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RESEARCH ARTICLE

Maternal food supplementation and perceived predation risk modify egg composition and eggshell traits but not offspring condition

Chiara Morosinotto[1,2,*], Robert L. Thomson[2,3], Erkki Korpimäki[2], Rafael Mateo[4] and Suvi Ruuskanen[2]

ABSTRACT

Mothers may vary resource allocation to eggs and embryos, which may affect offspring fitness and prepare them for future environmental conditions. The effects of food availability and predation risk on reproduction have been extensively studied, yet their simultaneous impacts on reproductive investment and offspring early life conditions are still unclear. We experimentally manipulated these key environmental elements using a 2×2 full factorial design in wild, free-living pied flycatchers (*Ficedula hypoleuca*), and measured egg composition, eggshell traits and offspring condition. Eggs laid in food-supplemented nests had larger yolks and thicker shells independently of predation risk, while eggs laid in nests exposed to predator cues had lower levels of immunoglobulins, independent of food supplementation. In nests without predator cues, shell biliverdin content was higher in eggs laid in food-supplemented nests. Incubation was 1 day shorter in food-supplemented nests and shorter incubation periods were associated with higher hatching success, but there were no direct effects of maternal treatment on hatching success. To investigate the impact of maternal treatment (via egg composition) on the offspring, we performed full brood cross-fostering after hatching to unmanipulated nests. Maternal treatment did not significantly affect body mass and immunoglobulin levels of offspring. Our results suggest that although prenatal maternal cues affected egg composition, these egg-mediated effects may not have detectable consequences for offspring growth or immune capacity. Unpredictable environmental stressors may thus affect parental investment in the eggs, but parental care may level off costs and benefits of differential maternal egg allocation.

KEY WORDS: Biliverdin, Immune factors, Non-lethal effects, Environmental mismatch, Maternal allocation, Transgenerational effects

INTRODUCTION

Parents can affect the fitness of their offspring through resources allocated to the current breeding attempt, in terms of overall investment in eggs, embryos and parental care (Clutton-Brock, 1991; Mousseau and Fox, 1998). In species laying cleidoic eggs, all the resources necessary for the development of the embryo are provided by the mother via the eggs (Groothuis et al., 2005; Boulinier and Staszewski, 2008; Gil, 2008). Initial maternal investment may therefore be critical in determining the survival and fitness prospects of offspring. Maternal effects and other forms of non-genetic inheritance depend on the conditions experienced or cues perceived in the environment by parents. However, how combinations of different environmental conditions can affect maternal investment and to what extent these maternal effects persist in the offspring are less well understood.

Food availability during breeding can strongly affect parental investment, egg composition and, subsequently, offspring growth and condition. For example, food-supplemented birds have been found to lay larger clutches and eggs (Zanette et al., 2006a; Karell et al., 2008; Benowitz-Fredericks et al., 2013; Ruffino et al., 2014; Ruuskanen et al., 2016; Podofillini et al., 2019; but see Giordano et al., 2015a) or vary their transfer of hormones and immune factors to eggs (Gasparini et al., 2007; Benowitz-Fredericks et al., 2013; Morosinotto et al., 2016; but see Ruuskanen et al., 2016). Maternal food supplementation is also known to shorten incubation duration and lead to increased offspring mass, condition and immune defence (Pihlaja et al., 2006; Karell et al., 2008; Moreno et al., 2008; Vafidis et al., 2016; Podofillini et al., 2019; but see Verboven et al., 2003).

Predation risk is another key biotic factor affecting animal physiology and behaviour (Caro, 2005; Ibáñez-Álamo et al., 2015). Individuals living under high predation risk pay a cost in terms of reduced foraging activity or efficiency (Sinclair and Arcese, 1995) and may suffer a physiological stress response leading to elevated glucocorticoid levels (Boonstra et al., 1998; Clinchy et al., 2013). These impacts further accentuate the costs of reproduction. For example, when living under high predation risk, birds are known to have smaller clutches (Eggers et al., 2006; Thomson et al., 2006; Morosinotto et al., 2010; Zanette et al., 2011) and may vary in egg composition and size (Coslovsky et al., 2012; Morosinotto et al., 2013; Possenti et al., 2018). Predation risk exposure in vertebrates may affect growth (Scheuerlein and Gwinner, 2006; Thomson et al., 2006; Sheriff et al., 2009; Coslovsky and Richner, 2011a) and antipredator behaviours of the offspring (Giesing et al., 2011; McGhee et al., 2012; Bestion et al., 2014). Altered egg composition and parental behaviour in risky areas could be a result of the trade-offs parents face during breeding between self-foraging and offspring developmental needs. In contrast, the pattern observed could also be an indirect effect of stress perceived by the mother during egg formation (Ensminger et al., 2018), because perceived risk will also alter maternal physiology (Boonstra et al., 1998; Sheriff et al., 2009). These patterns could also be adaptive for the offspring if the environmental conditions at fledging match the

[1]Novia University of Applied Sciences, Bioeconomy Research Team, Raseborgsvägen 9, Ekenäs, 10600, Finland. [2]Department of Biology, Section of Ecology, University of Turku, Turku 20014, Finland. [3]FitzPatrick Institute of African Ornithology, DST-NRF Centre of Excellence, University of Cape Town, Rondebosch 7701, South Africa. [4]Instituto de Investigación en Recursos Cinegéticos (IREC), CSIC-UCLM-JCCM, 13005 Ciudad Real, Spain.

