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RESEARCH ARTICLE

Are there synergistic or antagonistic effects of multiple maternally derived egg components on offspring phenotype?

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ABSTRACT

Eggs are 'multivariate' in that they contain multiple maternally derived egg components (e.g. hormones, antibodies, mRNA, antioxidants) which are thought to influence offspring phenotype. However, most studies have focused on single egg components and on short-term effects. Here, we simultaneously manipulated two egg components, maternally derived antibodies (MAb) and yolk testosterone, to assess potential synergistic or antagonistic effects on zebra finch offspring phenotype from hatching to sexual maturity. We found no evidence for short- or long-term effects of either MAb or yolk testosterone alone, or their interaction, on hatching mass, size at fledging (tarsus length), body mass at sexual maturity (day 82), chick survival, humoral immune function or any measured female reproductive trait at sexual maturity. There was a positive effect of yolk testosterone, but not MAb, on offspring phytohaemagglutinin (PHA) response at 26 days of age but at 82 days of age, MAb, but not yolk testosterone, had a positive effect on PHA response. There was also a MAb×sex interaction on 30 day chick mass, and a positive effect of yolk testosterone on male courtship behaviour at sexual maturity. However, we found no evidence for synergy, i.e. where offspring treated with both MAb and yolk testosterone had higher trait values than offspring treated with either MAb or yolk testosterone alone for any measured trait. Similarly, evidence for antagonistic (compensatory) effects, where offspring treated with both MAb and yolk testosterone had intermediate trait values compared with offspring treated with either MAb or yolk testosterone alone, was equivocal.

KEY WORDS: Multivariate egg, Maternal effects, Maternal antibodies, Yolk testosterone, *Taeniopygia guttata*

INTRODUCTION

Eggs have a complex maternally derived composition with components that serve not only as nutrients (resources) but also as 'signals' from the mother. These can potentially affect embryonic and post-hatching development, which, in turn, potentially affects offspring and adult phenotype and fitness (Gil, 2008; von Engelhardt and Groothuis, 2011; Williams, 2012; Williams and Groothuis, 2015). All egg components are maternally derived via transfer from mother to egg during egg formation, and this is considered an important pathway for maternal effects. Maternal effects have traditionally been defined as non-genetic contributions of the mother to her offspring, i.e. effects not directly involving variation in nucleotide sequence (though these can include epigenetic effects; Ledón-Rettig et al., 2013). Consequently, maternal effects have been a major focus of life-history studies (Badyaev and Uller, 2009; Mousseau and Fox, 1998). There have been a large number of studies of effects of maternally derived egg components but almost all individual studies, and the many reviews of these studies, have considered variation in single egg components, e.g. macronutrients (Hill, 1995), hormones (Groothuis et al., 2005; Groothuis and von Engelhardt, 2005; von Engelhardt and Groothuis, 2011), antioxidants (Blount et al., 2000) or antibodies (Grindstaff et al., 2003). There is on-going debate about the extent to which females control 'allocation' of specific components to yolk or albumen during egg formation, and the mechanisms involved remain poorly resolved (for a critique, see Williams, 2012; Williams and Groothuis, 2015). However, many studies continue to assume that maternally derived egg components are under female control, allowing females to adaptively 'fine-tune' offspring phenotype. If this is the case, then it has been argued that there should be some level of 'coupling' or co-regulation of different egg components by the mother to maximise or optimise the combined fitness effects (Williams and Groothuis, 2015) or to avoid counter-acting effects (Boulinier and Staszewski, 2008; Postma et al., 2014) of specific components. Therefore, to fully understand maternal effects associated with different egg components (such as hormones and antibodies), we need to consider the 'multivariate egg' (Giraudeau et al., 2017; Postma et al., 2014; Williams and Groothuis, 2015).

Several correlational studies have addressed this idea of the multivariate egg by analysing covariance among different egg components. Groothuis et al. (2006) found little evidence for covariance of androgens, egg mass, antibodies (immunoglobulins, IgG), carotenoids and vitamin E in eggs of common black-headed gulls (*Larus ridibundus*). They suggested that evolution has not strongly selected for mechanisms that allow mothers to adjust 'deposition' of pro-immunomodulatory antioxidants and antibodies into eggs to compensate for possible immunomodulatory effects of maternal testosterone. Similarly, Safran et al. (2008) found no relationship between yolk androgens and carotenoids in barn swallow (*Hirundo rustica*) eggs, and Ruuskanen et al. (2011) found few significant correlations between albumen lysozyme activity, yolk IgG, yolk androgens and yolk total carotenoids in pied flycatcher (*Ficedula hypoleuca*) eggs. They also suggested that different egg components might be regulated by different mechanisms (see also Eeva et al., 2011). Postma et al. (2014) found no association between maternally derived yolk IgG and yolk androgens in the great tit (*Parus major*) within clutches but across clutches these components were negatively correlated, suggesting that selection has co-adjusted deposition of these two egg components. Several other studies have reported correlations within classes of egg components, e.g. carotenoids and vitamins (Hargatai et al., 2006; Rubolini et al., 2011). However, none of these studies disentangled the differential effects of different egg components on offspring phenotype. To our knowledge there

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have only been two experimental studies that have attempted to simultaneously manipulate more than one egg component to look for interacting, synergistic or antagonistic effects. Giraudeau et al. (2017) manipulated yolk carotenoids and testosterone, and Possenti et al. (2018) manipulated pro- and anti-oxidants (see Discussion).

