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MATERNALLY DERIVED ANTIBODIES IN AVIAN EGGS AND OFFSPRING: ECOLOGY, LIFE HISTORY, AND DEVELOPMENT

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MATERNALLY DERIVED ANTIBODIES IN AVIAN EGGS AND OFFSPRING:

ECOLOGY, LIFE HISTORY, AND DEVELOPMENT

by

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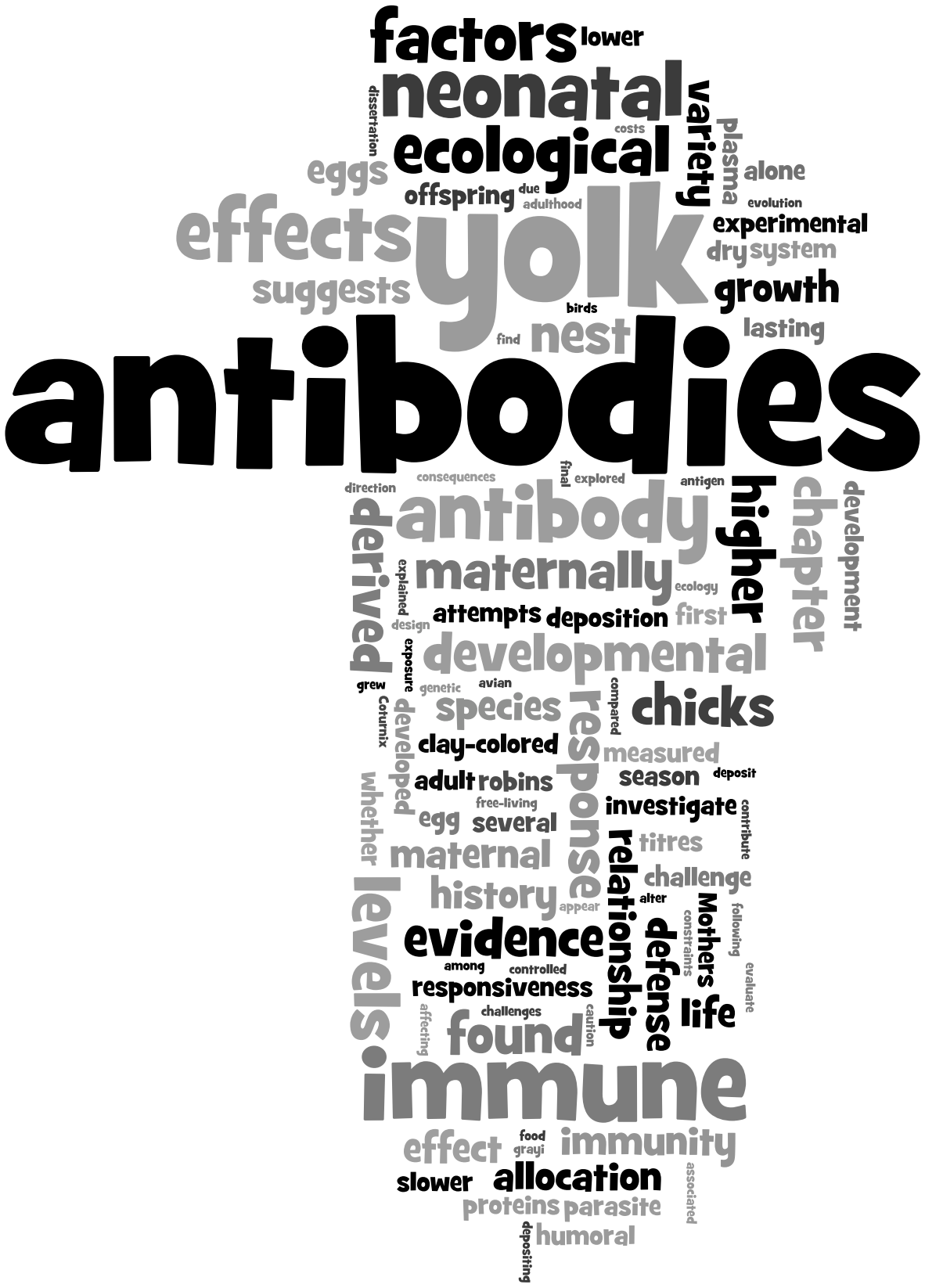
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DISSERTATION ABSTRACT

Mothers can alter offspring phenotypes through a variety of indirect effects, including the deposition of nutrients, hormones, and defense proteins in to the egg. Defense proteins, and antibodies in particular, may be tremendously important for neonatal defense against pathogens and the direction of resources into growth rather than immune responsiveness. Moreover, maternally derived immunoglobulins have been proposed to have an imprinting effect on the development of humoral immunity. In my dissertation, I explored a variety of ecological, life history, and developmental factors that could contribute to the evolution of yolk antibody allocation in a variety of avian species.

In the first chapter, I measured yolk antibodies in the eggs of 23 species of small Neotropical birds from lowland Panama, and using phylogenetic regression and model selection compared among several hypotheses for life history effects on yolk antibody levels. I found evidence that species with a slow-paced life history deposit lower levels of antibodies into egg yolks, and this strongly suggests that maternal antibodies impose developmental constraints on humoral immunity. There was little evidence for an effect of nestling ecology and nest parasite exposure on yolk antibody allocation.

In the second chapter, I investigate whether yolk antibodies have lasting effects on the immune response in a laboratory system of Japanese quail (*Coturnix japonica*). I developed a novel method of manipulating yolk antibodies in newly hatched chicks, and utilized this experimental system, while following individuals through a series of immune challenges into adulthood. I found no evidence of lasting effects of yolk antibodies on the immune response, but I did find a relationship between neonatal immune response and adult immune response. This relationship was independent of the

neonatal challenge antigen, and genetic factors were partially controlled for by the sibling set experimental design, suggesting that the observed relationship was due to a developmental effect of juvenile challenge on adult immune responsiveness.

In the third chapter, I evaluate ecological factors affecting yolk antibody allocation in free-living clay-colored robins (*Turdus grayi*), and test whether the same patterns appear in neonatal plasma levels of maternally derived antibodies. I found that eggs from first nest attempts had higher yolk antibody levels than subsequent nest attempts, but neonatal plasma antibodies were not predictable by any of the measured ecological factors. Most of the variability in maternally derived antibodies was explained by nest alone. This suggests that caution should be taken in the interpretation of results that measure antibodies in eggs or chicks alone.

In the final chapter, I investigate ecological variables and maternal effects on growth and immune development of clay-colored robins. My primary result was that in the dry season only, chicks with higher neonatal titres of maternally derived antibodies grew slower than chicks with lower antibody titres. This suggests that mothers depositing higher levels of yolk antibodies are probably associated with nests with higher parasite loads, resulting in slower growth of the offspring in the food limited dry season.

Overall, I have developed several new techniques for the study of maternal effects on immunity in oviparous vertebrates, and found some evidence for developmental costs of yolk antibodies, and ecological factors influencing both the deposition and consequences of yolk antibodies.

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Thanks to all who helped in the field and lab, my friends and family who kept me afloat, the funding agencies that made the money happen, the staff that helped in the navigation of bureaucracy, and to my committee for their enthusiasm and guidance. This has been a great experience, and I hope to see you all again soon and often. ¡Salud!

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ECOLOGICAL AND LIFE HISTORY FACTORS INFLUENCING THE EVOLUTION OF MATERNAL ANTIBODY ALLOCATION: A PHYLOGENETIC COMPARISON

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running header: Evolution of yolk antibody allocation

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Abstract

Maternally derived yolk antibodies provide neonates with immune protection in early life at negligible cost to mothers. However, developmental effects on the neonate's future immunity are potentially costly, and thus could limit yolk antibody deposition. The benefits to neonatal immunity must be balanced against costs, which may depend on neonate vulnerability to pathogens, developmental trajectories, and the immunological strategies best suited to a species' pace of life. We measured yolk antibodies and life history features of 23 species of small Neotropical birds and assessed the evidence for each of several hypotheses for life history and ecological effects on the evolution of yolk antibody levels. Developmental period and yolk antibodies are negatively related, which possibly reflect the importance of humoral immune priming through antigen exposure, and selection to avoid autoimmunity, in species with a slower pace of life. There is also a strong relationship between body size and yolk antibody concentration, suggesting that larger species are architecturally equipped to produce and transfer higher concentrations of antibodies. These results suggest that developmental effects of maternally derived antibodies, such as imprinting effects on B-cell diversity or autoimmune effects, are important and deserve more consideration in future research.