*Author for correspondence (chiara.morosinotto@novia.fi)

C.M., 0000-0002-9172-894X; R.L.T., 0000-0002-6958-1259; E.K., 0000-0001-7596-1955; R.M., 0000-0003-1307-9152; S.R., 0000-0001-5582-9455

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conditions perceived by the mother (Marshall and Uller, 2007), leading, for example, to faster growth to reduce predation risk at the nest (Coslovsky and Richner, 2011a).

Predation risk and food availability affect individual fitness directly, but also indirectly, via maternal effects. These indirect maternal effects may even span across generations (Khan et al., 2016). Despite the extensive literature on the independent effects of food availability and predation risk on the breeding investment and success of wild animals (Salo et al., 2010; Ruffino et al., 2014), experimental studies simultaneously investigating these effects are still lacking (but see Zanette et al., 2006a,b; Ruuskanen et al., 2017). Understanding how animals respond to interactive effects of food availability and predation risk is essential, since the starvation–predation risk trade-off is fundamental in behavioural theory (McNamara and Houston, 1990). In particular, whether these environmental stressors in the early breeding phase, perceived during egg and embryo formation, can have interactive carry-over effects for the offspring is still unknown.

We used a full 2×2 factorial design to experimentally test the impact of food availability and predation risk on female reproductive investment in eggs (yolk mass, immunoglobulin concentration and lysozyme activity, shell thickness and biliverdin content). We exposed pied flycatcher [*Ficedula hypoleuca* (Pallas 1764), hereafter flycatcher] females to experimentally increased nest predation risk (odour and visual cues), to food supplementation or to both during early breeding. Treatments were applied from the nest-building and egg-laying phases until clutch completion. Our experimental approach is the first to test the independent, additive and/or interactive effects of both food availability and predation risk on resource allocation to eggs. This approach will allow us to differentiate the effect of predation risk on foraging success from the influence of physiological stress on egg composition. In addition, after hatching, we performed a full-brood cross-fostering experiment to test the indirect consequences of maternal allocation on the growth and condition of offspring. By transferring full broods to unmanipulated nests, we could disentangle the carry-over effects of prenatal maternal cues and egg composition on offspring growth and condition from the impact of parental care.

Using a factorial design is especially crucial to understand the dynamics driving the transfer of immune factors and shell characteristics, because their cost for breeding females and their link to female condition are currently still unclear. Indeed, females in good condition could either increase (Cucco et al., 2007; Karell et al., 2008) or reduce (Gasparini et al., 2007) the immune factors transferred to eggs and offspring, such as immunoglobulins, antibodies and lysozyme, an antimicrobial (Saino et al., 2002). However, females exposed to high predation risk could also transfer either high or low levels of immune factors to the eggs (Coslovsky and Richner, 2011b; Morosinotto et al., 2013). These different patterns could be explained by the condition of the females during the pre-breeding period, but could also be due to the trade-offs between costs and benefits of high immune factor levels in the eggs (i.e. maternal immune factors are beneficial in the early post-hatching phase but may have costs for the offspring later in life; Grindstaff et al., 2003, 2010). It is currently still unclear how predation risk and food supplementation combined would affect female condition and the transfer of immune factors to the eggs. Similarly, the deposition of biliverdin in the shell (i.e. the pigment that gives the blue-green coloration to pied flycatcher eggs; Kaur et al., 2003) is higher in females in good condition (Morales et al., 2006, 2011; Hanley et al., 2008) but has costs in terms of antioxidant defences for the breeding females (Morales et al., 2008). Yet, the impact of predation risk, alone and combined with food supplementation, on biliverdin transfer has not been investigated to date.

First, we focused on how treatments affected egg composition and incubation duration. We predicted that when food is abundant, (1) flycatcher females will invest more resources to their eggs, expressed as increased yolk mass, shell thickness and colour, and will reduce incubation duration, because reduced self-foraging costs allow higher reproductive investment. However, we could not formulate any clear predictions about immunoglobulin and lysozyme transfer in the eggs because females could either transfer more immune factors, owing to their better body condition, or reduce the levels of immune factors, to reduce the costs for the offspring later in life. We predicted that in high predation-risk nests, (2) females would allocate fewer resources to breeding, expressed as smaller eggs with altered immune factor levels owing to reduced self-foraging time and higher physiological predator-induced stress. Females exposed to combined predation risk and food-supplemented treatments were (3) expected to allocate more resources to the eggs compared with females in high predation-risk nests without additional food resources, because of the reduced self-foraging costs.

Second, we focused on how the maternal prenatal treatments would affect offspring size and immunoglobulin levels. To decouple parental treatments from growth conditions, we experimentally cross-fostered offspring to random unmanipulated nests. We predicted that if supplemented females invest more in their current clutch, offspring of fed mothers will be in overall better condition throughout the rearing period, with heavier hatchlings and fledglings and a better immune capacity, independently on the rearing conditions. In contrast, we expected offspring of stressed mothers to be small at hatching with reduced growth and condition before fledging.

MATERIALS AND METHODS

Experimental design

The experiment was conducted in the surrounding of Kauhava, western Finland (63°N, 23°E), from May to July 2012. Nest-boxes for pied flycatchers were settled in 23 forest patches, at least 1 km apart, and regularly checked to detect the presence of breeding pairs. The first nest-box occupied in a forest patch was randomly assigned to one of four treatments following a full factorial experimental design: predator only, food only, predator+food and control (no predator cues and no food supplements). The other nests in the same patch were randomly assigned to one of the remaining treatments so that if there were four or more nests per patch, each treatment was applied at least once.

