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Consistent variation in yolk androgens in the Australian Brush-turkey, a species without sibling competition or parental care

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Abstract

Maternal hormones are an excellent pathway for the mother to influence offspring development, and birds provide exceptional opportunities to study these hormone-mediated maternal effects. Two dominant hypotheses about the function of yolk androgens in avian eggs concern maternal manipulation of sibling competition and post hatching paternal care. In megapodes, however, neither sibling competition nor post hatching parental care exists. Eggs are incubated by external heat sources, and chicks dig themselves out of their underground nest and live independently of their parents and their siblings. In this first study on egg androgens of such a megapode, the Australian Brush-turkey *Alectura lathami*, we found nevertheless substantial amounts of maternal androgens. Since size of the incubation mound, incubation temperature, egg size and laying date greatly vary in this species, we analysed variation in testosterone (T), androstenedione (A4) and dihydrotestosterone (DHT) in relation to these factors. T concentrations were significantly higher in eggs from bigger mounds and laid at greater depth, which may compensate via anabolic effects for the longer duration and higher energetic requirements of chicks when digging themselves out. T concentrations were higher in smaller eggs, and both yolk A4 and T concentrations increased with laying date, perhaps as a compensatory measure, while DHT concentrations only varied across different mounds. These results indicate that maternal androgens may influence offspring development outside the contexts of sibling competition or parental care.
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Keywords: Yolk androgens; Maternal effects; Sibling competition; Parental care; Birds; Megapodes

1. Introduction

In many vertebrate species, embryos are exposed not only to their own steroid hormones, but also to those of their mother. Although this exposure has been viewed as a potentially detrimental perturbation of developmental programs (for a recent review see Carere and Balthazart, 2007), most evidence collated over the last decade suggests that such hormone-mediated maternal effects are adaptive (e.g. Mousseau and Fox, 1998; De Fraipont et al., 2000; Gluckman et al., 2005; Groothuis et al., 2005). Birds are excellent models for the study of these maternal effects because they deposit a substantial amount of androgens in their eggs, and early exposure to these hormones can strongly affect offspring survival, behaviour, morphology, physiology, and even sex (reviewed in Groothuis et al., 2005).

A predominant hypothesis about the function of maternal androgens in avian eggs concerns its possible regulating role in sibling competition and parental care. Mothers may adjust begging behaviour and sibling competition within broods by varying the amount of androgens in their eggs. Indeed, yolk androgens can affect hatching time, begging behaviour and sibling competition (Schwabl 1993, 1996; Lipar et al., 1999; Eising et al., 2001; Eising and Groothuis, 2003; for a review see Groothuis et al., 2005). Megapodes

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are, in this context, an interesting group since their chicks live completely independently and do not directly compete with siblings for resources (Jones et al., 1995). They dig themselves out of their underground nest, a process that may take up to two days. After emergence, they live solitarily and disperse widely (Göth, 2002; Göth and Vogel, 2003). Hence, they provide an ideal system in which to study possible functions of maternal androgens for the offspring without the biases of parental care and sibling competition.

Here, we describe variation in yolk androgen concentrations in the Australian Brush-turkey (*Alectura lathami*). Brush-turkey males build and tend large incubation mounds (henceforth mounds) of organic matter in which incubation heat is produced by microbial decomposition. Females restrict their reproductive efforts on the mound to digging a tunnel and burying a single egg at a time. Therefore, mothers have only few means by which they can influence offspring condition. First, by initial resource allocation such as egg size and egg composition, including the amount of maternal hormones they deposit into the yolk, and second by the choice of the incubation site and the male for fertilization.

The first aim of this paper is to provide baseline data for yolk androgen concentrations, as this is the first such study for any megapode. Furthermore, we tentatively test four hypotheses aimed at explaining potential variation in Brush-turkey yolk hormone concentrations.

First, we hypothesize that yolk androgen concentrations will positively correlate with the size of the incubation mound. Brush-turkey chicks receive no assistance when digging themselves out of the mound, and the deeper the egg is laid, the more energy chicks have to invest in digging. It is well known that androgens can have anabolic effects, and studies on other bird species have shown that maternal testosterone in the egg positively affects the size of the neck muscle, involved in begging and hatching behaviour (Lipar, 2001), and decrease time until hatching (Eising et al., 2001). Yolk testosterone also affects motivational aspects of behaviour, such as persistence, boldness, alertness and activity concentrations (e.g. Eising et al., 2001; Daisley et al., 2005) that may assist the chick in digging itself out. Females could use three different parameters as indicators of how far the chicks will have to dig themselves out, that is the overall size (volume) of the mound, the height of the mound (given that chicks move upwards in a predominantly vertical manner), or the actual depth at which eggs are laid. Therefore, we hypothesize that Brush-turkey eggs contain substantial concentrations of androgens that positively correlate with these mound parameters.

