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Published in:
General and Comparative Endocrinology

DOI:
[10.1016/j.ygcen.2012.01.003](https://doi.org/10.1016/j.ygcen.2012.01.003)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Vergauwen, J., Goerlich, V. C., Groothuis, T. G. G., Eens, M., & Muller, W. (2012). Food conditions affect yolk testosterone deposition but not incubation attendance. *General and Comparative Endocrinology*, 176(1), 112-119. <https://doi.org/10.1016/j.ygcen.2012.01.003>

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Food conditions affect yolk testosterone deposition but not incubation attendance

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ARTICLE INFO

Article history:

Received 29 July 2011

Revised 3 January 2012

Accepted 4 January 2012

Available online 13 January 2012

Keywords:

Yolk hormones

Incubation behaviour

Maternal investment

Environmental effect

Nutritional stress

Hatching asynchrony

ABSTRACT

In many bird species with hatching asynchrony, yolk androgens increase across the laying sequence. This has been hypothesized to represent a compensatory mechanism for disadvantages of later-hatching chicks – via positive effects of yolk androgens on early competitiveness and growth. However, the costs and benefits of this compensatory strategy probably depend on environmental factors determining the survival chances of the chicks such as the food conditions, which should, therefore, influence maternal yolk androgen deposition.

We studied the consequences of manipulated food conditions on the expected level of hatching asynchrony in canaries (*Serinus canaria*) assigning females to either a low (=LQ) or high quality (=HQ) diet. We measured the incubation behaviour (as incubation attendance) and the yolk androgen deposition in order to investigate whether and how females modulate hatching asynchrony in relation to the food conditions.

Females on a HQ diet laid larger and heavier clutches, showed a stronger increase in yolk testosterone content towards the last-laid eggs, but did not alter their incubation attendance. Thus, females on a HQ diet seem to favour the survival of later hatching chicks, as indicated by their yolk testosterone deposition pattern. However, females on a HQ diet laid larger clutches and might need to compensate more in order to achieve a similar degree of hatching asynchrony than females on a LQ diet, given the lack of plasticity in incubation attendance. This suggests that canary females respond to food manipulations mainly via changes in clutch size rather than by altering the degree of hatching asynchrony.

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1. Introduction

The phenotype of an individual is shaped by the interaction between genes inherited from the parents and the environment experienced during development. Part of the latter is shaped by the mother, and the respective environmental effects on offspring phenotype fall into the category ‘maternal effects’ [33]. In oviparous species the egg comprises the environment in which the embryo will develop and forms an important pathway for maternal effects, since its composition is determined by the mother. Bird eggs have been shown to contain many substances of maternal origin such as antibodies, carotenoids, and steroid hormones (e.g. [16,36,42,47]), all of which can modulate the chick’s development (e.g. [31,48, 51]). Among these components, yolk hormones, particularly yolk androgens, have received much attention (see recent reviews: [17,22,23,37,56]). In several species the yolk androgens levels systematically increase or decrease across the laying sequence [17,22,56]. This systematic within clutch variation has been interpreted as a maternal tool to modulate the competitive abilities

of the chicks, since yolk hormones influence traits that play important roles in sibling competition such as growth and begging [17,22,56]. This hypothesis is supported by experimental studies showing that an increase of yolk androgens across the laying sequence may act as a compensatory mechanism for hatching asynchrony [12,32].

At first sight it seems counterintuitive to postulate a compensatory mechanism for hatching asynchrony, since mothers may have introduced hatching asynchrony to adaptively adjust brood size when the food availability during the nestling stage is insufficient to raise the entire brood [27,32]. Parents influence the hatching pattern of their clutch by varying the onset of incubation, which is believed to be the most important mechanism determining the degree of sibling hierarchy in a brood (reviewed in [10,52]). By varying the start of incubation relative to clutch completion, parents decrease or increase the amount of hatching asynchrony between their siblings, altering the probability of the last chick’s survival.

