Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 10 [10] September 2021 : 123-132

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Egg Yolk Predictor Substances which related Offspring fitness, Phenotype of Wild Birds

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ABSTRACT
Recent research in birds have shown that various yolk chemicals interact to influence offspring phenotype, but the consequences for offspring fitness and phenotypic in wild populations have remained unknown. During two mating seasons, researchers evaluated spontaneous variation in the amount of 35 yolk components known to influence offspring phenotypes, such as steroid hormones, antioxidants, and fatty acids, in eggs of free-living great tits (Parus major). We looked for links between offspring fitness and phenotypic and yolk component groupings. Egg yolk fatty acids (including saturated, monounsaturated, and polyunsaturated fatty acids) explained the majority of variation in hatchling and fledgling numbers, but not androgen hormones or carotenoids, which were previously thought to be significant drivers of offspring phenotype. Fatty acids also outperformed androgens and carotenoids as indicators of variance in nestling oxidative state and growth. Our findings indicate that fatty acids are key yolk components in free-living populations that help shape offspring fitness and phenotype. These findings highlight potential mechanisms (e.g., weather, habitat quality, foraging ability) through which environmental variation may shape maternal effects and consequences for offspring, because polyunsaturated fatty acids cannot be produced de novo by the mother and must be obtained through the diet. Our research is a crucial first step toward understanding how various yolk chemicals combine to affect offspring fitness and traits in free-living populations. It lays the groundwork for future studies to determine the mechanisms through which yolk components, individually and/or in combination, influence maternal effects in wild populations.
KEYWORD: Egg Yolk, Substances, Offspring fitness, Phenotype, Wild Birds

Received 12.06.2021 Revised 05.07.2021 Accepted 22.09.2021

INTRODUCTION

In evolutionary biology, one of the most important goals is to figure out what generates phenotypic variation. Although the size and direction of maternal effects are still debated [1-3], they are widely acknowledged as significant variables that contribute to phenotypic variation (meta-analysis by [1]). Mothers influence their offspring's phenotype not only via the genes they pass on to their children, but also through the environment they grow up in [4-6]. Mothers in egg-laying species may affect the embryonic environment by providing various resources to the yolk, such as hormones, antioxidants, and fatty acids [7-14]. Several species have shown the significance of several of these maternally transmitted chemicals for phenotypic characteristics (e.g., [9, 15, 16]). In birds, yolk steroid hormones like androgens and glucocorticoids can influence offspring growth, competitive ability, and survival (e.g., *Ficedulaalbicollis;* [17, 18] and reviewed by [19]), but they can also make chicks more vulnerable to oxidative stress by increasing reactive oxygen species production or impairing antioxidant defences (e.g., *Gallus gallus*; [20, 21]). By scavenging the reactive oxygen species generated during development, maternally derived antioxidants such as carotenoids or vitamin E may enhance growth (reviewed by [22]) and minimize the detrimental effects of increasing oxidative stress (e.g., *Gallus gallus, Larus michahellis, Parus major*; [23-,25]). Fatty acids are an essential source of energy for both embryos and nestlings, since they may improve their overall viability and development (reviewed by [26, 27]). Polyunsaturated fatty acids (PUFAs) are particularly important for cell membrane production, heart function, and brain development (e.g., [28]). PUFAs, on the other hand, are vulnerable to lipid peroxidation by reactive oxygen species produced as a by-product of offspring metabolism [29]. So far, the evidence for the importance of fatty acids in embryo and nestling phenotypic development has mostly come from research on poultry or captive birds, where environmental circumstances are usually intended to be benign. Yolk fatty acids have been shown to vary with environmental conditions in free-living birds (*Cyanistes caeruleus, Parus major*), but it's unclear whether they play a role in shaping offspring fitness

and phenotype in natural populations where food availability and weather conditions are constantly changing..

