

3.2. Within-clutch variation in masses, yolk androgen concentrations and total yolk androgens

Tests of the within-clutch differences between A- and B-eggs showed that all measured egg characteristics were significantly different between the two egg categories (Table 1). B-eggs had significantly higher egg mass, yolk mass, yolk androgen concentrations and total yolk androgens than A-eggs (Fig. 1).

Nevertheless, between clutches, egg mass, yolk mass, yolk androgen concentrations and total yolk androgens were correlated between A- and B-eggs of the same clutch ($n = 20$, all $r > 0.510$ and $P < 0.022$). Only yolk DHT concentration and total yolk DHT were not correlated between A- and B-eggs from the same clutch ($n = 20$, $r = 0.226$ and $P = 0.338$ for yolk DHT concentration and $n = 20$, $r = 0.376$ and $P = 0.102$ for total yolk DHT).

3.3. Laying period effect on masses, yolk androgen concentrations and total yolk androgens

The laying period did not significantly affect egg mass, yolk mass, yolk androgen concentrations and total yolk androgens (all $|t_{18}| < 0.773$ and all $P > 0.450$, Fig. 1) for A-eggs. For B-eggs, the laying period did not significantly affect egg mass ($t_{18} = 0.832$, $P = 0.416$), yolk mass ($t_{18} = -0.435$, $P = 0.669$), yolk T concentration ($t_{18} = 1.031$, $P = 0.316$), total yolk T ($t_{18} = 0.683$, $P = 0.503$) and total yolk A4 ($t_{18} = 1.897$, $P = 0.074$) but significantly affected yolk A4 concentration ($t_{18} = 2.372$, $P = 0.029$), yolk DHT concentration ($t_{18} = 3.237$, $P = 0.005$) and total yolk DHT ($t_{18} = 2.992$, $P = 0.008$). In all three cases, concentrations and totals were higher in late clutches than in early clutches (Fig. 1).

Consequently, among the 20 broods, the differences in egg mass and yolk mass between A- and B-eggs were not influenced by the laying period (egg category × laying period: $F_{1,9} = 0.079$, $P = 0.785$ for egg mass, $F_{1,9} = 0.017$, $P = 0.899$ for yolk mass, Fig. 1). However, increases in yolk androgen concentrations and total yolk androgens between A- and B-eggs were significantly affected by the laying period (egg category × laying period: $F_{1,9} = 5.294$, $P = 0.047$ for yolk T concentration, $F_{1,9} = 3.924$, $P = 0.079$ for total yolk T, $F_{1,9} = 7.457$, $P = 0.023$ for yolk A4 concentration, $F_{1,9} = 7.191$, $P = 0.025$ for total yolk A4, $F_{1,9} = 27.439$, $P = 0.001$ for yolk DHT concentration and $F_{1,9} = 70.224$, $P < 0.001$ for total yolk DHT, Fig. 1). These results suggest that late clutches had proportionally more androgens in the B-egg compared to the A-egg than early clutches (Fig. 1).

4. Discussion

In the present study, we analyzed variability in three yolk androgens. We found consistent differences with laying order and that laying date affected androgen deposition into A- and B-eggs differently.

4.1. Androgen content of eggs

This is the first study showing substantial amounts of the three main androgens (T, A4 and DHT) in the eggs of crested penguins, an avian group exhibiting both reversed hatching asynchrony and brood reduction. We observed the highest concentrations and total amounts per yolk for A4, intermediate values for T and the lowest values for DHT. This is consistent with their rank in terms of androgenic effects, since A4 is much weaker than T and T is much weaker than DHT (see [43]).

