The multivariate egg: quantifying within- and among-clutch correlations between maternally derived yolk immunoglobulins and yolk androgens using multivariate mixed models

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Abstract: Egg components are important mediators of prenatal maternal effects in birds and other oviparous species. Because different egg components can have opposite effects on offspring phenotype, selection is expected to favour their mutual adjustment, resulting in a significant covariation between egg components within and/or among clutches. Here we tested for such correlations between maternally-derived yolk immunoglobulins and yolk androgens in great tit (Parus major) eggs using a multivariate mixed model approach. We found no association between yolk immunoglobulins and yolk androgens within clutches, indicating that within clutches the two egg components are deposited independently. Across clutches, however, there was a significant negative relationship between yolk immunoglobulins and yolk androgens, suggesting that selection has co-adjusted their deposition. Furthermore, an experimental manipulation of ectoparasite load affected patterns of covariance among egg components. Yolk immunoglobulins are known to play an important role in nestling immune defence shortly after hatching, whereas yolk androgens, although having growth-enhancing effects under many environmental conditions, can be immunosuppressive. We therefore speculate that variation in the risk of parasitism may play an important role in shaping optimal egg composition and may lead to the observed pattern of yolk immunoglobulin and yolk androgen deposition across clutches. More generally, our case study exemplifies how multivariate mixed model methodology presents a flexible tool to not only quantify, but also test patterns of (co)variation across different organisational levels and environments, allowing for powerful hypothesis testing in ecophysiology.

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The multivariate egg: quantifying within- and among-clutch correlations between maternally derived yolk immunoglobulins and yolk androgens using multivariate mixed models

Erik Postma¹, Heli Siitari², Hubert Schwabl³, Heinz Richner⁴ and Barbara Tschirren¹*

¹Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland
²Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland
³School of Biological Sciences, Centre for Reproductive Biology, Washington State University, Pullman, WA 99164-4236, USA
⁴Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland

*Correspondence: Barbara Tschirren, Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland; Tel: +41 44 635 47 77, Fax: +41 44 635 68 18; Email: barbara.tschirren@ieu.uzh.ch

Author Contributions: BT designed the study and performed data collection; HS, HSch and HR developed methodology; BT and HS performed laboratory analyses; EP performed statistical analyses; BT and EP wrote the manuscript, all other authors commented on the manuscript
Abstract

Egg components are important mediators of prenatal maternal effects in birds and other oviparous species. Because different egg components can have opposite effects on offspring phenotype, selection is expected to favour their mutual adjustment, resulting in a significant covariation between egg components within and/or among clutches. Here we tested for such correlations between maternally-derived yolk immunoglobulins and yolk androgens in great tit (*Parus major*) eggs using a multivariate mixed model approach. We found no association between yolk immunoglobulins and yolk androgens within clutches, indicating that within clutches the two egg components are deposited independently. Across clutches, however, there was a significant negative relationship between yolk immunoglobulins and yolk androgens, suggesting that selection has co-adjusted their deposition. Furthermore, an experimental manipulation of ectoparasite load affected patterns of covariance among egg components. Yolk immunoglobulins are known to play an important role in nestling immune defence shortly after hatching, whereas yolk androgens, although having growth-enhancing effects under many environmental conditions, can be immunosuppressive. We therefore speculate that variation in the risk of parasitism may play an important role in shaping optimal egg composition and may lead to the observed pattern of yolk immunoglobulin and yolk androgen deposition across clutches. More generally, our case study exemplifies how multivariate mixed model methodology presents a flexible tool to not only quantify, but also test patterns of (co)variation across different organisational levels and environments, allowing for powerful hypothesis testing in ecophyiology.
Key words: maternal antibodies, yolk testosterone, egg composition, parasites, maternal effects, adaptation
Introduction

The environment a mother provides for her young early in their life – even before they are born – can have significant and long-lasting consequences for offspring phenotype and performance (Mousseau and Fox 1998; Christians 2002; Monaghan 2008). Such transgenerational phenotypic alterations in response to environmental conditions have been suggested to play a key role in the adaptation to variable, but predictable environments, and to potentially accelerate evolutionary responses to environmental change (Kirkpatrick and Lande 1989; Cheverud and Moore 1994; Wolf et al. 1998; Räsänen and Kruuk 2007).

