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Are there synergistic or antagonistic effects of multiple maternally-derived egg components (antibodies and testosterone) on offspring phenotype?

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Short title: Multivariate egg composition and offspring phenotype

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Summary

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 (most often yolk testosterone, or corticosterone), 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 offspring phenotype from hatching to sexual maturity. We used lipopolysaccharide treatment to generate a secondary immune response in female zebra finches (*Taeniopygia guttata*), which produced clutches of eggs with high (LPS-treated) or low (control) MAb. We then used a split design manipulating yolk testosterone within clutches of high- and low-MAb eggs using *in ovo* egg injection. 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 i/ males (sons) using standardise mating trials (courtship, song rate, etc); ii/ females (daughters) by measuring reproductive traits during breeding (egg size, clutch size etc), and iii/ cell-mediated and humoral immunity in both sexes.

Introduction

Eggs have complex macro- and micronutrient composition that serves not only as nutrients (resources) but potentially as 'signals', from the mother, that affect embryonic and post-hatching development and which, in turn, potentially affects offspring and adult phenotype and fitness in myriad ways (Gil 2008; von Engelhardt & Groothuis 2011; Williams 2012; Williams & Groothuis 2015). All egg components are maternally-derived, i.e. they are transferred from mother to egg during egg formation, and this is considered an important pathway for maternal effects: non-genetic contributions of the mother to her offspring, that are a major focus of life-history studies (Mousseau & Fox 1998; Badyaev & Uller 2009). There have been a very large number of studies on 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, or groups of related components, e.g. macronutrients (Hill 1995), hormones (Groothuis *et al.* 2005; Groothuis & von Engelhardt 2005; von Engelhardt & Groothuis 2011), antioxidants (Blount, Houston & Møller 2000), or antibodies (Grindstaff, Brodie & Ketterson 2003). However, given the complex compositional nature of eggs, if females do use maternally-derived egg components to fine-tune offspring phenotype to prevailing environmental conditions then there should be some 'optimum' *combination* of macro- and micronutrients with which females provision their eggs, i.e. we need to consider the "multivariate egg" (Postma *et al.* 2014; Williams & Groothuis 2015; Giraudeau *et al.* 2017). For example, as Boulinier and Staszewski (2008) pointed out it would appear counter-intuitive to produce an egg with high immunoglobulin content, perhaps to protect the naïve embryo in an immunologically challenging environment, that also had high yolk testosterone content which might simultaneously cause immunosuppression (see also, Postma et al. (2014). Similarly, an egg with high levels of both testosterone and thyroid hormone would presumably be more advantageous than one with high concentrations of only one of these hormones, given that both have potential growth-promoting effects.

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*) and suggested that evolution has not strongly selected for mechanisms that allow mother 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 immunoglobulins, yolk androgens and yolk total carotenoids in pied flycatcher (*Ficedula hypoleuca*) eggs, perhaps suggesting that different components might be regulated by different mechanisms (see also Eeva et al. (2011). Conversely, although Postma et al. (2014) found no association between maternally derived yolk IgG and yolk androgens in the great tit (*Parus major*) within clutches, across clutches these components were negatively correlated and they suggested 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 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*) to assess potential synergistic or antagonistic effects on offspring phenotype. 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 x 2 split design manipulating yolk testosterone (yolk-T) within clutches of high- and low-MAb eggs using *in ovo* egg injection. 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 standardise mating trials (courtship, song rate, etc) and females (daughters) by measuring reproductive traits during breeding (egg size, clutch size etc), and adult cell-mediated and humoral immunity in both sexes. We made a number of specific predictions based on previous literature studies for these two egg components (see Table 1 and Discussion).

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 x 39 cm x 43 cm) under controlled environmental conditions (temperature 19–23 °C; humidity 35–55%; constant light/dark schedule, 14L:10D, lights on at 07.00). 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 x 39 cm x 43 cm), each with an external nest box (14 cm x 14.5 cm x 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 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-sex communal cages with visual and acoustical contact with birds of the opposite sex, until essays to estimate breeding success were performed. The sex of survival nestlings was determined at 60-80 days of age base on their sexually dichromatic plumage.

Experimental procedure

To manipulate both, the amount of maternal antibodies and the level of androgens in the eggs, 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 manipulating yolk testosterone within clutches of high- and low-MAb eggs using *in ovo* egg injection of T on day 3 after the eggs were laid (Müller et al. 2005, 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 lipopolysaccharide (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) 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 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). Females' body mass (± 0.1g) was measured before each immune challenge, and when the first egg was laid, while tarsus length (± 0.1mm) was measured only during the first immune challenge.