Keywords: disease ecology; immune development; maternal effects; life history physiology; parasites; yolk antibodies

Introduction

Maternal influences on offspring phenotype, termed maternal effects (Mousseau & Fox 1998), have received special attention as an indirect genetic effect influencing an individual's life history traits. The recent literature has focused on maternal allocation of hormones that direct resources to influence offspring behavior, growth, and development (Gil 2003; Schwabl 1996). However, few studies to date have investigated maternal provisioning of antibodies, which protect offspring from pathogens, influencing resource allocation and development of immunity (Boulinier & Staszewski 2008; Grindstaff et al. 2003; Hasselquist & Nilsson 2009).

Maternally derived yolk antibodies protect neonates against environmental pathogens during early life (Baintner 2007; Graczyk et al. 1994; Pravieux et al. 2007). Yolk antibodies are deposited during yolk formation by the active transport of circulating immunoglobulins across the follicle (Linden & Roth 1978; West et al. 2004). The primary antibody present in avian eggs is immunoglobulin Y (IgY is for yolk, Rose et al. 1974; Tizard 2002), which is usually secreted by B-cells in direct response to an immune challenge. IgA and IgM appear at much lower concentrations and are primarily in the albumen of the egg. Chicken embryos undergo a period of rapid IgY uptake from their residual yolk starting about three days prior to hatch, and continuing for about two days post-hatch (Rose & Orlans 1981). Because most IgY is produced in a specific response to an immune challenge, neonates receive protection against pathogens likely to be relevant within their lifetime, and thus their passive immunity is shaped by their mothers' previous disease exposure. Maternally derived antibodies benefit chicks through two documented routes; first, they directly interfere with pathogenicity by

coating pathogens (Carlier & Truyens 1995; Leuridan & Van Damme 2007; Nemeth et al. 2008), and second, they reduce the demands on the neonate immune system, and thus free resources to be directed towards other growth or maintenance needs (Gallizzi et al. 2008; Grindstaff 2008; Pihlaja et al. 2006). There has also been a proposed imprinting effect of maternal antibodies on immune development of offspring (Lemke & Lange 1999), where the B-cell repertoire is proposed to be modeled after the antibodies present during development. This is usually considered a potential benefit as there is some evidence that "naïve" offspring of non-naïve mothers have higher responses to an antigen long after maternal antibodies have dissipated (Reid et al. 2006), though it can also have negative effects if mothers transfer autoantibodies (Greeley et al. 2002), or if imprinting mechanisms interfere with other immune system arms (Lemke & Lange 1999).

Despite their protective function, yolk antibodies make up only a small proportion of the total protein in the yolk. Presumably maternal provisioning of antibodies also imposes costs that limit the amount allocated to eggs. These costs could be imposed on the hen during egg formation, or they may be imposed on the developing chick. The direct cost to the hen of producing antibodies is relatively low and is not likely to be higher than producing other egg proteins (Klasing 1998). Physiological factors might also constrain antibody synthesis by the mother during reproduction. During reproduction the immune system may be generally suppressed, or reproductive resource use prioritized. For instance, during egg production in waterfowl antibody synthesis changes to an alternate isotype (called IgYΔFc) which reduces the inflammatory response but is also not transferred to yolks (Humphrey et al. 2004). Two distinct developmental costs to chicks of maternal antibodies have been proposed.

During B-cell development, lineages of pre-B-cells are positively selected if they are functional, and bind to environmental antigens presented in the bursa, or bind very loosely to self-antigens that might have similar molecular patterns to pathogenic organisms (Baumgarth et al. 2005). Pre-B-cells that are not functional, or that bind tightly to self-antigens are negatively selected. The resulting set of B-cells recognizes a diverse set of antigens, but should not inflict damage via auto-immunity. However, large volumes of maternally derived antibodies could coat, and effectively hide, antigens that are important to B-cell development (Carlier & Truyens 1995). There is evidence that maternal antibodies coat antigens, inhibiting the juvenile immune response (Lung et al. 1996; Staszewski et al. 2007). If environmental and self-antigens were hidden from the B-cell selection process, antibody diversity in later life might be compromised by a failure of positive selection at the pre-B-cell stage. The hiding of self-antigens could also cause autoimmune disorders through a failure of the negative selection process. The influence of maternal antibodies on diversification of the B-cell repertoire (Fink et al. 2008) or autoimmune disease has a relatively short history in the medical literature (Agarwal & Agarwal 2006; Victora et al. 2007; Von Herrath & Bach 2002; Zinkernagel 2001). While the costs remain poorly understood, we can assume that these costs do influence yolk antibody allocation decisions.

Many ecological and life history factors influence total and specific maternal antibody allocation to eggs (Grindstaff et al. 2003). Antibody allocation should match the exposure of offspring to pathogens, which will be determined by characteristics of the rearing habitat, the nest type, and the amount of time spent in the nest exposed to parasites. The developmental strategy will not only influence exposure period, but could alter the relative importance of allocation of resources to immune responses or

maturation (Lee 2006). We investigated ecological and life history patterns of maternal antibody allocation to eggs in a variety of lowland tropical bird species. If mothers adaptively allocated antibodies in response to ecological or life history factors, we would expect support for two non-mutually exclusive hypotheses. (1) Developmental life history hypothesis: a fast life history strategy, where growth is rapid and adaptive immunity is less important (Lee 2006), will select for higher yolk antibody levels because this will help to divert resources towards growth, despite costs to adaptive immune development. (2) Nestling ecology hypothesis: open nests and long nestling periods, where offspring are relatively exposed to environmental pathogens and vectors of disease, will select for higher yolk antibody levels relative to enclosed nests or short nestling periods. Alternatively, enclosed nests have been suggested to harbour more ectoparasites, and so may select for higher yolk antibodies compared to open nests.

Methods

We located nests of passerine birds in and around Gamboa, Panama, from 15 March – 30 June 2006, and 27 February – 25 May 2007. Gamboa is adjacent to Neotropical lowland dry forest in the Panama Canal Zone. The study period from February to May covers the dry to wet season transition (usually around mid-April) during the onset of the breeding season for most species in the area. Nests located during the building stage, or with only one egg, were used in the present study. Nests were checked daily, and first eggs were collected on the day the second egg was laid to prevent nest abandonment (except in clay-colored robins *Turdus grayi*, where whole clutches were collected for another study). Collected eggs were stored at 4° C for up to 24 hours until IgY

extraction. All collections were under permit from Panamanian ANAM authorities, University of Missouri IACUC, and the Smithsonian Tropical Research Institute.

Life history and ecological traits

We included the following life history and ecological variables in the analyses: clutch size; incubation and nestling periods; nest type (open or enclosed); and adult body mass. We collected life history data by locating nests and weighing adult birds captured during the breeding season. We found nests of most species during the nest-construction and egg-laying phases of the breeding cycle. Nests were visited daily during egg laying and near expected hatch and fledging dates. Eggs were numbered individually as they were laid and chicks were marked with non-toxic markers to facilitate individual identification. In this study, clutch size is the mean number of eggs per nest. Incubation period is the time from onset of incubation to hatching of the first egg; onset of incubation in most species was on the evening the penultimate egg of a clutch was laid. Nestling period is the time between hatching of the first egg and fledging of the first-hatched chick. We categorized nests as open when they were open cups, platforms, or scrapes on the ground. If nests were burrows, tree cavities, gourd- or pendant-shaped nests, or cavities in termitaria we categorized them as enclosed. Body masses were obtained from mist-netted birds at and near Gamboa.