The impact of single yolk components (in particular androgens) or groups of related components (e.g., steroid hormones) on offspring fitness and phenotypic characteristics is frequently used to study maternal effects via egg deposition. This method has been critical in furthering our understanding of how mothers influence offspring phenotypic. Maternal impacts, on the other hand, are multimodal by nature (reviewed by [33, 34]), with distinct groups of yolk components affecting comparable nestling characteristics, i.e., by having interaction effects on offspring phenotype. In Japanese quails (*Coturnix japonica*) hatching from eggs treated with testosterone or carotenoids, for example, hatchling mass was decreased and oxidative stress increased. However, neither hatchling mass nor oxidative stress were changed when both components were given simultaneously. After eggs were concurrently injected with corticosterone and vitamin E, yellow-legged gulls (*Larus michahellis*) showed similar compensatory effects. If the existence and activities of additional components are also taken into account, the impacts of maternal influences on offspring phenotypic that have been seen in research that concentrate on single components may be missing, reduced, or potentiated.

Over the course of two years, researchers investigated the link between multi-substance yolk composition and nestling fitness and phenotypic in a natural population of great tits. This observational study is useful for evaluating natural variation in yolk composition as well as offspring fitness and phenotypes, and it serves as a solid foundation for future experimental research. In the fourth egg from 69 clutches, we evaluated the amounts of 31 yolk components, including 4 steroid hormones, 3 antioxidants, and 24 fatty acids. We used principal component analysis to group yolk components, although a description of the relationships is beyond the scope of this paper. The connections between these yolk component groups and offspring fitness proxies like hatchling and fledgling number were next investigated. We also discovered connections between yolk components and phenotypic characteristics like as growth (body mass and tarsus length) and oxidative status of nestlings from a specific nest. The quantities of pro-oxidants (reactive oxygen species) and antioxidants (non-enzymatic and enzymatic substances) present in cells and tissues characterise an individual's oxidative state . A shift in the oxidative system's chemical components in favor of pro-oxidants may harm important molecules including lipids, proteins, and DNA (reviewed), with possible fitness implications (e.g., and meta-analysis by). The oxidative state of nestlings has therefore been suggested as a mediator of their survival and health (e.g., *Fregatamagnificens*). We measured chick phenotypic traits at two time points: before and after nestlings reached exponential growth, because the strength of maternal effects declines throughout offspring ontogeny (meta-analysis by [1]) and because the nestling oxidative status can change during the growth period (e.g., antioxidant defences; *Taeniopygia guttata*;).

MATERIAL AND METHODS

Species under investigation and field location

In the Indian forest with a mix of deciduous and coniferous trees, we investigated a nest-box population of great tits from April through July in both 2016 and 2017. Great tits lay one egg each day on average. In our population, the average clutch size is 8.45 1.13 eggs, incubation begins after the final egg is deposited and lasts about 14 days (mean SD = 14.07 2.77), and nestlings spend around 20 days in the nest (mean SD = 20.60 1.43; unpublished data). Only the first clutches were examined in this research.

Monitoring the nest and collecting the eggs

From the beginning of the mating season to the end, we visited nests every other day. We visited nests every day once egg laying began and marked eggs with a pencil to determine the laying order. The mean allocation of yolk components and their plasticity along the laying sequence vary significantly among Great Tit females (e.g., [10]); however, we previously reported that yolk components have medium repeat abilities for androgens (R = 0.30–0.64) and antioxidants (R = 0.36–0.39), and medium–high repeat abilities for fatty acids (R > 0.5 for SFAs and MUFAs and R > 0.9 for PFAs) in our population. These estimations of repeatability indicate that yolk component concentrations in eggs produced by the same female are more comparable than in eggs laid by different females. These medium–high repeat abilities also indicate that the average yolk quantity for each clutch is represented by the middle egg of each clutch. We collected the fourth egg in our research because it represents, on average, the middle egg of a great tit clutch (see for a similar method in this species). On the day they were laid, we retrieved eggs between 8:00 and 13:00 h and replaced them with a fake egg. Except for two focal females who were included in both years, all collected eggs from a total of 69 nests were deposited by separate females. We weighed and opened each newly laid egg in the laboratory on the day of collection, then separated the yolk from the albumen by rolling it on a piece of paper. The yolk was then homogenized with an equivalent quantity of distilled water (1 l per mg of yolk) and kept at 80 °C for further examination.