Parasites are an environmental factor that can strongly impair host fitness and that requires alterations in host morphology, physiology and/or behaviour to mitigate parasite impact. Maternal effects have been shown to play an important role in the adaptive alteration of offspring phenotype in response to parasitism (e.g. Heeb et al. 1998; Curno et al. 2009; Ewen et al. 2009). In particular maternally transferred antibodies are crucial for parasite defence early in life. Shortly after birth, the immune system of vertebrates is not yet fully functional and juveniles depend, at least partly, on antibodies received from their mother through placenta, milk or yolk to fight parasites and pathogens (reviewed in Grindstaff et al. 2003; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009). There are now a number of studies showing that female birds increase yolk antibody concentrations in response to parasitism or experimental immune challenges (Gasparini et al. 2001; Grindstaff 2008), and that maternal antibodies improve specific immune responses and/or provide partial disease protection in the young (Gasparini
Maternal antibodies are, however, not the only resources mothers can provide to alter offspring fitness. Maternal yolk hormones (in particular androgens), deposited by female birds during egg formation, have been shown to increase growth rate, begging vigour, competitive abilities, aggressiveness and ornamentation (reviewed in Groothuis et al. 2005b; Gil 2008). These traits will, under many social and environmental circumstances, increase offspring survival and/or reproduction. At the same time, however, exposure to high concentrations of maternal yolk androgens before birth may come at a cost in the form of reduced offspring immunocompetence (Hirota et al. 1976; Groothuis et al. 2005a; Müller et al. 2005; Navara et al. 2005, but see e.g. Tschirren et al. 2005; Kankova et al. 2012). Depositing low concentrations of yolk androgens into the eggs when parasite prevalence is expected to be high may therefore increase the offspring’s immunological defence capacity. Thereby, immunological costs of prenatal exposure to high yolk androgen concentrations might contribute to the maintenance of variation in yolk hormone deposition in natural populations experiencing variation in parasite prevalence (Tschirren et al. 2009a). Indeed, parasites have been identified as an environmental factor that influences yolk hormone deposition in the wild (Tschirren et al. 2004; Gil et al. 2006).

Hen fleas (Ceratophyllus gallinae) are common, nest-based ectoparasites of hole-breeding passerines (Tripet and Richner 1997). Because fleas overwinter in the old nesting material, and are therefore present before the female starts egg laying (Tripet et al. 2002), females can, at least in theory, predict the
future flea load of their offspring. This makes the bird-flea host-parasite system an ideal model to test for adaptive maternal effects in response to parasitism. In line with the hypothesis that mothers adaptively alter egg composition in response to parasitism, previous studies have shown that an experimental manipulation of flea load in the nests of great tits (Parus major) before egg laying lead to the deposition of higher concentrations of maternal yolk immunoglobulins (Buechler et al. 2002) and lower concentrations of maternal yolk androgens (Tschirren et al. 2004). Based on these findings, we hypothesise that females should either produce eggs with high immunoglobulin and low androgen concentrations or eggs with low immunoglobulin and high androgen concentrations, and that the optimal strategy depends on parasite prevalence. We thus predict that selection has co-adjusted the deposition of these two egg components to increase offspring and/or maternal fitness, resulting in a negative correlation between immuno-enhancing immunoglobulins and immunosuppressive androgens (see also Groothuis et al. 2006; Hargitai et al. 2009). Because nestlings within a nest are likely to be exposed to similar levels of parasitism, whereas parasite load can vary substantially among nests, we expect this negative correlation to be particularly pronounced at the among-clutch level. Here we tested this prediction by quantifying among- and within-clutch correlations of yolk immunoglobulin and yolk androgen deposition in great tit eggs using a multivariate mixed model approach. This approach provides a powerful and flexible statistical framework to both quantify and test patterns of variation and covariation across different organisational levels and environments.
Material and Methods