Yolk IgY extraction and measurement

Eggs were weighed whole, and then broken open, and the yolks separated and weighed to the nearest 0.001g. For extraction, we aliquoted 0.1g of yolk into a 1.5mL microcentrifuge tube and removed the lipid by vortexing with 400 uL of 3.5% PEG-

6000 in 0.9% NaCl, incubating overnight at 4° C, and centrifuging for 30 mins at 8000g and 4° C. The yolk pellet was discarded, and the supernatant reserved at 4° C for analysis. For yolk samples processed fresh, extraction efficiency is close to 100%, as validated with a stripped yolk pool spiked with known quantities of chicken IgY (B Addison, unpublished data).

Yolk IgY was quantified by SDS-PAGE on 5% gels run at 90 volts for 30 mins. Chicken IgY (purified polyclonal, Sigma-Aldrich, St Louis) standard dilutions were run simultaneously to generate a standard curve. Protein was stained with gelcode blue (coomassie) stain (Pierce Biotechnology, Rockford, IL) for 1 hour, and washed in distilled water overnight. Gels were then photographed on a white light table, and the images were analyzed for band density using the gel tool in ImageJ (NIH, figure S1, supplementary material). Integrated density values were converted to concentration, in mg/ml, using the chicken IgY standard curve, and the values multiplied by 5 to account for the dilution during extraction. We used yolk IgY concentration in the analysis because our species varied greatly in egg size.

Statistical analysis

For all species we calculated the mean value of IgY concentration for our comparative analysis (response variable). We selected nine statistical models from the parameters we measured including a null model and global model for AICc model ranking and multimodel inference (Burnham & Anderson 2002). Our candidate model set included a global model with all parameters, a nestling ecology model, a development model, the bivariate models for each of the individual parameters, and a null model (table 1).

We used a tree pruned from Cohen et al. (2008) to test for the effect of phylogeny. Using the regressionv2.m package (Lavin et al. 2008) in Matlab (R2007a), we first fit the best model of evolution for our traits of interest, and then selected a set of candidate models for explaining life history and ecology effects on yolk IgY, accounting for phylogeny and our model of evolution. The regressionv2.m package selects the best model of evolution for the traits of interest by fitting the saturated model for each of several models of evolution, including species values (ordinary least squares, no phylogenetic signal), Brownian motion (phylogenetic generalized least squares, equivalent to Felsenstein's contrasts), Ornstein-Uhlenbeck process (OU, drift about a fitness peak), or with branch lengths transformed using Pagels λ parameter. The OU and Pagels λ fitting procedures calculate branch length transformation parameters, d for the OU process that is a function of time, and λ for Pagels transformation, which is constant across the tree, by REML. Values close to 1 indicate evolution is close to Brownian motion, whereas values close to 0 approximate a star phylogeny.

Results

We obtained life history and yolk antibody values for 23 species including dove, sub-oscine and oscine species (figure S2, supplementary material). All species included have an altricial developmental mode, with incubation periods ranging from 12-23 days, nestling periods from 9-28 days, clutch size from 2-4 eggs, mass 7.3-68 g, and yolk antibody concentrations from 0.12-0.90 mg/mL. The within species variability in IgY varied greatly by species.

In AICc evaluation of the evolutionary models for phylogenetic signal, the OLS star phylogeny was the best fit to the data for the global model with an AICc weight of

0.9 (table 2). To be conservative, we also evaluated evolutionary models for the null model. The OU, and OLS trait evolution models both had ΔAICc values under 2 and can be considered to have support. Furthermore, the REML estimates of phylogenetic signal were $\lambda = 0.75$ (Pagel's lambda evolution model), and $d = 0.35$ (OU evolution model) for the null model and $\lambda = 0.39$, and $d = 0.15$ for the global model, such that the λ and d transformed trees had shorter interior branches for the traits of interest. We report AICc scores for all life history ecology models of yolk IgY for both OLS and OU evolutionary models.

The strongest model for both the OLS and OU analyses was the development model of yolk IgY (AICc weights of 0.9 and 0.8 respectively, table 3). The models that followed in support were the bivariate models of the parameters contained within the development model. Because the results for the OLS and OU analyses were qualitatively similar, we used only the OLS analysis for calculating parameter estimates and AICc weights by model averaging (table 4). The relationships between yolk IgY and both incubation period and nestling period were negative, but the interaction term was positive, indicating that the slopes are not equal. For species with longer incubation periods the relationship with nestling period was weaker (figure 1). These patterns hold even with the exclusion of the common tody-flycatcher (*Todirostrum cinereum*) and grey-breasted martin (*Progne chalybea*), the species with the longest incubation and nestling periods, respectively, in our dataset. There was also a strong positive relationship between yolk IgY and adult body mass (figure 1).

We found no support for the effects of clutch size or nest type on yolk IgY, and the relationship with nestling period was opposite to the direction expected for the nestling ecology hypothesis.

Discussion

We evaluated models representing two hypotheses for the evolution of yolk IgY deposition strategies. Both in AICc model evaluation, and in multimodel inference, factors related to life history and development weighted substantially more important than factors related to nestling ecology. Life history, specifically development period, has a strong effect on yolk IgY allocation in the resident Neotropical birds sampled in our study. Moreover, the effect of phylogenetic history on this trait was weak, suggesting it is highly labile. The effect of development on yolk IgY could be operating through several pathways, which are not mutually exclusive.

Larger adults deposit higher concentrations of yolk IgY in their eggs, which could reflect a greater capacity for antibody synthesis in larger animals (Davis et al. 1999; Lee et al. 2008; Magnadóttir et al. 1999), or some other allometric reason (Hasselquist 2007). Alternatively, the offspring of larger species may have a longer development period (Gillooly et al. 2008), however there was no relationship between body mass and developmental period in our species set. The effect of body size appears to reflect the architectural costs of yolk antibody deposition to the hen. It would be interesting to investigate whether these differences are indeed driven largely by plasma cell density and productivity, or if there are also differences in IgY receptor expression on the yolk follicle.

The strong negative relationship with incubation period most likely derives from incubation period serving as an index of development rate and/or pace of life. Since yolk IgY remains relatively inert until uptake by the embryo a short time before hatching (Kaspers et al. 1991; Tressler & Roth 1987), incubation period per se should not be related to yolk IgY deposition. If degradation of IgY over time prior to embryonic uptake were of concern, we would predict a positive relationship between incubation period and yolk IgY, which is the opposite of what we found. Thus, the relationship between IgY and this index of pace of life fits well with a developmental cost to maternal antibody allocation (see below). There is little information on comparative rates of immune development in avian species, and what is known is largely restricted to precocial species (Apanius 1998).

Although species with longer nestling periods also deposit less IgY into their eggs, the relationship was weaker for species with long incubation periods, generating a positive interaction between incubation and nestling periods in their effect on yolk IgY. This could reflect a compromise between long development periods and long exposure periods, suggesting weak effects of nestling ecology. For instance, species with long incubation periods tend to have correspondingly long nestling periods (Lack 1968), which make the nestling more vulnerable to parasites normally found in high densities in the nest, such as philornid flies and mites. Nestling period is a poor index of developmental period in the species in our dataset because many tropical species leave the nest at an early developmental state (Remes & Martin 2002; Ricklefs 1976). However, it might be a good indication of developmental priorities in very early life, as species leaving the nest early likely expend more resources prior to nest departure on traits, such as feather growth and muscle maturation, required to achieve that early nest

departure. As such, the benefits of maternal antibodies could be of greater importance, while the potential developmental costs are diminished, for these species.