We applied treatments by experimentally adding biotic variables at nests soon after nest building was initiated. Nests assigned to the predator-only treatment were provided with both olfactory (infusion of predator urine) and visual (predator faeces) cues of a common nest predator of passerines in the study area, the stoat (*Mustela erminea*; see Korpimäki et al., 1991). The urine was obtained by infusing stoat litter material in water for a minimum of 24 h and was sprayed daily at the entrance of the nest box, while small pieces of faeces were placed on the roof (more was added if these disappeared because of the rain). Nests assigned to the food-only treatment were food-supplemented with 7 g of mealworms (*Tenebrio molitor*) every day; the mealworms were placed in a plastic feeding container inside the nest-box. The daily food consumption was recorded by collecting and weighing the remaining mealworms in each nest-box. Nests assigned to the predator+food group were treated with both

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approaches, whereas control nests were sprayed daily with water and contained an empty plastic feeding container in the nest-box. Nests in the food-only and predator-only treatments were also sprayed with water or contained an empty feeder, respectively, thus the daily disturbance at the nest was similar in all four treatments (see details on the methods in Ruuskanen et al., 2017).

Duration of nest building and the date of laying the first egg (hereafter 'laying date') were recorded in each nest. The first three eggs were marked with a non-toxic black marker and, on the following day, the fourth unmarked egg was collected and substituted with a plasticine egg. This egg was chosen because in this species, egg mass and hormonal levels (testosterone and androstenedione) in the fourth egg are highly correlated with the average levels in the clutch (Morosinotto et al., 2016; Ruuskanen et al., 2017). Collected eggs were weighed and then stored at −18°C. During the first week of incubation, all females were trapped at the nest, measured and blood samples were collected. Females that received food supplementation were heavier and had lower antioxidant levels, whereas perceived predation risk did not affect female condition (Ruuskanen et al., 2017). Laying date, clutch size and egg mass did not vary between treatment groups (Ruuskanen et al., 2017), and female immunoglobulin levels were also not affected (see Table S1 and Fig. S1).

The date of hatching of the first offspring in the brood (hereafter 'hatching date'=0) and hatching success (i.e. number of offspring hatched from the total clutch, minus the egg collected) were recorded. Two days after hatching, all offspring were weighed and full broods were cross-fostered. Foster nests were unmanipulated nests, situated in neighbouring forest patches to the treatment nests but where no treatments were performed. Foster nests were matched for brood size and offspring age to treated nests. At 13 days old, offspring (hereafter 'fledglings') were weighed, measured and ringed (three out of 54 broods were measured at 12 days old), and blood samples (up to 25 µl) were collected from the brachial vein. All the samples were refrigerated for 6–8 h before centrifuging 10 min at 8000 rcf to separate red blood cells and plasma (Morosinotto et al., 2016), and then stored at −18°C. Unfortunately, it was not possible to also investigate how the treated parents may have impacted the 'unmanipulated' offspring owing to logistic constraints during fieldwork. Experiment and sample collection were conducted under licenses of the Animal Experiment Committee of the State Provincial Office of Southern Finland and the Environmental Centre of Southwest Finland (license number EPOELY/456/07.01/2012).

Laboratory analyses

We separated yolk, albumen and shell of the collected eggs in the laboratory. Albumen was collected for lysozyme measurements and yolk was weighed (0.1 mg), homogenised and used to measure immunoglobulin levels. After separation, all samples were stored at −18°C until laboratory analyses. Shells were kept in darkness until they were analysed for shell thickness and biliverdin content.

Immunoglobulin and lysozyme analyses

The antibody concentration in egg yolk and in the plasma were measured using an indirect enzyme-linked immunosorbent assay (ELISA) using a protocol modified from Pihlaja et al. (2006), details in Ruuskanen et al. (2011). The wells were coated with an anti-chicken IgG, while an anti-chicken IgY was used to conjugate secondary antibody (alkaline phosphates conjugated, 1:2000 dilution). To each sample, *p*-nitrophenylphosphate was added and the absorbance was measured at 405 nm; immunoglobulin level (IgY) was measured as U ml^{-1}. The standard stock solution, prepared separately for yolk and plasma, was prepared by pooling 5 µl of either the supernatant (for yolk analyses) or plasma from each sample (corresponding to concentration of 10^6 U ml^{-1}) and adding glycerol (1:1; see methods in Ruuskanen et al., 2011). The intra-assay variation for the analyses of immunoglobulin (IgY) in the yolk was <10% whereas the inter-assay variation was between 7 and 13%.

The lysozyme in the albumen was calculated through a turbidometric assay, by measuring the change in absorbance after adding a suspension of *Micrococcus lysodeikticus* (see methods in Jokinen et al., 2003; Ruuskanen et al., 2011). The absorbance was measured at 450 nm for 30 min using a microplate reader and the results on lysozyme activity were given as change in absorbance units (dAbs) ×1000 min^{-1}. Intra- and inter-assay variation were both <10%.

Shell measurements

Eggshells were carefully washed with distilled water, freeze-dried and weighed (±0.1 mg) for biliverdin analysis. Shell thickness was measured, excluding inner shell membranes, in three places on each eggshell using a Mitutoyo digital tube micrometer (model 395-271) with ball-point ends and precision of 0.001 mm (see detailed methods in Morales et al., 2013). Shell thickness was repeatable within samples among the three measurements (repeatability: R=0.86, s.e.=0.027, P<0.001; package rptR in R; Stoffel et al., 2017; https://www.r-project.org/) and thus the mean shell thickness is used.