For egg T treatment, on the day of laying eggs from the same clutch were randomly assigned to either the testosterone (hereafter, yolk-T 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 T-treated group were injected with 500 pg of testosterone (T, Fluka) 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 floats 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 lumens) and the needle was pushed through the shell at an upward angle. The hole in the shell was closed with a drop of cyanocrylate glue (Loctite gel control) and eggs were placed back in their nest once the glue was dry (~ 10 minutes). Eggs in the control group were injected 2 μl of sesame oil, but otherwise were treated in a similar way than eggs in the T treatment.

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

Cell-mediated and humoral immune response

Cell-mediated immune response of fledglings was evaluated, near independence (26 days) and when the birds reached sexual maturity (82 days), using the phytohemagglutinin (PHA) method widely used in ecological immunology studies (e.g. Alonso-Alvarez et al. 2004, Salvante 2006, Love et al. 2008). Birds were intradermally injected in the right-wing web (patagia) with 30 µg PHA (PHA-p, Sigma: L-9132) in 30 µL of sterile phosphate-buffered saline (PBS) using a monoject insulin syringe with a 27-gauge ½ 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 hrs after injection using a gauge micrometer (Dyer Company, model number 304196). The cell-mediated immunity 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 hrs after the PHA challenge.

To assess the potential long-term effects of MAb and yolk-T level on the humoral immunity of offspring, all offspring were immune challenged with an injection of LPS at 26 and 82 days post-hatching as described above and blood samples (80-100 µl) were taken before injection and 10 days post-injection (age 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 (hereafter, Ab titre, see below).

Assessment of treatment effects on adult phenotype

At 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, a clean, 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, a 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 the female and male courtship behaviour was recorded for 15 min. All of the courtship trials were performed between 0900 and 1200 h, and males were re-tested if they didn't show any courtship behaviour on their first trial (see Results). Five typical male courtship displays

(described in Zann, 1996) were recorded during the experiment: a) invitation (Y or N, i.e. did the male court the female), b) time in seconds to the initial mounting attempt; c) number of successful copulations, defined as those with cloacal contact; d) bill wiping (number of wipes), and e) following (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 (number of days between pairing and first egg), clutch size, mean egg mass, brood size at hatch, brood size at 21 days, and brood size at 30 days were recorded. For those that successfully raised chicks, the resulting chicks were weighed (± 0.01 g) and their bill, tarsus, wing, and P9 lengths (± 0.01 mm) were recorded at 21 and 30 days post-hatching (average age for chicks in the nest). Chicks were sexed between days 30 and 60 post-hatching in order to determine sex ratio of each nest.

LPS-specific antibodies determination

To determined LPS-specific antibodies we used an enzyme-linked immunosorbent assays (ELISA) and followed the procedures described in previous studies in zebra finches and other small passerines (Bonneaud et al. 2003, Müller et al 2005, Grindstaff et al. 2006).

Statistical analyses

All analyses were carried out using SAS v. 9.4 (add ref). General linear models (proc GLM) were used to evaluate the effect of an immune challenge on female (moms) Ab titers, mass, latency between pairing and egg laying, and mean egg mass. Hatching success and fledging success (coded as o or 1 for each treated egg) were analysed using generalized linear mixed model (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 T) 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 28 and days 82 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 log10 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 maternal Ab treatment and egg T treatment only (Wilcoxon two-sample test). Reproductive traits of adult females 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; Fisher's exact χ^2 = 1.62, P > 0.20). For n = 64 egg-laying females, 24 females (15 control; 9 LPS) did not incubate or produced infertile clutches, 8 females (3 control; 5 LPS) failed before fledging, and n = 32 females successfully fledged chicks (15 control; 17 LPS). Breeding outcome was independent of treatment (χ^2 = 2.06, P > 0.35).

There was no difference in female body between LPS-treated and control females at the 1-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 fledged chicks, LPS-treated females had higher LPS-specific Ab titres than control females ($F_{1,26}$ = 5.70, P = 0.025; Fig. 1). There was no effect of maternal treatment on the latency between pairing and laying of first egg, clutch size, or mean egg mass or brood size at fledging (P > 0.11 in all cases; Table 2).