A second hypothesis predicts that yolk androgen concentrations are related to developmental time (Gorman and Williams, 2005; Schwabl et al., 2007; but see Gil et al., 2007), which is in turn determined by incubation temperature (Jones et al., 1995). We test this hypothesis

by linking hormone concentrations to incubation temperature.

In addition, we test for two relationships found in other bird species. First, Brush-turkey eggs vary in size more than in most other bird species (Göth, 2007), and in several avian species smaller eggs contain relatively high concentrations of maternal androgens to compensate for their lower quality (e.g. Mousseau and Fox, 1998; Groothuis and Schwabl, 2002). Second, yolk concentrations of maternal androgens have been found to vary with laying date (e.g. Sockman et al., 2001; Müller et al., 2004; Verboven et al., 2003; Tobler et al., 2007) and Brush-turkeys have extended laying seasons (Jones et al., 1995).

2. Methods

2.1. Study species

The Australian Brush-turkey is common on the east coast of Australia, where it inhabits rainforests as well as drier scrubby areas or even suburban gardens. During the breeding season (from July to February or March), males build and tend the incubation mounds of fresh and decomposed leaf litter, which have an average incubation temperature of 34 °C (Jones et al., 1995). Females usually lay their eggs in the mounds of several males, and each mound receives eggs from several females; the species is thus polyandrous as well as polygynous (Jones, 1990; Birks, 1997). In captivity, females lay one egg every 2–5 days, and some lay more than 20 eggs per year (Baltin, 1969; Birks, 1996). Females have to mate with the male mound owner before being allowed to lay an egg into his mound, and males thus ensure that they sire the next egg to be laid (Birks, 1999). However, females may switch to a different mound when laying that egg a few days later, and males thus sometimes receive eggs that are not sired by them. DNA testing of 65 chicks found that up to 18 (28%) were not sired by the male tending the mound they hatched from (Birks, 1997).

2.2. Obtaining eggs, egg size and variables describing incubation conditions

Eggs were obtained by A.G. from natural incubation mounds in the Central Coast region north of Sydney (27°S, 153°E). Mound height and mound diameter were measured by tape measure prior to egg collection. Height was measured from the base of the mound to its highest point; mound diameter was measured at the base. For analysis, mound volume was calculated using the formula for cone volume ($\pi r^2 h/12$), where r is half the diameter at the base and h is mound height. Although not perfect, this formula represents actual mound volume best (A.G personal observation after viewing more than 100 mounds).

Mounds were then excavated by hand, by groups of 2–3 people to minimize the length of time for which each mound was open. Each time an egg was discovered, the depth at which it was found was measured relative to a previously established marker indicating the original height of the top of the mound. In addition, the temperature of the material surrounding the egg was measured immediately after discovering the egg by inserting a temperature probe into the soil at a distance of 1–2 cm to the egg. The temperature probe was attached to a digital thermometer (Dick Smith Electronics, calibrated to ±0.2° at 0 °C and 20 °C). The sensor was placed into an area next to the egg that had not yet been excavated, i.e. where the soil had not yet been touched. The untouched soil served as a 'buffer' between the place of measurement and the outside temperature. Both this buffer, and the fast excavation by groups of people, minimized the likelihood that the temperature next to the eggs was reduced significantly during the excavation process. Following excavation, the mound was restored to its original shape.

In total, 39 eggs were collected from five different mounds. Eggs were transported in warm material and then incubated artificially, at 34 °C (details in Göth, 2001) for 0–4 days before small yolk biopsies (on average 47.2 ± 4.1 mg) were taken at either day 5 of incubation (12 eggs), day 10 (25 eggs) or day 14 (two eggs). Stages of incubation were determined by A.G. via an egg candling method following Wong (1998). As we could not obtain fresh egg weight, we used egg volume as a measure of egg size. Length and width of all eggs were measured with sliding callipers (nearest 0.1 mm), and egg volume (cm^3) was then estimated using the well-established Hoyt (1979) equation: $V = 0.00051 \times$ length $\times$ breadth^2. Following biopsy, the eggs were further incubated until chicks hatched or the embryos ceased to develop. The animal care protocol involved keeping hatchlings in brooder boxes, transferring them to outdoor aviaries on their second day of life, and releasing them at the place of origin when 2–14 days old. Chicks were provided with food and water *ad libitum*.