However, parents may be constrained in varying their onset of incubation, e.g. due to high predation risks or solar radiation/heat damage both of which require a high level of nest attendance to protect the eggs (e.g. [2,4,7,39]). A limitation in food resources may on the other hand force the parents to spend more time foraging thereby causing a delayed onset of incubation (‘Energetic

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constraint hypothesis', [49]). If parents are constrained in their onset of incubation, they may benefit from a compensatory mechanism to adjust the degree of hatching asynchrony, such as differential yolk androgen deposition [22]. Thus, parents constrained to an early onset of incubation may – under favourable conditions – lay clutches with a steep increase in the yolk androgens over the laying sequence, in order to improve the survival probabilities of last-hatching chicks. A steeper increase in yolk androgen levels under good food conditions has been observed in one [44], but not all studies [43]. However, neither of the studies investigated the incubation behaviour.

A compensatory mechanism for hatching asynchrony may also be adaptive if there is uncertainty about whether the food conditions during egg laying predict the food availability post-hatching [22]. Females breeding in an unpredictable environment may start incubating earlier, introducing a high degree of hatching asynchrony, thus assuring the survival of their first hatched chicks at the expense of the later hatched chicks when food availability proves to be low (brood reduction strategy, [25]). Under these conditions, allocating relatively high levels of androgens to last laid eggs of a clutch may promote the survival of the latest hatched chicks through positive effects on growth and competitiveness, when food conditions prove to be sufficient to rear the whole brood [12,34]. However, under harsh conditions high yolk androgen levels may reduce the survival of the last hatched chicks because of their associated costs, such as increased oxidative stress or enhanced growth rates which may cause a higher food demand [40]. The costs and benefits of high levels of yolk androgens consequently depend on the environmental circumstances and may, therefore, provide a flexible tool for the mother to adjust brood size to food conditions.

Finally, mothers may adjust the incubation pattern itself to the food conditions instead of the pattern of yolk hormone deposition, since age differences among siblings are thought to be a central factor determining the degree of brood reduction (reviewed in [52]).

Here, we investigated the effects of manipulated food conditions on yolk androgen deposition and incubation attendance (i.e. the time a female spends incubating) in canaries (*Serinus canaria*), a species known for the increase of yolk androgens over the laying sequence [18,47,53]. We hypothesize that females kept on low quality food start to incubate earlier (before clutch completion) to increase the degree of hatching asynchrony and thus the chances for quick brood reduction if food conditions remain low. These females on a low quality diet may at the same time deposit high levels of androgens in their last laid eggs to fine tune their brood size to the environmental conditions at hatching. But if females do not or are unable to alter their incubation pattern, we expect that the increase in yolk androgens across the laying sequence is more pronounced in females breeding on high quality food conditions to enable them to rear the whole brood. Thus, we expect that the pattern of yolk androgen deposition in relation to the food conditions will depend on the plasticity in incubation behaviour.

2. Materials and methods

2.1. Study species, housing and food manipulation

Domesticated canaries typically show a high level of hatching asynchrony, whereby eggs laid later in the laying sequence hatch 1–2 days after the first laid eggs (Vergauwen and Müller, unpublished). This has significant effects on growth and survival, even under ad libitum conditions as provided in captivity (Vergauwen and Müller, unpublished). Moreover, only females incubate which makes distinguishing between male and female incubation

attendance unnecessary. Canaries are, therefore, a highly suited species for the aim of our study. In January 2009, we selected 44 female and 44 male Fife fancy canaries from the local breeding population at the University of Antwerp and subsequently housed them indoors on a 14:10 (L:D) light regime. Canary seed mixture, cuttlefish bone and water were provided ad libitum. After 1 week, female birds were weighed and housed separately in cages of 60 * 40 * 50 cm (length * depth * height) at room temperature (between 16 and 22 °C). They were randomly assigned to the low or high quality diet group and were provided with the same diet for the entire duration of the experiment (9 weeks). The low quality group (=LQ, $N = 22$) received 20 g of standard canary seed mixture each week (=average individual seed consumption in this population, pers. obs.), while the high quality group (=HQ, $N = 22$) received the same standard canary seed mixture ad libitum (allowing selective seed feeding). In addition to this, the HQ group received 10 g of egg food (high protein content, supplemented with vitamin E) and a piece of apple, carrot, or germinated seeds every other day. The weight of the females was monitored weekly. Meanwhile, male birds were housed in one large indoor aviary (200 * 200 * 200 cm (length * depth * height)) in a separate room on a 14:10 (L:D) light regime at normal room temperature (between 16 and 22 °C). Males were provided with standard canary seed mixture, cuttlefish bone and water ad libitum, while egg food was provided twice weekly. This experiment was conducted under proper legislation of the Flemish and Belgian law and with approval by the animal experimentation committee of the University of Antwerp (number 2008–26). At the end of the experiment, birds were moved to our large, single-sexed outside aviaries.

