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Experimental manipulation of food availability leads to short-term intra-clutch adjustment in egg mass but not in yolk androgen or thyroid hormones

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In birds, mothers can affect their offspring's phenotype and thereby survival via egg composition. It is not well known to what extent and time-scales environmental variation in resource availability, either via resource constraints or adaptive adjustment to predicted rearing conditions, influences maternal effects. We experimentally studied whether egg and yolk mass and yolk hormone levels respond to short-term changes in food availability during laying in wild great tits *Parus major*. Our treatment groups were: 1) food supplementation (mealworms) from the 1st until the last egg; 2) food supplementation from the 1st until the 5th egg, where the effect of cessation of the supplementary food treatment could also be studied; 3) no food supplementation (controls). We analysed both nutritional resources (egg, yolk and albumen mass), and the important developmental signals, yolk androgens (testosterone and androstenedione), and for the first time in a wild population, yolk thyroid hormones (thyroxine and 3,5,3'-triiodothyronine). Egg mass is a costly resource for females, androgens most likely non-costly signals, whereas thyroid hormones may be costly signals, requiring environmental iodine. In the food supplemented group egg, yolk and albumen mass increased rapidly relative to controls and when food supplementation was halted, egg and albumen mass decreased, indicating rapid responses to resource availability. Yolk androgen and thyroid hormone levels were not affected by food supplementation during laying. Thyroxine showed an increase over the laying sequence and its biological meaning needs further study. The rapid changes in egg mass to variation in within-clutch food availability suggest energetic/protein/nutrient constraints on egg formation. The lack of a response in yolk hormones suggest that perhaps in this species the short-term changes in resource availability during egg laying do not predict offspring rearing conditions, or (for thyroid hormones) do not cause systemic changes in circulating hormones, and hence do not affect maternal signaling.

Maternal effects are a crucial mechanism in a wide array of taxa to create phenotypic plasticity, potentially adapting offspring to the prevalent or expected environment, and thereby enhancing offspring and maternal fitness (Mousseau and Fox 1998). Oviparous species are good study species for experimentally testing prenatal maternal effects as their eggs develop outside the mother's body, facilitating the measurement and manipulation of maternal resources.

Effects of egg size on offspring development have been extensively studied in birds: a recent meta-analysis reveals that egg size positively correlates with offspring quality from hatching to post-fledging (Krist 2011). Nevertheless, there is large variation in egg size among females (ca 70% of the variation) and even within clutches (Bernardo 1996, Christians 2002, Williams 2012, Ruffino et al. 2014). Resource limitation via trade-offs between self and eggs, especially on protein, has been suggested as one of the explanations for this variation in egg size (constraint hypothesis, Lack 1966, Christians 2002). Nevertheless, only 36% of

food supplemental studies showed an increase in egg mass (reviewed by Meijer and Drent 1999, Christians 2002, Williams 2012). However, most of these studies used feeding long before egg-laying, thus affecting laying dates and maternal condition or other maternal traits (Christians 2002), which can potentially confound the effects on resource allocation to eggs: this could be the case when e.g. altered laying dates are accompanied with altered allocation to eggs. Importantly, many groups like passerines gather most resources for eggs during egg laying (i.e. income breeders, eggs take ca 40% of daily resources, Perrins 1996, Meijer and Drent 1999). Thus studies on fast changes in food availability during laying should reveal more direct causality of the short-term energetic/resource constraints: for example a rapid increase in egg size to short-term increase in food availability during egg laying would support the constraint hypothesis. However, food supplementary studies only during laying on egg mass are few and results are mixed: either no effect (Nilsson and Svensson 1993) or positive effects

(Williams 1996, Saino et al. 2010, see Discussion) of protein supplement have been found.

In addition to nutrients and lipids, eggs contain several hormones from maternal origin which can have a potentially strong influence on offspring fitness. Yolk androgens (such as testosterone, T and androstenedione, A4) can affect both early (Schwabl 1996) and later phenotype and fitness (Strasser and Schwabl 2004, Ruuskanen et al. 2012, Tschirren et al. 2014, reviewed by Groothuis et al. 2005, von Engelhardt and Groothuis 2011) and vary substantially among clutches due to social and environmental factors (Gil et al. 1999, Tschirren et al. 2004, reviewed by von Engelhardt and Groothuis 2011). In contrast to egg size, energetic costs of androgen production are likely to be negligible as its precursor comes from the highly abundant cholesterol (Groothuis and Schwabl 2008). However systemic costs and constraints via varying concentration in female circulation need to be considered, although there is inconsistent data for this hypothesis and evidence for independent regulation has been put forward (steroids being locally produced in the ovaries, Groothuis and Schwabl 2008, Okuliarova et al. 2011, see also Tschirren et al. 2014). Importantly, the effect of yolk androgens on offspring may be dependent on environmental conditions (Groothuis et al. 2005, Müller et al. 2010, Tschirren et al. 2005): females are expected to adjust deposition of maternal hormones in their eggs depending on prevailing environmental conditions or their own condition when these conditions are predictive for the conditions during offspring development, in order to adjust offspring phenotype to these conditions (predictive adaptive response, Lack 1954). Indeed, food supplementation before and during laying can affect yolk androgen levels but again the results are mixed, with some studies finding lower yolk androgen levels and/or an effect on the within clutch pattern (Verboven et al. 2003, Gasparini et al. 2007, Sandell et al. 2007, Vergauwen et al. 2012), and others no effect (Verboven et al. 2010, Benowitz-Fredericks et al. 2013). Lower yolk androgen levels under good rearing conditions have been interpreted as to enable the chick to better bear the potential costs of androgens such as higher immunosuppression and oxidative damage whereas the stimulating effect of the hormone on begging behavior would be less important under such conditions (Verboven et al. 2003, Groothuis et al. 2005). However, the effect of food availability per se as a cue, independent of female condition, on within-clutch variation in androgen concentrations has not been studied. Also it is not known how flexibly and fast the hormonal changes within the clutch may occur in response to environmental variation, although within-clutch variation in yolk androgens strongly affects sibling competition (Tschirren et al. 2005, Müller et al. 2010, Boncoraglio et al. 2011). One study indicated fast changes in androgen concentrations within clutches to social cues (Kingma et al. 2009).