2.3. Obtaining yolk samples

Yolk biopsies were conducted in a manner that allowed the egg to develop, using the following procedure: the egg surface was washed clean using cotton wool dabbed in a 70% ethanol solution. A sterile winged needle (25 G × 19 mm, Baxter Miniset Infusion Set AHC 1310, Baxter, Australia) was gently inserted through the egg shell near the blunt end of the egg and into the yolk. Yolk was sampled equally for all eggs as we used a standard length needle with a butterfly attachment inserted at the same location for each egg, at the same angle and the same depth. Approximately 50 mg of yolk was sucked into the 30 cm long silastic tubing fixed to the needle by very slowly lifting the syringe plunger. The hole in the eggshell was sealed with Opsite®. Yolk was transferred to Eppendorf tubes and weighed. Equal aliquots of water were added to the yolk sample and homogenized. Samples were kept frozen at −80 °C until shipped on dry ice from Sydney to Groningen. All procedures for egg collection and yolk sampling were approved by the Macquarie University Animal Ethics Committee (Protocol No. 2002/013) and the New South Wales Department of Environment and Conservation (Licence No. S10473).

2.4. Hormone analysis

We measured the concentrations of three different androgens of maternal origin: testosterone (T), 5-α-dihydrotestosterone (DHT) and androstenedione (A4). Hormone analyses were carried out by C.E. in the laboratory of T.G. at the University of Groningen, using a competitive-binding radioimmunoassay (RIA; see Wingfield and Farner, 1975; Schwabl, 1993). Homogenized samples were enriched with 2000 cpm of tritiated A4, DHT and T (NEN, The Netherlands) for calculation of recoveries. After overnight equilibration, samples were extracted twice using four ml diethyl/petroleum ether (70:30 vol), dried under nitrogen and reconstituted in 1 ml 90% ethanol. Samples were stored at –26 °C for 24 h and then spinned down to remove neutral lipids. Extracts were dried, reconstituted in 1 ml of 2% ethylacetate in 2,2,4-trimethylpentane and transferred to chromatography columns to elute each hormone fraction. Respective concentrations of 2%, 10% and 20% ethylacetate were used to wash out A4, DHT and T fractions. Androgen concentrations (pg steroid per mg yolk) were determined using a competitive-binding RIA with hormone specific antibodies (Endocrine Science, USA), in a single assay. The average recovery rate for A4 was 66.5%, 33.0% for DHT and 59.8% for T. The intra-assay coefficients of variation were 4.37%, 4.54% and 3.90%, respectively, for A4, DHT and T.

2.5. Statistics

Biopsies were taken from 39 eggs and analysed for hormone content. Hormone concentrations were normally distributed (Kolmogorov–Smirnov, all $P > 0.17$) after the exclusion of one outlier (sample no. 23 for all three hormones), which deviated 5.2 SD for both A4 and DHT and 4.8 SD for T. As the amount of yolk sampled from this egg was only 7.5 mg (overall average 48.33 mg) we felt this sample was unreliable and

hence excluded it from the analyses. In addition, two T samples were below the detection limits of the assay (10 pg/ml). Including these samples as zero values would mean introduction of two data points that deviated 2.04 and 3.32 standard deviations within their respective mounds. We therefore treated these two samples as outliers and excluded them from the dataset. However, re-running the models with these two zero data points included did not alter the outcome of the analyses in any way.

For analyses, eggs were nested within mounds, by incorporating mound number as a random term in all General Linear Models (GLM). Mound height ($r = 0.45$, $P = 0.024$) and mound volume ($r = -0.48$, $P = 0.019$) were significantly related to laying date and mound height correlated positively to burying depth. ($r = 0.42$, $P = 0.009$). Predictors that correlated significantly were not entered in the model simultaneously. The basic model contained egg volume, soil temperature, burying depth and laying date as co-variables. We used a backward elimination procedure by starting with the fully saturated model and eliminating all non-significant terms until model significance was obtained ($P < 0.05$). Only non-correlated parameters were entered into the model simultaneously. In subsequent models, we substituted burying depth with either mound height or mound volume (removing date because of intercollinearity).

Even though eggs were sampled at different stages of incubation (day 5–day 14, see above), incubation stage did not affect the distribution of any of the three hormones tested (all $P > 0.49$) and has therefore been excluded from the analyses.