Within eggs, yolk androgen levels were significantly positively inter-correlated, and correlated with egg mass and yolk mass. These different parameters were also correlated between A- and B-eggs of the same clutch. Only DHT levels did not correlate significantly with egg mass and yolk mass and between A- and B-eggs. DHT was also the androgen the most influenced by the laying period. However, similarly to T and A4 levels, DHT levels were higher in B-eggs than in A-eggs. Tschirren et al. [56] observed that, within clutches, A4 and T content increased significantly with laying order whereas DHT content significantly decreased. These results may suggest that DHT has a different means of deposition from the fe-

Table 1
Mean ± SD values of egg and yolk masses (in g), yolk androgen concentrations (in pg/mg) and total yolk androgens (T, A4 and DHT, in ng) of 20 pairs of A- and B-eggs collected from the same clutches in 2007. Within-clutch differences between A- and B-eggs were tested with paired *t*-tests.

Parameter	A-eggs	B-eggs	t_{19}	*P*
Egg mass	94.75 ± 10.02	118.94 ± 11.36	−14.441	<0.001
Yolk mass	20.02 ± 2.55	22.45 ± 2.56	−4.275	<0.001
Yolk T concentration	9.71 ± 2.59	15.85 ± 2.31	−11.652	<0.001
Total yolk T	198.01 ± 70.00	355.62 ± 65.80	−11.450	<0.001
Yolk A4 concentration	186.63 ± 53.92	352.39 ± 89.70	−9.979	<0.001
Total yolk A4	3813.2 ± 1337.8	7929.4 ± 2312.3	−10.446	<0.001
Yolk DHT concentration	3.00 ± 0.73	4.59 ± 1.11	−5.984	<0.001
Total yolk DHT	60.85 ± 19.06	102.21 ± 24.11	−7.559	<0.001

male to the egg and/or a different effect on the embryo than T and A4 (see [17,18] for more information on this issue). This is intriguing since Gil [9] suggested that it was A4 that was different from the two other androgens. Since no study so far has examined the differential effects of each of these hormones in development [9], it is difficult to discuss further the differences among the three androgens. However, the high inter-correlation among these three hormones within eggs and the minor differences among them elsewhere in the results allow us to combine the three androgens when discussing the other findings of the present study.

4.2. Differences between A- and B-eggs

In the present study, rockhopper penguin eggs had large within-clutch differences in egg mass, yolk mass and in the three yolk androgen concentrations and total yolk androgens.

A-eggs were collected at the same time as the B-eggs. The lower hormone concentrations in A-eggs may therefore be due to this strategy in egg collection. Elf and Fivizzani [7] observed a significant decrease in androgens during embryonic development. Eising et al. [5] showed that testosterone levels in chicken eggs do not change with developmental time and that A4 levels decrease between 3 and 5 days of development. Gilbert et al. [15] observed no decline in yolk androgen concentration at three days development for a species with an even shorter developmental time than penguins. Therefore, we would expect a higher ratio between B- and A-eggs in androgen concentration in our dataset than if both eggs had been collected on their laying dates. However, comparison with unpublished data on androgen concentrations for A- and B-eggs collected on their laying dates showed an inverse pattern. Moreover, as incubation in rockhopper penguins typically does not start before clutch completion [59], neither A- nor B-eggs were incubated for longer than approximately 24 h at collection. The fact that we observed no embryo development during the egg preparation appears to confirm this. We therefore assumed that embryo development and (potential) decrease in androgens had not yet begun and that the difference between A- and B-egg androgen concentrations was not due to the procedure of egg collection.