Blood samples and monitoring of nestling growth

We examined nests until the clutch was complete, as shown by the lack of newly deposited eggs and female incubation behavior. We carefully watched each nest two days before anticipated hatching to record the hatching date (day 0 = the day the first hatchling was seen). On the first day, we clipped few down feathers from each chick to identify it and record its body mass (to the nearest 0.1 g). We evaluated the oxidative status of nestlings on day 6 or 7 after hatching (hereinafter referred to as day 6) and day 12 or 13 after hatching (hence referred to as day 12). These two times were chosen because they occur shortly before and after great tit nestlings achieve exponential development (i.e., on day 9–10 post-hatching;) and because the nestlings are big enough to collect the minimal blood volumes needed to analyses their oxidative state. On day 6, we took a tiny blood sample (20 l) from the brachial vein with heparinized capillaries to evaluate the oxidative state of the nestlings. We took body mass and tarsus length measurements on all nestlings (N = 182) from the same nest (N = 51) within 15 minutes of disturbance (mean SD = 12.72 6.93). (to the nearest 0.1 mm). On day 12, we took a second blood sample (80 l) to evaluate the oxidative state of each nestling. We collected body mass and tarsus length measurements from two or three randomly chosen chicks (N = 96) each nest (N = 36) within 3 minutes of disruption (mean SD = 1.86 0.73). Each nestling was then given a numbered metal ring. We measured ultimate body mass and tarsal length on day 15, and fledging was tracked until all of the young had left the nest. Finally, we documented the environmental circumstances that great tits encountered throughout incubation and nesting.

Egg Yolk analyses

We counted 31 yolk components in all (Additional file 2). Additional file 6 describes the equipment, techniques, and assay details utilized to quantify each yolk component (see also [10]). We used diatomaceous earth columns to separate steroid hormones (androstenedione, 5-dihydrotestosterone, testosterone, and corticosterone). We used radio-immunoassays to measure androgen concentrations, assaying each sample in duplicate and correcting hormone concentrations for each sample's individual extraction efficiency. Two assays were used to examine all of the samples. The intra-assay coefficients of variation for androstenedione, 5-dihydrotestosterone, and testosterone were 9.6%, 14.9 percent, and 23.8 percent, respectively, as assessed from positive controls using stripped chicken plasma with a known amount of hormone added. We used enzyme immunoassays to assess corticosterone levels (lot numbers: 12041402D and 04281702, Enzo Life Sciences, Germany). Each sample was tested twice and samples were spread among five tests. The intra-assay coefficients of variance were 6.2, 11.4, 19, 7, and 12.3 percent, respectively, while the inter-assay coefficient of variation was 10.8 percent, as measured from stripped chicken plasma with a known amount of corticosterone injected.

Following [10], we used high-performance liquid chromatography (HPLC) to extract and quantify antioxidants (lutein, zeaxanthin, and vitamin E). Standard curves for lutein, zeaxanthin, and vitamin E (-tocopherol) were used to determine antioxidant concentrations, along with adjustments for their respective internal standards. One and two eggs, respectively, were lacking data on lutein and zeaxanthin concentrations from the 69 eggs examined. The value of the average population of each antioxidant was given to the eggs that had missing data.

Measurements of oxidative biomarkers

We measured antioxidant concentrations and oxidative damage in nestling plasma on days 6 and 12. (details on the equipment and methods used are described in Additional file 6). We used the OXY-Adsorbent test (Diacron International SRL,) to measure OXY (expressed as mM HOCl neutralised) and the d-ROMs test kit to assess oxidative damage (ROMs: generated by the oxidation of lipids, proteins, and nucleic acids, and expressed as mM H2O2 equivalents) (Diacron International SRL, Grosseto, Italy). Each sample was tested twice, and samples were analyzed on 21 plates. The inter-assay coefficients of variation were 5.1 percent and 2.8 percent for OXY and ROMs, respectively, as calculated from the calibrators (OXY) or known standards (ROMs). On day 12, we used the Ransel assay to assess the enzymatic activity of GPX (expressed as U/ml) in red blood cells (Randox Laboratories).