Finally, as we studied three androgens for which we have similar predictions, we took a conservative approach by applying a Bonferroni correction to the results to account for multiple testing.

3. Results

Table 1 presents concentrations of all three hormones, averaged per mound, as well as mound characteristics. A4 concentrations were significantly and positively correlated with DHT concentrations ($r = 0.44$, $P = 0.006$, $N = 38$), and likewise, DHT concentrations with T concentrations ($r = 0.56$, $P < 0.001$, $N = 36$).

3.1. Testosterone concentrations

Yolk testosterone concentrations did not differ significantly between mounds ($F_{4,28} = 1.80$, $P = 0.157$) and mound number was therefore no longer included as a random term. The final model for yolk testosterone concentrations was highly significant ($F_{3,32} = 7.55$, $P = 0.001$) and included significant effects of burying depth ($F_{1,32} = 11.70$, $P = 0.002$), egg volume ($F_{1,32} = 9.71$, $P = 0.002$) and date ($F_{1,32} = 12.60$, $P = 0.001$; Fig. 1 A–C, respectively). Yolk testosterone concentrations increased with deeper burying depths and later dates in the season while they decreased with egg volume. There were no significant one-way interaction effects (all $P > 0.17$).

Testing the alternative model including mound height and egg volume only yielded a significant model ($F_{2,33} = 9.19$, $P = 0.001$) with a significant effect of mound height ($F_{1,33} = 15.14$, $P < 0.001$, Fig. 1D) while egg volume also remained significant ($P = 0.003$). Substitution of mound height with mound volume did not yield a significant model ($P = 0.31$). All of the statistical significant models remained significant after Bonferroni correction.

Table 1
Average levels of yolk hormones (A4, DHT, T; in pg/mg yolk ± standard errors) in the five different incubation mounds sampled, and average values for four parameters describing incubation conditions: (1) mound volume (m^3); (2) mound height (m); (3) incubation temperature (°C) and (4) depth at which the egg was buried (m)

Mound number	A4	DHT	T	Mound volume and height	Temperature	Burying depth
1	63.1 ± 13.0 (8)	13.1 ± 2.5 (8)	3.9 ± 0.8 (8)	8.4 1.10	33.9 ± 0.4	68.9 ± 4.8
2	54.3 ± 23.8 (3)	5.7 ± 2.3 (3)	5.3 ± 1.1 (2)	9.2 2.00	35.5 ± 0.9	56.7 ± 6.7
3	85.1 ± 11.2 (18)	7.6 ± 0.8 (18)	3.4 ± 0.4 (17)	4.2 1.40	33.9 ± 0.2	46.7 ± 1.6
4	94.7 ± 27.5 (4)	11.8 ± 1.3 (4)	5.4 ± 0.7 (4)	8.9 0.90	33.7 ± 1.3	47.5 ± 4.8
5	62.7 ± 12.1 (5)	9.0 ± 1.7 (5)	6.7 ± 1.4 (5)	5.3 4.20	34.4 ± 0.2	74.0 ± 2.5
Overall	76.1 ± 7.1 (38)	9.2 ± 0.8 (38)	4.3 ± 0.4 (36)	6.2 1.68	34.1 ± 0.2	56.2 ± 2.3