So far the literature on maternal hormones in bird eggs has focused on steroid hormones. However, from poultry it is known that bird eggs contain the thyroid hormones thyroxine (T4) and 3,5,3'-triiodothyronine (T3, the biologically active form) (McNabb 2007). Thyroid hormones are indispensable for normal development and metabolism in vertebrates (Norris and Carr 2013), including birds (McNabb 2007). In mammals and fish maternal thyroid hormones are

crucial for normal growth, development and even survival (reviewed by Power et al. 2001, Patel et al. 2011). The avian embryo possesses thyroid hormone receptors before starting its own thyroid hormone production (Geysens et al. 2012, Van Herck et al. 2012, 2013). Furthermore, experimental elevation of thyroid hormones increased growth and hatching speed in quail and chicken (Wilson and McNabb 1997) and body mass in pigeons and great tits *Parus major* (Hsu et al. pers. comm., Ruuskanen et al. unpubl.) emphasizing the potentially important role of yolk thyroid hormones.

Thyroid hormones differ in several aspects from androgens in ways that may affect how the environment affects their deposition in the egg. First, thyroid hormones contain iodine, which needs to be taken up from the environment (iodine deficiency potentially altering thyroid function; McNabb et al. 1985, McNichols and McNabb 1987) and if in limited supply, a potential trade-off between allocating the hormone to the egg vs self exists (McNabb 2007). Second, in contrast to androgens, yolk thyroid hormones are derived directly from maternal circulation, and thus correlate with circulating plasma thyroid levels (McNabb and Wilson 1997), and therefore, environmental effects on maternal thyroid hormone production, e.g. to food or temperature, may more directly affect yolk thyroid hormone concentrations (epiphenomenon hypothesis, Groothuis and Schwabl 2008). Indeed, plasma thyroid hormone levels in birds are affected by food availability due to their role in metabolism (Klandorf and Harvey 1985, Darras et al. 1995, Reyns et al. 2002), but to what extent this is translated to short-term variation in yolk thyroid hormone levels among and within clutches in a wild population remains to be studied.

We studied the extent and flexibility in variation in maternal deposition of nutrients (yolk and albumen) and endocrine signals (androgens and thyroid hormones) in eggs over the laying sequence of a clutch in relation to short-term changes in food availability during laying in great tits *Parus major*. This species was chosen as it is an income breeder so that daily fluctuations in food availability are expected to affect the egg quickly. It also produces large clutches, enabling us to analyse changes in within-clutch patterns of egg quality. Our treatment groups were: 1) food supplementation (mealworms) from the 1st until the last egg; 2) food supplementation from the 1st first until 5th egg, where the effect of removal of the supplementary food was also studied; 3) no food supplementation as a control.

Our main questions were: is egg quality affected by adding a food supplement, how is egg quality affected by removal of the food supplement, and how fast can these changes occur? We predict that 1) if females are resource limited during egg laying and thus are faced with a trade-off in allocating resources to self vs offspring, egg mass and yolk mass should rapidly increase and decrease in relation to supplementary food and its cessation; 2) if females reduce the costs of androgens on offspring in good rearing conditions (via increased female condition or cues for good conditions), increased food supply could be expected to decrease yolk androgen concentrations; 3) if yolk thyroid hormone concentrations reflect plasma values, and plasma T3 levels decrease and T4 levels increase rapidly to food availability due their function in energy homeostasis, similar fast changes of these hormones are expected in the yolk.

Methods

The experiment was conducted in a nest box population in Bennekom in the Netherlands (51°59'53N, 5°40'35E). The forest was mostly deciduous, including European oaks, American oaks and beeches and had 125 nest-boxes. In early March, small feeding trays were inserted inside all boxes, attached to the back wall of the box. Nest-building was monitored from the end of March twice a week. As soon as the nest box bottom was covered with material, nests were checked every day for eggs. On the day the first egg was laid, the nest was assigned either to control, or one of the two feeding regimes (see below). In controls, an empty feeding tray was in the nest box, and the box was visited daily. In the first feeding regime (hereafter '5 days food'), 7 g of fresh mealworms was placed inside the nest box in the tray every day starting from the day when the 1st egg was laid until the day the 5th egg was laid. The amount of mealworms (g) remaining was weighed, removed, and 7 g of fresh mealworms given. For the rest of the clutch, no additional food was provided anymore, but nests were visited daily (i.e. from the day the 6th egg was laid). In the second feeding regime (hereafter 'long food'), 7 g of fresh mealworms was placed inside the nest box in the tray every day from the day when the 1st egg was laid until the last egg was laid. Mealworms were selected as food items as are rich in both protein (which is supposedly limiting during egg formation) and lipids (i.e. energy) (see e.g. Finke 2002 for composition). The 7-g daily supplement was chosen, as this is ca 40% of the energy needed for an egg-laying great tit (assuming 65% assimilation efficiency by insectivorous birds and 8.5 kJ g⁻¹ energy content in mealworms [Moreno 1989], and daily energy consumption 80–90 kJ during laying [Nager et al. 1997]).

