Chapter 4

Within-clutch patterns of yolk testosterone vary with the onset of incubation in black-headed gulls

Wendt Müller, Corine M. Eising, Cor Dijkstra, Ton G.G. Groothuis

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Abstract

Hatching asynchrony in birds produces an age and size hierarchy among siblings. Later hatching chicks have a competitive disadvantage and brood reduction may occur when food availability is insufficient to raise all chicks. When early-hatched chicks fail to survive or if the circumstances allow raising all chicks, mothers should reverse the disadvantage to late-hatched chicks. Increasing deposition of maternal androgens with the laying sequence has been suggested to compensate for detrimental effects of hatching asynchrony, allowing a more precise adjustment of the survival probabilities of each chick.

Here, we show for black-headed gulls that the increase in yolk testosterone with each successive egg is greater when the mother incubates more before clutch completion, which is the major determinant of the degree of hatching asynchrony. This finding supports the idea that yolk testosterone has a compensatory function in the context of hatching asynchrony. Our data further show that if the time needed to complete a clutch is lengthened, the developmental differences due to incubation between the first- and the last-laid egg increase. In addition, the onset of incubation before clutch completion occurs sooner as the breeding season progresses.

Both long inter-egg intervals and the seasonal shift in incubation behavior enhance the necessity of compensation for later-hatching chicks. Indeed, yolk levels of testosterone increased more steeply over the laying order with a longer duration of the egg laying period and in later-laid clutches. We suggest that prolactin plays a key role in the adjustment of testosterone allocation to the incubation pattern.

Introduction

Hatching asynchrony, as reported for a variety of avian species, results in broods that show a hatching-order-dependent size hierarchy amongst siblings (Clark and Wilson 1981, Stockland and Amudsen 1988). Due to the competitive disadvantage of later-hatched chicks, they are less likely to survive (Mock et al. 1990, O'Connor 1978). Several hypotheses have been proposed to explain its adaptive value, of which Lack's brood reduction hypothesis was the first and most prominent (Lack 1947). This hypothesis proposes hatching asynchrony as a means of adjusting brood size when food availability during the nestling stage is insufficient to raise the whole brood. However, experimental tests have provided little support for this and several other hypotheses have been put forward to explain hatching asynchrony (Stoleson and Beisssinger 1995). In Herring Gulls *Larus argentatus* survival of the last-hatched chick has been shown to be extremely rare unless one of the older siblings fails to hatch or survive (Graves et al. 1984). The last-laid egg has therefore been suggested to be an insurance against hatching failure or early loss of the first- or second-laid egg or its chick (Forbes et al. 1997, Graves et al. 1984, Stoleson and Beisinger 1995). In case of no such failure, hatching asynchrony should facilitate the reduction to the optimal brood size.

The main mechanism how birds induce hatching asynchrony is the timing of the onset of incubation before clutch completion (e.g. Mead and Morton 1985). However, birds, especially open field breeders such as gulls having high predation rates of unattended eggs (e.g. Brouwer and Spaans 1994, Parsons 1972) and the risk of sun radiation that may lower the viability of their eggs (Webb 1987), might face a constraint in the onset of incubation. Thus, they might be forced to an early onset of incubation in order to avoid egg predation or a decline in egg viability (Bollinger et al. 1990, Dunlop 1910, Parsons 1976, Webb 1987). This may preclude a complete control of the hatching pattern, thereby calling for additional mechanisms that allow the shaping of the optimal brood size.

The transfer of maternal hormones into the eggs of avian species has been suggested to provide such a mechanism. Maternal hormones have been shown to vary systematically with the laying order within a clutch (e.g. French et al. 2001, Gil et al. 1999, Lipar et al. 1999, Royle et al. 2001, Groothuis and Schwabl 2002, Schwabl et al. 1997) and also affect embryonic development. In particular, increased yolk androgens enhance the development of the hatching muscle, which is needed for breaking the eggshell (Lipar and Ketterson, 2000, Lipar, 2001), and lead to earlier hatching (Eising et al. 2001). Therefore, enhanced allocation of yolk androgens to later-laid eggs might reduce asynchrony in hatching and hierarchies within nests. Furthermore, yolk androgens promote competitiveness of the chick in the nestling stage (Eising and Groothuis 2003, Schwabl 1993, 1996). Thus, yolk androgens may serve as a mechanism to

mitigate detrimental effects of hatching asynchrony for later-hatched chicks in a brood.