Here, we simultaneously manipulated two egg components, maternally derived antibodies (MAb) and yolk testosterone in female zebra finches, *Taeniopygia guttata* (Vieillot 1817), to assess potential synergistic or antagonistic effects on offspring phenotype. We focused on these two egg components because (a) they have been the focus of a large number of maternal effects studies in birds (Grindstaff et al., 2003; von Engelhardt and Groothuis, 2011), (b) previous studies have suggested there are interactions or cross-talk between testosterone and immune function (Deviche and Cortez, 2005; Müller et al., 2005; Tobler et al., 2010), and (c) these two traits are relatively easy to manipulate in females. Although poorly understood, potential mechanisms do exist for coupling of transfer of steroid hormones and antibodies into egg yolk: there might be co-transport of lipophilic hormones via receptor-mediated endocytosis of lipoproteins during yolk formation and a mechanism for selective transfer of IgG has been identified in birds that also involves receptor-mediated endocytosis in ovarian follicles (Murai, 2013; West et al., 2004). Adult females were treated with lipopolysaccharide (LPS) to generate a secondary immune response so that females produced clutches of eggs with high (LPS-treated) or low (control) MAb. We then used a 2×2 split design manipulating yolk testosterone within clutches of high- and low-MAb eggs via *in ovo* egg injection (Fig. 1). We investigated (a) short-term effects of experimental manipulation of both egg components at 30 days post-hatching on chick growth and immune function at fledging, and (b) long-term effects at sexual maturity (>90 days post-hatching) on phenotypic quality of males (sons) using standardised mating trials (courtship, song rate, etc.) and females (daughters) by measuring reproductive traits during breeding (egg size, clutch size, etc.), and adult immune responses in both sexes. We tested predictions based on previous literature studies for these two egg components (see Table 1 and Discussion). In general, (a) if there were synergistic effects, then MAb+testosterone-treated offspring should have higher trait values (e.g. growth rates, immune function) than controls and offspring treated with either MAb or testosterone alone, and (b) if there were equal, antagonistic effects, then MAb+testosterone-treated offspring should have lower trait values than offspring treated with either MAb or testosterone alone, with trait values similar to those of controls. Specifically, we predicted synergistic effects of MAb and yolk testosterone for nestling and immature growth traits, and for adult 'quality' traits, but compensatory or even additive negative effects for immune function traits (Table 1).

MATERIALS AND METHODS

General husbandry and breeding

Research was conducted on a captive-breeding zebra finch population housed at Simon Fraser University, BC, Canada. Non-breeding birds were kept in single-sex cages (102 cm×39 cm×43 cm) under controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light/dark schedule, 14 h light:10 h dark, lights on at 07:00 h). Birds were fed with a mixed seed (millet) diet, water, grit and cuttlefish bone (calcium) provided *ad libitum*, and a multivitamin supplement in the drinking water once per week. Experienced adult male and female birds (i.e. birds that had been paired or laid eggs previously) were randomly paired and housed in individual breeding cages (51 cm×39 cm×43 cm), each with an external nest box (14 cm×14.5 cm×20 cm). Breeding pairs received 6 g of egg food supplement (20.3% protein, 6.6% lipid) per day during paring, laying and chick rearing. All breeding pairs were checked daily for egg laying to record laying date, egg mass and clutch size. Freshly laid eggs were weighed (±0.001 g) and individually numbered to identify laying order. Around hatching, nests were monitored daily to identify hatching order. Hatchlings were marked by uniquely clipping down plumage for individual identification, and at 8–12 days post-hatching, all birds were banded with a numbered aluminium ring. Body mass (±0.01 g) and the length of the tarsus were recorded (±0.01 mm) at hatching (day 0) and independence (30 days post-hatching). Juveniles (30 days of age) were then removed from their natal cages and were housed in same-

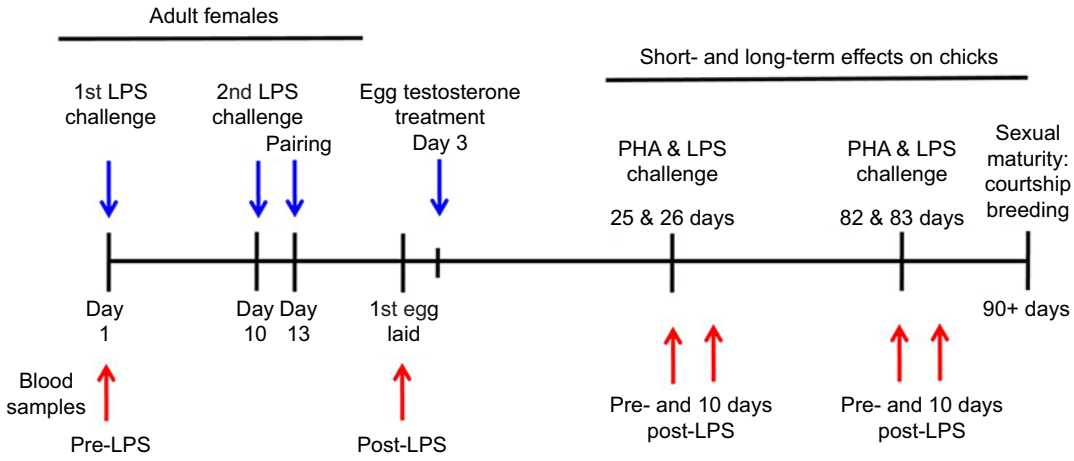

Fig. 1. Experimental manipulation of maternal antibodies (MAb) and level of testosterone in zebra finch eggs. Adult females in the experimental group were immune challenged with two injections of lipopolysaccharide (LPS; day 1 and day 10) while females in the control group were injected with vehicle. Male–female pairs were established 3 days after the second injection. Using a split design within clutches of high- and low-MAb eggs, 3 days after each egg was laid, eggs were injected with either testosterone dissolved in sesame oil (testosterone-treated eggs) or sesame oil alone (control eggs). Chicks were immune challenged at 25 and 82 days with phytohaemagglutinin (PHA) and 24 h later (after the response to PHA was measured) with an injection of LPS. At sexual maturity (90 days of age), female breeding traits and male courtship behaviour were evaluated. Red arrows below the timeline indicate times at which blood samples were taken to determine LPS-specific antibodies.

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

Table 1. Predictions for effects of manipulation of zebra finch eggs with yolk testosterone and maternally derived antibodies (MAb), either alone or in combination, on offspring and adult phenotype

		Immature phenotype		
Treatment	Sex	Growth	Immune function	Adult quality
Yolk testosterone	M	+	–	+
	F	+	–	–
MAb	M	+	+/–	+
	F	+	+/–	+
Yolk testosterone+MAb	M	+++	0/––	+
	F	+++	0/––	+

+++ indicates an additive positive effect if testosterone alone and MAb alone both have positive effects.
+/– indicates that previous studies have reported positive and negative effects of MAb-only treatment.
0/––– indicates either no net effect where the effect of testosterone alone is negative and that of MAb alone is positive, or an additive negative effect if the effect of testosterone alone and MAb alone is negative.

sex communal cages with visual and acoustical contact with birds of the opposite sex, until assays to estimate breeding success were performed. The sex of surviving nestlings was determined at 60–80 days of age based on their sexually dichromatic plumage. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 1074B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

Experimental procedure

To manipulate both the amount of MAb and the level of androgens in the eggs, adult females were first immune challenged before egg laying to generate a secondary immune response and to induce a greater transfer of MAb to eggs (Boulinier and Staszewski, 2008; Grindstaff, 2008). We then used a split design (Fig. 1), manipulating yolk testosterone within clutches of high- and low-MAb eggs by *in ovo* egg injection of testosterone on day 3 after the eggs were laid (Müller et al., 2005) using the general technique described in Winter et al. (2013).