Each eggshell was then homogenised and the powder was used for the extraction of biliverdin pigment; detailed methods are described in Morales et al. (2013). Briefly, biliverdin concentration was measured from 10–20 mg of each eggshell sample, by adding to each sample 250 µl of 3 mol l^{-1} HCl and 500 µl of acetonitrile. Samples were then vortexed and sonicated for 1 min and centrifuged for 10 min at 10,000 rcf, and 400 µl of the supernatant was then collected. These steps were performed 3 times per sample to obtain a total of 1200 µl of extract. High performance liquid chromatography (HPLC) was then performed following the protocol described by Mateo et al. (2004), with few modifications (Moreno et al., 2006; Morales et al., 2013). The samples were maintained at 63°C and the UV detection was performed at 377 nm wavelength (which is the peak of absorbance for biliverdin). Concentration of biliverdin was expressed as nmol g^{-1} of dry mass of eggshell. The concentration of biliverdin was then multiplied by eggshell mass to obtain a measure of biliverdin content in the whole eggshell, and thus of female allocation per egg (all analyses use biliverdin content, expressed as nmol egg^{-1}).

Statistical analyses

The response variables analysed were: egg characteristics (yolk mass, immunoglobulin level, lysozyme activity, shell thickness and shell biliverdin content), incubation period (the interval between laying date and hatching date, minus the number of eggs in the clutch), hatching success (the number of offspring hatched from the total clutch, minus the egg collected), hatchling (2 days old) and fledgling (13 days old) mass, and immunoglobulin levels (see Table 1 for means±s.e.m. of all the variables and details on the samples size per group for each of the variables considered). All variables were analysed using generalised linear mixed models (GLMMs; fitted by PROC GLIMMIX, SAS 9.3, Kenward–Rogers as the method for calculating degrees of freedom). Gaussian GLMMs were fitted to all the variables, except for hatching success, which was analysed using a binomial GLMM (with log link function, event/trials model). Immunoglobulin levels in eggs and in fledglings, as well as in females (see Table S1), were $\log_{10}$ transformed to improve the normality of residuals.

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Table 1. Means±s.e.m. of all the variables considered for egg characteristics, time of breeding, offspring mass and immune factors according to the 2×2 factorial design (four treatments: control, food only, predator only and predator+food)

	Control	Food only	Predator only	Predator+food
Yolk mass (mg)	336.91±6.82 (17)	347.21±4.88 (15)	323.30±6.37 (16)	344.87±7.86 (19)
Egg IgY (U ml^{-1})	91.60±11.25 (17)	94.06±10.96 (15)	81.38±14.53 (16)	66.82±13.90 (18)
Lysozyme activity (dAbs×1000 min^{-1})	9.10±0.29 (17)	8.51±0.45 (15)	8.85±0.25 (16)	8.89±0.27 (19)
Shell thickness (mm)	0.064±0.0006 (17)	0.065±0.0008 (15)	0.065±0.0009 (16)	0.067±0.0008 (19)
Biliverdin content (nmol egg^{-1})	16.12±0.75 (17)	18.66±0.88 (15)	17.62±1.28 (16)	16.78±1.14 (19)
Incubation duration (days)	13.50±0.36 (14)	12.46±0.24 (13)	13.47±0.32 (15)	12.32±0.31 (19)
Hatching success (%)	0.90±0.07 (13)	0.93±0.37 (12)	0.88±0.07 (13)	0.91±0.03 (18)
Hatchling mass (g)	3.28±0.08 (13; 73)	3.18±0.09 (12; 62)	3.24±0.11 (12; 62)	3.21±0.08 (18; 90)
Fledgling mass (g)	14.12±0.21 (10; 52)	13.78±0.16 (11; 54)	14.22±0.11 (13; 67)	14.48±0.09 (18; 85)
Fledgling IgY (U ml^{-1})	61.40±6.79 (10; 46)	71.70±7.72 (11; 46)	77.68±18.17 (13; 60)	112.11±21.93 (18; 76)

Sample size is presented in parentheses for each treatment. For hatchling and fledgling measures, where there are multiple measurements per nests, we present first the number of nests and then the overall number of offspring measured. IgY, immunoglobulin Y.

Forest patch was used as random factor in all models for egg characteristics, incubation period and hatching success, because nests with different treatments could be present in the same patch. In the models for hatchling and fledgling mass and fledgling immunoglobulin levels, the 'nest of origin' (i.e. the nest where the eggs were laid) was included as random factor because offspring from the same nests were not independent of each other.

In all models, food supplementation ('food'), predation risk treatment ('predator') and their interaction were included because they represent the 2×2 experimental design. The interaction was removed if $P>0.1$ to allow us to interpret the main effects of the two treatments. When discussing the main effects of treatments, the comparison for 'food' is between fed (food only and predator+food combined) and unfed (predator only and control), whereas the comparison for 'predator' is between nests with predator cues (predator only and predator+food) and nests without cues (food only and controls).