This leads to our following prediction: Mothers should adjust the hormone allocation to her incubation pattern in order to obtain a certain degree of hatching asynchrony. In other words, the steepness of the increase in androgen content with egg laying sequence should increase with an earlier onset of incubation since the latter leads to a larger degree of hatching asynchrony (e.g. Mead and Morton 1985).

We tested this prediction for the black-headed gull *Larus ridibundus*, a species with a fixed clutch size, which typically hatches asynchronously (Cramp and Simmons 1983). As an open field breeder black-headed gulls face the typical high egg predation of *Larus* gulls (e.g. Dunlop 1910). In addition a clear increase in yolk androgens within the laying order has been described previously (Eising et al. 2001, Groothuis and Schwabl 2002).

In addition we looked at two other factors that may influence hatching asynchrony and androgen allocation to the eggs. First, the degree of hatching asynchrony might not only depend on the onset of incubation, but also on the time intervals between laying of the subsequent eggs, which vary considerably between individuals of the same species (e.g. MacRoberts and MacRoberts 1972). Given the need for early onset of incubation, an increasing time span between the laying of the first and last egg potentially enhances the degree of hatching asynchrony. This makes a compensatory process such as elevated androgen levels in the last laid eggs particularly important.

Secondly, in several avian species the tendency to start incubation earlier after laying of the first egg increases in the course of the breeding season (e.g. Beukeboom et al. 1988, Meijer et al. 1990, Sharp et al. 1979). Both could be responsible for the enhanced hatching asynchrony later in the year in species with a constant clutch size (e.g. Courtney 1979, Hebert and McNeil 1999). Therefore, the androgen allocation to the last-laid egg should increase over the time of year to compensate for the increased disadvantage of the last-hatched chick in nests of late breeders.

Material and Methods

(a) Study species and egg collection

Black-headed gulls are monogamous, colonial breeders. The clutch typically consists of three eggs, which are laid over a three- to five-day period (Cramp and Simmons 1983). In 2000 and 2001, nests of several neighbouring black-headed gull colonies (300-1000 breeding pairs) along the northeast coast of the Netherlands were checked once a day for egg laying. In 2001 we also collected late clutches that were laid after the whole colony area was flooded at a time that the first chicks just hatched. These clutches therefore are most likely replacement clutches (laying date >165). Freshly laid eggs were marked with a non-toxic

marker referring to the position within the laying order and date of laying ([day of the year]). In order to obtain as accurate laying dates as possible nests were visited between 10.00-11.00 each day since laying normally takes place during the early morning. After clutch completion all eggs of the clutch were collected on the same day and the eggs were weighed to the nearest 0.1 g. Based on the laying date the laying interval was defined as the difference in laying date between last and first egg (laying interval = 2: all three eggs are laid on consecutive days).

(b) Estimation of hatching asynchrony

In order to be able to obtain both, estimation whether and how long the eggs had been incubated prior to clutch completion and the hormone data of the same clutch, we incubated all eggs artificially (37.5 °C with 60 % humidity) a standard time to obtain measurable embryo weights. The incubation procedure differed slightly between years. In 2000 all eggs of a clutch were incubated for 72 h and subsequently stored at –20 °C. However, we observed that sometimes the developmental stage of the eggs varied quite markedly within a clutch indicating that incubation had started well before clutch completion. To avoid effects of differences in incubation time on yolk levels of androgens, in 2001 we tried to compensate for these differences by incubating last-laid eggs for one day longer than first-laid eggs. By doing so we tried to equalize the developmental stage of the first-laid and the last-laid egg within a clutch. This also reduced a potential effect of incubation on hormone levels. In a later study, we found for our study species that hormone levels drop from day 0 to day 1 of incubation and subsequently remain stable till day 8 (Eising CM, unpublished data; see also Elf and Fivizanni 2002 for similar data on the domestic chicken). Endogenous production of androgens, another potential confounding factor, does not start before day five of development (e.g. Woods 1975) and there are no indications that endogenous androgens are transferred into the yolk (Elf and Fivizanni 2002). Since the mean incubation time in our study was 4.2 +/- 0.09 days and all our eggs were incubated for more than one day, differences in the hormone levels of subsequent eggs in a clutch cannot be explained by incubation time and/or endogenous production.