The assignment of nests to treatments was randomly done within blocks of three (treatments in random order within a block to remove laying date and daily fluctuations in environmental factors as confounding variables). Furthermore, to increase the number of control clutches (data collected for another research question) also every 7th nest (i.e. after 2 blocks of 3 treatments) was assigned as a control. There was no difference in the mean laying date (GLM, treatment: $F_{2, 54} = 0.5$, $p = 0.57$) between the treatment groups. The sample size in each group was: control $n = 22$ nests; '5 days food' $n = 16$; 'long food' $n = 17$.

We visited the nests daily to collect eggs on the day of laying, recorded the laying order, and froze them at -20°C until dissection. Collected eggs were replaced with dummy eggs, which were readily accepted. Already in the first day, most of the females consumed the majority of the mealworms. During egg laying of eggs 2–6, females consumed on average 29.2 g (SD 6.8, range 7.9–35 g, '5 days food' average 29 g, 'long food' average 30 g) mealworms of the maximum 35 g (maximum = 5 d × 7 g = 35 g). Start of incubation was determined as the day when eggs were found warm the first time or when a female was found incubating. When incubation started and there was no additional egg for one day, we still visited the nest once to check that the clutch was really complete. After clutch completion, females were allowed to incubate the dummy eggs for 12 d after which the dummy eggs were removed. This was done in order to avoid laying a replacement clutch in another nest box which would

have confounded our sample by collecting eggs of different clutches of the same pair (pseudo-replication) as well as by mixing first clutches with replacement clutches.

Egg collection and dissection

As yolk formation takes 2.5–4 d in passerines (Badyaev et al. 2005), and food supplement was started on the day the 1st egg was laid, we decided to analyse egg 1 (i.e. pre-treatment egg), egg 4 and 6 (either food supplemented or control), and egg 8 (either after removal of food supplementation, still during food supplementation or control). Clutch sizes were relatively small in the study year (mean 7.2 eggs), with only 4 clutches of 10 eggs, limiting further egg collection.

Shell, albumen and yolk were separated by thawing about 3 months after collection. Yolk mass was weighed (~ 0.001 g), frozen on dry ice and then cut in two halves, and again in a quarter. In this way, we ended up with 4 samples that all contained the same yolk layers, so that this cannot be a confounding factor in the hormonal analyses (due to layering of yolk hormones, Hackl et al. 2003). One quarter of the yolk (ca 80 mg) was used for androgen analyses: it was mixed with water (MilliQ 1:1) with the help of glass beads, and frozen until hormone analyses. For thyroid hormones (based on pilot analyses) we needed ca 400–500 mg of yolk for accurate measurements (and the average yolk mass is ca 250–350 mg): thus we decided to pool yolks from eggs 5 and 6 (hereafter egg 5) and eggs 8 and 9 (hereafter egg 8) for thyroid hormone analysis. The yolk mass taken from each of the two pooled yolks was kept as similar as possible (average 15% difference in the mass between the two yolks).

Yolk steroid analysis methods

Steroids were analysed using extractions and radioimmunoassay as described in e.g. Tschirren et al. (2004) and Ruuskanen et al. (2009). Aliquots of yolk/water emulsion (see above, approximately 80 mg) were taken, weighed (to the nearest 0.1 mg), and mixed with 100 µl of distilled water and 20 µl of 3H Tracer T (~ 2000 counts min⁻¹) to assess extraction efficiency. The samples were extracted 3 times with 2.5 ml of 70% diethyl ether/30% petroleum ether (vol:vol) and dried under a stream of nitrogen. The extracts were then redissolved in 1 ml 70% methanol to precipitate any lipids and samples were stored at least overnight at -20°C, centrifuged, and decanted. The supernatant was dried under a stream of nitrogen and redissolved in phosphate-buffered saline. Testosterone concentrations were measured using Spectria radioimmunoassay kits purchased from Orion Diagnostica ('Spectria 68628', Espoo, Finland) and A4 using Immunotech/Beckman Coulter kits purchased from Beckman Coulter GmbH ('DSL-3800', Sinsheim, Germany), following the manufacturer's protocol. The average recovery rate was 82% (SD 3.3%, range 53–92%). We corrected the measured androgen concentrations (pg mg⁻¹ yolk) for extraction efficiency. Parallelism of dilution curves with the standard curve confirmed reliability of extraction and assay protocols. Samples from different treatments were evenly divided over 8 extractions and 5 assay batches. Inter-assay variation based on standard pools for T and A4 was 3.3 and 7.7% respectively. One out of 8 A4 assays showed an outlier

in the pool values. Part of the eggs in this assay were redone, and their values were very close to the values obtained earlier when corrected for the average pool values of the other 7 assays. Therefore we corrected all values from the 8th assay based on the average pools of the other 7. Cross reactivities of the kits, according to their manufacturer, were the following: Testosterone (Spectria 68628) kit: T 100%, A4 1.7%, 5 α -dihydrotestosterone 2.6% methyltestosterone 0.3%, others < 0.1%. A4 Beckman-Coulter DSL3800 kit: A4 100% and low (< 1%) cross reactivities with several related molecules (incl. Androsterone, 17 OH progesterone, cortisone).