Neither food supplementation nor perceived predation risk affected laying date and clutch size (see results in Ruuskanen et al., 2017), but they were included in all models as covariates. Laying date was included in all models to control for time of breeding, whereas clutch size was included to control for the initial reproductive investment in models of egg composition and incubation length. Incubation length was included in the model of hatching success and hatchling mass, because the duration of incubation may affect embryo development. In the model of hatchling mass, the number of offspring hatched, i.e. the brood size at hatching, was also included as a covariate. In the model of fledgling mass, brood size and wing length were included, because body mass and wing length are highly correlated in birds, and offspring condition depends on the number of siblings in the nest. In the model of fledgling immunoglobulin levels, fledgling mass was included to control for individual condition. For yolk mass, we ran also an additional model including egg mass as covariate to clarify the effect of treatments on relative yolk mass. See Table 2 for the full models. Items in parentheses were excluded from the final model.

RESULTS

Egg composition

Yolk mass was significantly larger when females received food supplements, whereas predation or the interaction between food and predation had no effect (estimates±s.e.: unfed, 329.85±4.86 mg; fed, 346.74±4.80 mg; see Tables 1 and 2, Fig. 1A). When correcting for egg mass, there was a non-significant tendency to have larger yolks among food-supplemented nests (predator, $F_{1,61}=2.00$, $P=0.16$; food, $F_{1,61}=2.93$, $P=0.09$; food estimates±s.e.: unfed, 332.57±4.67 mg; fed, 344.11±4.61 mg); yolk mass was significantly explained by egg mass ($F_{1,61}=8.88$, $P=0.004$, slope±s.e.=0.08±0.03).

Immunoglobulin levels were significantly lower in eggs laid in nests with predator cues (Tables 1 and 2, Fig. 2A). In contrast, food supplementation and the interaction between food and predation risk appeared not to affect immunoglobulin transfer to eggs (log estimates±s.e.: without predator cues, 1.91±0.05 U ml^{-1}; with predator cues, 1.78±0.05 U ml^{-1}; Tables 1 and 2, Fig. 2A). Lysozyme activity levels were unaffected by either treatment or the interaction between them (Table 2).

Eggs laid by food-supplemented females had significantly thicker shells (estimates±s.e.: unfed, 0.06±0.0006 mm; fed, 0.07±0.0006 mm; see Table 2, Fig. 1B), whereas predation treatment and the interaction between food and predation treatment had no impact on shell thickness. Shell biliverdin content (i.e. biliverdin concentration multiplied by eggshell mass) was significantly explained by the predator×food interaction (Table 2, Fig. 1C). *Post hoc* analyses, performed to untangle this interaction, showed that in nests without experimental predator cues, eggs laid by fed females contained on average 10% more biliverdin in the shell compared with eggs laid by unfed females (*post hoc* test: control versus food only, estimate±s.e.: −3.29±1.44, $t=-2.28$, $P=0.03$, all other *post hoc* comparisons had $P>0.05$; Fig. 1C).

Incubation behaviour and offspring traits

In food-supplemented nests, the duration of incubation was more than 1 day (8.6%) shorter than in unfed nests (estimates±s.e.: unfed, 13.52±0.23; fed, 12.36±0.22 days), independent of the perceived predation risk or of the interaction between food and predation (Table 3). Hatching success (i.e. the number of offspring hatched per clutch) was significantly lower in nests where incubation lasted longer (Table 3), and there was a non-significant tendency for higher hatching success in nests of fed females, independent of the level of predation risk (Table 3). Hatchling mass was higher in nests with a long incubation duration, but was not directly affected by maternal treatment. Fledglings did not vary in their mass or immunoglobulin levels (Fig. 2C) according to maternal treatment (Table 3).

DISCUSSION

We found that food availability and predation risk experienced by females during early breeding affected aspects of egg composition via direct/indirect maternal effects. In particular, food-supplemented females strongly increased initial investment in several egg parameters, while those exposed to predation risk altered investment in eggs to a smaller degree. Surprisingly, in most traits there were no

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Table 2. Results of the generalised linear mixed models of egg composition in relation to food supplementation (fed versus unfed) and predation risk (with or without predator cues) and their interaction

	Variable	Slope±s.e.	Denominator d.f.	*F*	*P*
Yolk mass	Predator	9.00±6.87	62	1.71	0.20
	Food	−16.89±6.90	62	5.99	**0.02**
	Laying date	−2.14±1.51	62	2.01	0.16
	Clutch size	−0.76±5.81	62	0.02	0.90
	(Predator×Food)		61	0.65	0.42
Egg IgY	Predator	0.14±0.07	48.99	3.97	**0.05**
	Food	0.04±0.07	54.02	0.33	0.57
	Laying date	−0.003±0.02	60.88	0.02	0.88
	Clutch size	−0.03±0.06	59.04	0.23	0.64
	(Predator×Food)		55.87	1.39	0.24
Egg lysozyme	Predator	−0.09±0.31	47.85	0.09	0.77
	Food	0.27±0.32	54.26	0.70	0.41
	Laying date	−0.002±0.07	60.3	0.10	0.76
	Clutch size	0.21±0.29	61.13	0.54	0.47
	(Predator×Food)		56.27	0.74	0.39
Shell thickness	Predator	−0.001±0.0008	62	2.05	0.16
	Food	−0.002±0.0008	62	4.93	**0.03**
	Laying date	−0.00004±0.0002	62	0.00	0.98
	Clutch size	0.0005±0007	62	0.52	0.48
	(Predator×Food)		61	0.51	0.48
Shell biliverdin content	Predator	2.30±1.35	48.8	0.08	0.77
	Food	0.75±1.33	53.4	1.67	0.58
	Laying date	−0.34±0.22	56.6	2.26	0.14
	Clutch size	−0.26±0.29	60.9	0.08	0.77
	(Predator×Food)	−4.05±1.96	55.1	4.25	**0.04**