The idea that maternally derived antibodies have a blocking effect on offspring immunity has been important for the development of vaccination schedules in human and veterinary medicine (Block et al. 2007; Siegrist 2007). While the effect of maternal antibodies on immune responses is well documented, it is not clear whether this blocking effect is exclusive to T-cell mediated responses, or whether it might also affect lymphocyte development (Carlier & Truyens 1995). The recognition of antigen is an important component of B-cell development (Baumgarth et al. 2005; Tizard 2002), presenting a potential mechanism for a negative effect of maternal antibodies on immune system development. However, some evidence also supports a positive effect via immunological imprinting (Gasparini et al. 2006; Grindstaff et al. 2006; Reid et al. 2006). The exact mechanism is unclear, but the B-cell repertoire may somehow model itself after antibodies in circulation (Fink et al. 2008).

The relationship between maternal antibodies, development time, and immunity is likely complex, and dependent on the arm of the immune system of interest, and possibly even the route of response to a specific antigen. The evidence for an effect of developmental period, or pace of life in general, on immunity is mixed, and much more needs to be done to understand what predictions should be made for different arms of the immune system. Tella et al. (2002) found that slow pace of life species have higher cell-mediated immunity assayed by PHA wing web swelling, whereas Palacios and Martin (2006) found the opposite relationship using the same measure of immunity. Tieleman et al. (2005) found that slow pace of life species had higher innate immunity measured by bactericidal activity of blood. Lee et al. (2008) found that slow pace of life species

had higher natural antibodies (relationship with incubation period) and lower complement activity (relationship with clutch size) in a set of species that includes many of the same species as this study. Different arms of the immune system could evolve independently and the exact directional predictions probably depend a great deal on what aspect of immunity is measured (Blount et al. 2003). Perhaps one of the best illustrations of this is a study of immune function in high (fast pace of life) and low latitude (slow pace of life) populations of house sparrows (*Passer domesticus*, Martin et al. 2006) which found that the slow pace of life population had a higher speed of humoral response measured as secondary antibody response to wing web injections of keyhole limpet hemocyanin and a higher magnitude of cell-mediated response measured by PHA wing web injection, but a lower magnitude peak of humoral response and lower T-cell response to KLH challenge compared to the fast pace of life population. It has been suggested that long developmental periods, and a slow pace of life, permit and perhaps necessitate the development of a stronger adaptive immune system (Lee et al. 2008). Species with long incubation periods have previously been shown to have lower parasite induced nestling mortality (Møller 2005) and lower adult stage hematozoan infection prevalence (Ricklefs 1992) suggesting potentially important effects of pace of life on immune development.

Yolk IgY concentrations measured in this study were lower than levels measured in chickens (1.15-2.26 mg/mL, Hamal et al. 2006) or quail *Coturnix japonica* (3.27-4.35 mg/mL, Grindstaff et al. 2005), and comparable to estimates reported in *Ficedula albicollis* (0.63 mg/mL relative to chicken standard, Hargitai et al. 2006). Several factors could contribute to the low levels measured in wild altricial birds. Domesticated fowl have better nutrition and thus more easily produce antibodies for allocation to eggs.

Alternatively, the species studied in the wild are generally of small body size and this appears to be a very important factor. Also, the altricial developmental mode, where much of the immune development likely occurs after hatch when maternal antibodies are active, may contribute to lower levels. Many of the studies of maternal antibodies in wild species have not attempted to quantify actual concentrations, and have instead used relative values measured in immunoassays. This inhibits further comparative analysis, and researchers are encouraged to quantify actual values alongside relative values whenever possible.

Previous studies have found that hen plasma antibody levels and egg yolk antibodies are correlated (Hamal et al. 2006). For specific antigens, the slope of the correlation between hen plasma and yolk antibodies differs for different idiotypes (Tizard 2002), which could indicate a degree of selectivity for the movement of antibodies across the follicle membrane. There are two possible ways for adjustment of yolk antibody levels. Adjustment of circulating plasma antibodies allows control both of the total antibodies deposited in yolks as the molecules will tend to move down a concentration gradient, and the control of the proportion of different antibody idiotypes in the yolk. The second way to modify yolk antibodies is by adjustment of yolk follicle receptor density. It is not clear to what degree different species employ these two possible strategies.

Much of the ecoimmunology literature has addressed almost exclusively benefits of maternal antibody allocation (Boulinier & Staszewski 2008; Hasselquist & Nilsson 2009) but the low levels of yolk IgY in wild species strongly indicates important costs. We suggest three physiological avenues of investigation that remain unexplored in a comparative context. First, we need to understand the role of maternally derived

antibodies in B-cell development, in particular during pre-B-cell selection in the bursa, and in T-cell development. Studies should consider the different modes of immune response, T-cell dependent versus independent, for different antigen patterns. Second, we need more information on the time course of immune development for different arms of the immune system, and among different life history strategies. For instance species investing little in humoral immunity should prioritize development of the innate arms of the immune system, with B-cell development occurring later and more rapidly. Third, we need to understand the long-term consequences of maternal antibodies for B-cell and T-cell repertoires, immunologic responsiveness and memory, immunosenescence, and autoimmunity.

Overall, our analysis suggests a developmental cost limiting yolk IgY deposition, at least in small Neotropical birds. While life history appears to be an important determinant of the evolution of this maternal effect, outstanding questions remain about the causes of inter-individual variability of this trait, and the physiological activity of maternal antibodies, especially with respect to costs.

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Table 1. List of models included in the AICc analysis of life history effects on yolk IgY deposition. The parameters included are clutch size (species mean, clutch), incubation period (species mean in days, incp), nestling period (species mean in days, nestlp), adult body mass (mean in g, mass), nest type (**o**pen or **c**losed, nesttype), and an evolutionary model (evolmodel).

ID	Model
Global 1	clutch, incp, nestlp, mass, nesttype, nestlp*nesttype, incp*nestlp, evolmodel
Nestling ecology 2	clutch, nestlp, mass, nesttype, nestlp*nesttype, evolmodel
Development 3	incp, nestlp, mass, incp*nestlp, evolmodel
4	clutch, evolmodel
5	incp, evolmodel
6	nestlp, evolmodel
7	mass, evolmodel
8	nesttype, evolmodel
null	evolmodel

Table 2. Evaluation of the best fit evolutionary model for the global model (A) and the null model (B). Non-phylogenetic analysis (OLS) was best fit for the global model, but second to models of a weak phylogenetic signal (OU) in the null model.

A. global = clutch, incp, nestlp, mass, nesttype, nestlp*nesttype, incp*nestlp, evolmodel

Model	*K*	n	Lg*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*	evidence ratio	r^2
OLS	9	23	-6.825	45.495	0.000	1.000	0.948	1.000	0.71
OU	10	23	-7.012	52.357	6.862	0.032	0.031	30.904	0.67
Pagels l	10	23	-7.511	53.355	7.860	0.020	0.019	50.909	0.67
PGLS	9	23	-12.762	57.370	11.875	0.003	0.003	378.986	0.57

B. null = evolmodel

Model	*K*	n	Lg*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*	evidence ratio	r^2
OU	3	23	-19.359	45.982	0.000	1.000	0.440	1.000	0
OLS	2	23	-20.940	46.480	0.499	0.779	0.343	1.283	0
Pagels l	3	23	-20.434	48.132	2.150	0.341	0.150	2.930	0
PGLS	2	23	-22.562	49.724	3.742	0.154	0.068	6.496	0

Table 3. AICc ranking, delta values, likelihoods, and weights, and model r^2 for models of life history effects on yolk IgY deposition. Analysis was done assuming a star phylogeny (A., OLS), and assuming stabilizing selection with drift (B., OU) using a tree compiled from the literature and presented in Cohen et al. 2008.