Statistical analysis

We used a principal component analysis (PCA) to categories the 31 yolk components that were examined. The three discovered principal components (PC) accounted for 58% of the total variation in our data. Between years, the mean values of the main components varied. We normalized the loadings to adjust for year variations since ‘year' could not be included as a random (we only had two levels) or fixed component (limited sample size). As a result, we divided the loading of each main component by the mean value of that component in the corresponding year for each nest. We were able to evaluate the significance of each yolk component regardless of year variations using this approach.

We first ran two generalized linear models (we initially ran two generalized linear mixed effect models with ‘female identity' as a random factor, but since this factor did not explain any variance, we excluded it

from all final models) to investigate the relationship between yolk components in the fourth egg of clutches and fitness proxies for these clutches. The response variables were the number of hatchlings and fledglings, while the covariates were PC1, PC2, PC3, and the date of egg collection. In great tits, body mass during fledging is a significant predictor of post-fledging survival. On day 15, we looked at the connection between yolk components, nestling mass, and tarsus length as additional fitness indicators. Because yolk components may affect structural body size (i.e., *tarsus length*) independently of mass, these morphological variables were investigated in separate models. We used the residuals of a linear regression between these two estimations of body condition and clutch size as response variables in two independent linear mixed-effect models due to sample size limitations and to minimize the number of explanatory factors. The model contained PC1, PC2, PC3, and capture date as variables, while nest ID was fitted as a random factor. Environmental factors were originally included in the analysis; however, they were removed from the final models since they had no effect on the response variable.

We used linear mixed-effect models to investigate the association between egg components and the physiological state of individual nestlings from a particular brood. As response variables, we used OXY, GPX (only for day 12), ROMs, nestling mass, and tarsus length (adjusted for brood size). PC1, PC2, PC3, and the day of egg collection were used as covariates, whereas nest identification was used as a random factor. Other covariates were originally included in the model based on their biological relevance to the research topic, however due to sample size limitations, only factors that affected the response variables were included in the final model. On day 12, total sampling time was fitted as a covariate in the model predicting ROM concentrations, while clutch size was maintained as a covariate in models for OXY concentrations.

R statistical software R-3.3.3 was used for all statistical studies. The 'prcomp' software was used to conduct principal component analysis. In a Bayesian framework with non-informative priors, statistical models were run using the 'lme4' and 'arm' packages. For generalized linear models, we used a Poisson error distribution, and for linear mixed-effect models, we used a Gaussian error distribution. The residuals were visually verified for model fit in all instances. Response variables were converted wherever required (details on transformations are provided in the tables). Because the scales of magnitude of the variables varied, we mean-centered all of them (i.e., mean value = 0, standard deviation = 1). Rather than model selection, we based model structure on the research topic and the biology of the species. After that, we utilised the 'sim' function to simulate values from model parameter posterior distributions. The 95 percent Bayesian credible interval (CrI) around the mean was derived from 10,000 simulations, and statistical support was evaluated by getting the posterior distribution of each parameter. CrIs provide additional information than p-values, such as the level of uncertainty around the estimates. When the calculated impact varied from zero with a posterior probability greater than 0.95, we label it statistically significant. In a frequentist paradigm, a 5% threshold is equal to the significance level (for more information on statistical inference, see here).