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

Hatching mass was independent of maternal Ab treatment ($F_{1,54}$ = 0.04, P > 0.80) and egg T treatment ($F_{1,54}$ = 0.00, P > 0.90; Supplemental Table 1). 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 significant maternal treatment*sex interaction ($F_{1,52}$ = 6.09, P = 0.017; egg mass, $P <$ 0.001; Supplemental Table 1), so mass was analysed for each sex separately. For females there was no main effect of maternal Ab treatment ($F_{1,13}$ = 1.05, $P >$ 0.30), egg T 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 egg T treatment or the interaction term (P > 0.20) but there was a marginally significant effect of maternal Ab treatment ($F_{1,12}$ = 4.22, P = 0.06; Fig. 2a,b): males chicks from MAb egg were heavier than those from control eggs (13.6 ± 0.2 g vs. 13.1 ± 0.2 g). In a reduced model, excluding egg T treatment, there was a significant maternal Ab treatment*sex interaction ($F_{1,56}$ = 8.49, $P <$ 0.01): female offspring from MAb eggs had lower 30 day mass than controls, and the opposite pattern occurred in males (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; Supplemental Table 1).

Overall hatching success was independent of maternal Ab treatment ($F_{1,254}$ = 1.62, $P >$ 0.20), egg T treatment ($F_{1,254}$ = 1.16, $P >$ 0.25) and the interaction ($F_{1,254}$ = 0.26, $P >$ 0.60; egg mass, $P >$ 0.30; Table 3). Similarly, fledging success was independent of maternal Ab treatment ($F_{1,254}$ = 1.06, $P >$ 0.30), egg T treatment ($F_{1,254}$ = 0.51, $P >$ 0.45) and the 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 ($F_{3,31}$ = 0.68, $P >$ 0.55; Table 3).

Effects of MAb and yolk T treatment on offspring immune response

For PHA response at fledging (day 26) there was a marginally significant main effect of egg T treatment ($F_{1,52}$ = 3.25, P = 0.08; Fig. 3a,b) but PHA response was independent of all other main effects and interactions ($P >$ 0.20 in all cases; body mass, P = 0.09; Supplemental Table 2). In contrast, for PHA response at maturity (day 82) there was a main effect of maternal Ab 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; Supplemental Table 2)

Log10 LPS antibody titres in offspring at fledging (day 26) and at maturity (day 82) were independent of all main effects and interactions (P > 0.15 in all cases; Supplemental Table 3).

Effect of MAb and yolk T treatment on adult male courtship behaviour

Courtship trials were conducted on n = 49 male offspring at sexual maturity (day 90), and 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 maternal Ab treatment ($F_{1,18}$ = 0.68, P > 0.40) or a treatment interaction ($F_{1,18}$ = 0.18, P > 0.60). However, fewer males showed courtship behaviour in control eggs (46%, n = 28) compared with T-treated eggs (81.0%, n = 21; $F_{1,18}$ = 5.46, P = 0.031). Of the 19 males tested in a second trial, 8 performed some courtship. 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 1st copulation among the four combined maternal Ab/yolk T treatments (χ^2 = 4.83, df = 3, P = 0.18; Table 4). However, pooling data by egg treatment, time to 1st copulation was significantly shorter in T-treated eggs compared to control eggs (Z = 2.09, P = 0. 037; Fig. 4a). Conversely, pooling data by maternal Ab treatment there was no difference in time to 1st copulation (P > 0.90). For number of successful mounts/15 min there was a significant overall treatment effect (χ2 = 8.15, df = 3, P = 0.043). Males from control eggs had a higher number of successful mounts compared with males from T-treated eggs (Z = 2.06, P = 0.039; Fig. 4b). However, number of successful mounts was independent of maternal Ab 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 maternal Ab and egg T treatment were analysed separately (P > 0.05 in all cases).

Effect of MAb and yolk T treatment on adult female reproduction

Among females surviving to sexual maturity (n = 45) there was no effect of MAb, yolk T 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 (% females laying ≥ 1 egg) was high for all treatments: >80% (Table 5). There was no effect of MAb, yolk T treatment or their interaction on latency between pairing and laying of first egg, clutch size, or egg mass (P > 0.12 in all cases; Table 5). Hatching success was independent of maternal Ab treatment ($F_{1,189}$ = 0.41, P > 0.50), egg T treatment ($F_{1,189}$ = 1.83, P > 0.15) and the interaction ($F_{1,189}$ = 0.12, P > 0.70; Table 5). Similarly, fledging success was independent of maternal Ab treatment ($F_{1,189}$ = 0.13, P >

0.70), egg T treatment ($F_{1,189}$ = 0.01, P > 0.90) and the interaction ($F_{1,189}$ = 0.73, P > 0.35; Table 5).