A. evolmodel=OLS

Model	*K*	n	Lg*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*	evidence ratio	r^2
3	6	23	-8.666	34.582	0.000	1.000	0.940	1.000	0.66
5	3	23	-17.457	42.178	7.596	0.022	0.021	44.611	0.26
7	3	23	-17.622	42.507	7.926	0.019	0.018	52.603	0.25
6	3	23	-18.560	44.383	9.801	0.007	0.007	134.381	0.19
8	3	23	-18.874	45.010	10.429	0.005	0.005	183.879	0.16
1	9	23	-6.825	45.495	10.914	0.004	0.004	234.341	0.71
null	2	23	-20.940	46.480	11.899	0.003	0.002	383.485	0.00
2	7	23	-12.856	47.179	12.598	0.002	0.002	543.937	0.51
4	3	23	-20.629	48.521	13.939	0.001	0.001	1063.668	0.03

B. evolmodel=OU

Model	*K*	n	Lg*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*	evidence ratio	r^2
3	7	23	-8.525	38.516	0.000	1.000	0.785	1.000	0.63
7	4	23	-16.155	42.532	4.016	0.134	0.105	7.447	0.24
5	4	23	-16.953	44.128	5.612	0.060	0.047	16.543	0.19
6	4	23	-17.680	45.582	7.066	0.029	0.023	34.219	0.14
null	3	23	-19.359	45.982	7.465	0.024	0.019	41.794	0.00
8	4	23	-18.407	47.035	8.519	0.014	0.011	70.780	0.08
4	4	23	-19.317	48.857	10.341	0.006	0.004	175.964	0.01
2	8	23	-11.538	49.362	10.845	0.004	0.003	226.495	0.50
1	10	23	-7.012	52.357	13.841	0.001	0.001	1012.759	0.67

Table 4. Parameter weights and weighted estimates of effect on yolk IgY deposition based on all OLS models (assuming star phylogeny).

	AICc*W*	*W* mean	(±SE)
Intercept	1.000	3.826	(1.543)
incp	0.965	-0.285	(0.093)
mass	0.963	0.008	(0.003)
nestlp	0.953	-0.279	(0.093)
incp*nestlp	0.944	0.017	(0.006)
nesttype	0.011	0.003	(0.005)
clutch	0.007	0.000	(0.001)
nestlp*nesttype	0.006	0.000	(0.000)

Figure 1. Life history predictors of yolk IgY deposition. Points are species means ± SE. a) Incubation period is strongly negatively related to yolk IgY deposition, even if the common tody-flycatcher (*Todirostrum cinereum*, incubation period of 23 days) is excluded. Marker shade is coded by nestling period, darker colors correspond to longer nestling periods. b) Nestling period is also negatively related to yolk IgY deposition, again, even with the exclusion of the extreme value of the grey-breasted martin (*Progne chalybea*, nestling period of 28 days). Marker shade corresponds to incubation period. c) Adult body mass is positively related to yolk IgY deposition. d) Predictive surface showing the relationship between incubation period, nestling period, and mass corrected yolk [IgY], generated without the two extreme value species.

Figure S1. SDS-PAGE lanes and density profiles generated in ImageJ for yolk extractions from each species.

Figure S2. Phylogenetic relationship and species values for life history and yolk immunoglobulin concentrations (including sample size and coefficient of variation) used in the analysis. Abbreviations as in table 1.

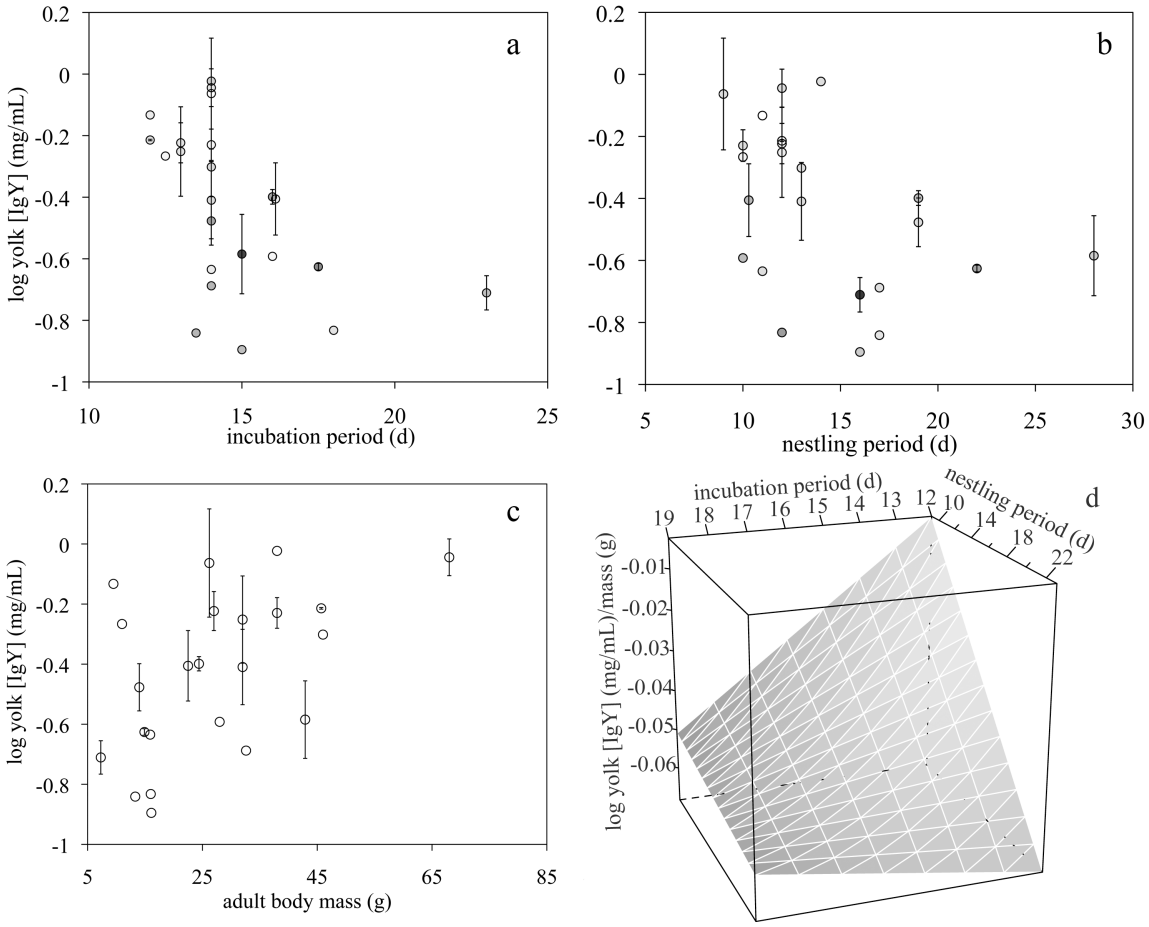

a
b
c
d
log yolk [IgY] (mg/mL)
incubation period (d)
nestling period (d)
adult body mass (g)
log yolk [IgY] (mg/mL)/mass (g)