RESULTS AND DISCUSSIONS

- *Components of the yolk's covariation*

We used PCA to determine the connections between all 31 yolk components. PC1 was shown to be adversely correlated with vitamin E, one monounsaturated fatty acid (MUFA; 20:1n 9) and all six PUFAs (16:2n 6, 18:2n 6, two 18:3n 6, 20:2n 6, 20:3n 6, 20:4n 6, 22:4n 6). PC2 was linked to four saturated fatty acids (SFAs; 15:0, 16:0, 17:0, and 18:0), all but one MUFA (18:1n 9, 16:1n 9, 16:1n 7, 18:1n 7) and all three PUFAs (18:3n 3, 20:5n 3, 22:5n 3, 22:6n 3). Carotenoids (lutein and zeaxanthin) and androgens (androstenedione, 5-dihydrotestosterone, and testosterone) loaded favorably onto PC3, while two MUFAs (18:1n 9, 16:1n 9) loaded adversely onto PC3. To make the findings easier to understand and visualize, all PCs will be described in terms of positive loadings from now on (i.e., we transformed PC1 into positive values). Also, since PC2 contains nearly all MUFAs, while PC1 and PC3 include just one or two, the impacts of various types of fatty acids are more relevant to our study topic. The effects of MUFAs on nestling phenotype are mainly addressed based on PC2 findings, rather than particular fatty acids. Only modest levels of corticosterone were found on the PCs for this yolk component. Because corticosterone concentration is the least repeatable characteristic in yolk (R 0.18; [10]), yolk corticosterone concentrations in the fourth egg do not consistently predict corticosterone concentrations in the other eggs in the same clutch. As a result, we chose not to investigate corticosterone further in this research.

- *Components of eggs and fitness proxy*

Nests with greater SFA, MUFA, and 3 PUFA concentrations had more hatchlings and fledglings (Fig. 1, Additional file 2) than nests with lower concentrations of these fatty acids (Fig. 1, Additional file 2). Furthermore, the number of fledglings was lowest in nests with eggs containing higher levels of vitamin E

and 6 PUFAs. Neither androgens nor carotenoids were shown to be responsible for fitness proxies. There was no link between fledgling mass and tarsus length and any component of the yolk.

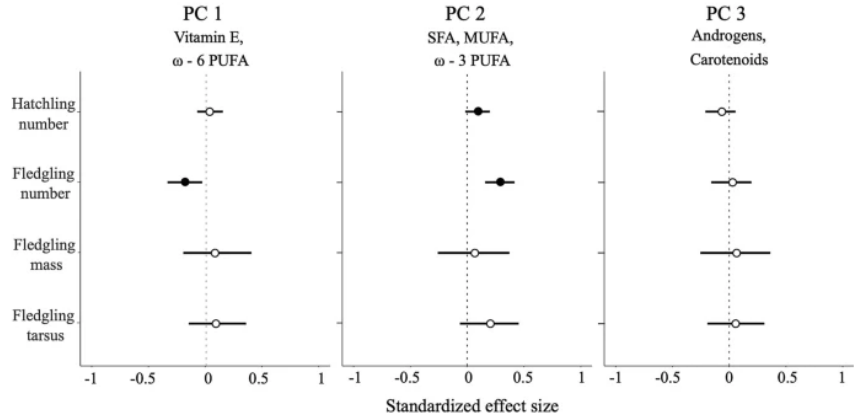

Figure 1 In great tit nestlings, relationships between egg yolk components and fitness proxies were investigated.

- *Phenotypic characteristics and egg components*

Nestlings that hatched from clutches with high concentrations of SFAs, MUFAs, and 3 PUFAs had longer tarsi on day 6 than nestlings that came from clutches with low concentrations of these fatty acids (Fig. 2a). Nestling non-enzymatic antioxidant concentrations (i.e., OXY) were adversely linked to yolk androgen and carotenoid concentrations, while mass was not correlated with any group of components. All yolk component groupings, although in distinct ways, described the oxidative state of great tit nestlings on day 12 (Fig. 2b,. Nestlings hatching from eggs rich in vitamin E and 6 PUFAs, in particular, exhibited significant levels of reactive oxygen metabolites (ROMs) in plasma and low amounts of the enzymatic antioxidant glutathione peroxidase (GPX) in red blood cells. Plasma OXY concentrations were adversely linked to high levels of SFAs, MUFAs, and 3 PUFAs, as well as high levels of androgens and carotenoids, as shown on day 6. There was no link between body mass and tarsal length and any of the yolk components.