2.2. Pair formation and recording of incubation attendance

Females were provided with a randomly selected unrelated male partner ("pair formation") after 5 weeks of food manipulation. The duration of food manipulation is based on previous studies on captive passerines manipulating the food conditions between 3 and 6 weeks prior to pair formation (e.g. [43,44]). The breeding pair received the same diet as the female was assigned to before, however, adjusted for the number of birds in the cage (=40 g seeds in case of the low quality group). The pairs were provided with nest boxes and nesting materials and nests were checked daily for egg laying. After being replaced by a dummy egg, freshly laid eggs were individually marked, weighed (to the nearest 0.0001 g) and immediately stored at –18 °C until hormone analysis.

When the first egg was detected in the nest, a water/soil temperature sensor (TMC6-HD, Onset®, USA), was fitted into the dummy egg, which replaced the first laid egg. The sensor was connected to a data logger (HOBO U12, Onset®, USA), which was set to record the temperature every 15 s. Among nests, the probes differed in the extent of direct contact between temperature probe and brood patch of the female, due to their fixed position in the nest. Among nest variation in incubation temperature was, therefore, not used for analysis. Nevertheless, the nest temperature on the first day after the probe was inserted was 28.55 ± 3.22 °C ($N = 10$), while mean ambient temperature was 17.77 ± 0.01 °C, indicating embryonic development could take place [9]. However, the probes gave, based on the temperature fluctuations in the nest, accurate measures of the onset and duration of incubation (for similar methods see also [24,57]). Temperature was measured per 24 h periods, as the data showed us that even during night females alternated presence and absence on the nest. An incubation bout started when the increase in nest temperature was larger than the increase in ambient temperature (which was constantly measured) and stopped when temperature decreased for longer than two consecutive temperature measurements. The incubation

behaviour of each female (=incubation attendance) was estimated as sum of all 15s bouts with incubation behaviour. Incubation attendance was measured and analysed until clutch completion.

2.3. Hormone analysis

2.3.1. Yolk testosterone and androstenedione extraction

Yolk testosterone was measured following the protocol as described in Goerlich et al. [19]. The yolk was weighed to the nearest 0.1 mg, diluted in an equal volume (1:1) of demineralised water and thoroughly homogenised. Approximately 100 mg of this yolk mixture were weighed for the extraction. Prior to the extraction ca. 5000 cpm radioactive labelled testosterone was added to each sample in order to account for losses due to the extraction protocol. The extraction was performed using 2.5 mL of diethyl ether/petroleum benzene, 70:30 (vol/vol). After vortexing (30 s) and centrifugation (3 min, 2000 rpm, 4 °C), tubes were snap frozen, the ether layer was decanted and subsequently dried under a nitrogen stream. This step was repeated once. Next, 1 mL 70% methanol was added to the dried tubes, which were then vortexed until the complete dried pellet was dissolved. This solution was stored at –20 °C over night. Next day the samples were centrifuged (5 min, 2000 rpm, 4 °C), decanted and dried again under a nitrogen stream. The pellet was resuspended in 400 µL of phosphate buffered saline solution with 1% gelatine (PBSG). Recoveries of the initially added labelled testosterone were measured in a subsample of this solution. Average recoveries were $72.4 \pm 0.5\%$ (range 60.6–86.8%).