For activation of maternal immune function, adult females were randomly assigned to an experimental (n=41) or control group (n=38). Females in the experimental group were injected intraperitoneally with 0.01 mg of a LPS (*Escheriachia coli*, serotype 055:B5; Sigma, St Louis, MO, USA) diluted in 0.1 ml of phosphate-buffered saline solution (PBS, concentration 0.1 mg ml^{-1}) to initiate a primary immune response. Females in the control group were injected with the same volume of PBS (0.1 ml). Ten days after the first injection, females received a second injection to obtain a stronger, secondary immune response. Three days after the second immune challenge, male–female pairs were established and birds were allowed to breed as described above. To determine the level of female antibodies, a blood sample (80–100 µl) was taken immediately before the first injection and on the day the first egg was laid (approximately 10 days after the second LPS injection). Female body mass (±0.1 g) was measured before each immune challenge, and when the first egg was laid, while tarsus length (±0.1 mm) was measured only during the first immune challenge.

For yolk testosterone treatment, on the day of laying, eggs from the same clutch were randomly assigned to either the testosterone (yolk testosterone group) or the control group (thus controlling for variation among females and genetic background). On day 3 after each egg was laid, eggs in the testosterone-treated group were injected with 500 pg of testosterone (Fluka, Sigma) dissolved in 2 µl of sesame oil. Before injection, the side of the egg was cleaned with 100% ethanol, and the egg was held vertically with the apex at the top and the cap (air cell) at the bottom, until the yolk floated to the top of the egg. The vehicle was injected into the yolk using a 10 µl removable needle Hamilton syringe (gastight 1700 series) and 26 gauge ½ inch small hub removable needle with a bevel tip. To reach the yolk, eggs were candled with a high-powered LED flashlight (900 lm) and the needle was pushed through the shell at an upward angle. The hole in the shell was closed with a drop of cyanoacrylate glue (Loctite gel control) and eggs were placed back in their nest once the glue was dry (~10 min). Eggs in the control group were injected with 2 µl of sesame oil, but otherwise were treated in a similar way to eggs in the testosterone treatment.

Assessment of short- and long-term effects on offspring immune function

Offspring immune response to a novel antigen was evaluated near independence (26 days) and when the birds reached sexual maturity (83 days) using the phytohaemagglutinin (PHA) method (e.g. Alonso-Alvarez et al., 2004; Salvante, 2006; Love et al., 2008). Birds were intradermally injected on days 25 and 82 in the right-wing web (patagia) with 30 µg PHA (PHA-p, Sigma: L-9132) in 30 µl of sterile PBS using a monoject insulin syringe with a 27 gauge ½ inch needle. The point of injection was marked with an indelible marker and three repeated measurements of the height of the swelling (±0.01 mm) were taken prior to injection and 24 h after injection using a gauge micrometer (Dyer Company, Lancaster, PA, USA; model number 304196). Repeatability of the successive measurements of the swelling was high (age 26 days: pre-injection r=0.95, post-injection r=0.93; age 83 days: pre-injection r=0.92, post-injection r=0.95, all P<0.001); hence, we used the mean value of the three measurements for analyses. Immune response to PHA was estimated as the change in thickness (mm) of the wing web 24 h post-injection. Body mass (±0.1 g) was measured on the day of injection, and 24 h after the PHA challenge.

To assess the potential long-term effects of MAb and yolk testosterone level on the humoral immunity of offspring, all offspring were immune challenged with an injection of LPS at 26 and 83 days post-hatching as described above and blood samples (80–100 µl) were taken before injection and 10 days post-injection (at 36 and 92 days, respectively). Blood samples were collected and plasma separated to determine antibody levels. Plasma samples were stored in Eppendorf tubes at −80°C until analysis. The humoral immune response of offspring was evaluated as the LPS-specific antibody production (Ab titre, see below).

Assessment of treatment effects on offspring phenotype at adulthood

After chicks reached 90 days of age (sexual maturity), we assessed phenotypic quality of all males and females that had been manipulated *in ovo*, by breeding females and conducting mating trials in males.

Male courtship behaviour was measured as previously described (e.g. Wada et al., 2008; Yu et al., 2016). Briefly, an experienced wild-type female was randomly chosen and placed in a cage (61×46×41 cm) for 5 min to acclimate alone. Different females were randomly chosen for each male and trial. The cage contained a perch, grit, a cuttlefish bone, but no water or food, and was visually but not acoustically isolated from other cages. Each *in ovo*-treated or control male from our experiment was then placed in the cage with

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

the female, and male courtship behaviour was recorded for 15 min. All of the courtship trials were performed between 09:00 h and 12:00 h, and males were re-tested if they did not show any courtship behaviour on their first trial (see Results). Five typical male courtship displays (described in Zann, 1996) were recorded during the experiment: (1) invitation (Y or N, i.e. did the male court the female?), (2) time in seconds to the initial mounting attempt; (3) the number of successful copulations, defined as those with cloacal contact; (4) bill wiping (number of wipes) and (5) following (the number of times the male followed the female from perch to perch).

Adult females were paired at 90 days post-hatching with a random, unrelated, clean experienced male under the same conditions as described above for breeding pairs. If a female did not lay any eggs within 15 days of pairing, she was un-paired and recorded as a 'non-breeder'. For the remaining females (those that laid eggs within 15 days), laying interval (the number of days between pairing and the first egg), clutch size, mean egg mass, brood size at hatching, brood size at 21 days and brood size at 30 days were recorded. For females that successfully raised chicks, we recorded body mass (±0.01 g) and tarsus length (±0.01 mm) at 21 days post-hatching (average fledging age for chicks in the nest).

LPS-specific Ab determination

To determine LPS-specific antibodies, we used an enzyme-linked immunosorbent assay (ELISA) and followed the procedures described in previous studies on zebra finches and other birds (Bonneaud et al., 2003; Grindstaff et al., 2006; Müller et al., 2005). Ninety-six-well ELISA plates were coated with 100 µl of LPS at a concentration of 5 µg ml^{-1} suspended in carbonate buffer (0.15 mol l^{-1}, pH 9.6). Plates were incubated at 4°C overnight to coat. The next day, plates were blocked with 3% milk solution diluted in 0.01 mol l^{-1} phosphate-buffered saline and Tween-20 for 2 h at room temperature. Plasma samples were diluted 1/500 in diluent (1% powdered milk in PBS–Tween-20). After washing the plate with PBS–Tween-20, the diluted samples were added to the plate in duplicate. The plate was then incubated overnight at 4°C. On the third day, after washing, 100 µl of alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma, A-9171, 1:1000 in diluent) was added to every well of the plate. The plates were incubated for 1 h at 37°C. After washing, 100 µl of peroxidase-labelled rabbit anti-chicken serum antibody (Sigma, A9046, 1:2000 in diluent) were added to every well and the plates were incubated for another 30 min at 37°C. The plates were washed and 100 µl of a solution consisting of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and concentrated hydrogen peroxide diluted to 1:1000 was added to each well. The plates were immediately transferred to an Epoch-2 plate reader (BioTek Instruments, Winooski, VT, USA) and read at 30 s intervals for 14 min at 405 nm.