For the analysis, eggs were defrosted and the yolk and embryo separated. The embryos were weighed to an accuracy of 0.1 μg. Based on a dataset of embryo weights resulting from known incubation times of 0-13 days (N = 137, Eising unpublished data), we derived a formula to calculate incubation times from embryo weights using the curve estimation function in SPSS 11.0, 2002 (see Parsons 1972 for a similar approach). The following formula was obtained: incubation time = 3.3652+ (4.8382 * embryoweight) + (-0.6073* (embryoweight)^2, (df=134, r^2=0.91). This formula was subsequently used to estimate the incubation time of our samples on the basis of embryo weight. We decided not to use

published formulas (e.g. Ricklefs 1987) since these estimations were done for embryos of much older age while there were large differences between species. Since embryos do not have any androgen receptors before they are at least one week old (see e.g. Godsave et al. 2002), embryonic development at that stage can not be influenced by maternal yolk androgens. Embryonic development therefore is very likely to depend mainly on maternal heat transfer.
We were interested in the duration of incubation prior to clutch completion, since this determines the degree of hatching asynchrony. Therefore we subtracted from the estimated incubation duration the time that the eggs had been incubated artificially. The difference in natural incubation time between the last-laid egg and the first-laid egg was taken as estimate for how long maternal incubation had taken place before the last egg was laid. This estimated onset of incubation before clutch completion (subsequently OIC) was used for the statistical analysis.

(c) Hormone analysis

The yolks were homogenized with 1 ml water per gram of yolk. About 150 mg of the yolk/water emulsion was used for hormone analysis, keeping all eggs of a clutch in the same assay. Each assay contained clutches of the two different laying date categories. We followed a standard procedure according to Schwabl (1993), with a slight modification. Briefly, samples were extracted twice with 4 ml petroleum ether/diethylether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently, the hormones were separated on diatomaceous earth chromatography columns. Androgen concentrations were measured in double competitive-binding radioimmunoassays (RIA) with tritiated hormone (NEN, the Netherlands) and hormone-specific antibodies (Endocrine Science, USA). The average recovery was 49.2 % for testosterone and 58.6 % for androstenedione. The inter-assay coefficients of variation were 15.3 % for testosterone and 19.2 % for androstenedione; for testosterone, intra-assay variation was 12.6 %, for androstenedione 18.6 %.
As a measure of the steepness of hormone change over the laying order of a clutch we used $(H_{\text{last-laid egg}} - H_{\text{first-laid egg}})/H_{\text{first-laid egg}}$, where H_x = yolk hormone titer of egg x. This corrected for between-clutch variation in hormone levels since we were interested in within-clutch variation. For all statistical analyses we used this obtained relative value for the increase of testosterone with laying order.

(d) Statistical analyses

All variables were checked for normality using the Kolmogorov-Smirnov test. Subsequently, variables were tested using parametric statistics (Pearson Correlation) or univariate linear regression. All tests were carried out using SPSS 11.0, 2002.

Results

Sixty-four clutches (22 in 2000, 42 in 2001) were included in the analysis. The onset of incubation (OIC) varied from 0.24 to 5.05 days before laying of the last egg of the clutch. Hormone concentrations of testosterone and androstenedione were within the range of a previous study (Eising et al. 2001, for details see table 1), except for last-laid eggs of late clutches, but clutches of this laying date category were not included in the previous study. Due to extraction failure of either the first or the last laid egg for androstenedione only 56 out of the 64 clutches could be analyzed. Both years showed the same pattern and did not differ significantly in relative increase in yolk hormones or estimated onset of incubation before clutch completion (t-test, $p>0.167$ in all cases). We therefore combined both years in all subsequent analyses.