2.3.2. Radioimmunoassay (RIA)

Yolk testosterone and androstenedione were measured using commercial RIA kits (*Active*[®] Testosterone Coated-Tube RIA DSL-4000 kit, Diagnostic Systems Laboratories, Beckman Coulter Nederland B.V., Woerden, The Netherlands; detection limit: 0.08 ng/mL, antibody cross-reactivity: 100% testosterone, 5.8% 5 α -dihydrotestosterone, and 2.3% androstenedione; *Active*[®] Androstenedione Coated-Tube RIA DSL-3800 kit, Diagnostic Systems Laboratories; detection limit: 0.03 ng/mL, antibody cross-reactivity: 100% androstenedione, 0.33% Androsterone, and 0.08% 5 α -dihydrotestosterone). The assay kits were validated by ensuring parallelism of serial dilutions of yolk samples with the standard curve. Samples were measured in two RIAs per hormone, with all eggs from one nest within one RIA and treatment groups distributed equally. RIA concentrations were corrected for initial yolk mass and calculated as pg/mg yolk. Standard curves reached from 20 ng hormone/mL to 0.156 ng hormone/mL in both assays and were measured in duplicate, as well as assay controls. Based on these we calculated the intra- and interassay coefficients of variation. Average inter-assay CVs were 11.67% (testosterone) and 8.07% (androstenedione), average intra-assay CV's were 2.23% for the first RIA and 5.83% for the second RIA (testosterone), and 2.72% for the first RIA and 2.06% for the second RIA (androstenedione).

2.4. Statistical analyses

Egg mass, yolk mass, female body mass, incubation attendance and hormone data were analysed in MLwiN 2.18 via random slope models [46]. Factors were removed from the models in a backward elimination procedure by removing least significant highest interactions first with a significance level of $\alpha \leq 0.05$. Significance was calculated based on the increase in deviance (Δ deviance, which follows a χ^2 distribution, with corresponding change in degrees of freedom) when a variable was removed from a model, while the amount of data was kept constant. When interactions were significant, the data were split for one of the interacting parameters and separate models were run on the split file.

Female body mass changes during the pre-laying period were analysed in a two-level model in MLwiN 2.18, using (i) female identity and (ii) time point of the measurement as hierarchical levels (=random effects). We tested the effect of treatment (HQ = 1, LQ = 2), the time point of the measurement (week), and their interaction. Egg mass, yolk mass and yolk hormones were analysed in a two-level model in MLwiN 2.18, with (i) female identity and (ii) egg position as hierarchical levels. We tested the main effect of treatment, laying order and the interaction of laying order and treatment as predictors. Instead of the absolute laying position we calculated the relative laying position in order to obtain a better representation of the changes across the laying sequence and to avoid confounding clutch size effects (each first egg relative laying position 0 and each last egg relative laying position 1; all other laying positions between the first and the last were equally distributed between 0 and 1). In addition we centred the relative laying order to obtain meaningful treatment main effects at an average laying position in the presence of a significant interaction [45].

Incubation attendance was analysed in a two-level model, with (i) female identity and (ii) day (after clutch initiation) as hierarchical levels. We included the effect of treatment, days after clutch initiation, their interaction as well as clutch size and its interaction with the number of days after clutch initiation.

All other analyses were done in SPSS 16.0. When required, we applied non-parametric statistics or transformed the data in order to be normally distributed. The increase in yolk hormones within each clutch was calculated as an index of dimorphism using the formula $\log_{10}(\text{yolk hormones first egg/yolk hormones last egg})$ [21]. Data are shown as mean/estimate $\pm$ SE, unless indicated otherwise. One HQ female laid eggs prior to the start of the experiment and laid only two eggs at the beginning of the experiment. This was not considered to represent a full clutch. Another 6 females did not lay any eggs (2 LQ females, 4 HQ females). These females were excluded from the statistical analyses.