Statistical analyses

All analyses were carried out using SAS v.9.4 (SAS Institute, 2013). General linear models (proc GLM) were used to evaluate the effect of an immune challenge on female (mothers) Ab titre, body mass, latency between pairing and egg laying, and mean egg mass. We treated clutch and brood size as count data and used proc GENMOD with Poisson distribution. Hatching success and fledging success (coded as 0 or 1 for each treated egg) were analysed using generalised linear mixed models (proc GLIMMIX), with binomial distribution, egg mass as a covariate and nest as a random factor. Hatching and 30 day mass were analysed using proc MIXED with maternal treatment (LPS or control), egg treatment (control or testosterone) and their interaction as main effects, egg mass as covariate, and pair number and egg sequence as random factors. For 30 day mass we also initially included sex as a main effect with all two- and three-way interactions. PHA response and LPS Ab titre at days 26 and days 83 were analysed using proc MIXED with the same model structure, except that body mass at time of injection was used as the covariate instead of egg mass. PHA data were normally distributed (Shapiro–Wilks test) but we $\log_{10}$ transformed Ab data to approximate normality.

Male courtship behaviour at sexual maturity, for birds treated as offspring, was analysed using non-parametric statistics (proc NPAR1WAY), first for all four combined treatments (Kruskal–Wallis test) and then for MAb treatment and yolk testosterone treatment alone (Wilcoxon two-sample test). Reproductive traits of adult female offspring at sexual maturity, for birds treated as offspring, were analysed as described above for mothers.

RESULTS

Effect of LPS treatment on adult females

Before the LPS immune challenge, females in the control and experimental group did not differ in mass ($F_{1,78}$=0.28, P>0.50) or tarsus length ($F_{1,78}$=0.36, P>0.50). Similarly, male partners of control and experimental females did not differ in mass ($F_{1,78}$=0.99, P>0.30) or tarsus length ($F_{1,78}$=0.32, P>0.50).

Of 79 adult females initially paired, 15 females did not lay eggs (5 control, 10 LPS treated; Fisher's exact χ^2=1.62, P>0.20). For the 64 egg-laying females, 24 females (15 control, 9 LPS treated) did not incubate or produced infertile clutches, 8 females (3 control, 5 LPS treated) failed before fledging and 32 females successfully fledged chicks (15 control, 17 LPS treated). This breeding outcome was independent of treatment (χ^2=2.06, P>0.35).

There was no difference in female body mass between LPS-treated and control females at the one-egg stage of egg laying ($F_{1,29}$=0.06, P>0.80). However, at the time of egg laying (approximately 10 days after the second LPS injection), among females that hatched at least one chick, LPS-treated females had higher LPS-specific Ab titres than control females (2.13±0.35 versus 0.95±0.37 OD; $F_{1,33}$=5.29, P=0.028). There was no effect of maternal treatment on the latency between pairing and laying of the first egg, clutch size, mean egg mass or brood size at fledging (P>0.11 in all cases; Table 2).

Effects of MAb and yolk testosterone treatment on chick growth and survival

Hatching mass was independent of MAb treatment ($F_{1,54}$=0.04, P>0.80) and yolk testosterone treatment ($F_{1,54}$=0.00, P>0.90; Table S1). There was a marginally significant maternal×egg treatment interaction ($F_{1,54}$=3.32, P=0.07); however, no *post hoc* pair-wise comparisons among treatments were significant (P>0.15 in all cases). In the full model, for day 30 body mass there was a

Table 2. Reproductive traits in control and lipopolysaccharide (LPS)-treated adult females (mothers)

Trait	Control (n=15)	LPS treated (n=17)	F or χ^2	P
Laying latency (days)	7.4±0.9	5.4±0.9	2.67	0.11
Mean egg mass[a] (g)	1.114±0.025	1.107±0.024	0.04	0.84
Clutch size[b]	5.9±0.4	5.5±0.3	0.25	0.62
Brood size at fledging	3.5±0.3	3.6±0.3	0.01	0.93

Values are least squares means±s.e., n=sample size.
[a]Controlling for female body mass at the one-egg stage. [b]Controlling for laying interval.

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

significant maternal treatment×sex interaction ($F_{1,52}$=6.09, P=0.017; egg mass, P<0.001; Table S1), so mass was analysed for each sex separately. For females, there was no main effect of MAb treatment ($F_{1,13}$=1.05, P>0.30), yolk testosterone treatment ($F_{1,13}$=0.69, P>0.40) or their interaction ($F_{1,13}$=0.65, P>0.40) on day 30 body mass (Fig. 2A). For males, there was no effect of yolk testosterone treatment or the interaction term (P>0.20) but there was a marginally significant effect of MAb treatment ($F_{1,12}$=4.22, P=0.06; Fig. 2): male chicks from MAb eggs were heavier than those from control eggs (13.6±0.2 versus 13.1±0.2 g). In a reduced model, excluding yolk testosterone treatment, there was a significant MAb treatment×sex interaction ($F_{1,56}$=8.49, P<0.01): female offspring from MAb eggs had a similar 30 day mass to controls (t_{56}=0.86, P=0.39), but male offspring from MAb eggs had a higher body mass than controls (t_{56}=2.60, P=0.012; Fig. 2B). For tarsus length at day 30 and body mass at maturity (day 82; Table 3), there was no effect of maternal treatment, egg treatment, sex, or any interaction (P>0.09 in all cases; Table S1).

Overall hatching success was independent of MAb treatment ($F_{1,254}$=1.62, P>0.20), yolk testosterone treatment ($F_{1,254}$=1.16, P>0.25) and their interaction ($F_{1,254}$=0.26, P>0.60; egg mass, P>0.30; Table 3). Similarly, fledging success was independent of MAb treatment ($F_{1,254}$=1.06, P>0.30), yolk testosterone treatment ($F_{1,254}$=0.51, P>0.45) and their interaction ($F_{1,254}$=0.44, P>0.50; egg mass, P=0.029; Table 3). Mean brood size at fledging did not vary among treatments (χ^2=1.05, d.f.=3, P>0.75; Table 3).