Unfed and nests without predator cues were considered as reference levels, while control was the reference level for the interaction. Numerator d.f. was always equal to 1, thus only denominator d.f. is presented. Terms in bold are statistically significant ($P<0.05$). The terms in parentheses were removed from the final model.

strong additive or interactive effects between these key biotic factors, although biliverdin content was higher in nests without predator cues when females were food supplemented. We also did not detect long-term effects on offspring growth and immune condition after full-brood cross-fostering. It appears that when the conditions perceived by the mother in early breeding are not maintained throughout the breeding period, parents might be able to counterbalance the initial maternal programming with parental care. Exposure to environmental stressors during early breeding alone might thus not have long-term carry-over effects on offspring condition and may not translate to transgenerational consequences.

Egg composition

Yolk mass

Larger yolks laid by food-supplemented mothers show that fed females invested more resources in their eggs, which is in line with our predictions. Females breeding under high food availability have lower self-foraging costs and thus would have more resources to invest in their eggs, but not all studies have found this result (Verboven et al., 2003). Nevertheless, our experimental result supports correlative work showing that females living in areas with rich food resources lay eggs with high yolk mass (Ardia et al., 2006). The apparent lack of impact of predation risk and its interaction with food supplementation on yolk mass, suggests that self-foraging costs of predation did not limit resource allocation. This result is in line with previous findings when the risk is experienced by the females only during egg-laying phase (Coslovsky and Richner, 2011a; Morosinotto et al., 2013; Ruuskanen et al., 2017; but see Possenti et al., 2018). We need to consider the possibility that the predation risk cues were not perceived as a stressor, or that the treatment was not strong enough, although this seems unlikely given the effect we found on other response variables and the successful use of this treatment in other studies (e.g. Mönkkönen et al., 2009).

Shell characteristics

Thicker egg shells were produced by food-supplemented females. This suggests that food-rich environments and/or higher female body condition permits higher allocation or more efficient foraging for calcium-rich sources (Tilgar et al., 1999; Hargitai et al., 2011). Producing eggs with a thicker shell should be fitness enhancing as these eggs have a lower risk of shell breakage and thus higher hatching success (Graveland and Berends, 1997). Maternal investment in the eggshell is limited by the availability of calcium. Normal prey of insectivorous birds can provide only up to 20% of the calcium needed to form eggshells (Graveland and Van Gijzen, 1994), and thus, there is a trade-off between time spent for foraging and time spent searching for calcium-rich food resources (Graveland and Berends, 1997). Our study was the first to test predation risk impacts on eggshell thickness of passerine birds, but, opposite to our predictions, the lack of effect suggests that females exposed to predator cues did not pay a cost in terms of reduced calcium intake compared with controls.

Biliverdin content, i.e. the concentration corrected by eggshell mass, was significantly affected by both food supplementation and predation risk. In nests without predator cues, fed females allocated more biliverdin to the shell than unfed mothers. Biliverdin is the main pigment that gives the blue-green coloration to flycatcher eggs, and has strong antioxidant properties (Kaur et al., 2003). Previous studies have found that females in good condition and/or that were food supplemented transferred more pigment to eggshells (Morales et al., 2006, 2011; Hanley et al., 2008), but at the cost of reduced general antioxidant defences (Morales et al., 2008). There seems to be a trade-off between the biliverdin allocated to the shell, antioxidant capacity of the females and antioxidant transferred to the eggs (Hanley et al., 2008; Morales et al., 2008). In support of this trade-off, our study showed that fed females transferred more biliverdin to the shell of their eggs, but also showed lower

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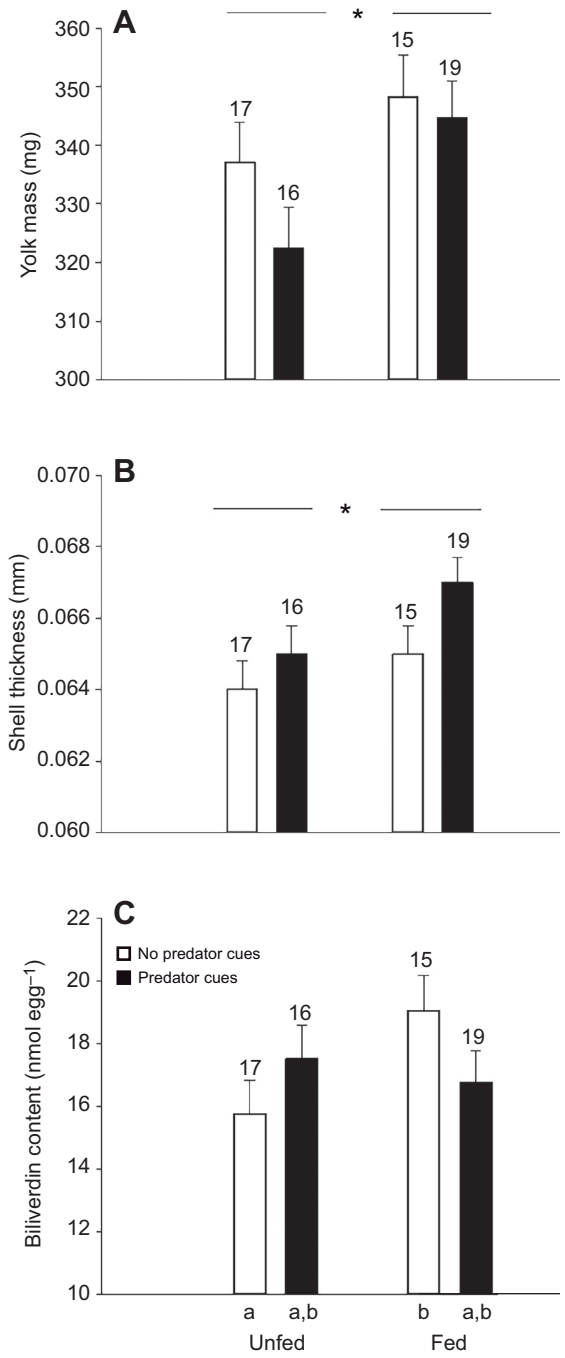