Mean brood size at fledging did not vary among treatments ($F_{3,38}$ = 0.48, P > 0.60; Table 5). For chick mass at fledging (day 21) there was a maternal Ab*egg T 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 were significant (P > 0.30, with Bonferroni adjustment). For chick tarsus there was also a maternal Ab*egg T 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 Ab + T-treated females had longer tarsi than offspring of Ab + control-treated 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, maternally-derived antibodies (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 + T offspring should have higher trait values (e.g. growth rates, immune function) than controls and offspring treated with either Mab or T only (Table 1). If there were equal, antagonistic effects then MAb + T treated offspring should have lower trait values than offspring treated with either MAb or T only, 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 T 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 + T treated offspring might be intermediate between those of offspring treated independently with MAb or T-only (see below).

Although we did not measure LPS antibody titres in eggs, LPS-treated females did have higher plasma antibody 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 & Staszewski 2008; Grindstaff 2008). Numerous studies have shown positive relationships between circulating antibody concentrations in mothers (but not fathers) and antibody concentrations in eggs or offspring (Kowalczyk *et al.* 1985;

Gasparini *et al.* 2002; Grindstaff 2008). In chickens, a secondary immune response to *E. coli* LPS peaked ~28 days after the first immunization (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 T, the dose of T we injected corresponds to the difference in T + 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). Furthermore, this method of manipulating yolk T has been widely used in zebra finches (Rutkowska *et al.* 2005; Tobler & Sandell 2007), and other small passerines (Tschirren *et al.* 2005; Ruuskanen *et al.* 2009; Ruuskanen & Laaksonen 2010) in studies of effects of maternal T on offspring phenotype.

LPS-immunisation has no effect of body mass, breeding propensity or breeding productivity in adult females, even though LPS-treated females had higher LPS-specific Ab titres than control females at the time of laying. Several previous studies also found no effect of LPS on immunised females themselves (Grindstaff 2008; Bowers *et al.* 2012; Burness *et al.* 2018; Martyka *et al.* 2018). Collectively, these studies support the idea that effects 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 maternal antibodies 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 T, or their interaction, on hatching mass, size at fledging (tarsus), 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 manipulation of maternal immunoglobulins had no effect on offspring growth in other studies (Grindstaff *et al.* 2006;

Burness *et al.* 2018). 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 immunized mothers were heavier and had longer tarsi (although chicks were measured at 12 days only half way through the nestling growth period).

In our study, there was a positive effect of yolk T on offspring antibody titers at 26 days of age but at 82 days of age MAb had a positive effect on offspring antibody titers. 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-enhancing in offspring of vaccinated mothers up to one year after maternal treatment in free-living song sparrows, *Melospiza melodia*, but Addison et al. (2010) found no long-term effects on MAb in Japanese quail (*Coturnix c. japonica*). Most previous studies involving manipulation of yolk T 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 T on male courtship behaviour at sexual maturity, where yolk T-treated males had much shorter times to first mounting, and fewer successful mounts. Although this result might seem counter-intuitive 'better' males which mount female quickly, might need fewer attempts for functionally successful copulation (we defined successful mounts only as mounts with cloacal contact and could not determine if successful sperm transfer occurred). This would be consistent with positive, organisational effects of yolk androgens on dominance, competition, and male sexual traits (Strasser & Schwabl 2004; Eising, Muller & Groothuis 2006; Partecke & Schwabl 2008; Schweitzer *et al.* 2013); 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 T on female traits. In a large-scale field study, Ruuskanen et al. (2012a; 2012b) found no long-term effect of yolk T 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 T-treated eggs had a lower egg-laying rate than controls, higher rates of egg infertility but no difference in egg size 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 if there were synergistic or antagonistic (compensatory) effects of multiple maternally-derived egg components. We predicted synergistic effects of Mab and yolk T 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 both with MAb and yolk T had *higher* trait values than offspring treated with either MAb or yolk T alone. We found no statistically significant 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 T should involve offspring treated both with MAb and yolk T having *intermediate* trait values compared with offspring treated with either MAb or yolk T 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 has only been two experimental study that has 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 pre-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) and Possenti et al. (2018) have failed to provide compelling evidence for strong, and long-term, synergistic effects of multiple, maternally-derived egg components; though the latter two studies provide some evidence for short-term antagonistic effects. Although the task of fully investing 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). Nevertheless, it seems 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 generalizable patterns of single maternal effects in avian studies will magnify the challenge of understanding the multivariate egg.