Fig. 1

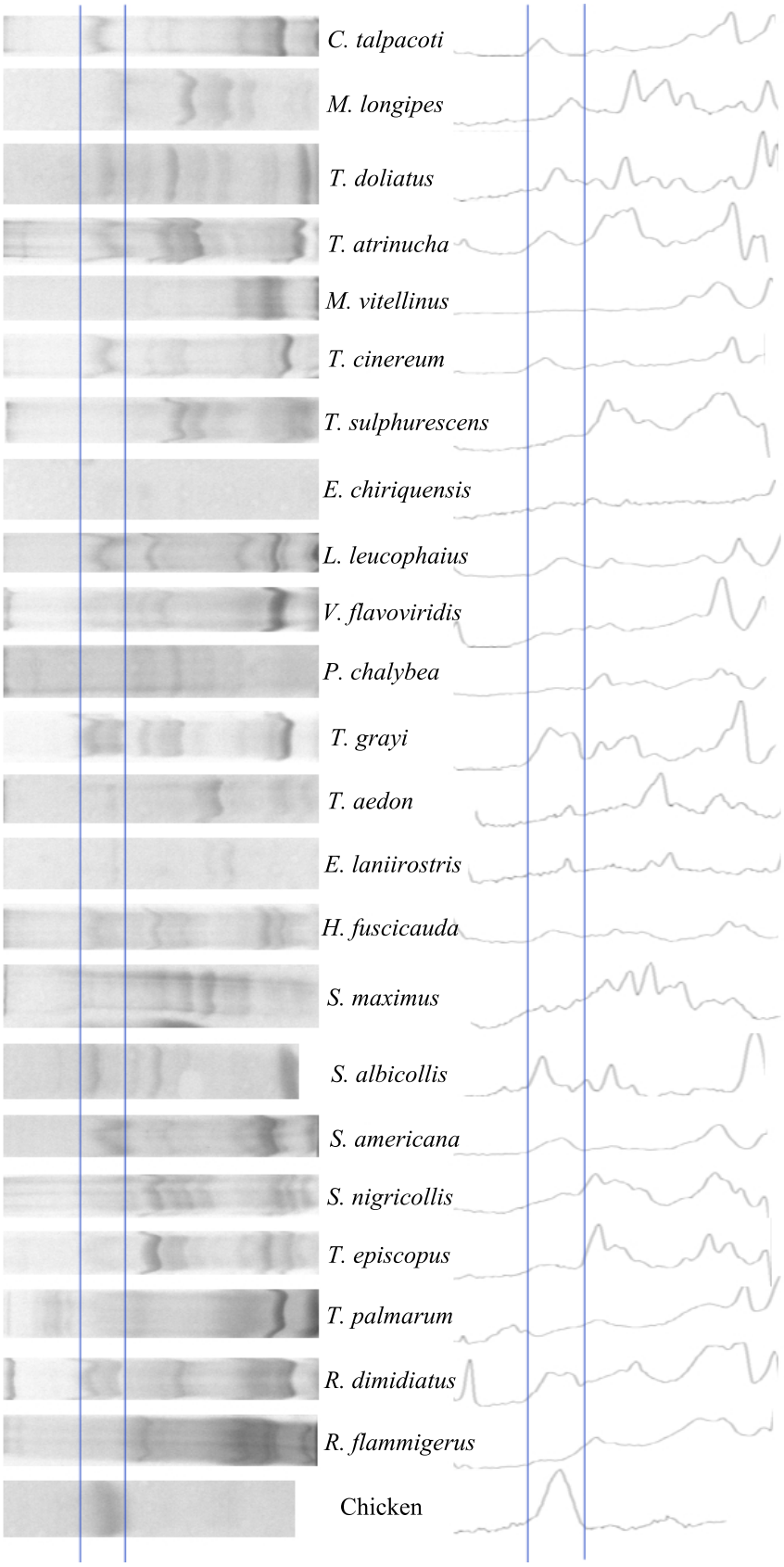
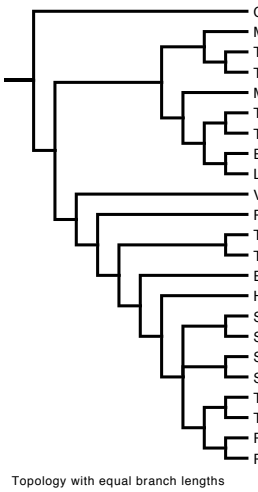

C. talpacoti
M. longipes
T. doliatus
T. atrinucha
M. vitellinus
T. cinereum
T. sulphurescens
E. chiriquensis
L. leucophaius
V. flavoviridis
P. chalybea
T. grayi
T. aedon
E. laniirostris
H. fuscicauda
S. maximus
S. albicollis
S. americana
S. nigricollis
T. episcopus
T. palmarum
R. dimidiatus
R. flammigerus
Chicken

Fig. S1



	[IgY] (mg/mL)	n	cv	clutch	incp (d)	nestlp (d)	nesttype	mass (g)
Columbina talpacoti	0.6107	2	0.01	2	12	12	o	45.7
Myrmeciza longipes	0.2558	1	-	2	16	10	o	28
Thamnophilus doliatus	0.8644	2	0.56	2	14	9	o	26.2
Thamnophilus atrinucha	0.3930	3	0.42	2	16.1	10.3	o	22.5
Manacus vitellinus	0.1471	1	-	2	18	12	o	16
Todirostrum cinereum	0.1383	7	0.33	2.5	23	16	c	7.3
Tolmomyias sulphurescens	0.2366	2	0.04	2.5	17.5	22	c	14.9
Elaenia chiriquensis	0.1273	1	-	2	15	16	o	16.1
Legatus leucophaius	0.3992	2	0.42	3	16	19	c	24.4
Vireo flavoviridis	0.2319	1	-	3	14	11	o	15.9
Progne chalybea	0.2602	2	0.41	3	15	28	c	42.9
Turdus grayi	0.9030	16	0.62	3	14	12	o	68
Troglodytes aedon	0.1442	1	-	3	13.5	17	c	13.3
Euphonia laniirostris	0.3334	10	0.44	4	14	19	c	14
Habia fuscicauda	0.5894	4	0.23	3	14	10	o	38
Saltator maximus	0.4993	1	-	2	14	13	o	46
Saltator albicolis	0.9486	1	-	2.5	14	14	o	38
Sporophila americana	0.5416	1	-	2	12.5	10	o	11
Sporophila nigricollis	0.7366	1	-	2	12	11	o	9.5
Thraupis episcopus	0.3894	6	0.61	3	14	13	o	32
Thraupis palmarum	0.2052	1	-	3	14	17	o	32.6
Ramphocelus dimidiatus	0.5981	5	0.31	2	13	12	o	27
Ramphocelus flammigerus	0.5603	4	0.54	2	13	12	o	32

Topology with equal branch lengths

Fig. S2

DO MATERNALLY DERIVED ANTIBODIES AND EARLY IMMUNE EXPERIENCE SHAPE THE ADULT IMMUNE RESPONSE?

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running header: maternal antibodies and immune development

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Summary

1. Immunological imprinting by maternally derived antibodies has been proposed to have both positive and negative consequences for offspring immunity in early and adult life. However, few studies of maternal effects on immunity have followed individuals past the juvenile stages.

2. Using laboratory Japanese quail, we developed a novel method of directly manipulating yolk antibodies of neonates, and then followed individuals through a series of immune challenges until they were of reproductive age. We utilized two antigens for this study, keyhole limpet hemocyanin, which induces a T-cell dependent immune response, and lipopolysaccharide, which induces a T-cell independent immune response.

3. Our method of directly injecting purified antibodies into the yolk sac of newly hatched chicks successfully elevated the plasma titres of specific anti-KLH IgY in neonates. This allows us to test whether differences in neonatal anti-KLH IgY affect immunity at the juvenile and adult stages of life.

4. We found little evidence for an effect of maternal antibodies on juvenile stage immune response, in contrast to results from previous studies. Adult immune response depended largely on the magnitude of the juvenile immune response regardless of the identity of the antigen in the juvenile immune challenge, and did not depend on neonatal IgY titres.

5. Our results are consistent with a priming effect of early immune experience on adult stage immune responsiveness, but we found no evidence of carryover effects of yolk-derived antibodies on adult immunity.

6. This study employs new methodology for investigation of maternal antibodies and presents results suggesting that further studies of maternal effects on immunity will

require careful consideration of the numerous ways maternally derived yolk components can impact the different types of immune response.

Key-words: maternal effects, yolk antibodies, immunological imprinting

Introduction

Maternally derived antibodies have been shown to benefit neonates by markedly improving immunity (Grindstaff, Brodie & Ketterson, 2003, Tizard, 2002), reducing acute phase responses to challenge (Goldyne, 2000), and increasing growth rate (Grindstaff & Ketterson, 2001, Pihlaja, Siitari & Alatalo, 2006, Grindstaff, 2008). Moreover, the nutritional demands of maternal antibody allocation have been shown to be negligible (Grindstaff, Demas & Ketterson, 2005, Klasing, 1998). Given these substantial benefits with little cost, the observed levels of maternally derived antibodies in avian eggs are remarkably low.