Fig. 1. Yolk mass and eggshell traits according to the combined maternal treatments: food supplementation and perceived predation risk. Estimates (marginal means)±s.e. of (A) yolk mass, (B) shell thickness and (C) biliverdin content. The interaction predator×food is presented even when not statistically significant (A,B) because it is representative of the experimental design. Numbers on top of each bar represent the sample size per group. Black bars represent nests with predator cues, while white bars represent nests without cues, both in fed and unfed groups. In C, letters below the bars denote statistical differences within the predator×food interaction; bars with the same letter are not statistically different (see *post hoc* Tukey's test in Results and Table 2).

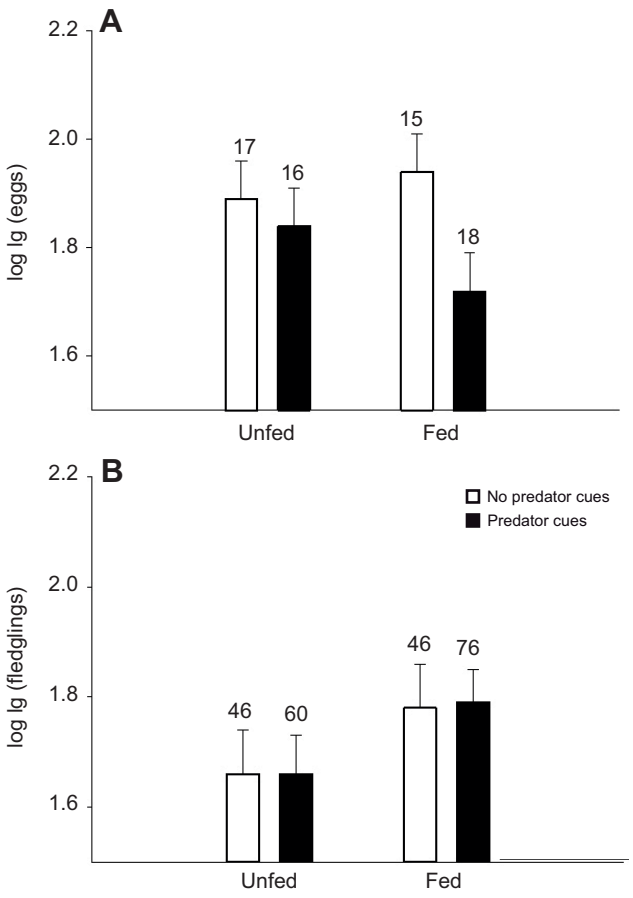

Fig. 2. Immunoglobulin levels in eggs and in fledglings according to the combined maternal treatments: food supplementation and perceived predation risk. Estimates (marginal means)±s.e. of immunoglobulin levels in (A) eggs and (B) fledglings. The interaction predator×food is presented even if not statistically significant because it is representative of the experimental design. Numbers on top of each bar represent the sample size per group. Black bars represent nests with predator cues, while white bars represent nests without cues, both in fed and unfed groups (see Tables 2 and 3).

antioxidant enzyme activity (see results in Ruuskanen et al., 2017). Contrary to the results in nests without predator cues, in nests with predator cues there were no significant differences in biliverdin content between fed and unfed females, which may suggest that exposure to high predation risk may have negated any positive effect of food supplementation. To the best of our knowledge, the impact of predation risk alone, as well as combined with food supplementation, on avian eggshell thickness and coloration has not been studied previously in a cavity nester, and thus further studies are needed to fully understand how environmental stressors affect eggshell traits.

Immune factors

Females exposed to predator cues laid eggs with lower immunoglobulin levels, independent of food supplementation, whereas lysozyme activity was unaffected by either treatment. This contradicts our previous findings of higher immunoglobulin levels and altered lysozyme activity in the eggs of flycatchers under both natural and experimentally increased predation risk (Morosinotto et al., 2013), but matches the results of Coslovsky and Richner (2011b), who found lower immunoglobulin levels in offspring of great tit mothers exposed to predator cues. However, blood immunoglobulin levels in incubating females were unaffected by either of our treatments (see Table S1, Fig. S1) and were not correlated with the levels transferred to the fourth laid egg (Pearson correlation: $r=-0.15$, $P=0.27$), suggesting that the transfer of lower

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Table 3. Results of the GLMMs of incubation length, hatching date and success, offspring mass and immune factors according to food supplementation (fed versus unfed), predation risk (with or without predator cues) and their interaction