3. Results

3.1. Female body mass

Body mass at the start of the experiment did not differ between HQ and LQ females (HQ: 20.85 ± 0.57 g; LQ: 20.87 ± 0.53 g; independent samples *t*-test: $t = -0.04$, $p = 0.97$, $N = 44$). The interaction between treatment and time had a significant effect on the body mass of the females (treatment $\times$ time: estimate = -0.20 ± 0.09 [g], $df = 1$, Δ deviance = 4.56, $p = 0.03$), since body mass decreased over time in the LQ group (time: estimate = -0.22 ± 0.06 [g], $df = 1$, Δ deviance = 15.12, $p < 0.001$), but not in the HQ group (time: estimate = -0.02 ± 0.07 [g], $df = 1$, Δ deviance = 0.10 $p = 0.76$; after 5 weeks (=pair formation): HQ: 21.05 ± 0.69 g; LQ: 19.61 ± 0.42 g).

3.2. Egg laying, egg mass and yolk mass

HQ females initiated egg laying sooner after pair formation than LQ females (Mann Whitney *U*: $Z = -2.94$, $p < 0.01$; median LQ = 6 days, 25% = 5.25 days, 75% = 7.75 days, $N = 20$; median HQ = 5 days, 25% = 4.75 days, 75% = 5.25 days, $N = 17$). HQ females laid significantly larger clutches than LQ females (Mann Whitney *U*: $Z = -4.33$, $p < 0.001$) (Fig. 1a).

There was a significant interaction effect between treatment and relative laying order on egg mass [g] (treatment $\times$ relative laying order: estimate = -0.22 ± 0.09 , $df = 1$, Δ deviance = 5.60, $p = 0.02$) (Fig. 1b). The treatment main effect was significant as well (treatment: estimate = -0.15 ± 0.05 , $df = 1$, Δ deviance = 8.72, $p < 0.01$). The effect of relative laying order on egg mass was not

Fig. 1. Females on a high quality diet (HQ, $N = 17$) laid significantly larger clutches (a). Their egg mass did not significantly change with laying order, while the egg mass of females that received a low quality diet (LQ, $N = 20$) tended to decrease with laying order (b). The dashed vertical line symbols the mean laying order at which the treatment main effect was estimated. 4 HQ females and 2 LQ females did not lay, while 1 HQ female did not complete her clutch. These data were excluded. ** $p < 0.01$.

significant in HQ-females (relative laying order: estimate = 0.02 ± 0.04 , $df = 1$, Δ deviance = 0.21, $p = 0.65$), but significant in LQ-females (relative laying order: estimate = -0.21 ± 0.08 , $df = 1$, Δ deviance = 6.76, $p = 0.01$) (Fig. 1b).

Yolk mass [g] did not significantly differ between treatments in interaction with relative laying order (treatment $\times$ relative laying order: estimate = 0.01 ± 0.02 , $df = 1$, Δ deviance = 0.25, $p = 0.62$) or between treatments (treatment: estimate = -0.003 ± 0.012 , $df = 1$, Δ deviance = 0.06, $p = 0.80$). Yolk mass did not vary with relative laying order (relative laying order: estimate = 0.01 ± 0.01 , $df = 1$, Δ deviance = 0.41, $p = 0.52$).

3.3. Incubation attendance

There was no effect of the food manipulation on the daily incubation attendance (treatment: estimate = -5537.44 ± 5540.86 [s], $df = 1$, Δ deviance = 0.96, $p = 0.33$). The increase in incubation attendance with time also did not differ between HQ- and LQ-females (treatment $\times$ days: estimate = 539.35 ± 2384.69 , $df = 1$, Δ deviance = 0.05, $p = 0.83$) (Fig. 2). The daily incubation attendance increased more strongly across females laying smaller clutches (clutch size $\times$ days: estimate = -2007.83 ± 882.99 , $df = 1$, Δ deviance = 4.54, $p = 0.03$).

3.4. Yolk hormones

3.4.1. Testosterone (T)

There was a trend for yolk T concentrations [pg T/mg yolk] to increase more strongly across the relative laying order in clutches laid by HQ females compared to clutches laid by LQ females (treatment $\times$ relative laying order: estimate = -23.71 ± 13.11 , $df = 1$, Δ deviance = 3.09, $p = 0.08$). In both groups the increase over the relative laying order was significant (HQ: relative laying order: estimate = 55.51 ± 7.13 , $df = 1$, Δ deviance = 60.59, $p < 0.001$; LQ: relative laying order: estimate = 31.53 ± 11.28 , $df = 1$, Δ deviance = 7.82, $p < 0.01$) (Fig. 3a), while treatment had no significant effect on the yolk testosterone concentration (treatment: estimate = -10.93 ± 7.93 , $df = 1$, Δ deviance = 1.70, $p = 0.19$).