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Table 1. Predictions for effects of manipulation of zebra finch eggs with either yolk testosterone (yolk-T) or maternally-derived antibodies (MAb) only, and the combined effect, on offspring and adult phenotype

Treatment	**Sex**	**Immature phenotype**		**Adult 'quality'**
		Growth	Immune function	
Yolk-T –only	M	+	-	+
	F	+	-	-
MAb-only	M	+	+/- [b]	+
	F	+	+/-	+
Yolk-T + MAb	M	+++ [a]	0/--- [c]	+
	F	+++	0/---	+

[a] +++ Indicates additive positive effect if T-only and MAb-only both have positive effects

[b] +/- Previous studies have reported positive and negative effects of MAb-only treatment

[c] 0/--- indicates either no net effect where T-only is negative and MAb-only is positive, or an additive negative effect if T-only and MAb-only are negative

Table 2. Reproductive traits in control and LPS-treated adult females (mothers). Values are lsmeans ± S.E. with sample size in parentheses.

Trait	Control (n = 15)	LPS-treated (n = 17)	F	P
Laying interval (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	0.72	0.40
Brood size at fledging	3.5 ± 0.3	3.6 ± 0.3	0.01	0.91

[a] Controlling for female body mass at 1-egg stage

[b] Controlling for laying interval

Table 3. Hatching success, fledging success and brood size at fledging in relation to MAb and yolk T treatment. Values are percentages or lsmeans ± S.D.

Variable	**Egg treatment**			
	Cont + Cont	**MAb + Cont**	**Cont + T**	**MAb + T**
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
Body mass at maturity (day 82, g)	14.2 ± 0.4	14.7 ± 0.4	14.8 ± 0.4	14.4 ± 0.3

Table 4. Variation in courtship behaviour in male offspring at sexual maturity (day 90) in relation to their MAb and yolk T treatment as chicks. Values are means ± S.E. with sample size in parentheses.

Trait	**Cont + Cont (n = 11)**	**Cont + T (n = 9)**	**MAb + Cont (n = 8)**	**Mab + T (n = 10)**
% invitation	68.8	100	66.7	83.3
Time to 1st mount (sec)	138.1 ± 49.3	29.6 ± 13.8	155.0 ± 53.9	83.1 ± 43.2
No. successful mounts/15 min	1.6 ± 0.4	0.4 ± 0.4	3.9 ± 1.2	1.6 ± 0.5
No. 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

Table 5. Variation in reproductive traits in female offspring at sexual maturity (day 90) in relation to their MAb and yolk T treatment as chicks (brood size at fledging includes nests where brood size = 0). Values are lsmeans ± S.E. with sample size in parentheses, or percentages.

Trait	**Cont + Cont** (n = 8)	**Cont + T** (n = 11)	**MAb + Cont** (n = 9)	**MAb + T** (n = 17)
Breeding propensity (%)	87.5	81.8	88.9	88.2
Laying interval (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 (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

Figure legends

Fig. 1. Effects of LPS treatment on adult female (mothers) antibody titres at the time of egg-laying. Values are lsmeans ± S.E.

Fig. 2. Effects of a) MAb and yolk T treatment and b) MAb treatment only, on body mass of male (open triangles) and female chicks (closed circles) at day 30 post-hatching. Values are lsmeans ± S.E.

Fig. 3.

Fig. 4.

Fig. 1.