Effects of MAb and yolk testosterone treatment on offspring immune response

For the PHA response at fledging (day 26), there was a marginally significant main effect of yolk testosterone treatment ($F_{1,52}$=3.25, P=0.08; Fig. 3A,B) but the PHA response was independent of all other main effects and interactions (P>0.20 in all cases; body mass, P=0.09; Table S2). In contrast, for the PHA response at maturity (day 83), there was a main effect of MAb treatment ($F_{1,42}$=7.19, P=0.010; Fig. 3C,D) but there were no other significant main effects or interactions (P>0.25 in all cases; body mass, P>0.55; Table S2). In both cases, the PHA response was higher in testosterone-treated eggs (Fig. 3B) or eggs from MAb-treated mothers (Fig. 3D) compared with controls.

The $\log_{10}$ LPS Ab titres in offspring at fledging (day 26) and at maturity (day 83) were independent of all main effects and interactions (P>0.15 in all cases; Table S3).

Effect of MAb and yolk testosterone treatment on adult male (sons) courtship behaviour

Courtship trials were conducted on n=49 male offspring at sexual maturity (day 90); on the first trial, n=30 males showed some courtship behaviour (i.e. 'invitation') and n=19 showed no courtship behaviour. For this first mating trail, there was no effect of MAb treatment ($F_{1,18}$=0.68, P>0.40) or a treatment interaction ($F_{1,18}$=0.18, P>0.60). However, a greater proportion of males from testosterone-treated eggs showed courtship behaviour (81.0%, n=21) compared with males from control eggs (46%, n=28; $F_{1,18}$=5.46, P=0.031). Of the 19 males tested in a second trial, 8 performed some courtship behaviour. Data were therefore pooled for these 8 males from the second trial and the 30 first trials for subsequent analysis of specific courtship behaviour (n=38 males; Table 4).

There was no overall difference in time to first copulation among the four combined MAb/yolk testosterone treatments (χ^2=4.83, d.f.=3, P=0.18; Table 4). However, after pooling data by egg treatment, time to first copulation was significantly shorter in testosterone-treated eggs compared with control eggs (Z=2.09, P=0.037; Fig. 4A). Conversely, after pooling data by MAb treatment, there was no difference in time to first copulation (P>0.90). For the number of successful mounts per 15 min there was a significant overall treatment effect (χ^2=8.15, d.f.=3, P=0.043). Males from control eggs had a higher number of successful mounts compared with males from testosterone-treated eggs (Z=2.06, P=0.039; Fig. 4B). However, the number of successful mounts was independent of MAb treatment (P>0.09). Male bill wiping behaviour and following behaviour were independent of combined treatments (P>0.30 in both cases; Table 4) and when MAb and yolk testosterone treatment were analysed separately (P>0.05 in all cases).

Effect of MAb and yolk testosterone treatment on adult female (daughters) reproduction

Among females surviving to sexual maturity (n=45) there was no effect of MAb, yolk testosterone treatment or their interaction on adult body mass ($F_{3,44}$=0.86, P>0.40) or tarsus length ($F_{3,35}$=1.06, P>0.30) at pairing.

Breeding propensity (percentage of females laying ≥1 egg) was high for all treatments: >80% (Table 5). There was no effect of MAb, yolk testosterone treatment or their interaction on the latency between pairing and laying of the first egg, clutch size or egg mass (P>0.12 in all cases; Table 5). Hatching success was independent of

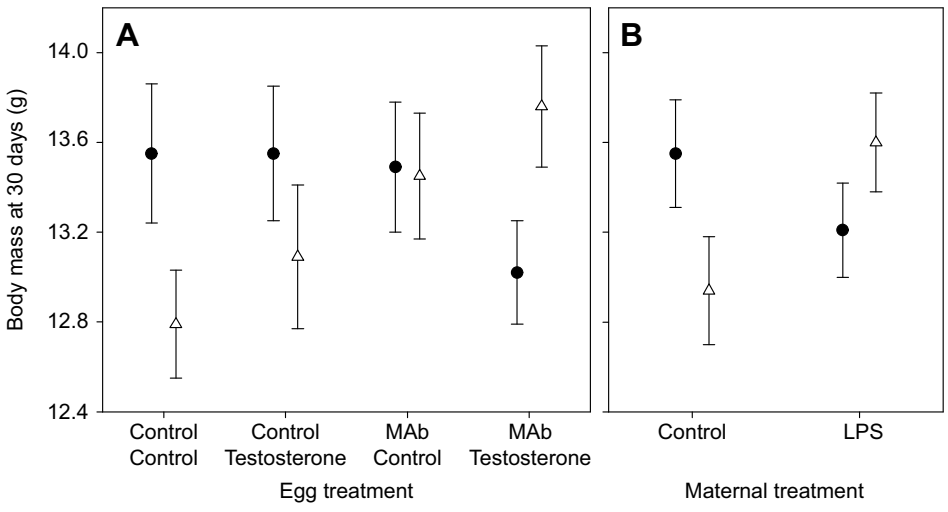

Fig. 2. Effects of treatment on offspring body mass at day 30 post-hatching. (A) Least squares mean (±s.e.m.) body mass for all four treatments from the full model with MAb and testosterone as main effects and the interaction, analysing sexes separately. (B) Least squares mean (±s.e.m.) body mass for a reduced model including MAb treatment and sex only. Males: open triangles; females: filled circles.

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

Table 3. Hatching success, fledging success and brood size at fledging in relation to maternal antibody (MAb) and yolk testosterone treatment

	Egg treatment			
Variable	Control+control	MAb+control	Control+testosterone	MAb+testosterone
No. of eggs	84	81	76	80
Hatching success (%)	48.8	58.0	50.0	66.3
Fledging success (%)	35.7	45.7	34.2	48.8
Mean brood size	3.4±1.1	4.2±1.9	4.4±1.3	3.7±1.3
No. of broods	7	6	8	11
Tarsus length (day 30, mm)	16.8±0.2	17.2±0.2	17.0±0.2	17.1±0.1
Body mass at maturity (day 82, g)	14.2±0.4	14.7±0.4	14.8±0.4	14.4±0.3

Values are percentages or least squares means±s.d.