The eggs with the shortest developmental time (B-eggs) were heavier, had heavier yolks and had higher yolk androgen concentrations and total yolk androgen amounts. In a previous study we observed similar developmental times for rockhopper penguin A- and B-eggs when they were incubated alone [34]. This finding may mean that egg characteristics such as yolk androgen concentrations and total yolk androgens had no significant impact on developmental time. However, the developmental time increased for A-eggs but not for B-eggs when they were incubating with a sibling [34]. As the hypothesis of a preferential incubation treatment for B-eggs was not validated in crested penguins [46,25], we could alternatively suggest that A- and B-eggs do not have the same response in sub-optimal conditions (i.e. incubated with a sibling). Since egg mass itself has no effect on developmental time in rockhopper penguin eggs incubated with a sibling [47], this "behavioural" difference between A- and B-eggs should be linked to other intrinsic egg characteristics. For example, high yolk androgen concentration and/or total yolk androgens in B-eggs may help to compensate physiologically for sub-optimal incubating conditions while A-eggs, having low yolk androgen concentrations and/or total yolk androgens, may not be able to offset this handicap. Although this compensatory role of yolk androgens and its context dependency have also been emphasized for canaries in the context of hatching asynchrony [29], this hypothesis needs to be further explored and requires further experimental tests to be validated.

4.3. Differences between early and late clutches

Female rockhopper penguins only arrive at the colony ten to fifteen days before laying [52] and personal observations) while egg formation takes approximately 25 days for A-eggs and a little less for B-eggs (see [35] for chronology). Therefore, most of the yolk formation (around 18 days for A-eggs and 15 days for B-eggs) occurs at sea for A-eggs while the B-egg yolk is mostly formed at the colony. As yolk androgen concentrations and total yolk androgens can vary according to breeding conditions, often increasing with nest density and social interactions in many bird species [31]; and see [17] for a review; but see [6,1,20], higher yolk androgen concentrations and total yolk androgens in B-eggs than in A-eggs may be a consequence of the increase in social interactions with time. In this case, since the arrival at the colony is generally synchronised between breeding females, we would additionally expect yolk androgen concentrations and total yolk androgens to increase with laying date. We indeed observed this increase for B-eggs but it did not exist for A-eggs. Consequently, the within-clutch increase in yolk androgens between A- and B-eggs was higher in late than in early clutches. This increase with laying date is not in line with some previous studies on birds [32,14,55] but is similar to the observation on black-headed gull (*L. ridibundus*) showing a steeper increase in yolk testosterone levels over the laying order in later-laid clutches [30]. These results suggests that only part of the differences in yolk androgen concentrations and total yolk androgens between A- and B-eggs can be explained by increased nest density and social interactions with time [26].

Several hypotheses regarding the mechanisms of yolk hormone transfer have recently been reviewed and addressed in Groothuis and Schwabl [18]. Without more evidence, we cannot assume that females actively transfer androgens in such a way as to manipulate offspring phenotypes. However, these androgens can be adaptive even if there is no active transport to the yolk by females. In this way, the different prevalence of brood reduction between species, places and/or even laying dates may have driven different degrees of yolk androgen transfer between and/or within species. In rockhopper penguins, brood reduction is obligate [57] except in a few