Thyroid hormone analysis methods

T₄ and T₃ concentrations were measured by radio-immuno assay (RIA) following extraction of the tissues as described in detail earlier (Darras et al. 1990, Reyns et al. 2002). In short, 400–500 mg of yolk was homogenized in a methanol volume 3 times the tissue's weight. As individual internal recovery tracers, 1500–2000 cpm of outer ring labeled [¹³¹I] T₃ and [¹²⁵I] T₄ were added. A volume of chloroform, twice the volume of methanol, was added. After centrifugation (15 min, 1900 g), the pellet was re-extracted in a mixture of chloroform and methanol (2:1). Back-extraction into an aqueous phase (0.05% CaCl₂) was followed by a re-extraction with a mixture of chloroform:methanol:0.05% CaCl₂ (3:49:48) and this phase was further purified on Bio-Rad AG 1-X2 resin columns. The iodothyronines were eluted with 70% acetic acid, evaporated to dryness and resuspended in RIA buffer. The average recovery rate was 33.5% (SD 8.1, range 17.8–54.6%) for T₄ and 64.6 (SD 9.4, range 41.4–81.8%) for T₃. For each sample, total hormone levels were calculated based on the individual recovery rate for that sample. As experimental groups were evenly divided over the 4 batches (each batch containing eggs from all treatments, all eggs of the same female in the same batch), there should be no systematic effect of variation in recovery rates over different batches. The assay batch was included as a random effect in the statistical analyses (see below). Antisera for the RIAs were purchased from Fitzgerald Industries International (US-Ireland). The reference numbers were 20-TR40 for the T₄ antibody and 20-TR45 for the T₃ antibody. The T₃ RIA had a detection limit of 388.4 pg ml⁻¹ and an intra-assay variability of 2.2%. The T₄ RIA had a detection limit of 65.1 pg ml⁻¹ and an intra-assay variability of 2.8%. For the T₃ RIA cross-reactivity with T₄ was 0.1–0.5%, whereas for the T₄ RIA cross-reactivity with T₃ was 3.5%. All samples were measured within a single RIA assay.

Statistics

All statistics were conducted with SAS 4.2. Our response variables were egg mass (g), yolk mass (mg), albumen mass (mg), T, A4, T₄ and T₃ concentrations (pg mg⁻¹) and T₃/T₄ ratio. All hormone concentrations were log-transformed for normality. Sample sizes among treatments and eggs (egg 1, 4, 6, 8 respectively) for egg, albumen and yolk mass and yolk androgens were the following: control: 22, 22, 20, 7; '5 days food': 16, 16, 16, 6; 'long food': 17, 17, 15, 8 respectively. Sample sizes for yolk thyroids (combined eggs 1 and

2, 5 and 6, 8 and 9) were: control: 20, 20, 4; '5 days food': 15, 15, 4; 'long food': 14, 14, 7; respectively.

We conducted the analysis in three parts, each reflecting a specific question and hypothesis: first we analysed whether adding supplementary food affected egg size and quality. In this model we used data from egg 1 (pre-treatment) and egg 4. The predictors in this model were treatment (two levels: food vs no food), egg number (as a class variable: egg 1 and 4) and their interaction. We also controlled for laying date and clutch size by including these as covariates. Female identity was included as a random factor to control for non-independence of the eggs from the same clutch and these estimates are also reported as repeatability/consistency between females. Kenward–Roger method was used for calculating degrees of freedom (Kenward and Roger 1997). For thyroid hormones we also included the assay batch as a random effect since recoveries varied among the batches. Covariates were removed from the model ($p > 0.05$), starting from the highest p -values. Treatment, egg number and their interaction were always included, as this was our main question. Non-significant effects were reintroduced to the reduced model and these statistics reported in Table 1. Tukey type post-hoc tests using model estimates (thus controlling for all the factors in the model) were used to further study differences among and within treatment groups and eggs. There was no initial difference in any of the measured egg components at egg 1 (pre-treatment) among the three treatments (GLM/GLMMs, all p -values > 0.09, Fig. 1a–g).

Secondly, we tested the speed of the potential change in egg quality to supplementary food by analyzing the differential effect of the food supplement on egg 4 versus 6. The predictors in this model were treatment (two levels: food vs no food), egg number (as a class variable: egg 4 and 6) and their interaction, and otherwise the model was similar as above. For thyroid hormones, hormone levels of egg 4 were not determined, so this analysis could not be conducted for these hormones.

Thirdly, we analysed if removal of the food supplement affects egg quality, by analyzing only the clutches where both egg 6 and 8 could be sampled. The predictors in this model were treatment (three levels: control, 5 days food or long food), egg number (as a class variable: egg 6 and 8) and their interaction. The models were again similar as above. We report raw means $\pm$ SE from the full data in Fig. 1, and data from clutches with clutch size ≥ 8 in Supplementary material Appendix 1, Fig. A1.

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.hb349>> (Ruuskanen et al. 2015).

Results

Egg, yolk and albumen mass

The mean egg mass ($\pm$ SD, including all eggs) was 1.67 ± 0.13 g (range 1.32–2.04 g) and the mean yolk mass was 323 ± 42 mg (range 219–453 mg). Egg and yolk mass significantly varied among females (estimate (female id) $\pm$ SE; egg mass: 0.008 ± 0.002 , $Z = 4.34$, $p = 0.003$; yolk

Table 1. Results of linear mixed models analyzing the effect of food supplementation on egg composition in great tits *Parus major*: a) data from eggs 1 and 4, i.e. testing the effect of adding the food supplement, using two treatment levels (food vs no food); b) data from eggs 4 and 6, i.e. testing the speed of the response to adding the food supplement, using two treatment levels (food vs no food); c) data from eggs 6 and 8, i.e. testing the effect of food removal or continuing food supplement, three treatment levels (control, 5 d food, long food), including only clutches with clutch size ≥ 8 . Nest identity, and for yolk thyroids, batch number, were included as random factors. The model always included treatment, egg number and their interaction. Statistically significant terms are marked with underlining. For thyroid hormones, only eggs 1, 5, 8 were analysed and thus data in table b is lacking (NA). R^2 is estimated for the fixed effects.