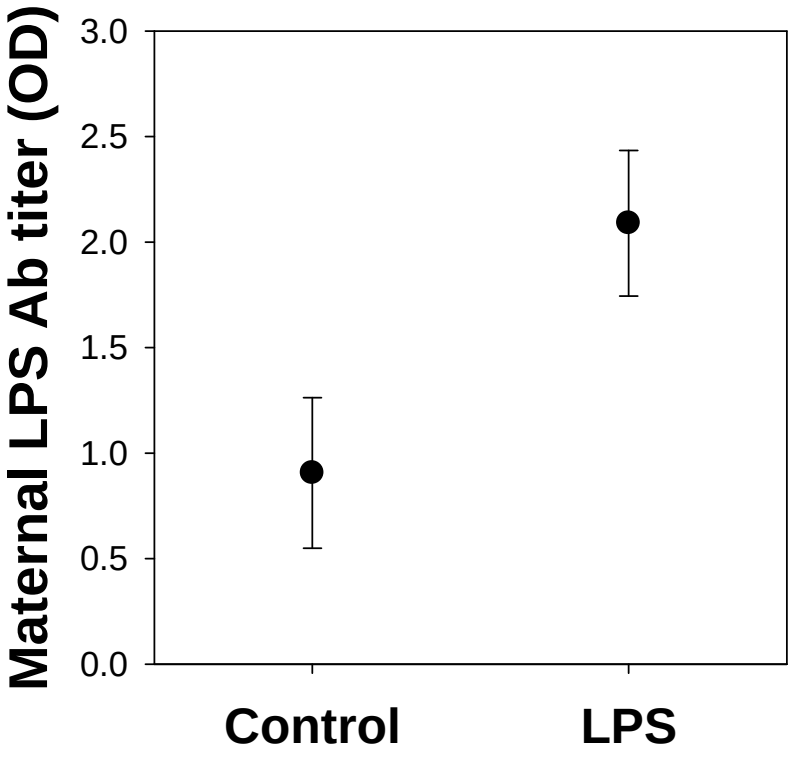


Fig. 2.