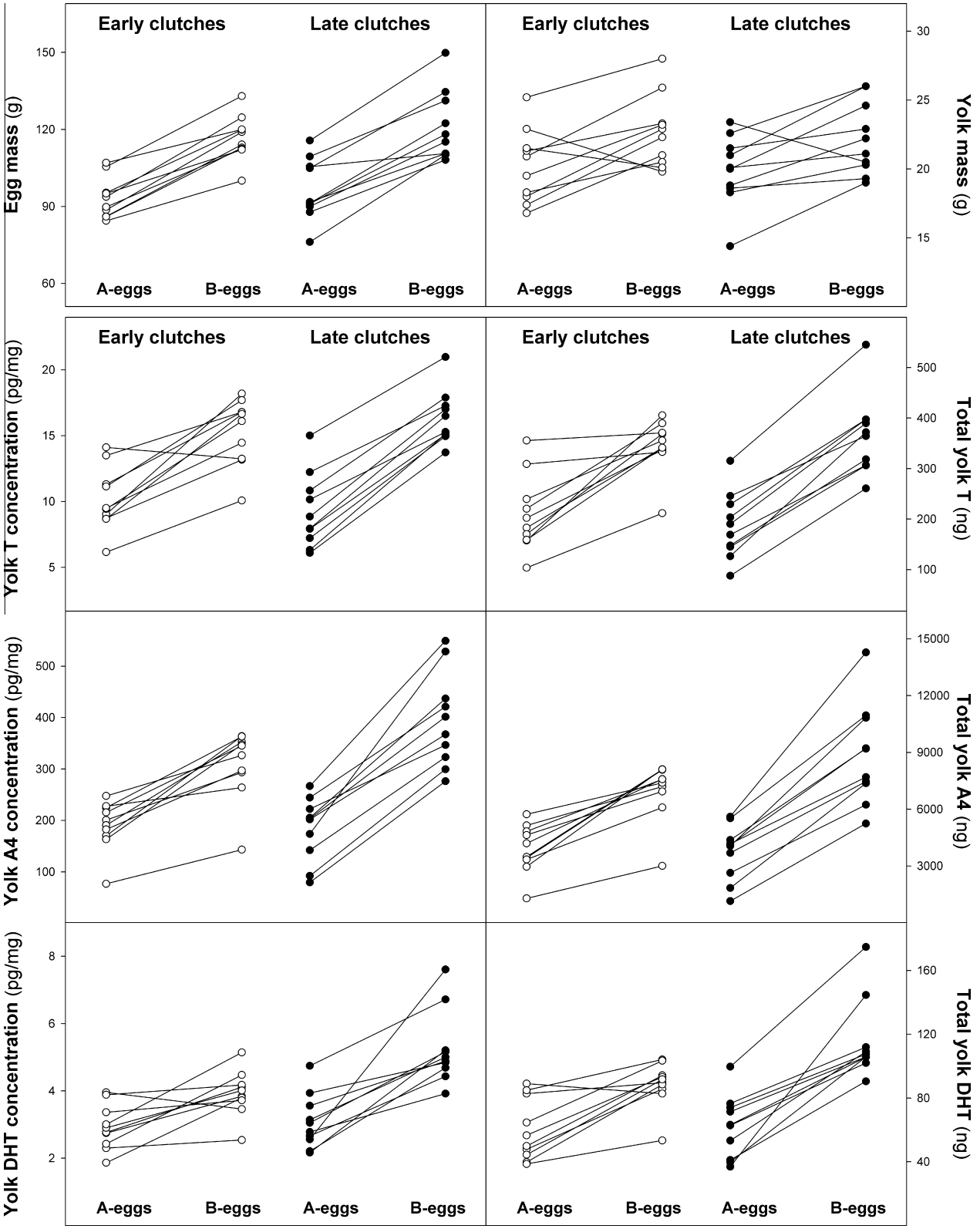

Fig. 1. Within-clutch variation in egg mass (in g), yolk mass (in g), yolk androgen (T, A4 and DHT) concentrations (in pg/mg) and total yolk androgens (in ng) according to laying period (left sides: early clutches; right sides: late clutches). $n = 20$ nests.

rare cases [34,3]. Therefore, eggs should have evolved to increase the effects of the hatching asynchrony in favour of the first egg to hatch (i.e. the second egg laid because of the reversed hatching asynchrony). This would be especially important for late breeders, enabling a quicker elimination of A-chicks in late clutches than in early clutches. Our results are in line with the idea that chick mass and androgens could positively influence begging, food competitiveness and growth in chicks. However, in a previous study [34] we found that the hatching and survival rates of rockhopper penguin chicks did not differ between early and late nests for both

egg categories, thus not supporting this hypothesis. However, environmental conditions were favourable, resulting in high breeding success in that study [34], while the adaptive value of quick brood reduction might be apparent only under more adverse environmental conditions.

In conclusion, we have shown here that rockhopper penguins are an interesting model species to explore further the adaptive significance of yolk androgens. As yolk hormone concentrations are correlated between siblings, it is possible to collect one egg for hormone measurements and follow the fate of the sibling. Future studies should explore the influence of environmental conditions on female condition, hormonal status and yolk androgen patterns.

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