Study population and parasite manipulation

The study was performed in a great tit population breeding in nest-boxes in a deciduous forest in Switzerland. During winter, all old nesting material was removed from the boxes to ensure that boxes were parasite-free. When the new breeding season started, we monitored nestbuilding activity and manipulated the load of nest-based ectoparasites (hen fleas, *Ceratophyllum gallinae*) once the nest cup was constructed (7.7 ± 0.6 days before the female laid the first egg). Thereto, we heat-treated all nests in a microwave oven following Richner et al. (1993) to kill fleas that immigrated into the boxes during nest building. Afterwards, nests were randomly assigned to be either infested with 25 female and 15 male hen fleas or to remain free of parasites. Hen fleas used for the manipulation were extracted from old nests collected in the study area during winter. After the manipulation, we visited the boxes daily to determine the start of egg laying. The first, third, sixth and then every other egg, and the last egg of a clutch was collected on the day it was laid. Collected eggs were immediately replaced with an artificial egg. In total, we collected 300 eggs from 27 parasitized and 27 parasite-free nests. Yolks were separated from the albumen and frozen at −20 °C until hormone and immunoglobulin (IgG) analysis. The experiments were conducted under a license provided by the Ethical Committee of the Office of Agriculture, Bern, Switzerland.
Yolk androgen assays

Yolks were homogenized with an equal (1µl per mg of yolk) amount of distilled water. Aliquots of this yolk–water emulsion (250 mg) were used for the quantification of yolk androstenedione (A4), yolk testosterone (T) and yolk 5α-dihydrotestosterone (DHT) concentrations using radioimmunoassay (RIA). The extraction of androgens and RIA procedures were performed according to published protocols (see Schwabl 1993; Tschirren et al. 2004 for details).

Yolk IgG assays

Yolk IgG concentrations were determined using enzyme-linked immunosorbent assay (ELISA) as described in Tschirren et al. (2009b). In brief, we centrifuged the homogenised yolk samples and collected the clear supernatant containing the immunoglobulins for analysis. 96-well ELISA-plates (Nunc™ Immunoplate) were coated with anti-chicken IgG (Sigma C-6409) 1:180 in 50mM carbonate buffer (pH 9.6). The plates were then incubated at 4°C overnight. After emptying the wells, they were masked with 1% BSA-PBS (Roche Diagnostics) for 1 hour and washed 3 x 200µl with PBS-Tween. Samples and their replicates (50µl / well) were diluted with 1% BSA-PBS and added to the wells (dilutions of 1:2000 and 1:4000 of the supernatant).

To determine total IgG levels, a standard of pooled yolks (50 µl / well; diluted with 1% BSA-PBS) was added to each plate, and was assigned a concentration of 10⁶. All values were subsequently expressed relative to this standard. Samples and standards were incubated for 3 hours at room temperature. After washing the plates (3 x 200µl with PBS-Tween), an
alkaline phosphatase conjugated antibody (Sigma A-971 anti-chicken IgG; diluted with 1% BSA-PBS 1:10000) was added to the wells and incubated overnight at 4°C. Finally, after washing the wells (3 x 400µ with PBS-Tween) an alkaline phosphatase substrate PNPP (p-nitrophenyl phosphate, Sigma 104® phosphatase substrate) in 1M diethanol amine buffer (1 mg/ml) was applied (50µl / well). The absorbance of the wells was read in an ELISA reader at 405 nm for up to 1 hour (or until the highest standard reached the absorbance 2.0).