	Egg mass (g)		Yolk mass(mg)		Albumen mass (mg)		Yolk T (pg mg ⁻¹)		Yolk A4 (pg mg ⁻¹)		Yolk T4 (pg mg ⁻¹)		Yolk T3 (pg mg ⁻¹)		T3:T4 ratio	
	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p	F _{ndf, ddf}	p
a) Eggs 1, 4																
Treatment	4.44 _{1,53}	0.04	3.90 _{1,53,5}	0.05	3.90 _{1,66}	0.05	0.00 _{1,55}	0.96	0.48 _{1,54}	0.49	0.43 _{1,43,9}	0.51	0.00 _{1,45}	0.97	0.03 _{1,45}	0.87
Egg number	1.2 _{1,51}	0.24	6.63 _{1,26}	0.01	2.77 _{1,53}	0.10	10.7 _{1,55}	0.002	2.89 _{1,52}	0.09	18.0 _{1,41,2}	0.001	0.08 _{1,47}	0.77	9.92 _{1,46}	0.003
Treat × egg number	4.4 _{1,52}	0.04	0.07 _{1,53}	0.78	10.82 _{1,53}	0.002	0.54 _{1,54,5}	0.46	0.74 _{1,52}	0.39	1.2 _{1,47}	0.28	1.61 _{1,47}	0.20	0.02 _{1,47}	0.87
Clutch size	0.60 _{1,52}	0.44	9.4 _{1,52}	0.003	0.15 _{1,51}	0.70	3.37 _{1,53,3}	0.07	2.4 _{1,53,3}	0.12	6.51 _{1,43}	0.01	0.20 _{1,43}	0.60	2.03 _{1,45}	0.16
Laying date	0.66 _{1,70}	0.41	3.9 _{1,53}	0.05	0.62 _{1,68}	0.43	5.64 _{1,53,8}	0.02	1.79 _{1,53}	0.18	1.27 _{1,44}	0.26	0.32 _{1,44}	0.57	2.48 _{1,43}	0.12
R ²	12%		17.3%		20%		12.1%		2.5%		23.3%		0%		4.9%	
	n = 109		n = 110		n = 109		n = 113		n = 113		n = 98		n = 98		n = 98	
b) Eggs 4, 6																
Treatment	16.6 _{1,29}	0.001	8.1 _{1,36}	0.006	19.1 _{1,60}	<0.001	0.01 _{1,53}	0.92	0.76 _{1,54}	0.38	NA		NA		NA	
Egg number	0.35 _{1,49}	0.56	0.22 _{1,50,8}	0.64	0.08 _{1,52}	0.78	0.26 _{1,51}	0.61	0.01 _{1,52}	0.92						
Treat × egg number	0.25 _{1,49}	0.62	0.51 _{1,56}	0.47	0.00 _{1,52}	0.96	0.01 _{1,51}	0.92	0.09 _{1,52}	0.78						
R ²	11.9%		18.9%		13.0%		0%		0%							
	n = 107		n = 107		n = 107		n = 108		n = 108							
c) Eggs 6, 8																
Treatment	1.83 _{2,18}	0.18	0.19 _{2,18}	0.82	3.19 _{2,17}	0.07	0.69 _{2,18}	0.52	0.54 _{2,18}	0.58	1.75 _{2,15}	0.20	0.73 _{2,13}	0.49	0.63 _{2,15}	0.54
Egg number	4.44 _{1,18}	0.05	3.14 _{1,18}	0.09	0.43 _{1,16}	0.52	3.94 _{1,18}	0.06	2.89 _{1,18}	0.02	20.90 _{1,12}	0.002	0.01 _{1,10}	0.90	8.46 _{1,13}	0.01
Treat × egg number	9.39 _{2,18}	0.002	0.65 _{2,18}	0.53	4.89 _{2,16}	0.02	0.77 _{2,18}	0.47	0.14 _{2,18}	0.86	0.02 _{2,12}	0.97	0.28 _{2,9}	0.76	0.40 _{2,13}	0.67
R ²	46.8%		3.7%		29.3%		7.8%		15.1%		59.5%		0%		37.4%	
	n = 42		n = 42		n = 42		n = 42		n = 42		n = 36		n = 36		n = 36

Figure 1. Egg composition in relation to food supplementation experiment and egg number in great tits (mean $\pm$ SE from full data) (a) egg mass; (b) yolk mass; (c) albumen mass; (d) yolk testosterone (T) concentration; (e) yolk androstenedione (A4) concentration; (f) yolk thyroxine (T4) concentration; (g) yolk triiodothyronine (T3) concentration; (h) T3/T4 ratio. Control = no food supplement. '5 days food' = food supplement from the 1st egg to the 5th egg after which supplement was halted. 'Long food' = food supplement from the 1st egg to the last egg. For T4 and T3, the samples are combined from eggs 1 and 2 (shown as egg 1), 5 and 6 (shown as egg 5), and 8 and 9 (shown as egg 8). For statistical testing, data from the two food supplementary treatments was pooled separately for eggs 1, 4 and 6 (see Methods). Data from clutch sizes ≥ 8 were used in the statistical test comparing egg 6 and 8 (Supplementary material Appendix 1, Fig. A1).

mass: 334 ± 169.4 , $Z = 1.9$ $p = 0.024$, albumen mass: 6350 ± 1868 , $Z = 3.40$, $p = 0.003$).