Potential indirect costs of maternal antibody provisioning include compromised offspring immune development (Carlier & Truyens, 1995) and autoimmune disorders triggered by maternal antibodies (Greeley, Katsumata, Yu *et al.*, 2002, Von Herrath & Bach, 2002, Lemke, Tanasa, Trad *et al.*, 2009). Environmental and self-antigen exposure is essential to the diversification and development of B-cells (Baumgarth, Tung & Herzenberg, 2005), influencing the maturation and refinement of natural immunity (Apanius, 1998). Maternally derived antibody circulating in neonates binds antigens introduced via infection or environmental exposure, masking those antigens from the developing B-cells, and possibly preventing positive selection for matched variable-region B-cells. The resulting cost could be two-fold: offspring may develop a

less diverse set of B-cells due to a reduction in positive selection, and they may have reduced resistance to specific infectious agents upon re-exposure later in life.

Early immune experience has lasting effects on the immune response, through the production of memory cells (Tizard, 2002) or desensitization to antigens (Wills-Karp, Santeliz & Karp, 2001). Exposure to some antigens early in life provides lifetime immunity against those pathogens. Vaccination scheduling is designed to take advantage of this feature of the immune system, while taking into account potential interference of maternal antibodies (for discussion Pastoret, 2007). Despite an extensive literature on maternal antibodies and vaccination schedules, individuals are seldom followed past the juvenile stage to reproductive age, and so little is known about whether maternal antibodies influence the adult immune response of birds.

We initiated this study to determine whether high concentrations of maternally-derived antibodies in neonates could depress the immune response later in life. We generated a harvestable pool of yolk antibodies (IgY) by hypervaccinating Japanese quail (*Coturnix coturnix japonica*) hens against one of two well defined challenge antigens: bacterial lipopolysaccharide (LPS) or dendroaspis natriuretic peptide conjugate – keyhole limpet hemocyanin (KLH). These antigens represent both T-cell independent (LPS) and T-cell dependent (KLH) antigens, thus presenting the opportunity to assess the impacts of maternal IgY on both types of immune response. Harvested IgY was then used to manipulate yolk IgY levels of newly hatched chicks, which were subsequently given a series of challenges as juveniles and adults to test immune competency against these antigens.

We predicted that yolk IgY treatment would reduce endogenous IgY production at both the juvenile and adult stages in a dose-dependent fashion. Moreover, we

expected these effects to be strongest for challenges with antigens that match the yolk IgY treatments at the juvenile- and adult-stage challenges, and diminished for the antigen familiar from juvenile-stage challenges at the adult stage. If maternal antibodies have a negative affect on B-cell development, the effects will occur for both antigens; effects only for KLH would suggest an affect on T-cell development.

Methods

Harvest of quail yolk IgY

We created two IgY types by harvesting yolk IgY from quail hens vaccinated with either LPS or KLH. Eight hens for each antigen received four vaccinations, spaced every three days, with either LPS or KLH (1 mg/kg) antigen in Freund's incomplete adjuvant to promote B-cell responses. One week after the first vaccination, we started to collect eggs, which we continued for two weeks to harvest sufficient IgY for our yolk injection treatments. Yolk IgY was purified by two-step PEG-6000 (polyethylene glycol) extraction. First, yolk was measured into a conical centrifuge tube and four volumes of 3.5% PEG in saline was added, and thoroughly vortexed. We incubated the solution at 4C for four hours, and then centrifuged at 5 000g for 30 mins at 4C. The supernatant was reserved in a new conical tube, and the lipid discarded. One volume of 24% PEG in saline was added to the supernatant, and the solution was vortexed and incubated at 4C for one hour. The solution was again centrifuged at 5 000g for 30 mins at 4C, the supernatant poured off and the white IgY pellet reserved. The IgY pellet was briefly rinsed with –20C ethanol to remove residual PEG, and dried in a fume hood. The dry precipitate of all eggs in each antigen treatment was pooled, and the extraction process repeated to further purify the IgY. Dry IgY from all eggs in each treatment was

weighed, and rehydrated in saline at a concentration of 800mg/mL, and stored at 4C. We checked IgY concentrations of stock solutions by direct ELISA using a chicken IgY (Sigma Aldrich) standard curve.

Effects of yolk IgY on humoral immune development

We tested the effect of yolk IgY treatment in a complete block design using siblings matched across treatments (Table 1). Our experiment had four treatment combinations: injection of 20 mg albumin (control), 2 mg anti-KLH IgY with 18 mg albumin resulting in an equivalent protein level (low KLH), 20 mg anti-KLH IgY (high KLH), and 20 mg anti-LPS IgY (high LPS). Note that our treatments are not monoclonal antibodies, but a mixture of antibodies deposited into eggs after vaccination of the hens. Injections of 0.05 mL volume protein dissolved in sterile saline were made into the yolk sac of newly hatched chicks using 0.5cc tuberculin syringes with 27g needles. The treatment levels of yolk IgY were selected to reflect near natural levels of IgY in quail eggs from unvaccinated hens (about 2-4 mg, unpublished data and (Grindstaff *et al.*, 2005), bringing yolk IgY levels to 4-6 mg for chicks in the low KLH group) and levels well outside of the vaccinated range (of 10 mg (Grindstaff *et al.*, 2005), bringing yolk IgY levels to about 22-24 mg for high KLH and LPS groups, more on this in results). Small blood samples (50 uL) were drawn at two days of age for ELISA confirmation of IgY incorporation into circulation. At seven days of age, half of the sibling sets were challenged with LPS and the other half with KLH. Blood was sampled again ten days post-challenge for ELISA measurement of anti-LPS or anti–KLH antibodies. All birds were challenged again with KLH or LPS in alternate challenges at five and seven weeks of age (table 1), and blood was sampled for IgY response 10 days later. All challenge

doses were 1 mg/kg of body weight administered in solution concentrations adjusted to approximately 0.05 mL vaccination volumes injected subcutaneously over the shoulders.

Serum samples were stored at -20C until ELISA analysis. Antibody titers were measured by sandwich ELISA as in Hasselquist, Marsh, Sherman *et al.*, (1999) by binding 2ug/mL solutions of LPS or KLH antigens in carbonate-bicarbonate binding buffer to 96 well plates, blocking with 1% BSA, adding 100 uL of 1:100 dilutions of plasma in PBS-tween, blocking with 1% BSA, and adding a 1:20 000 dilution of HRPO-anti-bird IgY (Bethyl Laboratories) secondary antibody in blocking buffer. Colorimetric detection employed TMB PO substrate (Thermo Scientific, Rockford IL) stopped with 1N sulfuric acid after 15 mins, and the plate was read at 450 nm. A standard was run using serial dilutions of pooled serum samples from all birds collected after the second immune challenge. Samples were run in duplicate and duplicates with CVs greater than 0.15 were rerun and the outlier discarded. All IgY values are from the averaged duplicates and expressed as titres relative to the pooled serum standard and then log transformed; thus each sample titre is a log proportion of the pooled serum titre.

Statistical analysis

Data were analyzed in three steps using AICc model selection and goodness of fit tests (Burnham & Anderson, 2002). First, we determined the efficacy of the yolk sac injection treatments. Second, we assessed whether yolk derived antibodies affected the response to challenge as a juvenile. Third, we assessed whether yolk derived antibodies or juvenile challenge antigen affected the adult stage response. All models included sibling group as a random effect and were estimated using the lmer() function in the lme4 package of R (v. 2.7.2).

The efficacy of yolk sac injections was evaluated by simple AICc comparison of mixed models for differences between groups against a null model. Separate analyses were conducted for anti-KLH and anti-LPS IgY titres.

To assess the effects of yolk derived IgY and challenge antigen on juvenile response, we compared seven models including a null model, which included the terms yolk treatment, anti-KLH and anti-LPS neonatal titres, vaccination antigen, and the interactions between vaccination antigen and neonatal IgY titres. If yolk derived antibodies affected juvenile response we would expect the models including the interaction terms to assume the highest weight, and neonatal IgY titres (yolk antibodies, yAbs) additionally to be important.