	2000 (N=11)	2001 (early) (N=31)	2001 (late) (N=11)
Testost. A [pg/mg]	7.11 +/- 1.05	15.90 +/- 1.38	14.46 +/- 3.40
Testost. C [pg/mg]	10.53 +/- 1.23	19.78 +/- 1.47	32.78 +/- 5.30
A_4 A [pg/mg]	338.59 +/- 54.82	557.63 +/- 77.25	434.60 +/- 93.5
A_4 C [pg/mg]	896.90 +/- 187.15	968.78 +/- 149.25	654.65 +/- 133.77
Embryo size A [g]	0.39 +/- 0.10	0.16 +/- 0.02	0.36 +/- 0.07
Embryo size C [g]	0.08 +/- 0.04	0.05 +/- 0.01	0.13 +/- 0.04

Table 1: Embryo size [g], testosterone (Testost.) and androstenedione (A_4) concentrations [pg/mg] for first-laid (A-egg) and last-laid (C-egg) eggs (Mean+/-std. error) subdivided according to year and laying date category (early < 140 [day of the year], 2000: N= 22, 2001: N=31, except for A_4: N= 23; late > 163 [day of the year], 2001: N=11).

First, we tested our prediction that the increase of yolk androgens should be steeper with a larger OIC. In line with our hypothesis, there was a significant positive correlation between OIC and the relative increase in yolk testosterone (Pearson correlation: 0.525, $p < 0.001$, Figure 1). This was not the case for the relative androstenedione increase in relation to the OIC (Pearson correlation: 0.052, $p = 0.68$).

Furthermore, we hypothesized that variation in OIC should depend on the time span between the laying of the first and the last egg, and on laying date. In a linear regression, we tested the effect of laying interval and laying date on OIC. Laying interval and laying date were not correlated ($t = 22.737$, $df = 63$, $p = 0.795$) and could therefore be used in the same model.

There was a positive correlation between duration of the laying interval and OIC [laying interval of two days (mean): 1.47 days of incubation (N=3); three days: 1.31 (N=19); four days: 1.55 (N=28); five days: 2.03 (N=12); t = 2.511, p = 0.015]. Furthermore, OIC increased with a progressing breeding season (t = 3.353, p = 0.001; Figure 2).

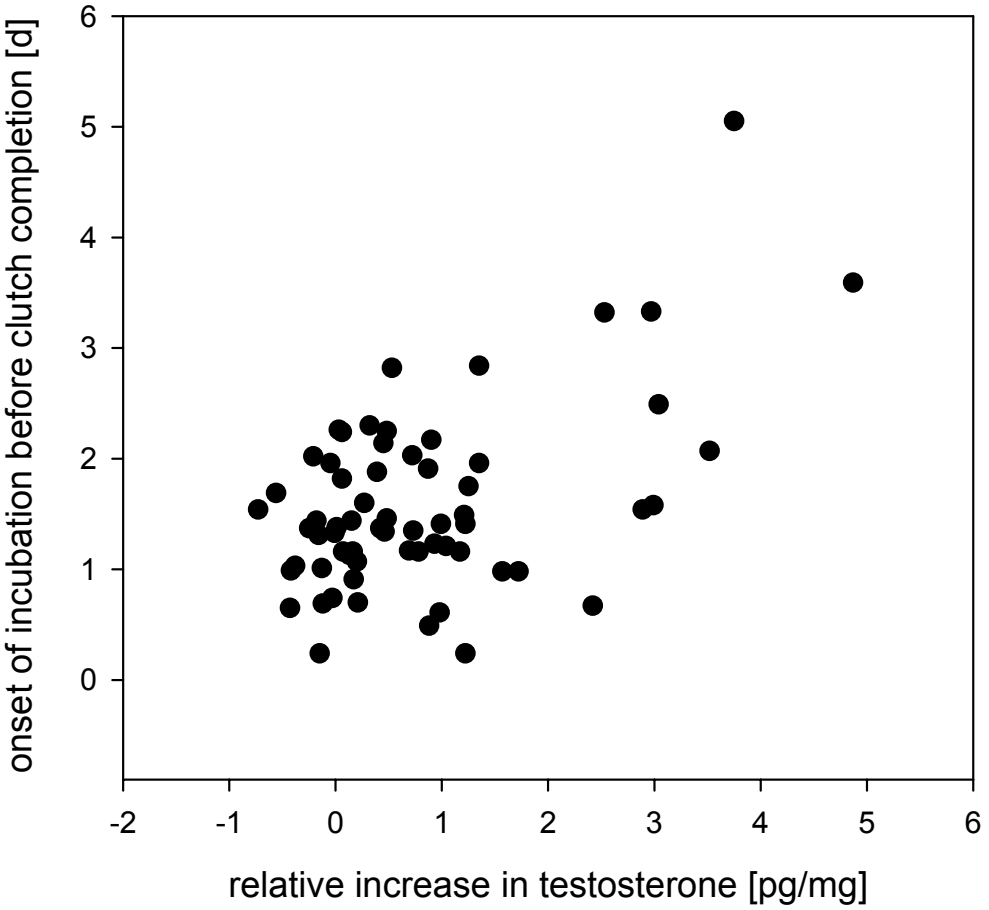

Figure 1: Relative increase in testosterone concentration [pg/mg] between the last-laid and the first-laid egg, against the duration that incubation has already taken place before clutch completion in days [d]