Adding supplementary food increased egg mass already at egg 4 (egg number $\times$ treatment interaction, Table 1a, Fig. 1a). Post hoc tests using model estimates revealed that in food treatment egg mass increased from egg 1 to egg 4 ($t = -2.47$, $p = 0.02$) but showed no change in the control group ($t = 0.53$, $p = 0.60$). Egg 4 was larger in the food treatment than control ($t = -3.06$, $p = 0.003$). The effect of food supplement on egg mass was similar for eggs 4 and 6, i.e. both eggs were larger in the food treatment than in control (Table 1b). When analyzing large clutches where the effect of food removal could be studied, we found an interaction between treatment (control, 5 days food, long food) and egg number (egg 6 and 8, Table 1c, Fig. 1a, Supplementary material Appendix 1, Fig. A1a): egg mass in '5 days food' treatment decreased from egg 6 to 8 (egg 6 vs egg 8, $t = 3.66$, $p = 0.002$) and thus mass of the egg 8 in '5 days food' was not significantly different from the egg 8 in the control treatment (egg 8: control vs '5 days food': $t = 0.30$, $p = 0.76$). In 'long food' treatment egg mass still increased from egg 6 to egg 8 ($t = 3.66$, $p = 0.002$) and egg 8 was larger than in control or '5 days food' (egg 8: control vs 'long food': $t = -2.22$, $p = 0.04$, '5 days food' vs 'long food': $t = 2.5$, $p = 0.01$), so there seemed to be a cumulative positive effect of the food supplementation on egg mass. For comparison to other egg quality measurements, we primarily report here the results of egg mass of eggs 1, 4, 6 and 8. However, as we do have egg mass data from all the eggs in the laying sequence, we further analysed the speed of the response in more detail, i.e. if the response to supplementary food occurs even earlier than in egg 4 (analyzing egg 1 in comparison with egg 2 or 3) and if the effect of food supplement removal occurs earlier than in egg 8 (analyzing egg 6 in comparison to egg 7). However, no statistically significant effects were found (egg number $\times$ treatment interactions p -values > 0.30) in these analyses, thus 2–3 d was needed to elicit a response in egg mass in this study.

Adding supplementary food did not differently affect yolk mass of egg 4 (no egg number $\times$ treatment interaction, Table 1a, Fig. 1b). However there seemed to be generally slightly higher yolk mass in the food treatment (Table 1a). Also when analyzing eggs 4 and 6, the eggs in the supplementary food treatment were generally larger than controls (Table 1b). When analyzing large clutches where the effect of food removal could be studied, we found no differential effect of food removal, control or food supplement (Table 1c, Fig. 1b, Supplementary material Appendix 1, Fig. A1b). Yolk mass increased with laying date ($\beta \pm \text{SE}$: 1.96 ± 0.98 , Table 1a), clutch size ($\beta \pm \text{SE}$: 7.33 ± 2.39 , Table 1a), and egg number in laying sequence from egg 1 to 4 (mean $\pm \text{SE}$ mg, egg 1: 315.6 ± 5.3 , egg 4: 325.6 ± 5.1).

Adding supplementary food increased albumen mass already at egg 4 (see egg number $\times$ treatment interaction, Table 1a, Fig. 1c): similarly as for egg mass, since post hoc tests revealed that egg mass increased from egg 1 to egg 4 in food supplement treatment ($t = -3.8$, $p = 0.003$) but not in the control ($t = 1.06$, $p = 0.29$). Egg 4 was larger in the food treatment than control ($t = -3.39$, $p = 0.001$). The effect of food supplement on egg mass was similar for eggs 4 and 6 (Table 1b, Fig. 1c). When analyzing the effect of food

removal, we found an interaction between treatment and egg number (Table 1c, Fig. 1c, Supplementary material Appendix 1, Fig. A1c), albumen mass in '5 days food' treatment decreased from egg 6 to 8 (egg 6 vs egg 8, $t = 2.63$, $p = 0.02$) and thus mass of the albumen 8 in '5 days food' was not significantly different from the egg 8 in the control treatment (egg 8: control vs '5 days food': $t = 0.75$, $p = 0.45$). Albumen in egg 8 was heavier in 'long food' than in control or '5 days food' (egg 8: control vs 'long food': $t = -2.57$, $p = 0.02$, '5 days food' vs 'long food': $t = 3.24$, $p = 0.003$).

Yolk androgen and thyroid hormone concentrations

The mean T concentration ($\pm \text{SD}$, including all eggs) was 72.2 ± 22.4 pg mg^{-1} (range 24.5–151.7 pg mg^{-1}) and the mean A4 concentration 107.1 ± 31.2 pg mg^{-1} (range 40.4–237.8 pg mg^{-1}). Yolk testosterone and androstenedione significantly varied among females (estimate (female id) $\pm \text{SE}$; yolk T: 0.0065 ± 0.001 , $Z = 3.8$, $p < 0.001$; yolk A4: 0.0065 ± 0.002 , $Z = 3.84$, $p < 0.0001$). There was no effect of the food treatment on yolk testosterone or androstenedione concentrations in any of the eggs nor by itself or by interaction with egg number (Table 1a–c, Fig. 1 d, e), Yolk testosterone concentration decreased from egg 1 to egg 4 (Table 1, Fig. 1d, mean $\pm \text{SE}$ pg mg^{-1} , egg 1: 78.4 ± 3.0 , egg 4: 68.9 ± 2.6). Yolk testosterone concentration decreased with increasing laying date ($\beta \pm \text{SE}$: -0.0094 ± 0.004 , Table 1a).