Statistical analyses
Ecophysiology frequently deals with inherently multivariate data, yet multivariate mixed model approaches are still relatively rarely used to test for differences in the associations among multiple physiological traits across different organisational levels and environments. Here we used a multivariate mixed model approach to test whether correlations between maternally derived yolk IgG and yolk androgens are significantly different from zero, whether the within- and among-clutch correlations are significantly different from each other, and whether they are affected by the presence of parasites. To this end, we simultaneously estimated all within- and among-nest (co)variances and correlations by fitting a restricted maximum likelihood (REML) multivariate mixed model, with yolk IgG and the three yolk androgens A4, T and DHT as the dependent variables, and female ID (nested in parasite treatment) as a random effect.

Yolk IgG (U / mg yolk) and androgen concentrations (pg / mg yolk) were log transformed and standardized for the statistical analyses. Significance of each
within- and among-clutch correlation was tested by constraining it to zero, and
testing whether this resulted in a significantly worse fit of the model to the data
using a likelihood-ratio test with one degree of freedom (Pinheiro and Bates
2000). To avoid the statistical complications associated with multiple testing
and to maximise statistical power, we furthermore compared a model in which
all within- or among-clutch correlations involving yolk IgG and the three yolk
androgens were constrained to zero to an unconstrained model using a
likelihood-ratio test with three degrees of freedom.
Using a similar approach, we tested whether the within- and among-clutch
correlations were significantly different from each other by comparing a model
in which within- and among-clutch correlations were constrained to be the
same to an unconstrained model. Again, we did this both for all three
correlations involving yolk IgG and yolk androgens at once (likelihood-ratio
test with three degrees of freedom) and for all combinations of traits
separately (likelihood-ratio test with one degree of freedom).
In a last step, we tested whether the relationship between yolk IgG and yolk
androgens was affected by the parasite treatment, providing insights into the
environmental (i.e. parasite) dependency of within- and among-clutch patterns
of (co)variance. To this end, (log-transformed and standardised) yolk IgG and
yolk androgen concentrations from nests with and without parasites were
considered separate traits, resulting in a total of eight dependent variables.
Because each clutch was either from a parasitized or from a parasite-free
nest, the correlations among yolk IgG and yolk androgen concentrations
across treatments cannot be estimated and had to be constrained to zero.
This results in a total of eight variances and twelve correlations to be
estimated. Although this provides us with (co)variance and correlation estimates that are identical to those from two separate treatment-specific models, our approach makes it possible to directly test whether the correlations between yolk IgG and yolk androgens are significantly different in the absence or presence of parasites (i.e. similar to testing for an interaction with parasite treatment). As in the overall analyses outlined above, this is achieved by constraining one or more correlations with and without parasites to be the same, and comparing its logLikelihood value to that of an unconstrained model.

Although all significance testing is based on likelihood-ratio tests, approximate standard errors for all variance ratios and correlations were estimated for completeness. All mixed model analyses were performed in ASReml 3 (Gilmour et al. 2009). Means ± 1SE are presented.

Results

Correlations between yolk IgG and yolk hormones within and among clutches

The complete correlation matrix (including approximate standard errors) is given in Table 1. In short, clutch (i.e. female) identity explained a large proportion of variation in maternal yolk IgG (70.1 ± 4.8 %), yolk A4 (62.0 ± 5.7 %), yolk T (63.8 ± 5.5 %) and yolk DHT (66.1 ± 5.3 %) concentrations. There was a consistent positive relationship between A4 and T, whereas the relationship between DHT and the other androgens was weaker and more variable.

Among clutches, there was a significant negative association between maternal yolk IgG and yolk androgen concentrations ($\chi^2 = 10.38, DF = 3, P =$
Androgen-specific estimates revealed that this negative association was to a large degree driven by a negative correlation between yolk IgG and yolk DHT ($\chi^2 = 4.67$, $DF = 1$, $P = 0.031$). However, although the associations with yolk T ($\chi^2 = 0.30$, $DF = 1$, $P = 0.585$) and yolk A4 ($\chi^2 = 1.87$, $DF = 1$, $P = 0.171$) were weaker and by themselves not significantly different from zero, they were negative as well (Fig. 1).