To investigate the individual contributions of laying date, laying interval and OIC itself on the steepness of the increase of yolk testosterone we performed the following linear regression in which the residuals of incubation onset (Res-OIC) on laying date and laying interval as well as the latter two variables were included as predictors for the relative increase in testosterone.
With a longer laying period, the relative increase of testosterone over the laying order was enhanced [laying interval of two days: 0.59 (pg/mg) testosterone increase (N=3); three days: 0.51(pg/mg) (N=19); four days: 0.70 (pg/mg) (28); five

days: 1.36 (pg/mg) (N=12); t = 2.197, p = 0.03]. The relative increase of testosterone was also positively correlated with the laying date (t = 2.774, p = 0.007; Figure 2). Res-OIC values were still positively correlated with the relative increase in testosterone (t = 3.761, p < 0.001).
In a third linear regression, we analyzed the relative increase of androstenedione in relation to laying interval, laying date and the residuals of incubation onset (Res-OIC) over laying date and duration of the laying period. There was no relationship (linear regression, laying date: t = -0.901, df = 53, p = 0.251; duration of the laying period: t = -0.035, df = 53, p = 0.972; Res-OIC: t = 0.465, df = 53, p = 0.465)

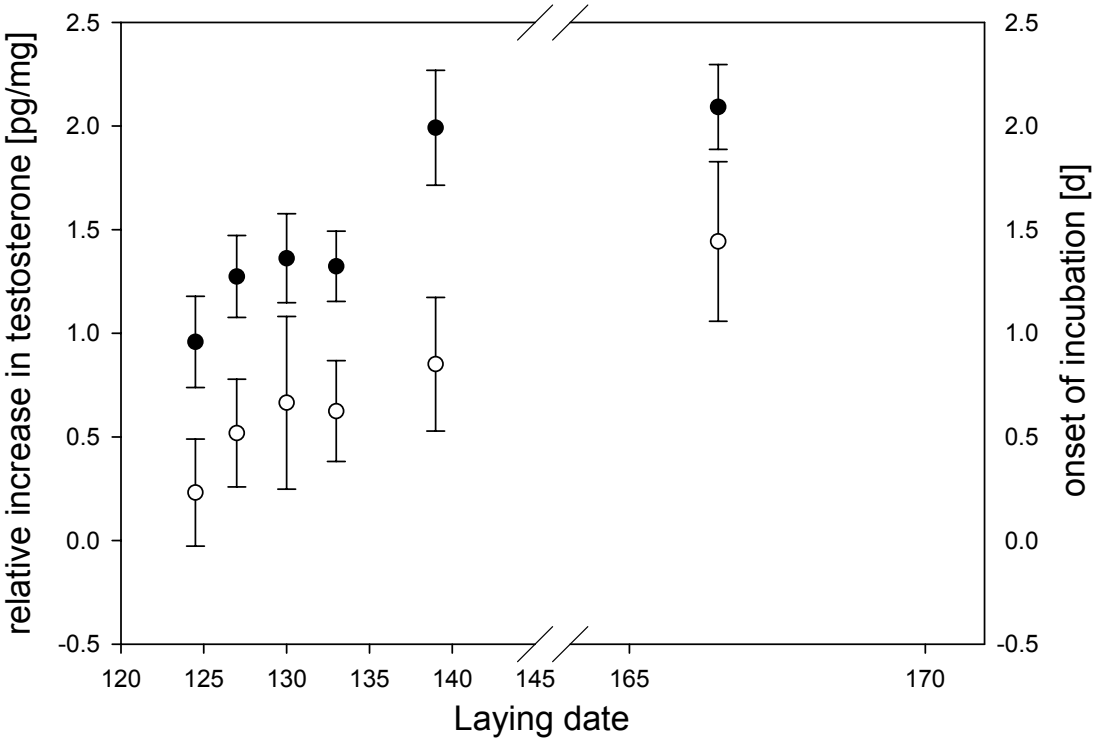

Figure 2: Correlation between laying date ([day of the year], subdivided in categories: category 1:124-126, N=7; category 2: 126-128, N=11; category 3: 129-131, N=12; category 4: 132-134, N=7; category 5: 135-142, N=14; category 6 (after flooding): 164-170, N=11) and (a) onset of incubation before clutch completion (open symbols, mean +/- s.e.) (b) relative increase in testosterone (filled symbols, mean +/- s.e.)