The absolute yolk T content [ng T/yolk] increased more steeply with the relative laying order in clutches laid by HQ females than in clutches laid by LQ females (treatment $\times$ relative laying order: estimate = -6.89 ± 3.35 , $df = 1$, Δ deviance = 3.95, $p = 0.046$). The

absolute yolk T content of the yolk increased with relative laying order in both groups (HQ: relative laying order: estimate = 16.46 ± 1.98 , $df = 1$, Δ deviance = 69.06, $p < 0.001$; LQ: relative laying order: estimate = 9.48 ± 2.73 , $df = 1$, Δ deviance = 12.05, $p < 0.001$). The absolute yolk T levels tended to be higher in eggs from HQ than from LQ females (treatment: estimate = -4.41 ± 2.29 , $df = 1$, Δ deviance = 3.53, $p = 0.06$).

We repeated the analysis without the data for the highest laying position for LQ (laying position 5, $N = 1$) and for HQ (laying position 6, $N = 2$) given the small sample sizes. However, this made only minor differences to the outcome (yolk T concentrations: treatment $\times$ relative laying order: estimate = -26.68 ± 13.23 , $df = 1$, Δ deviance = 3.79, $p = 0.05$, absolute yolk T content: treatment $\times$ relative laying order: estimate = -7.69 ± 3.29 , $df = 1$, Δ deviance = 4.91, $p = 0.03$).

3.4.2. Androstenedione (A_4)

There was no difference in yolk androstenedione concentrations [pg A_4 /mg yolk] between HQ and LQ females in interaction with relative laying order (treatment $\times$ relative laying order: estimate = -7.42 ± 5.39 [pg A_4 /mg yolk], $df = 1$, Δ deviance = 1.83, $p = 0.18$) (Fig. 3b), neither was there an overall difference between treatments (treatment: estimate = -1.89 ± 2.61 , Δ deviance = 0.49, $p = 0.48$) (Fig. 3b). The yolk A_4 concentrations significantly increased with relative laying order (relative laying order: estimate = 6.65 ± 2.77 [pg A_4 /mg yolk], $df = 1$, Δ deviance = 5.77, $p = 0.02$).

There was neither a significant difference in absolute yolk A_4 content [ng A_4 /yolk] between HQ and LQ females in interaction with laying order (treatment $\times$ laying order: estimate = -2.11 ± 2.02 , $df = 1$, Δ deviance = 1.07, $p = 0.30$), nor an overall treatment effect (treatment: estimate = -0.93 ± 0.85 , $df = 1$, Δ deviance = 1.13, $p = 0.29$). Absolute A_4 content significantly increased with relative laying order (relative laying order: estimate = 2.16 ± 1.02 , $df = 1$, Δ deviance = 4.48, $p = 0.03$).

The total time a female spent incubating before the last egg was laid, which determines the leeway in developmental time of the last chick to hatch, was correlated with the dimorphism index of the yolk T concentrations, a measure for increase in yolk T concentrations towards the last egg (Pearson's $R = -0.44$, $p = 0.02$). This was the same in the case of the dimorphism index of the yolk A_4 concentrations (Pearson's $R = -0.37$, $p = 0.05$). Similarly, the

Fig. 2. Females on a high quality diet (HQ, $N = 17$) did not differ in their incubation attendance (i.e. the time a bird spends incubating) from females that received a low quality diet (LQ, $N = 20$). Incubation attendance was estimated until clutch completion.

dimorphism index of the absolute yolk T content correlated with the time a female spent incubating before the last egg was laid (Pearson's $R = -0.46$, $p = 0.01$) while such a tendency was observed for the dimorphism index of the absolute yolk A_4 content (Pearson's $R = -0.31$, $p = 0.09$). Given the gradual increase in incubation attendance (Fig. 2) it is not possible to use the onset of incubation as a measure for the degree of introduced hatching asynchrony.