MAb treatment ($F_{1,189}$=0.41, P>0.50), yolk testosterone treatment ($F_{1,189}$=1.83, P>0.15) and their interaction ($F_{1,189}$=0.12, P>0.70; Table 5). Similarly, fledging success was independent of MAb treatment ($F_{1,189}$=0.13, P>0.70), yolk testosterone treatment ($F_{1,189}$=0.01, P>0.90) and their interaction ($F_{1,189}$=0.73, P>0.35; Table 5).

Mean brood size at fledging did not vary among treatments (χ^2=1.48, d.f.=3, P>0.60; Table 5). For chick mass at fledging (day 21), there was a MAb×yolk testosterone treatment interaction ($F_{1,71}$=5.18, P=0.026; Table 5) but no other main effects or interaction terms were significant, and no pair-wise contrast among treatments was significant (P>0.30, with Bonferroni adjustment). For chick tarsus length, there was also a MAb×yolk testosterone treatment interaction ($F_{1,71}$=7.13, P<0.01; Table 5) but no other main effects or interaction terms were significant (P>0.10). Offspring of MAb+testosterone-treated females had longer tarsi than offspring of MAb+control females (t_{71}=3.07, P=0.018; Table 5), and but no other pair-wise contrasts were significant (P>0.30).

DISCUSSION

We manipulated two maternally derived egg components, MAb and yolk testosterone, alone and in combination, to assess potential synergistic or antagonistic effects on offspring phenotype. If there were synergistic effects, then MAb+testosterone offspring should have higher trait values (e.g. growth rates, immune function) than controls and offspring treated with either MAb or testosterone alone (Table 1). If there were equal, antagonistic effects, then MAb+testosterone-treated offspring should have lower trait values than offspring treated with either MAb or testosterone alone, with trait values similar to controls (Table 1). We found some evidence for main effects, and sex-specific effects, of either maternally derived component independently, but little direct evidence for synergistic or antagonistic effects of MAb and testosterone combined based on significant interaction terms. However, our study highlights the difficulties of detecting antagonistic effects where there is little variance in phenotypic trait values and where traits for MAb+testosterone-treated offspring might be intermediate between those of offspring treated independently with MAb or testosterone alone (see below).

Although we did not measure LPS Ab titres in eggs, LPS-treated females did have higher plasma Ab titres at egg laying. We immune challenged females twice before egg laying to generate a higher, secondary immune response and to induce a greater transfer of MAb to eggs (Boulinier and Staszewski, 2008; Grindstaff, 2008).

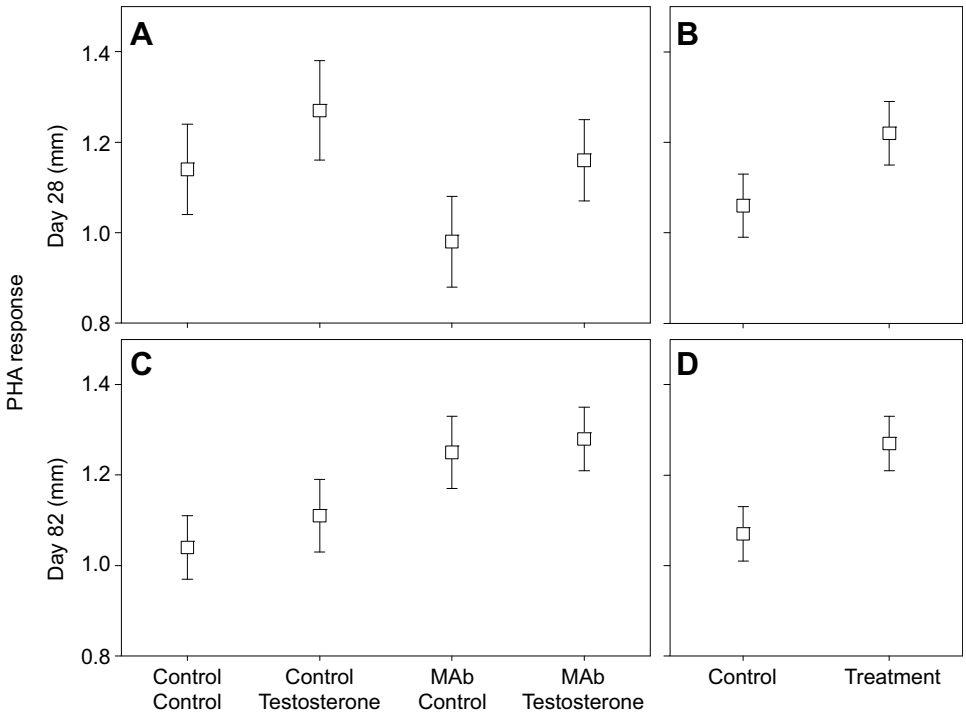

Fig. 3. Effects of MAb and yolk testosterone treatment on immune response to PHA injection. (A) All treatments at day 28 post-hatching. (B) The main effect of yolk testosterone at day 28: P=0.07. (C) All treatments at day 82 post-hatching. (D) The main effect of MAb at day 82: P=0.01. Values are least squares means±s.e.m. There was no sex effect or sex interaction in the model (see Results), so sexes have been pooled.

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

Table 4. Variation in courtship behaviour in male offspring at sexual maturity (day 90) in relation to their MAb and yolk testosterone treatment as chicks

Trait	Control+control (*n*=11)	Control+testosterone (*n*=9)	MAb+control (*n*=8)	MAb+testosterone (*n*=10)
% Invitation	68.8	100	66.7	83.3
Time to 1st mount (s)	138.1±49.3	29.6±13.8	155.0±53.9	83.1±43.2
No. of successful mounts/15 min	1.6±0.4	0.4±0.4	3.9±1.2	1.6±0.5
No. of bill wipes/15 min	15.3±5.6	20.9±3.1	13.5±3.6	20.7±5.3
Following behaviour/15 min	11.9±4.1	14.4±6.3	24.9±6.1	21.7±7.2

Values are means±s.e.m., *n*=sample size.

Numerous studies have shown positive relationships between circulating antibody concentrations in mothers (but not fathers) and antibody concentrations in eggs or offspring (Gasparini et al., 2002; Grindstaff, 2008; Kowalczyk et al., 1985). In chickens, a secondary immune response to *E. coli* LPS peaked ∼28 days after the first immunisation (Sunwoo et al., 1996); hence, by the time our females laid their first egg (∼21 days after the first injection), most of them should have been near the peak response phase to the immune challenge. Similarly, although we did not measure yolk testosterone, the dose of testosterone we injected corresponds to the difference in testosterone+dihydrotestosterone measured in yolks of eggs from females paired with attractive males versus unattractive males and thus mimics natural variation (Gil et al., 1999; von Engelhardt et al., 2006). This method of manipulating yolk testosterone has been widely used in zebra finches (Rutkowska et al., 2005; Tobler and Sandell, 2007) and other small passerines (Ruuskanen et al., 2009; Ruuskanen and Laaksonen, 2010; Tschirren et al., 2005) in studies of effects of maternal testosterone on offspring phenotype.