To our knowledge, this was the first time yolk thyroid hormone levels are reported from an altricial wild bird from a wild population: The mean T4 concentration ($\pm \text{SD}$, including all eggs) was 2.96 ± 0.83 pg mg^{-1} (range 1.46–6.6 pg mg^{-1}) and the mean T3 concentration was 0.32 ± 0.13 pg mg^{-1} (range 0.12–0.59 pg mg^{-1}). Yolk T4 and T3 concentrations varied significantly among females (estimate (female id) $\pm \text{SE}$; yolk T4: 0.007 ± 0.0019 , $Z = 3.68$, $p < 0.002$; yolk T3: 0.011 ± 0.0029 , $Z = 3.93$, $p < 0.001$). There was no effect of the food treatments on yolk T3 or T4 concentration or on the T3/T4 ratio (Table 1, Fig. 1f–h). However, yolk T4 concentration increased and T3/T4 ratio decreased significantly with egg number (Table 1a, c; Fig. 1f–h; mean $\pm \text{SE}$ pg mg^{-1} : T4 egg 1: 2.69 ± 0.09 ; T4 egg 5: 2.95 , T4 egg 8: 3.50 ± 0.32 ; T3/T4 ratio egg 1: 0.144 ± 0.006 , egg 5: 0.130 ± 0.010 ; egg 8: 0.10 ± 0.01). In addition, yolk T4 concentration decreased significantly with clutch size ($\beta \pm \text{SE}$: -0.017 ± 0.007 ; Table 1a), and T3/T4 ratio increased with clutch size ($\beta \pm \text{SE}$: 0.008 ± 0.003 ; Table 1a).

At individual egg level, yolk T and A4 were positively correlated (Pearson $r = 0.29$, $p < 0.001$), and yolk T3 and T4 were positively correlated (Pearson $r = 0.66$, $p < 0.001$). Furthermore, T was positively correlated with T3 (Pearson $r = 0.30$, $p < 0.0012$), but there were no other correlations between yolk steroid and thyroid hormones.

Discussion

Our results showed that egg and albumen mass increased and decreased rapidly in relation to food supplementation and cessation of supplementation whereas yolk hormones showed no response. A novel finding was that the increase in egg and albumen mass was found in just three days after

starting the food supplement and also that there was a fast decrease in egg size when the supplementation was discontinued. It suggests that within-clutch variation in egg mass reflects day-to-day variation in resource availability, and thus energetic or protein/nutrient constraints via trade-offs on egg laying (constraint hypothesis, Lack 1966, Christians 2002).

The large body of food supplementary studies providing extra food before and during laying, testing the energy/protein limitation hypothesis, have found no consistent effect of food availability on egg mass (reviewed by Christians 2002, Williams 2012, Ruffino et al. 2014). Also in passerine income breeders (like the great tit), the effects of pre-laying food supplementation, even on a protein supplement, which may be the most limiting resource, are unclear: there was a small increase (7%) in egg mass due to protein supplement in blue tits *Cyanistes caeruleus* (Ramsay and Houston 1997) and in zebra finches *Taeniopygia guttata* (Selman and Houston 1996, Williams 1996), but not in great tits (Nager et al. 1997) or pied flycatchers *Ficedula hypoleuca* (Sanz and Moreno 1995) although in both cases clutch size increased. However far fewer studies supplemented food during laying only, thus not confounded by e.g. laying date effects, and testing more direct causal effects of food availability on egg mass: contrary to our results, Nilsson and Svensson (1993) found no effect of mealworm supplement (i.e. protein and lipids) during laying on egg mass in blue tits, but females started incubation earlier. Williams (1996) found that feeding a protein diet in zebra finches during laying increased egg mass, similarly to our results, but lipid-rich supplement had no effect. Saino et al. (2010) also found a positive effect of protein supplement, but only on albumen mass, in gulls. Interestingly, similarly as our results, a correlational study in swallows showed an association between natural insect availability 2–3 d prior to egg laying and egg size (Ardia et al. 2006).

Furthermore the environmental conditions during study years may affect the outcome of food supplementation: the effects are likely to be stronger in a cold, poor year than a warm year when insect food is more available (threshold hypothesis, Nager et al. 1997). During our study year (2013), the breeding season was ca 2 weeks later than in an average year in our study system (date of caterpillar biomass peak in 2013 on 30 May vs 1994–2014 on 16 May) and temperatures were several degrees colder (average March temperature in 2013; 2.5°C vs 2003–2012: 6.0°C; 2013 April 8.3°C vs in 2003–2012 10.5°C), thus the low temperatures and low natural food availability may have contributed to the fact that birds were more energy/protein-restricted, and therefore food supplementation had a clearer effect. However, on average, the egg mass in control clutches was similar to values previously reported for this species in the Dutch study populations (1.65 g in 2013 vs 1.60 g in Lessells et al. 2002, van Noordwijk 1984), but the cold season led to smaller than average clutch sizes (7.2 eggs in 2013 vs 8–9 eggs on average in this species).

Even though within-clutch changes in egg mass were small (ca 10%) compared to among-clutch variation (between clutches, the largest eggs were 1.5 times the smallest eggs) as repeatedly reported (Christians 2002, Schaper and Visser 2013), these small within-clutch differences in egg size may affect sibling competition (Forbes and Wiebe 2010). Given that deviation from the optimal for each female and chick

may be more important than absolute egg size within the clutch (Williams 1996, 2012, but see Krist et al. 2004), a response to day-to-day variation in resource availability via altered egg size within clutches may have consequences for offspring and maternal fitness, which may not always be positive.