Discussion

Based on the hypothesis that the increase of yolk androgens with laying sequence has a compensatory function in the context of hatching asynchrony we investigated whether yolk hormone allocation is adjusted to the extent of hatching asynchrony of the clutch. As predicted, the increase in yolk testosterone over the laying sequence was larger when the difference in embryonic development between first- and last-laid egg, which determines the degree of asynchrony at hatching, was enhanced. Furthermore, the differences in embryonic development and therewith the degree of hatching asynchrony depended on the inter-egg interval and the time of the year. In line with our hypothesis, also the allocation of testosterone was enhanced if the laying interval was lengthened and late in the year.

Testosterone accelerates embryonic development, reducing the time difference in hatching between chicks of the same brood (Eising et al. 2001, Lipar and Ketterson 2000, Lipar 2001). In addition, it enhances the competitive skills that are particularly important for the youngest chick if the age difference in a brood is large (Eising and Groothuis 2003, Schwabl 1993; 1996). Therefore, our findings support the idea that increasing testosterone concentrations with laying order, as reported previously for the black-headed gull (Eising et al. 2001, Groothuis and Schwabl 2002), play a compensatory role in the context of hatching asynchrony.

A possible causal explanation for the differential allocation of testosterone may be found in its relationship with prolactin levels. Increasing concentrations of plasma prolactin after the onset of laying enhanced the expression of incubation behavior in American kestrels (Sockman et al. 2000). Enhanced plasma prolactin also increased the deposition of yolk testosterone but not of androstenedione (Sockman et al. 2001). Although the detailed mechanism still has to be discovered, Sockman et al. (2001) suggested that prolactin could influence the activity of aromatase (inhibition), 3-hydroxysteroid dehydrogenase (activation) and 17- hydrosteroid dehydrogenase (activation). This could possibly lead to accumulation of testosterone but evoke only little changes in androstenedione concentrations. This very likely explains why for wrens a relationship between yolk androgens and hatching asynchrony could not be found (Ellis et al. 2001). Since an overall measure of androgens was used, rather than testosterone specifically, a potential correlation between testosterone and the degree of hatching asynchrony might be masked by much higher concentrations of androstenedione that did not show a relationship with OIC (e.g. Groothuis and Schwabl 2002, Schwabl et al. 1997, but see also Schwabl 1993).

Our results on the relationship between the onset of incubation and androgen allocation in black-headed gulls are consistent with the results of Sockman et al. (2001). As in the kestrel, increased prolactin levels might induce both an early onset of breeding and enhanced testosterone deposition in last-laid eggs.

Sockman et al. (2001) also showed a seasonal rise in plasma prolactin that correlated with a seasonal increase in yolk testosterone in the last eggs of a clutch. Also in our study, testosterone increment over the laying sequence was larger with a progressing breeding season. Finally, there is some evidence that a seasonal increase in plasma prolactin reduces the time between onset of egg laying and start of incubation during the laying period (Meijer et al. 1990). Indeed, we found that later in the breeding season incubation started significantly earlier compared to the earlier laying dates. Thus, prolactin may play a key role in the adjustment of incubation pattern (and thereby degree of hatching asynchrony) and the deposition of yolk testosterone (compensating effects of hatching asynchrony) to each other. The mechanism by which prolactin could elevate yolk testosterone concentrations is unclear, since prolactin is supposed to be antigonadotropic (e.g. Buntin et al. 1999, Goldsmith 1983).

Regardless of the precise mechanism, our results support the hypothesis that increasing levels of testosterone over the laying sequence are adjusted to the degree of hatching asynchrony. This supports the idea that yolk testosterone has a compensatory function in the context of hatching asynchrony.

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