We acknowledge some potential limitations of our experimental design. Firstly, because of logistic constraints (∼80 breeding pairs initially), we did not use a cross-fostering design, so eggs were laid and chicks reared by the same mother. However, we did use a within-clutch design to control for any differences in maternal body condition and quality/quantity of parental care. Within each clutch, both egg treatments were represented so all eggs/chicks in each clutch from the different treatments would have been raised by the same quality/condition mother. LPS immunisation can reduce activity levels and the frequency of nestling feeding (Bonneaud et al., 2003). In our study, LPS treatment had no effect on body mass, breeding propensity or egg and clutch size, i.e. primary reproductive effort for traits thought to be related to female 'quality'. Furthermore, LPS treatment did not affect the number of chicks reared in our study, and sons of LPS-treated females had higher body mass, i.e. endpoints reflecting the outcome of parental care were not consistent with a negative effect of LPS. Several previous studies also found no effect of LPS on immunised females themselves (Bowers et al., 2012; Burness et al., 2018; Grindstaff, 2008; Martyka et al., 2018). LPS can induce fever and the systemic inflammatory immune response can involve changes in oxidative stress and antioxidant levels (van de Crommenacker et al., 2010). Our within-clutch design would mean that all eggs of all treatments from the same mother would have had similar 'background' antioxidant levels, even if these varied among females as a result of LPS treatment. However, we acknowledge that there could have been complex multivariate interactions between traits we manipulated and measured with one, or more, unmeasured egg component. This certainly highlights the complexity of conducting experiments on 'multivariate' eggs. In general, however, we think most studies support the idea that effects of experimental manipulation of egg components on offspring phenotype are directly related to changes in maternally derived egg components rather than indirect effects mediated by maternal condition or changes in absolute egg size (Grindstaff, 2008).

Although not the primary goal of our study, we found mixed evidence for effects of either MAb or yolk testosterone alone on offspring phenotype (mirroring contradictory results from many studies in the literature; von Engelhardt and Groothuis, 2011; Williams and Groothuis, 2015; Martyka et al., 2018; Burness et al., 2018). We found no short- or long-term effects of MAb or yolk testosterone, or their interaction, on hatching mass, size at fledging (tarsus length), body mass at sexual maturity (day 82), chick survival, humoral immune function in response to an LPS challenge or any measured female reproductive trait at sexual maturity. There was a marginally significant, positive effect of MAb on fledging body mass (day 30) and a significant MAb×sex interaction, whereby female offspring of MAb-treated mothers had lower day 30 mass and male offspring had higher day 30 mass. Some previous studies have reported positive effects of MAb on nestling growth (e.g. Grindstaff, 2008; Gallizzi et al., 2008) but experimental

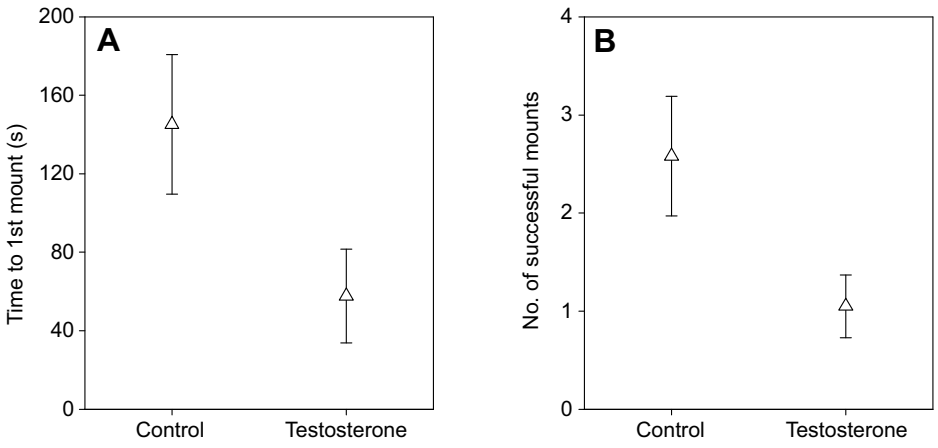

Fig. 4. Long-term main effect of yolk testosterone treatment on male courtship behaviour. (A) Time to first copulation: *P*=0.037. (B) Number of successful mounts in 15 min: *P*=0.043. Values are least squares means±s.e.m.

Journal of Experimental Biology (2019) 222, jeb196956. doi:10.1242/jeb.196956

Table 5. Variation in reproductive traits in female offspring at sexual maturity (day 90) in relation to their MAb and yolk testosterone treatment as chicks

Trait	Control+control (*n*=8)	Control+testosterone (*n*=11)	MAb+control (*n*=9)	MAb+testosterone (*n*=17)
Breeding propensity (%)	87.5	81.8	88.9	88.2
Laying latency (days)	7.6±1.1 (7)	8.9±1.0 (9)	8.6±1.1 (8)	7.6±0.8 (15)
Egg mass (g)	1.093±0.030 (47)	1.068±0.028 (47)	1.114±0.029 (47)	1.084±0.021 (88)
Clutch size	6.6±0.6 (7)	5.4±0.5 (9)	6.0±0.5 (8)	5.9±0.4 (15)
Brood size at fledging	2.7±0.8 (7)	1.8±0.7 (9)	1.4±0.8 (8)	1.9±0.6 (15)
Chick fledging mass (g)	12.7±0.3 (23)	11.9±0.3 (21)	12.0±0.3 (18)	12.5±0.2 (40)
Chick tarsus length (mm)	16.9±0.1 (23)	16.7±0.2 (21)	16.4±0.2 (18)	17.1±0.1 (40)
Hatching success (%)	61.7	59.8	74.5	55.7
Fledging success (%)	48.9	44.7	38.3	45.5

Brood size at fledging includes nests where brood size=0. Values are least squares means±s.e. with sample size in parentheses, or percentages.

manipulation of maternal IgG had no effect on offspring growth in other studies (Burness et al., 2018; Grindstaff et al., 2006). Martyka et al. (2011) reported a sex-specific effect of maternal immunisation with sheep red blood cell antigen, but in the opposite direction to our result: daughters, but not sons, of immunised mothers were heavier and had longer tarsi (although chicks were measured at 12 days in their study, only half-way through the nestling growth period).