Within clutches, maternal yolk IgG and maternal yolk androgen concentrations were not significantly correlated ($\chi^2 = 2.64$, $DF = 3$, $P = 0.450$). Androgen-specific estimates revealed weak positive, but non-significant correlations between maternal yolk IgG and yolk A4 ($\chi^2 = 0.61$, $DF = 1$, $P = 0.435$), yolk T ($\chi^2 = 0.07$, $DF = 1$, $P = 0.794$) and yolk DHT ($\chi^2 = 1.25$, $DF = 1$, $P = 0.264$) concentrations (Fig. 1; Table 1). When comparing the within- and among-clutch correlations between maternal yolk IgG and yolk androgen concentrations, we found that they were significantly different from each other ($\chi^2 = 12.67$, $DF = 3$, $P = 0.005$, Fig. 1).

Effects of parasite treatment on the correlations between yolk IgG and yolk androgen concentrations

The complete treatment-specific correlation matrices are given in Table 2. Among clutches, we found that the correlations between maternal yolk IgG and yolk androgens tended to be different between flea-free and flea-infested nests ($\chi^2 = 6.93$, $DF = 3$, $P = 0.074$). Androgen-specific estimates revealed that this difference was mainly due to a significantly negative correlation between maternal yolk IgG and yolk A4 in flea-free nests ($\chi^2 = 6.91$, $DF = 1$, $P = 0.009$), but a weak and non-significant positive correlation in flea-infested nests.
nests ($\chi^2 = 0.21, DF = 1, P = 0.644$; difference in the correlation between yolk IgG and yolk A4 between treatment groups: $\chi^2 = 4.72, DF = 1, P = 0.030$).

Although the correlation between IgG and yolk T was stronger in flea-free nests than in flea-invested nests, they were not significantly different from each other ($\chi^2 = 0.88, DF = 1, P = 0.348$). Finally, the correlation between maternal yolk IgG and yolk DHT was negative in both flea-infested and flea-free nests, and although the correlation was stronger in flea-invested nests, the two correlations were again not significantly different from each other ($\chi^2 = 1.23, DF = 1, P = 0.268$).

Within clutches, the correlation between maternal yolk IgG and yolk androgens tended to be more pronounced in flea-free than in flea-infested nests ($\chi^2 = 6.54, DF = 3, P = 0.088$). Androgen-specific estimates showed that this difference was mainly due to the correlation between maternal yolk IgG and yolk DHT, which was significantly positive in flea-free nests ($\chi^2 = 6.63, DF = 1, P = 0.010$), but weakly and non-significantly negative in flea-infested nests ($\chi^2 = 0.56, DF = 1, P = 0.453$; difference in the correlation between yolk IgG and yolk DHT between treatment groups: $\chi^2 = 5.55, DF = 1, P = 0.019$).