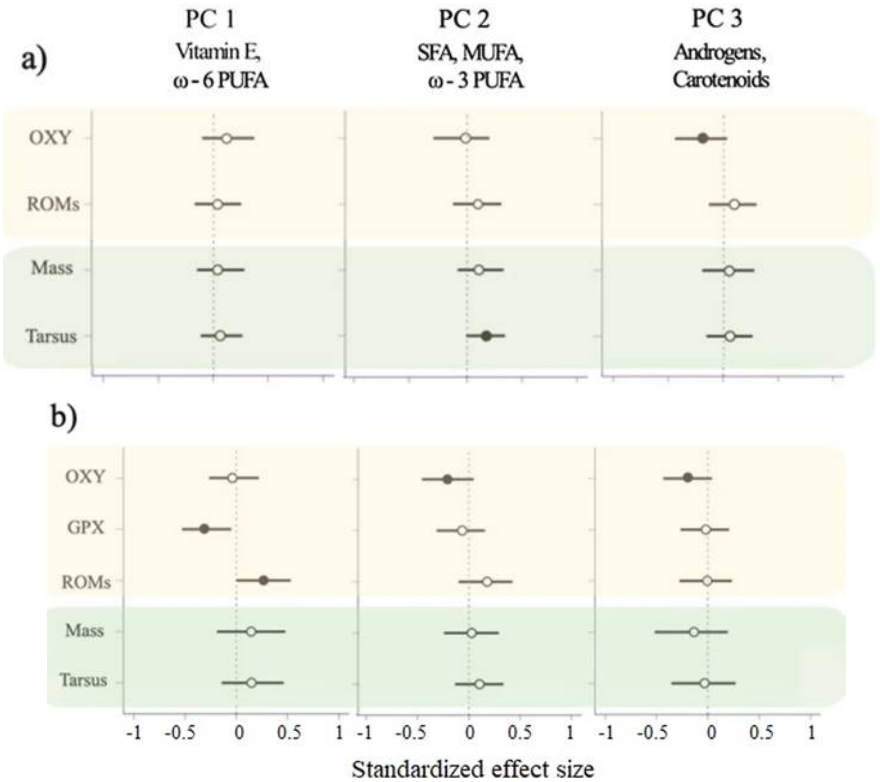

Figure 2 On days 1-3, relationships between egg yolk components and physiological characteristics in great tit nestlings were investigated (a) 6 and (b) 12.

DISCUSSION

The only category of yolk components that explained variance in nestling fitness proxies in a free-living population of great tits was natural variation in fatty acid concentration. Androgens and carotenoids, which have been demonstrated to influence chick growth and fitness in the past (e.g., [17, 18] and reviews by [19, 22]), could not explain for fitness variance. Furthermore, the two yolk component groups containing fatty acids were associated with more elements of the nestlings' physiological phenotype (size and oxidative state) than androgens and carotenoids (only one measure of oxidative status). As a result, spontaneous variation in yolk fatty acid concentration may play a key role in determining offspring fitness and phenotypic in a wild bird population.

- *Components of eggs and fitness proxy*

In our research, yolk fatty acids were linked to the quantity of hatchlings and fledglings. Yolk fatty acids have been shown to improve fitness in captive invertebrates (e.g., *Penaeus chinensis, Acartiaerythraea;*) and vertebrates (e.g., birds: *Gallus gallus*), but this is the first study to show a link with fitness-related traits in a free-living population exposed to natural environmental variation.

The significance of yolk fatty acid composition for hatchling and fledgling numbers may be influenced by the female's nutrition during egg laying in general. Great tits switch from eating seeds to mostly eating caterpillars during the nesting season [47, 48]. SFAs, 3 PUFAs (i.e., -linolenic acid), and antioxidants are abundant in caterpillar i.e., chemicals that are believed to be advantageous to progeny fitness. Adult great tits, on the other hand, depend on other invertebrates such arachnids [52], which are richer in 6 PUFAs, if caterpillars are rare due to low ambient temperatures or poor habitat quality. While this dietary flexibility helps parents to keep their weight in check, it may jeopardize the optimum nutritional balance that females can deposit into their eggs. Females may store and move all fatty acids from internal reserves to plasma, and subsequently to the yolk (reviewed by [53]). Females' PUFA and antioxidant composition is closely related to their diet (e.g., *Parus major*; [54]), thus changes in maternal food resources may have a direct impact on hatchling and fledgling counts.