For yolk mass, the effects of supplementary feeding were less clear: supplementation somewhat increased yolk mass, but its cessation had no statistically significant effect, although this latter might be caused by the limited statistical power due to small clutch sizes. Furthermore, as the supplement was removed on day 5, a large part of the egg may have already been formed by that day (Badyaev et al. 2005), limiting the effects on yolk mass (but see Kingma et al. 2009). However, changes in albumen mass were similar as egg mass and thus can explain more of the variation in egg mass (as in Williams 1996). Albumen is deposited on the last day before ovulation, thus it could indeed respond more rapidly to day-to-day changes than yolk (Badyaev et al. 2005). Albumen consists ca 80% of water and its solids are composed mainly of proteins (including antimicrobials): the increase in egg and albumen mass could thus be via increasing albumen water content or protein content. Both water content and proteins are important for offspring development: manipulation of albumen size causing e.g. decreased survival (Ferrari et al. 2006, Willems et al. 2014). All in all our results support the constraint hypothesis explaining the within-clutch variation in egg and yolk size.

We found no changes in yolk testosterone and androstenedione concentration in relation to food supplementation or its cessation during laying. This may be due to our artificial food supplementation, which only lasted for a short time, compared to other studies with changes in hormone levels that have used longer feeding regimes (see below): short-term changes may not have functioned as a proper cue for predicting rearing conditions to which yolk hormones could be adjusted to or food availability during laying may not generally be a reliable predictive cue for food conditions during the critical time of chick rearing. One could also speculate that the sampling was too soon after the change in treatment to detect a response in yolk hormones – however manipulation of male quality using similar timescales was found to affect yolk androgen levels (Kingma et al. 2009). Our results add to the equivocal set of results from studies on the relation between yolk androgens and food availability: food supplementation before and during laying decreased yolk androgen levels in gulls and kittiwakes (Verboven et al. 2003, Gasparini et al. 2007), but had no effect in two other studies on the same species (Verboven et al. 2010, Benowitz-Fredericks et al. 2013). In zebra finches under poor quality diet pre-laying, yolk androgen levels decreased over laying sequence whereas there was a flat pattern under high quality diet (Sandell et al. 2007). In canaries *Serinus canaria*, birds under poor quality diet showed less within-clutch increase of androgen concentrations compared to good quality diet (Vergauwen et al. 2012). Although the literature indicates that at least some bird species have short-term flexibility of yolk androgen allocation to environmental factors (see also Kingma et al. 2009), the potential differences in reaction to different environmental cues and among different species remains unclear.

Furthermore, we found that yolk T4 and T3 levels were not affected by food supplementation. This is contrary to what we expected based on correlations between circulating thyroid hormone levels and food availability, which is a general phenomenon across vertebrates (Eales 1988, Darras et al. 1995, Reyns et al. 2002). However again we need to be careful in the interpretation of lack of effect on egg 8, where sample size and power was low. We assumed that yolk thyroid hormone levels reflect maternal levels (epiphenomenon hypothesis), but we could not measure circulating thyroid hormone levels, as blood sampling during egg laying would have caused nest desertion and impeded collection of further eggs. So it is possible that food supplementation simply did not affect plasma thyroid hormone levels and thus also not yolk hormone levels. Most studies on thyroid hormones (conducted with captive birds) used food restriction or starvation versus normal feeding regimes and it could be that starvation causes stronger effects than supplementation (Harvey et al. 1981, Harvey and Klandorf 1983, Darras et al. 1995, McNabb 2007). Alternatively, it may also be that, as is the case for egg mass, the type of food matters: carbohydrate diet has been found to have a strong impact on plasma thyroid hormone levels (Klandorf and Harvey 1985) whereas our supplement was high in protein and fat content but low in carbohydrates. Furthermore, considering the potential for iodine limitation on thyroid hormone production and trade-offs between self and egg: it is not known whether great tits (or any temperate insectivores) are iodine limited in their natural diet. Our mealworm supplement contains moderate levels of iodine ($0.17 \text{ mg I kg}^{-1}$; Finke 2002), but without prior knowledge on the natural diet, its role is difficult to judge, although in general birds seem to be rather resistant to variation in iodine availability (when $> 0.15 \text{ mg I kg}^{-1}$) on circulating thyroid hormone levels (McNabb et al. 1985, McNichols and McNabb 1987).

In general, the thyroid hormone levels measured in great tit eggs were ca 3–4 times lower than those measured for chickens in the same laboratory (Van Herck et al. 2013) suggesting yolk thyroid hormone levels are higher in galliforms than in song birds. Interestingly, we found the novel results that yolk T4 levels increased with laying sequence and were negatively correlated with clutch size. Similar within-clutch patterns have been found in yolk androgens and other steroids in tens of species (Schwabl 1993, Groothuis et al. 2005): higher levels of androgens in the last eggs have been experimentally shown to compensate for hatching asynchrony via increasing growth of the small, last hatching chicks (Tscharren et al. 2005, Müller et al. 2010, Boncoraglio et al. 2011). We can only speculate whether yolk thyroid hormones would have a similar function, or whether this is an epiphenomenon reflecting changes in female circulating hormone levels. Certainly more data is needed from other species and environmental conditions to reveal how general this pattern is. Nevertheless the increase over the laying sequence suggests that there is no depletion of iodine so that females may not be faced with a trade-off between iodine supplementation between self and the egg.

In conclusion, we found rapid changes in egg and albumen mass in response to experimental manipulation of food availability, which suggest energetic or protein/nutrient constraints and trade-offs on egg formation. These

changes may have consequences for sibling competition and thus potentially on fitness. Yolk androgens or thyroid hormones did not show similar patterns, whereas yolk thyroxine increased over the laying sequence, but the proximate mechanisms and function of this pattern are currently unknown.

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Supplementary material (Appendix JAV-00728 at <www.avianbiology.org/appendix/jav-00728>). Appendix 1.