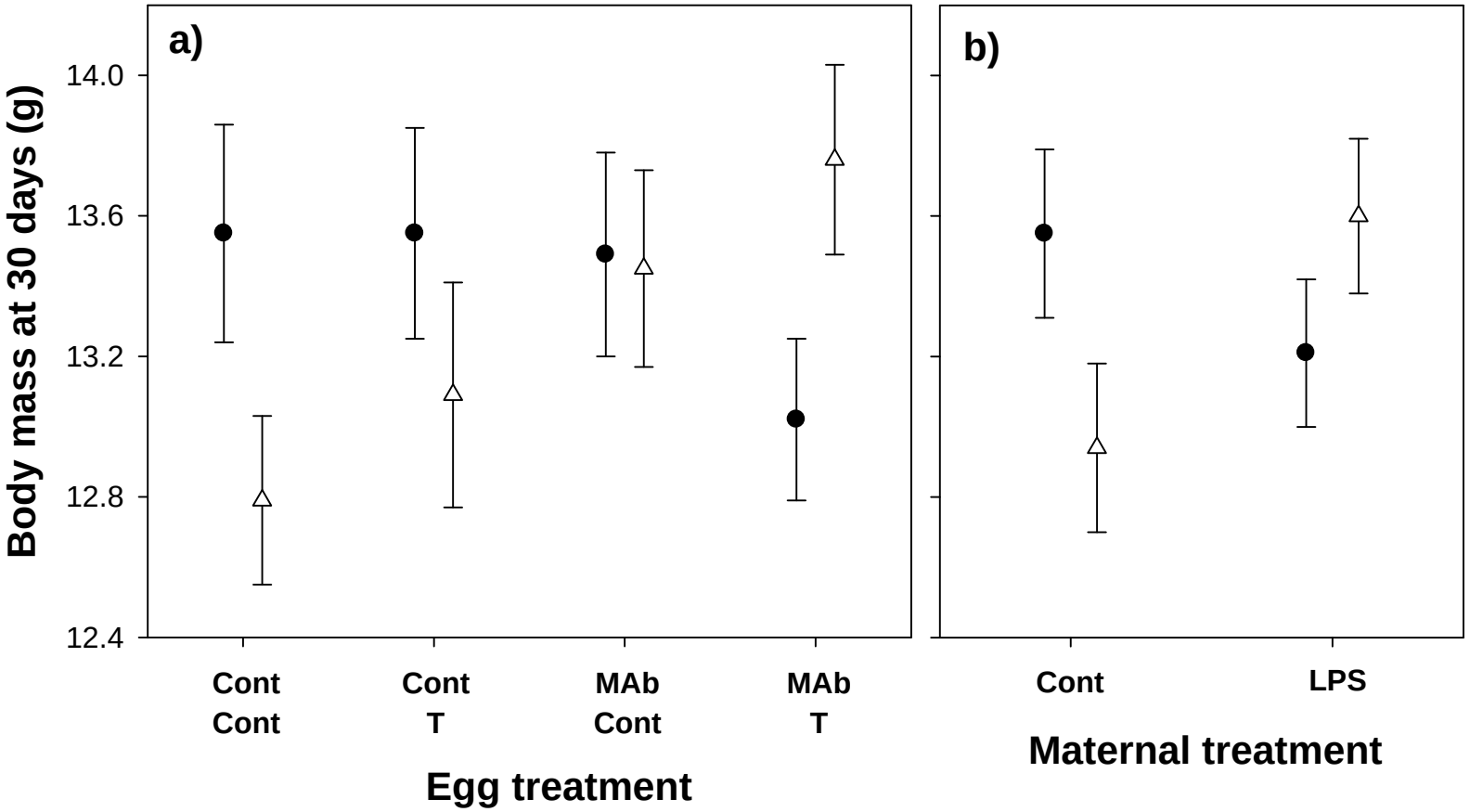

a)
b)
Body mass at 30 days (g)
14.0
13.6
13.2
12.8
12.4
Cont
Cont
Cont
T
MAb
Cont
MAb
T
Egg treatment
Cont
LPS
Maternal treatment

Fig. 3.