Hatchling and fledgling counts were greatest in the nests of great tit mothers who deposited eggs with high SFA, MUFA, and 3 PUFA concentrations (Fig. 1). SFAs and MUFAs, the most abundant fatty acids in the yolk (e.g., [10, 32]), may be generated endogenously (i.e., they are not necessary fatty acids; reviewed by [27]), and they are a significant source of energy. 3 PUFAs are especially essential in the early stages of life, when nestlings are developing and expanding rapidly, and may help with bone development and immunological function (in birds e.g., [55-57]). As a result, eggs rich in SFAs, MUFAs, and 3 PUFAs may have supplied the embryo with both the energy and necessary components needed for growth, thus increasing the number of chicks that hatched in our research. Nestlings who emerged from nests with such eggs had longer tarsi on day 6 in our research, suggesting that these fatty acids may have aided early development (Fig. 2a, Additional file 3). Long tarsi are an excellent proxy for survival during the nestling period in our population (see Additional file 5), therefore they are likely to contribute to higher fledgling numbers. Alternatively, eggs with high concentrations of SFAs, MUFAs, and 3 PUFAs may have had a high lipid content overall (reviewed by [26]). As a result, it will be crucial to determine if the number of hatchlings and fledglings is greater in eggs that have high concentrations of particular fatty acid groups or have a high total lipid content in the future.

High quantities of one kind of fatty acid, 6 PUFAs, and the antioxidant vitamin E, on the other hand, were adversely linked to the number of fledglings (Fig. 1). This result may be explained in two ways that are not mutually incompatible. First, given that 3 PUFAs and 6 PUFAs are derived from distinct dietary sources, one would anticipate to find an inverse connection between the two kinds of -PUFAs in the yolk in great tits. As a result, nestlings from nests with high concentrations of 6 PUFAs and vitamin E may have had lower concentrations of other resources, which may have improved fledging success. Second, poultry studies show that a high ratio of 6 PUFA concentrations to 3 PUFA concentrations is linked to the production of pro-inflammatory eicosanoids (i.e., inflammation mediators), while the inverse ratio is linked to the formation of anti-inflammatory eicosanoids. If chicks required to mount inflammatory processes during the nestling period, chicks from clutches with a high ratio of 6 vs 3 PUFAs in their yolks could have shown significant inflammatory responses, lowering their fledging success.

In contrast to previous studies indicating an effect of these yolk components on early development and survival in birds, offspring fitness proxies were not linked to yolk androgen and carotenoid concentrations in the present research (e.g., [17, 18] and reviews by [19, 22]). Three non-exclusive reasons for this finding are presented below, which may be explored in future studies. On the one hand, we simultaneously measured three different groups of yolk components, and some, such as fatty acids, explained more variation in offspring phenotype and fitness than androgens and carotenoids, in contrast to many previous studies that focused on one group of yolk components (e.g., androgens, e.g., [17]). As a result, a possibly little impact of androgens and/or carotenoids may have been obscured by the greater

influence of fatty acids. Our research design, on the other hand, placed two constraints. Using PC3, we first investigated the link between fitness and androgens/carotenoids. However, this PC3 also included two MUFAs, which may have disguised the connection between androgens and carotenoids—though we doubt this. Second, we took the middle egg as a representative for each clutch and let its siblings to develop normally in order to evaluate offspring fitness and phenotypes. Because alternative methods, such as taking a biopsy of the yolk from each egg, can result in a significant reduction in offspring fitness (e.g., hatching success; *Troglodytes aedon*,), and the effects on offspring phenotypes are unclear, this technique is commonly used to study the adaptive value of maternal yolk deposition (in this species see; see also e.g., *Ficedulaalbicollis* [18, Many yolk components have a high degree of repetition in our population of free-living great tits (androgens R = 0.30–0.64 and carotenoids R = 0.38–0.39; [10]), indicating that females are generally constant in their yolk deposition within a clutch (see also [62]). Despite the consistency, there is a significant amount of residual variation (36–70%; (1 R)), which is likely due to flexibility in the concentrations of yolk components that mothers deposit throughout the laying sequence [10]. As a result, the absence of a link between yolk androgen and carotenoid concentrations and fitness characteristics necessitates additional research in a multi-component study.