For maternal yolk IgG and yolk A4 ($\chi^2 = 0.23, DF = 1, P = 0.633$) and maternal yolk IgG and yolk T ($\chi^2 = 0.38, DF = 1, P = 0.540$), the correlations were weak and did not differ significantly between treatment groups.
We have shown that maternal yolk immunoglobulin and yolk androgen concentrations (and in particular yolk DHT, which has the highest affinity for the androgen receptor and hence the greatest potency of all three androgens (Groothuis and Schwabl 2008)) correlate negatively across great tit clutches, which is in line with selection having favoured the mutual adjustment of these egg components. Indeed, maternally derived immunoglobulins play an important role in immune defence against pathogens early in a bird’s life (reviewed in Grindstaff et al. 2003; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009, but see Tschirren et al. 2009b), whereas maternally-derived yolk androgens can be immunosuppressive (Groothuis et al. 2005a; Müller et al. 2005; Navara et al. 2005, but see e.g. Tschirren et al. 2005; Kankova et al. 2012). Parasite-mediated selection might therefore play an important role in shaping optimal egg composition, and lead to a negative correlation between these egg components. Alternatively to the scenario outlined above, the co-adjustment of immunoglobulins and androgens might be beneficial for the mother, rather than the offspring, and the patterns observed in the eggs might simply reflect a passive, and not necessarily adaptive transfer from the mother’s circulation (but see Heylen et al. 2012). Although the question whether the concentrations of maternally-derived immunoglobulins and androgens in the eggs are a reflection of the concentrations present in the mother’s circulation is still debated, there is evidence that the deposition of egg components is, at least to a certain degree, independent of the female’s own circulation (reviewed in Groothuis and Schwabl 2008; Hasselquist and Nilsson 2009).
Interestingly, a significantly different pattern between yolk immunoglobulins and yolk androgens was observed within clutches. For both yolk immunoglobulin as well as yolk androgen concentrations, most variation was found among clutches, whereas within-clutch variation was comparably low. This low variation reduces the potential for within-clutch covariation between egg components. However, it is important to note that it does not explain the lack of a correlation, which is independent of the within-clutch variances. Instead, the lack of a (negative) correlation within clutches may at least partly be explained by selection for different patterns of deposition at the within-clutch level. Indeed, whereas there is no change in IgG concentrations with laying order ($F_{1, 248.3} = 0.131, P = 0.718$), yolk A4 and T significantly increase with laying order, whereas yolk DHT concentrations significantly decrease (Tschirren et al. 2004). Differences in yolk hormone deposition with laying order have been suggested to promote brood reduction (in the case of decreasing hormone concentrations) or increase competitive abilities of late hatched nestlings (in the case of increasing hormone concentrations with laying order) (Schwabl 1993; Schwabl et al. 1997). In line with the latter hypothesis we found in a previous study that it was particularly nestlings late in the laying sequence that benefited from high yolk androgen concentrations (Tschirren et al. 2005). Unlike yolk immunoglobulins, yolk androgens may thus be strategically deposited within a clutch to optimise growth trajectories. Importantly, the lack of a correlation between yolk immunoglobulins and androgens within clutches shows that mothers can, at least within certain limits, vary each of the egg components independently, or that they are transferred randomly. It thereby provides indirect support for the hypothesis
that selection has favoured the mutual adjustment of yolk immunoglobulins and yolk androgens *among* clutches.

The mechanisms underlying the observed negative correlation among clutches are currently unknown. One possibility is a negative genetic correlation between yolk immunoglobulin and yolk androgen deposition (Schroderus et al. 2010). Extensive pedigree data (Postma and Charmantier 2007; Wilson et al. 2010), or the creation of selection lines for high/low yolk immunoglobulin deposition, and testing for correlated responses in yolk androgens (or vice versa) (Schroderus et al. 2010), would make it possible to estimate the degree to which the negative correlation between the two egg components has a genetic basis. Furthermore, environmental factors that simultaneously, but oppositely affect immunoglobulin and androgen deposition (e.g., parasite exposure or health status of the mother (Boonekamp et al. 2008)) might also play a role. Further experimental work is required to quantify the role of such factors in mediating the negative covariation of yolk androgens and antibodies observed across clutches. Possible approaches include the experimental manipulation of female health status or condition, and to test how this affects the correlation between immunoglobulins and androgens in the eggs (see e.g., Gasparini et al. 2007). As a further improvement to our current study, females could be injected with a novel antigen (e.g., Tetanus vaccine) to measure specific antibody responses, rather than total IgG.