	Variable	Slope±s.e.	Denominator d.f.	*F*	*P*
Incubation length	Predator	0.14±0.32	56	0.20	0.66
	Food	1.106±0.32	56	13.40	**0.0006**
	Laying date	−0.03±0.07	56	0.20	0.66
	Clutch size	−0.38±0.26	56	2.17	0.15
	(Predator×Food)		55	0.00	0.99
Hatching success	Predator	0.37±0.54	51	0.48	0.49
	Food	1.16±0.59	51	3.81	*0.06*
	Laying date	0.02±0.13	51	0.03	0.86
	Incubation length	−0.50±0.19	22.93	7.18	**0.01**
	(Predator×Food)		50	0.06	0.81
Hatchling mass	Predator	0.06±0.15	48.45	0.15	0.70
	Food	−0.07±0.18	48.75	0.16	0.69
	Laying date	0.02±0.03	49.32	0.25	0.62
	Incubation length	0.14±0.07	54.39	4.14	**0.05**
	No. hatchlings	−0.008±0.10	51.15	0.01	0.94
	(Predator×Food)		47.28	0.04	0.84
Fledgling mass	Predator	−0.30±0.25	46.10	1.50	0.23
	Food	−0.02±0.24	45.72	0.00	0.95
	Wing	0.10±0.02	252	26.88	**<0.0001**
	Laying date	−0.09±0.05	46.48	2.73	0.11
	No. offspring	−0.10±0.15	47.07	0.49	0.49
	(Predator×Food)		44.46	1.48	0.23
Fledgling IgY	Predator	−0.01±0.08	46.06	0.04	0.85
	Food	−0.12±0.07	44.46	2.50	0.12
	Laying date	0.0002±0.02	48.03	0.00	0.99
	Fledgling mass	0.09±0.03	166.9	9.77	**0.002**
	No. offspring	−0.05±0.05	47.16	1.05	0.31
	(Predator×Food)		44.76	0.01	0.91

Unfed and nests without predator cues were considered as reference levels, whereas control was the reference level for the interaction. Numerator d.f. was always equal to 1, thus only denominator d.f. is presented. Terms in bold are statistically significant ($P<0.05$). The terms in parentheses were removed from the final model.

immunoglobulin levels in the eggs was not explained by female condition. Together, these results suggest that the predation risk perceived by the mother during egg laying does alter the levels of immunoglobulin transferred to the eggs, but the pattern observed is not consistent. Differently from previous studies (Gasparini et al., 2007; Cucco et al., 2007), food supplementation did not affect the transfer of immune factors in the eggs, neither immunoglobulin or lysozyme, nor the levels in females blood circulation.

Incubation length, hatching success and offspring condition

In agreement with our predictions, food supplementation shortened the incubation period, whereas no effect of experimental predation risk was observed. A previous study also found that offspring of fed females hatched 1 day earlier (Vafidis et al., 2016). This suggests that fed females managed to reduce the time spent in self-foraging and thus optimise their incubation behaviour (Conway and Martin, 2000), leading to a more constant egg temperature and shorter development time (Hepp et al., 2006).

Egg composition had no direct detectable effects on offspring. Similar to earlier work (Giordano et al., 2014), hatching success, body condition of offspring, and immunoglobulin levels showed no treatment-mediated impacts. Altered egg immunoglobulin levels following predation risk treatment did not translate into altered fledgling immune defence. Immediately after hatching, offspring immunity is solely based on maternal immune factors, but within the first week, endogenous production will begin (King et al., 2010). Offspring of stressed mothers might have counterbalanced the low levels of maternal immunoglobulin through an overproduction of endogenous immunoglobulins. This is suggested also by the non-significant negative correlation in our dataset between the average immunoglobulin levels per brood and the levels measured in the collected fourth egg ($n=49$; Pearson correlation: $r=-0.27$, $P=0.06$).

Lack of an effect of the prenatal maternal treatments on offspring mass and immunoglobulin levels could be due to several reasons. First, a mismatch between the rearing environment and the maternal cues perceived during egg laying could have masked the effects of maternal food supplementation and/or perceived risk during growth. Maternal effects may be adaptive if the cues perceived by the mother in the early breeding phase persist in the environment, allowing mothers to 'prepare' their young for specific environmental conditions (Marshall and Uller, 2007). In our study, however, all the offspring were cross-fostered after hatching to unmanipulated nests, which likely caused environmental condition mismatches. Second, foster parents (via parental care) might counteract the effect of the original maternal allocation in the eggs, thus masking effects of the maternal treatment. Third, maternal treatments could have sex-dependent effects on offspring condition not investigated in our study. Sexes may vary in their vulnerability to poor early-life environments, with daughters of stressed mothers, but not sons, being smaller than controls (Coslovsky and Richner, 2011a), while daughters of unfed mothers have higher oxidative stress than male offspring (Giordano et al., 2015b). Overall, unpredictable environmental stressors in the early breeding season may affect parental investment in the eggs, but parental care (here offered by the foster parents) may level off costs and benefits of differential maternal egg allocation, especially when exposure to environmental stressors is limited to early life.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.M., R.L.T., E.K., S.R.; Methodology: C.M., R.L.T., R.M., S.R.; Formal analysis: C.M., R.M., S.R.; Resources: E.K.; Data curation: C.M.; Writing - original draft: C.M.; Writing - review & editing: C.M., R.L.T., E.K., R.M., S.R.; Funding acquisition: C.M., R.L.T., S.R.

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Data availability
Data are available from the Harvard Dataverse repository: https://doi.org/10.7910/DVN/CJJYLZ

Supplementary information
Supplementary information available online at
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