4. Discussion

We investigated whether canary females altered their incubation attendance (i.e. the time spend incubating) or yolk hormone deposition according to the food conditions in order to match the degree of hatching asynchrony to the survival probabilities of the chicks. The food conditions pre-laying had a significant effect on clutch initiation time, clutch size and egg mass, indicating that the experimental manipulation was successful. Females did not differ in their incubation attendance when breeding on a high quality (HQ) or low quality (LQ) diet, but the amount of yolk T in HQ clutches increased more strongly across the laying sequence than in LQ clutches. These topics will be discussed subsequently.

4.1. Egg laying

Females breeding on a HQ diet had a shorter laying latency, and made a larger investment in their clutch. The effects were substantial and similar to previous studies in both wild and captive populations (reviewed in [6]). Interestingly, yolk mass did not differ between the two treatment groups, which indicates that the larger egg mass was probably due to an increase in albumen content (see also [54]). The fact that the clutch size was smaller in the LQ group shows that LQ females adjusted their brood size via a reduction in clutch size – in addition to potential pathways for further brood reduction post-hatching (see below). Furthermore, the decrease in egg mass across the laying sequence in the LQ group (Fig. 1) indicates unequal distribution of resources. As a consequence, chicks of later laid eggs will not only be younger but also smaller at hatching, reinforcing their inferior position in sibling competition.

4.2. Incubation attendance

Females may, by introducing hatching asynchrony, postpone the decision of brood reduction until post-hatching, which may form a more flexible strategy compared to a reduction in clutch size at laying [1,27]. We hypothesized that LQ females would advance the onset of incubation to increase the hatching asynchrony between their chicks and thus the degree of brood reduction, as these females may not be able to raise the whole brood if the environmental conditions do not change until hatching. However, we did not find a significant difference in incubation attendance between HQ and LQ females. Most previous studies manipulating food conditions during incubation in passerines found an increase in incubation attendance under good food conditions (see e.g. [3,5,26] for a summary), indicating that females are energetically constrained [49]. But most of these studies were performed after clutch completion, when variation in incubation attendance has no further impact on hatching asynchrony. Yet, food supplementation during laying had a similarly positive effect on incubation attendance, degree of hatching asynchrony or both [11,38], which is actually contrary to our expectation that LQ females increase the degree of hatching asynchrony of their chicks to facilitate brood reduction.

However, our nest temperature measurements provide accurate information on nest attentiveness [15,30]. But this gives no reliable information on the exact temperature and the effectiveness of incubation, while it may affect the actual hatching pattern [24]. Yet, females typically raised their nest temperature above the physiological zero, at which embryo development starts [9]. Since we collected all eggs for hormone analysis we unfortunately could not link actual hatching patterns to the incubation attentiveness. Additional studies are needed to elucidate whether and why canary females may be constrained in varying incubation attendance during laying.

Interestingly, zebra finches in captivity appear to have more asynchronous broods than wild zebra finches [29,41,58]. It has been hypothesized that this relates to a release of energetic or other types of constraints for incubation during laying in captivity (see above) or that it may be the result of artificial selection for

Fig. 3. (a) The change in yolk testosterone content across the laying sequence differed significantly between clutches laid by HQ females (black bars) versus clutches laid by LQ females (white bars) (b) while this was not different in the case of the yolk androstenedione content. The dashed vertical line symbols the mean laying order at which the treatment main effect was estimated and the numbers above the symbols indicate sample sizes.

broodiness. In our study, a release of energetic constraints appears unlikely given the lack of an effect of the food manipulation on incubation attendance.