- *Phenotypic characteristics and egg components*

Our findings show that maternal yolk deposition influences phenotypic variance in offspring, but that its impact differs depending on characteristics and developmental stages (Fig. 2a, b). Physiological characteristics believed to be markers of nestling health (oxidative state) were shown to have a greater relationship with yolk substances than morphological traits. In fact, only one morphological feature (tarsal length) was described by yolk content, and that was only on day 6, before the nestlings achieved exponential development.

Fatty acids, antioxidants, and androgens have all been linked to the oxidative state of nestlings, although via distinct redox system components (Fig. 2a, b). On day 12, nestlings with high yolk concentrations of 6 PUFA and vitamin E, which are components inversely linked to fledgling number, had a poor oxidative state (i.e., nestlings had low enzymatic antioxidant concentrations in red blood cells and high concentrations of oxidative damage in plasma). On the other hand, high levels of SFAs, MUFAs, and 3 PUFAs, yolk components related to hatchling and fledgling numbers, were linked to low levels of non-enzymatic antioxidants in nestling plasma on day 12. (but were not associated with nestling oxidative damage). Finally, high levels of androgens and carotenoids in the yolk were linked to low levels of non-enzymatic antioxidants in the nestlings on days 6 and 12. These findings lead us to believe that yolk fatty acid contents affect the oxidative state of the nestlings on day 12, explaining the variations in fledgling numbers. However, in our population, the majority of the nestlings observed on day 12 (N = 160) survived to day 15 (N = 153), indicating that this is not the case.

When comparing day 6 to day 12, maternal yolk deposition predicted less elements of the nestlings' oxidative state (Fig. 2). These findings contradict the theory that the intensity of maternal influences diminishes with time as children grow. However, our findings may be explained if the great tit nestling antioxidant system develops at a comparable ontogenetic rate as chicken chicks. During the final week of incubation, carotenoids and vitamin E are transported from the egg yolk to the tissues of the chick. Nestlings are unable to efficiently absorb antioxidants from their food immediately after hatching, and their antioxidant system is mostly controlled by the content of yolk antioxidants deposited by the mother (reviewed). A chick's plasma concentrations of carotenoids and vitamin E drop significantly one to two weeks after hatching (reviewed) and a nestling's antioxidant protection is increasingly controlled by its own synthesis of enzymatic antioxidants like GPX (reviewed). The temporal dynamics of chick antioxidant protection given by maternal yolk resources against the chick's own antioxidant synthesis in free-living populations have yet to be investigated. However, if free-living great tit nestlings follow a similar temporal pattern of antioxidant protection as poultry chicks, a decrease in maternally derived antioxidants and an increase in metabolic demands during the nestling period could explain the stronger relationship between maternal yolk hormones and the nestlings' oxidative status observed on day 12 compared to day 6. This may be particularly true for nestlings born from clutches with high concentrations of 6 PUFA and vitamin E in the fourth egg, since the former is vulnerable to lipid peroxidation by reactive oxygen species produced as a by-product of offspring metabolism.

CONCLUSION

We discovered a link between average yolk component deposition and offspring fitness and phenotype by collecting the fourth egg. Other sources of variation in female yolk deposition (i.e., the plasticity of deposition throughout the laying sequence and the covariance between average deposition and plasticity; e.g., [10] should be investigated in future research to see how they influence offspring characteristics.

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CITATION OF THIS ARTICLE

D Saxena and F A Shergojri. Egg Yolk Predictor Substances which related Offspring fitness, Phenotype of Wild Birds. Bull. Env. Pharmacol. Life Sci., Vol 10[10] September 2021 : 123-132.