In contrast to our study, previous work did not detect a significant association between yolk immunoglobulin and yolk androgen concentrations in the eggs of free-living birds (Groothuis et al. 2006; Hargitai et al. 2009) or observed
positive relationship between IgG and A4 within clutches (Gasparini et al. 2007). These differences may be explained by ecological differences among species (e.g. differences in parasite-mediated selection pressures), or a focus on within-clutch variation in previous work.

Covariation among other egg components, however, is well documented. Total yolk carotenoid concentration, for example, is positively correlated with vitamin E concentration within collared flycatcher (Ficedula albicollis) clutches (Hargitai et al. 2006). Similar positive relationships were found between carotenoid, vitamin E and vitamin A concentrations within, and, to a lesser extent, among clutches of yellow-legged gulls (Larus michahellis) (Rubolini et al. 2011). Finally, there was a negative within-clutch correlation between yolk antioxidants and yolk androgens in black-backed gulls (Larus fuscus) (Royle et al. 2001).

In addition to the overall within- and among-clutch patterns of covariation between yolk immunoglobulins and yolk androgens, we have here also tested how an experimental parasite-infestation influences the strength and direction of this relationship, again both at the within- and among-clutch level. This provides us with first insights into the environmental dependency of these correlations. Indeed, we found some indication for correlations among egg components to depend on the presence or absence of parasites (e.g. the relationship between yolk IgG and yolk DHT becomes more negative in parasite infested nests), which is deserving of future study. In particular, it would be interesting to separate the among-clutch correlations into their genetic and environmental components (see above), and to test how the genetic correlation between yolk IgG and yolk androgens is shaped by the
environment (i.e. to test for G x E interactions, Sgrò and Hoffmann 2004). The latter is particularly interesting given that, if parasites are an important selective force acting upon these egg components, such a parasite-dependency of the genetic variance-covariance matrix could have major evolutionary implications. Having said this, it should be noted that overall the differences between the two treatment groups did not reach statistical significance ($P = 0.074$ and $P = 0.088$ for the among- and within-clutch comparisons, respectively), and in the absence of more data we have to be careful not to over-interpret the observed patterns.

The treatment-specific analysis of within- and among-clutch correlations provides an illustration of the possibilities offered by the multivariate mixed model approach taken here. Although its strengths when it comes to the estimation of within- and among-clutch patterns of variance and covariance have been highlighted before (e.g. Rubolini et al. 2011), we would here like to emphasise the advantages offered in terms of hypothesis testing. First, as the number of parameters to be estimated increases exponentially with the number of traits analysed, individual parameters are typically estimated with low precision. Although as a consequence none of the individual parameters may be significantly different from zero, being able to simultaneously constrain multiple elements to zero (in this case, all correlations involving yolk IgG and the different yolk androgens) allowed us to maximise statistical power and avoid problems associated with multiple testing. Second, by constraining particular elements of the (within- or among-clutch) correlation matrix to be identical, it is possible to explicitly test whether covariances and / or correlations differ significantly across organisational levels (here within-
versus among-clutch) or environments (here with or without fleas). Third, it is possible to simultaneously correct all traits for the same or a different set of fixed effects, which is not possible in a univariate regression analysis, in which confounding variation can only be accounted for in the dependent variable, but not in the explanatory covariates. Although routinely employed in other fields, including quantitative genetics (e.g. Postma et al. 2011), within the light of these advantages it is surprising that multivariate mixed model methodology is still relatively rarely used in other fields, including that of ecophysiology, which routinely deals with inherently multivariate and correlated phenotypes.

Acknowledgements

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Figure legend

**Fig 1** Correlation coefficients ($r \pm 1$ SE) of the relationship between maternal yolk immunoglobulin (IgG) and yolk androstenedione (A4), yolk testosterone (T) and yolk 5α-dihydrotestosterone (DHT) concentrations among (black bars) and within (gray bars) great tit clutches.
Table 1 The association between (log transformed and standardised) yolk IgG and yolk androgen concentrations. The percentage of variance among clutches is given on the diagonal. Among-clutch correlations are provided below the diagonal, and within-clutch correlations are provided above the diagonal. Approximate standard errors are given in brackets. Underlined correlations are significantly different from zero at the 5% level (based on likelihood-ratio tests). Correlations involving IgG are given in bold.