4.3. Hormone allocation

We hypothesized that HQ females – if limited in their ability to alter incubation attendance – should allocate higher levels of yolk androgens to later laid eggs to compensate for disadvantages of hatching asynchrony. Indeed, canary females did not vary their incubation attendance, but the increase in yolk content of T but not A₄ across the laying sequence was steeper in clutches of HQ females. Changes in yolk T deposition across the laying sequence in relation to food conditions have previously been found in zebra finches showing a decreased deposition of yolk T in last laid eggs under poor food conditions, potentially favouring the survival of the first hatched chicks [44]. This is in line with the outcome of our study. However, this pattern was not found in

a second study [43]. But these eggs had been incubated, which in turn may have affected their yolk hormone levels [13,14]. In a food manipulation experiment in lesser-black-backed gulls, Verboven et al. [54] did not find a change in the increase of yolk testosterone across the laying sequence either, but for A₄ this bordered statistical significance. This apparent inconsistency in the changes of yolk androgen deposition in relation to food conditions across species may relate to the different function of yolk androgens as compensatory mechanism for hatching asynchrony. For example, the last-laid egg in gulls has been suggested to represent a replacement egg for the frequent loss of earlier laid eggs [20], a hypothesis that may not apply to zebra finches [44] and canaries [this study].

Our results on yolk androgen deposition provide only very weak evidence that females on a low quality or low quantity diet deposited overall more yolk androgens in their eggs contrasting earlier studies [8,16,54]. Nevertheless, the functional consequences of the observed among clutch variation remain as yet unclear.

Unfortunately, these studies analysed only a single egg [8,16,54], which does not allow any conclusions as to whether the deposition patterns differ across the laying sequence to compensate for hatching asynchrony.

Furthermore, we did not find an effect of our food manipulation on yolk A_4 deposition, which may indicate that testosterone plays a more prominent role as compensatory mechanism for hatching asynchrony in canaries [34]. This is supported by the fact that yolk A_4 occurs in relatively low levels in canary eggs compared to other species such as gulls (e.g. [18,47,54], this study), while it is known that yolk testosterone modulates early growth in this species [34,35,48,55]. The latter has been shown to particularly benefit chicks that were competing with heavier and older siblings, as occurs in the context of hatching asynchrony [34].

Females may – in addition to the costs and benefits that females obtain through the effects of the maternal hormones on their offspring – face additional costs related to the process of yolk hormone deposition. This may for example be the case if the deposition of yolk hormones is not completely uncoupled from the females' plasma levels [23], yet this study does not allow conclusions on the (nature of) direct costs of yolk hormone deposition to the female. However, the weak effect of our food manipulation on the amount of yolk testosterone deposited (see also [8,16,54]) suggests that there may be some kind of energetic cost limiting the deposition of yolk androgens [44,54].

5. Conclusions

Canary females breeding on a high quality diet did not decrease egg mass throughout the laying sequence and allocated higher amounts of testosterone to last-laid eggs. Both allocation patterns particularly benefit the last-hatching chicks. However, including the incubation attendance in the interpretation, which was not possible in previous studies, sheds a slightly different light on the observed pattern. HQ females laid more eggs, but did not differ in their incubation attendance from LQ females – potentially increasing hatching asynchrony [28]. Thus, last-hatching chicks in clutches of HQ females may require more yolk T dependent compensation. The steeper increase in yolk testosterone content with laying order in clutches laid by HQ females may, therefore, not necessarily represent enhanced compensation, but may lead to a hatching pattern similar to the one observed under LQ conditions. Future studies should investigate the respective contributions of clutch size, extended incubation attendance and elevated yolk testosterone levels on the actual hatching pattern and chick survival (see [24]). The degree to which these traits are entwined and eventually regulated by the same hormonal mechanism determines whether and how females may alter these traits independently [50]. Both, the onset of incubation and the deposition of yolk testosterone are potentially regulated by a common mechanism (prolactin), while this may not be true for the regulation of clutch size [50].

Acknowledgments

We thank Geert Eens and Peter Scheys for taking care of the birds, Bonnie de Vries for her support during hormone analyses, Josie Meaney-Ward, Hector Rivera Gutierrez, Natalia Estramil, Alain Van Hout, and both reviewers for commenting on this manuscript. This research was funded by a Ph.D. grant to JV by the Institute for the Promotion of Innovation through Science and Technology (IWT) in Flanders and WM by a postdoctoral grant of the FWO Flanders (1.5.033.07). ME is funded by FWO Flanders and the University of Antwerp.

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