In our study, there was a positive effect of yolk testosterone on offspring immune response to PHA at 26 days of age, but at 82 days of age, MAb had a positive effect on offspring immune response to PHA. Previous studies have produced similarly mixed results; for example, Martyka et al. (2018) reported short-term immune-enhancing effects of MAb on nestling immune function, Merrill and Grindstaff (2014) reported immune-suppressing effects and Burness et al. (2018) found no effects of MAb on offspring immune function. Reid et al. (2006) reported long-term immune enhancement in offspring of vaccinated mothers up to 1 year after maternal treatment in free-living song sparrows, *Melospiza melodia*, but Addison et al. (2010) found no long-term effects of MAb in Japanese quail (*Coturnix c. japonica*). Most previous studies involving manipulation of yolk testosterone have found no or inconsistent effects on offspring immune function (reviewed in von Engelhardt and Groothuis, 2011; and see below).

The most compelling result we found was an effect of yolk testosterone on male courtship behaviour at sexual maturity, where a greater proportion of yolk testosterone-treated males engaged in courtship (81% versus 46%), and yolk testosterone-treated males had shorter times to first mounting and fewer successful mounts. Although the last two results might seem counter-intuitive, 'better' males which mount females more quickly might need fewer attempts for functionally successful copulation (we defined successful mounts only as mounts with cloacal contact as we could not determine whether successful sperm transfer occurred). This would be consistent with positive, organisational effects of yolk androgens on dominance, competition and male sexual traits (Eising et al., 2006; Partecke and Schwabl, 2008; Schweitzer et al., 2013; Strasser and Schwabl, 2004; although, again, many studies reported negative or no effect on the latter: von Engelhardt and Groothuis, 2011; Uller et al., 2005; Vergauwen et al., 2014). In contrast to males, we found no effect of experimental treatment on female reproductive traits at sexual maturity. Fewer studies have considered effects of yolk testosterone on female traits. In a large-scale field study, Ruuskanen et al. (2012a,b) found no long-term effect of yolk testosterone manipulation on any breeding parameter, in either sex, including laying date, clutch size, number of hatchlings and fledging success. Rubolini et al. (2007) reported that females hatching from testosterone-treated eggs had a lower egg-laying rate than controls, higher rates of egg infertility but no difference in egg size, and there was no sex difference in yolk size among the eggs laid by control females. In contrast, Uller et al. (2005) found that females experiencing relatively high levels of testosterone during embryonic development laid smaller eggs as adults.

The main goal of our study was to determine whether there were synergistic or antagonistic (compensatory) effects of multiple maternally derived egg components. We predicted synergistic effects of MAb and yolk testosterone for nestling and immature growth traits, and for adult 'quality' traits, but compensatory or even additive negative effects for immune function traits (Table 1). Synergy would be detected if offspring treated with both MAb and yolk testosterone had higher trait values than offspring treated with either MAb or yolk testosterone alone. We found no statistically significant evidence for this for any measured trait, and absolute trait values were only higher for one trait: 30 day mass in males (with the opposite pattern in females; see Fig. 2A). Compensatory effects of MAb and yolk testosterone should involve offspring treated with both MAb and yolk testosterone having intermediate trait values compared with offspring treated with either MAb or yolk testosterone alone. Although we found intermediate values for several traits, such as 26 day PHA response (see Fig. 3A) and brood size at fledging among manipulated clutches, none of these effects were significant. To our knowledge, there have only been two previous experimental studies that have attempted to simultaneously manipulate more than one egg component to look for interacting, synergistic or antagonistic effects of multiple egg traits. In Japanese quail, treatment of eggs with either exogenous carotenoids or testosterone had weak negative effects on offspring phenotype (e.g. lower hatching mass) but when eggs were treated simultaneously with both carotenoids and testosterone, the detrimental effects were mitigated (Giraudeau et al., 2017). Possenti et al. (2018) manipulated both an anti-oxidant (vitamin E) and a putative pro-oxidant (corticosterone) and found that administration of vitamin E or corticosterone alone caused a reduction in body mass relative to controls, whereas the combined treatment reversed the negative effects.

In summary, three studies (this study; Giraudeau et al., 2017; Possenti et al., 2018) have failed to provide compelling evidence for strong, or long-term, synergistic effects of multiple, maternally derived egg components, although the latter two studies provide some evidence for short-term antagonistic effects. Although the task of fully investigating the concept of the 'multivariate egg' (*sensu* Postma et al. 2014; Williams and Groothuis, 2015) is clearly challenging, these studies highlight the importance of being able to predict *a priori* the direction and magnitude of effects of single egg components, to enable clear predictions about multivariate effects (see Table 1). It seems, to us, likely that species-specific effects, sex-specific effects, differences among short- and long-term endpoints,

and the importance of developmental and ecological context, that have confounded generalisable patterns of single maternal effects in avian studies will magnify the challenge of understanding the multivariate egg. Evidence for strong, long-term maternal effects for single egg components remains equivocal in birds (von Engelhardt and Groothuis, 2011; Williams, 2012; Williams and Groothuis, 2015). With only three multivariate (or at least bivariate) experimental studies, it is premature to conclude that consideration of interactions among multiple egg traits will not reveal stronger, long-term maternal effects. Clearly, therefore, additional research on multivariate maternal effects is warranted and will prove useful, regardless of the outcome (i.e. either confirming and elucidating the evolutionary significance and mechanisms of interactions among multiple maternal effects, or verifying that studying the multivariate egg provides no additional insight than studying single parameters). However, future experimental studies where two, or preferably more, egg traits are manipulated simultaneously must include long-term, fitness-related end-points (survival, future reproduction) and large-scale field studies (*sensu* Ruuskanen et al., 2016) would be especially valuable.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.T., T.D.W.; Methodology: R.T., E.C., R.R., T.D.W.; Validation: R.T., E.C.; Formal analysis: R.T., E.C., R.R., T.D.W.; Investigation: R.T., R.R., T.D.W.; Resources: T.D.W.; Data curation: R.T., T.D.W.; Writing - original draft: R.T., T.D.W.; Writing - review & editing: R.T., E.C., R.R., T.D.W.; Visualization: T.D.W.; Supervision: R.T., T.D.W.; Project administration: R.T., T.D.W.; Funding acquisition: T.D.W.

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Data availability
Data are available from the Dryad Digital Repository (Torres et al., 2019): https://doi.org/10.5061/dryad.j348s75/1.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.196956.supplemental

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