<table>
<thead>
<tr>
<th></th>
<th>A4</th>
<th>T</th>
<th>DHT</th>
<th>IgG</th>
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<tbody>
<tr>
<td>A4</td>
<td>62.0 (5.7)</td>
<td>0.747 (0.028)</td>
<td>-0.075 (0.064)</td>
<td><strong>0.050 (0.064)</strong></td>
</tr>
<tr>
<td>T</td>
<td>0.808 (0.054)</td>
<td>63.8 (5.5)</td>
<td>0.100 (0.064)</td>
<td><strong>0.017 (0.064)</strong></td>
</tr>
<tr>
<td>DHT</td>
<td>0.007 (0.153)</td>
<td>0.227 (0.145)</td>
<td>66.1 (5.3)</td>
<td><strong>0.074 (0.065)</strong></td>
</tr>
<tr>
<td>IgG</td>
<td><strong>-0.204 (0.145)</strong></td>
<td><strong>-0.082 (0.153)</strong></td>
<td><strong>-0.317 (0.137)</strong></td>
<td>70.1 (4.8)</td>
</tr>
</tbody>
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Table 2 The association between (log transformed and standardised) yolk IgG and yolk androgen concentrations in the absence (A) and presence (B) of fleas. In both, the percentage of variance among clutches is given on the diagonal, correlations among clutches are provided below the diagonal and correlations within clutches above the diagonal. Approximate standard errors are provided in brackets. Underlined correlations are significantly different from zero at the 5% level (based on likelihood-ratio tests). Correlations involving yolk IgG concentration are given in bold.

A)

<table>
<thead>
<tr>
<th></th>
<th>A4</th>
<th>T</th>
<th>DHT</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>61.8 (8.2)</td>
<td>0.842 (0.027)</td>
<td>-0.007 (0.093)</td>
<td><strong>0.017 (0.093)</strong></td>
</tr>
<tr>
<td>T</td>
<td>0.753 (0.095)</td>
<td>58.6 (8.6)</td>
<td>0.185 (0.090)</td>
<td><strong>0.069 (0.092)</strong></td>
</tr>
<tr>
<td>DHT</td>
<td>-0.210 (0.220)</td>
<td>0.042 (0.230)</td>
<td>51.9 (9.1)</td>
<td><strong>0.238 (0.088)</strong></td>
</tr>
<tr>
<td>IgG</td>
<td><strong>-0.521 (0.159)</strong></td>
<td><strong>-0.279 (0.199)</strong></td>
<td><strong>-0.164 (0.213)</strong></td>
<td>82.1 (4.7)</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th></th>
<th>A4</th>
<th>T</th>
<th>DHT</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>60.2 (8.2)</td>
<td>0.665 (0.050)</td>
<td>-0.144 (0.087)</td>
<td><strong>0.079 (0.088)</strong></td>
</tr>
<tr>
<td>T</td>
<td><strong>0.847 (0.066)</strong></td>
<td>66.7 (7.4)</td>
<td>0.019 (0.089)</td>
<td><strong>-0.010 (0.089)</strong></td>
</tr>
<tr>
<td>DHT</td>
<td>0.225 (0.206)</td>
<td>0.388 (0.182)</td>
<td>75.6 (6.0)</td>
<td><strong>-0.070 (0.091)</strong></td>
</tr>
<tr>
<td>IgG</td>
<td><strong>0.103 (0.224)</strong></td>
<td><strong>0.004 (0.224)</strong></td>
<td><strong>-0.474 (0.177)</strong></td>
<td>48.5 (8.8)</td>
</tr>
</tbody>
</table>