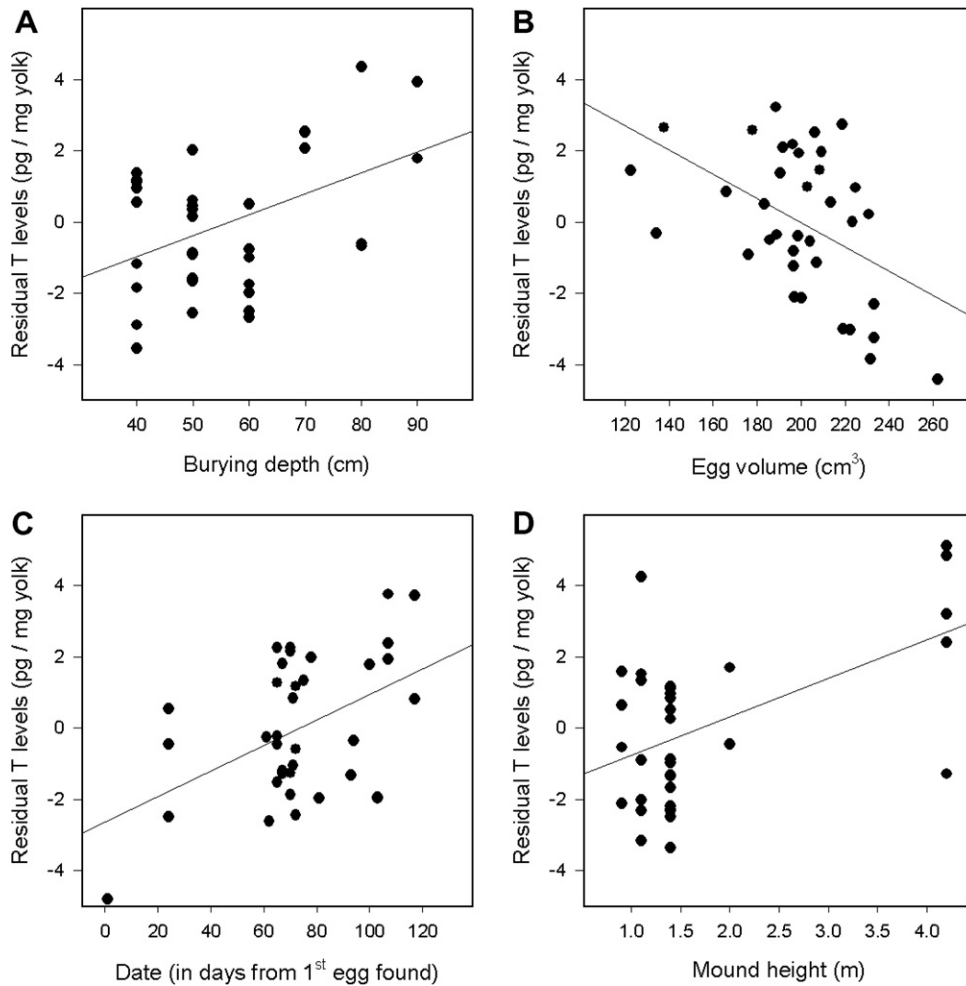Values in brackets represent the number of eggs sampled.

Fig. 1. (A) Residual yolk testosterone concentrations (corrected for egg volume and date) increase with burying depth in the mounds. (B) Residual yolk testosterone concentrations (corrected for burying depth and date) decrease with egg volume. (C) Residual yolk testosterone concentrations (corrected for burying depth and egg volume) increase with date. Date was measured in days from the date of first egg collection (29 September 2004). (D) Residual yolk testosterone concentrations (corrected for egg volume) increase with mound height. Residuals are un-standardized residuals calculated from the General Linear Models incorporating the two factors that needed controlling for. Lines represent regression lines.

3.2. DHT concentrations

Contrary to the effects found for T, DHT concentrations in the Brush-turkeys' eggs varied only significantly between mounds ($F_{4,33} = 2.76$, $P = 0.044$), although this effect did not remain after Bonferroni correction. Substitution of mound number with mound height as a co-variable yielded no significant result ($P = 0.45$) while there was a trend for a positive effect of mound volume ($F_{1,36} = 3.78$, $P = 0.06$). There was also a trend for a positive effect of burying depth ($F_{1,36} = 3.87$, $P = 0.057$), while egg volume ($P = 0.60$) and date ($P = 0.82$) had no effect what so ever.
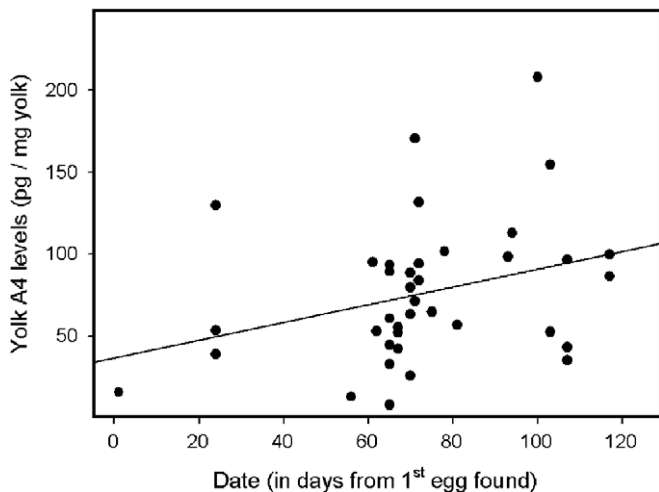
Fig. 2. Yolk androstenedione concentrations increase with day in the season. Date was measured in days from the date of first egg collection (29 September 2004).