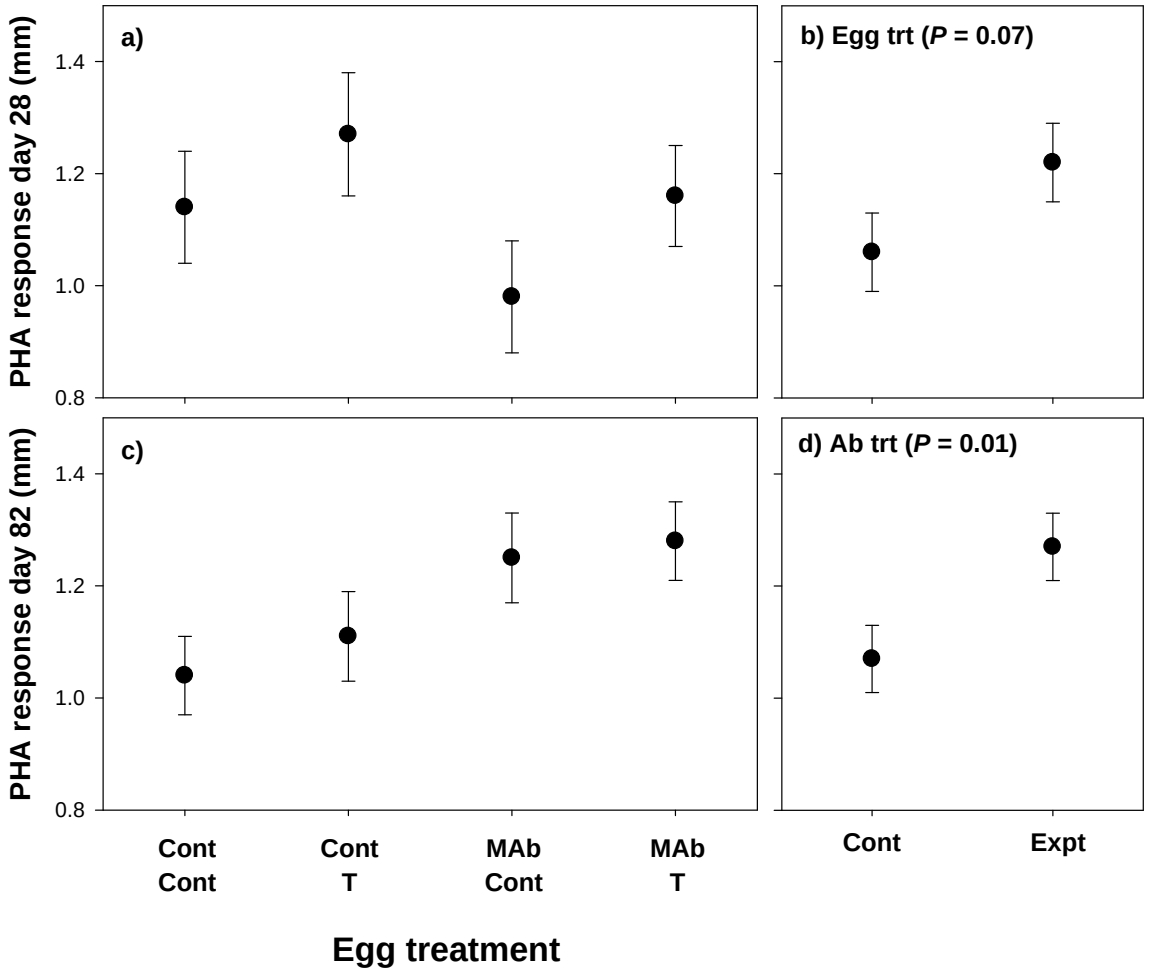
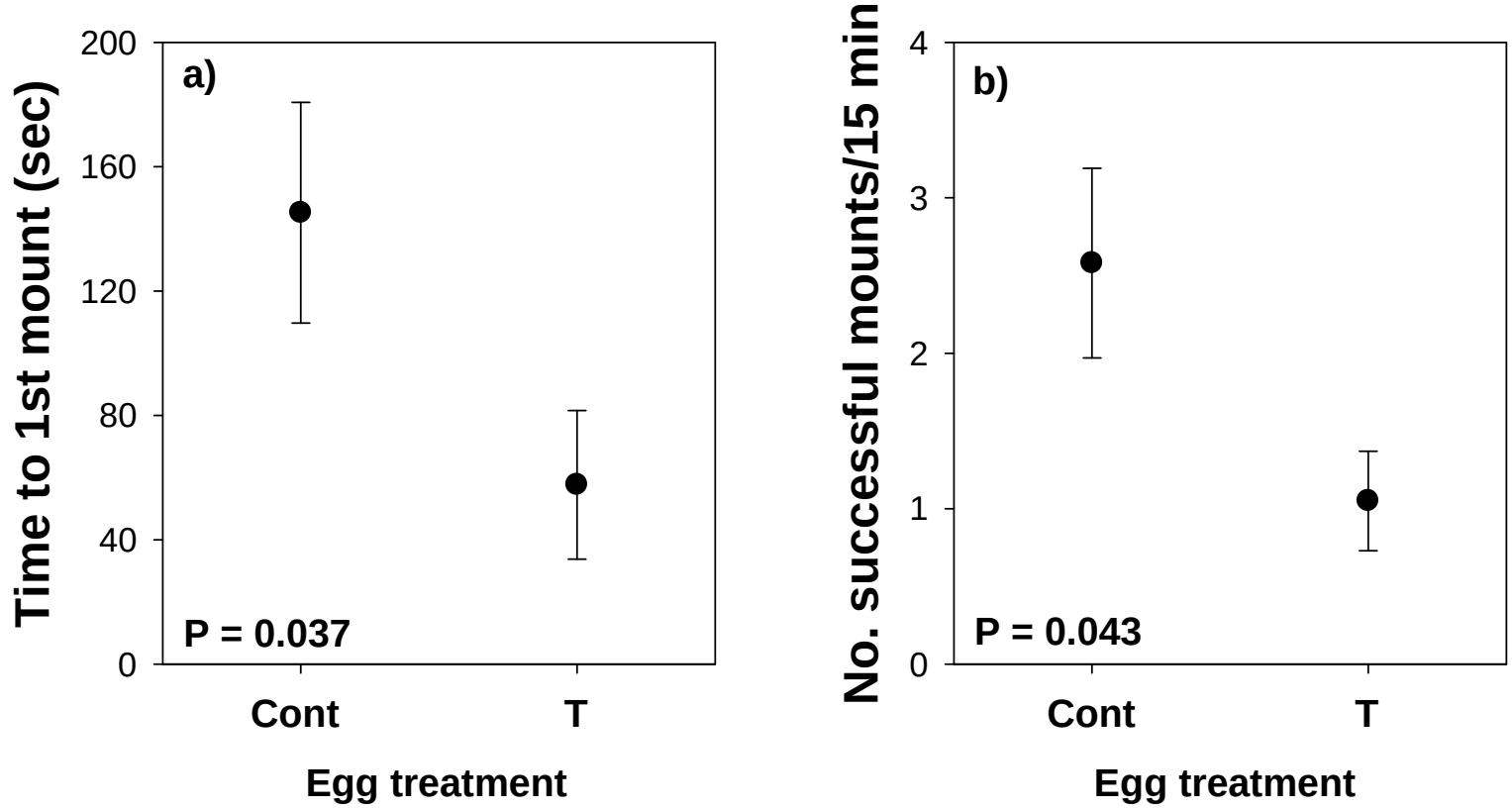

a)
b) Egg trt (P = 0.07)
c)
d) Ab trt (P = 0.01)
PHA response day 28 (mm)
PHA response day 82 (mm)
1.4
1.2
1.0
0.8
Cont Cont
Cont T
MAb Cont
MAb T
Cont
Expt
Egg treatment

Fig. 4.


a)
Time to 1st mount (sec)
200
160
120
80
40
0
P = 0.037
Cont
T
Egg treatment
b)
No. successful mounts/15 min
4
3
2
1
0
P = 0.043
Cont
T
Egg treatment