3.3. A4 concentrations

Yolk A4 concentrations did not differ significantly between mounds ($P = 0.51$). None of the other parameters tested here significantly influenced yolk A4 concentrations either (all $P > 0.44$) except date (Fig. 2, $F_{1,36} = 4,11$, $P = 0.05$). Yolk A4 concentrations increased as the season progressed. However, after Bonferroni correction the result does not remain significant.

4. Discussion

This is the first study showing substantial amounts of androgens in the eggs of an avian species that lays only one egg per clutch and does not provide parental care after hatching. The results strongly suggest that yolk androgens from maternal origin can have important functions other than those previously suggested in the context of sibling competition and parental care. Our study species is extraordinarily in that it uses incubation mounds to incubate the eggs. We therefore investigated whether the yolk androgens in this species may function to facilitate the chick to dig itself out of the incubation mound. Since these mounds vary in incubation temperature, affecting developmental time that correlates with yolk androgen concentrations in a comparative approach (Gorman and Williams, 2005; Schwabl et al., 2007), we tested also whether yolk concentrations of androgens are related to variation in mound temperature within this species. Since the Brush-turkey shows large variation in egg volume and an extended laying season, we also analysed the correlation between yolk androgen concentrations and these parameters that have been shown to exist in other species (see introduction). We found evidence in support for three of these four predictions.

We demonstrated a positive correlation between the height of incubation mounds and the depth at which eggs are buried in them and concentrations of T in the yolk of the Brush-turkey eggs. Females may thus prepare their offspring for this "digging challenge" after hatching by increasing T concentrations in the egg laid in higher mounds, thereby positively affecting motor development and digging persistence (see Section 1). A faster speed of digging may enable the chick to benefit from yolk for a longer time period during the first days of life. This hypothesis requires further experimental testing by means of in ovo injections of testosterone, but is supported by experimental manipulation of testosterone concentrations in eggs of other avian species where testosterone positively affected both muscle development (Lipar and Ketterson, 2000; Lipar, 2001), and behaviour (hatching time: Eising et al., 2001; begging behaviour: Schwabl 1996; Eising and Groothuis 2003; von Engelhardt et al., 2006; general activity: Daisley et al., 2001, 2005; Eising et al., 2001). Maternal androgens are transferred to the yolk during the rapid yolking phase, several days before egg laying. Our data suggest therefore that females choose mounds before the day they burry their eggs, which may explain the variation in the relationship between mound characteristics and yolk T concentrations, since the male may have changed the mound during this interval. Potentially, mound height may be an indication of male quality. Other studies in birds have shown a positive relation between maternal yolk hormones and male attractiveness (e.g. Gil et al., 1999; Gil et al., 2004; Tanvez et al., 2004; von Engelhardt, 2004; but see Marshall et al., 2005; Michl et al., 2005; Navarra et al., 2006). It would also explain why DHT concentrations tended to vary among different mounds, owned by different males. However, up to 25% of the eggs are fertilized by males other than the owner of the mound (Birks, 1997), making this hypothesis less likely.

We found that bigger eggs contained lower concentrations of testosterone. This is in line with other studies (e.g. Groothuis and Schwabl, 2002; Verboven et al., 2003), and is in line with the hypothesis that females compensate lower egg quality with higher concentrations of T (Groothuis and Schwabl, 2002). Egg volume did not change notably over the season, but yolk concentrations of T and A4 increase with day in the season. It is conceivable that in this way females compensate for either lower egg quality in terms of its composition, or for reduced survival opportunities later in the season, but further data are required to substantiate this speculation. In contrast to our expectation we did not find a relationship between mound temperature and yolk concentrations of androgens. Perhaps temperature fluctuates substantially due to mound care of the male.

The possibility that our measurements were confounded by endogenous production of androgens by the chick itself is unlikely. In chickens endogenous production starts at low concentrations at day 6, approximately one third of total incubation time (21 days), and slowly increases in the week thereafter. Incubation time of the Brush-turkey is 48 days, and we took biopsies on average 8.6 days after

start of incubation, (less than one fifth of incubation time) with only two eggs later than day 10. The short period of incubation ensured that the yolk layers, that may contain different hormone concentrations, were thoroughly mixed, avoiding the potential flaw of affecting androgen measurements.

In conclusion, our results indicate that maternal androgens are present in substantial amounts in a species without sibling competition or parental care. This suggests that maternal hormones may have important functions outside these contexts as well. One of these is to enhance motor abilities of the chick, facilitating the Brush-turkey chicks to dig itself out of the mound.

Acknowledgments

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