To assess the effects on adult stage response we compared nine models including a null model, which included the terms in the juvenile response models, the juvenile response itself, and the vaccination order of the two adult stage challenges. The responses of the week 5 and week 6 challenges were pooled as "adult response", but the two antigens were analyzed separately. The model hypotheses are outlined in table 2.

Best-fit models were assessed for goodness of fit using the rescaled generalized coefficient of determination (R^2) definition from Nagelkerke (1991) which utilizes likelihoods of the model of interest and the null model.

Results

Yolk injection treatments

The anti-KLH titres differed among yolk treatment groups (ΔAICc =28 for the null model, AICcw ~ 1 for the treatment model, R^2 = 0.91, fig. 1a). However anti-LPS titres did not differ among yolk treatment groups (ΔAICc = 10 for the treatment model,

AICc*w* ~ 1 for the null model, R^2 = -0.10, fig 1b). The absolute difference among treatments was not as large as would be expected given the differences in yolk sac injection amounts, anti-KLH titres were about 1.5x control for the low-KLH treatment, and 3x control for the high KLH treatment, and anti-LPS titres were about 2x control for the high LPS treatment.

Juvenile response

Of the juvenile response models, the model including only the challenge antigen was ranked above the null model (ΔAICc = 0, AICc*w* = 0.45 for vaccine antigen; ΔAICc = 0.35, AICc*w* = 0.38 for null model), and the model including only the neonatal anti-KLH titre ranked third (ΔAICc = 2.9, AICc*w* = 0.11). However, the R^2s were close to zero and so the model fits were poor. Because of the weak explanatory power of the models we do not report effect size, as the confidence intervals include zero. Thus, yolk antibodies have no measureable effect on antibody response at the juvenile stage.

Adult response

For both the KLH response and the LPS response, the best-fit models were the juvenile response alone, independent of juvenile antigen exposure; models including juvenile antigen exposure ranked relatively high with ΔAICc values in the range of plausible hypotheses (table 3). However, model fits for the juvenile response effect were poor, with R^2 = 0.12 for KLH response and R^2 = 0.14 for the LPS response (fig. 2).

Discussion

Yolk sac injections of newly hatched chicks have not previously been used in ecological research to study the effects of maternal antibody allocation (Boulinier & Staszewski,

2008). However, this is a useful technique for manipulating neonatal IgY because it allows more precise control over the maternal effect compared to maternal vaccination, which may alter inflammation (Tizard, 2002) and subsequently steroid hormone levels (Sapolsky, Rivier & Yamamoto, 1987) in addition to yolk IgY. One could use higher injection concentrations than in this study because not all the injected antibody was absorbed into the bloodstream. We have no information about whether excess maternal antibodies might be shunted to specific tissues rather than remaining in circulation. The use of ovalbumin as a sham is a reasonable control for nutritional effects of yolk IgY, and this might become more important if injection treatment concentrations were significantly increased. The major challenge with this technique is to obtain antibodies that behave like maternally derived antibodies. For studies in wild populations, it may be possible to use antibodies from donor eggs from the same population, or to explore whether commercially available bird IgY can be used in other species.

In contrast to previous studies on maternally derived antibodies, we found no effect of yolk antibodies on the juvenile immune response. Previous studies have reported both positive and negative effects of yolk antibodies on antigen-specific antibody production in chicks of domestic fowl (Grindstaff, Hasselquist, Nilsson *et al.*, 2006, Hassan & Curtiss, 1996, Reid, Arcese, Keller *et al.*, 2006, Staszewski, Gasparini, McCoy *et al.*, 2007). There are several possible explanations for a lack of effect. Manipulated levels of yolk antibodies probably were sufficient to cause a detectable impact, as differences in neonatal titres should have been physiologically relevant. Vaccination doses were probably insufficient to overcome the yolk antibody levels because the vaccine dosage selected was the minimum likely to cause a B-cell response (Koutsos & Klasing, 2001). Alternatively, chicks might respond to the particular

antigens utilized through a route not affected by maternal antibodies. Alternatively, the time window within which maternal antibodies affect immunity could occur prior to our yolk sac manipulation during pre-hatching uptake. Ours is the first study to indicate that the timing of the effects of maternal antibodies in birds might be so early in development.

The lack of a strong effect in our yolk antibody manipulations may also imply that synergistic effects of other changes involved with vaccination may be more important in the response suppression normally associated with maternal antibodies. Previous studies of maternal antibodies have manipulated offspring levels via vaccination of the mother (Reid *et al.*, 2006, Staszewski *et al.*, 2007). Relatively little attention has been paid to effects on other egg components after maternal vaccination; this may be an important direction for future research. Additionally, there has been no attempt to distinguish between types of antigens and whether the effects of maternal antibodies could differ depending on the pathogen of interest, despite clear differences in neonatal immunity against different antigens (Siegrist, 2007).

We found evidence for a positive relationship between juvenile response and adult response, regardless of whether the antigen of the adult vaccination matched the juvenile vaccination. This effect is not due to memory cell production because it was independent of the juvenile challenge antigen. It is likely due to a combination of factors including a generalized effect of early immune experience on development and subsequent reactivity of leukocytes. We partially controlled for genetic factors by including sibling group as a random effect on intercept in our models, suggesting that pleiotropic effects may not be the only factor contributing to this relationship. Some evidence suggests that genetic factors play a more important role in juvenile immune

response than in adult immune response (Kimman, Vandebriel & Hoebee, 2007), so this pattern might arise not from direct genetic factors but indirectly via carryover effects from the juvenile challenge response.

We found no evidence of carry-over effects of maternal IgY into adult immunity, at least when yolk antibody levels approached high vaccine response levels. However, yolk antibodies had little or no effect on juvenile immune response, and so it is not surprising that there were no long-lasting effects. Thus, we found no evidence that the costs of maternal antibody allocation are borne in the specific immune response. The ecoimmunology literature has so far addressed only the adaptive significance of yolk antibodies, and the costs remain largely unknown (Hasselquist & Nilsson, 2009). Potential developmental effects of yolk antibodies such as diversification of the B-cell repertoire, or autoimmunity, remain unstudied. Also, nutritional quality tradeoffs in the protein allocated to yolks may limit antibodies in favour of more complete proteins, or sulfur amino acids. Our study presents a novel technique for manipulating yolk antibodies, and this can now be utilized to test other hypothesized effects of maternally derived antibodies on offspring development.

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Table 1. Experimental design with four treatments and four challenge schedules using sibling sets from 46 hens. The numbers represent the n value for the particular treatment*challenge block. There are 12 sibling sets for each challenge schedule, with one sib from each set in each treatment (some sibling sets were incomplete), and a total experimental n of 160.

challenge schedule			treatments			
1 week	5 week	6 week	control albumin	high IgY (anti-LPS)	low IgY (anti-KLH)	high IgY (anti-KLH)
LPS	KLH	LPS	6	12	10	9
LPS	LPS	KLH	8	10	10	11
KLH	KLH	LPS	10	11	10	9
KLH	LPS	KLH	11	11	11	11

Table 2. Models for analysis of adult KLH and LPS responses.

	description	yolk treatment	neonatal antibody titre	juvenile challenge antigen	juvenile response titre	vaccination schedule	neonatal antibodies × juvenile antigen	juvenile response × juvenile antigen
1	global	•	•	•	•	•	•	•
2	yolk derived antibodies and age of vaccination	•	•			•		
3	yolk derived antibodies	•	•					
4	yolk derived antibodies and early immune experience		•		•			
5	early immune experience depending on antigen familiarity			•	•			•
6	antigen familiarity			•				
7	early immune response/pleiotropy				•			
8	age of vaccination (vaccination order)					•		
9	null							

Table 3. AICc model selection for response to vaccination at the adult stage. Early immune response, independent of the antigens involved, best predicted the adult antibody response to vaccination with LPS (a, n = 110) and KLH (b, n = 143).

a)	model	K	log*L*	AICc	ΔAICc	*L*ΔAICc	AICc*w*
	7. early immune response/pleiotropy	4	-95.76	199.88	0.00	1.00	0.69
	4. yolk derived antibodies and early immune experience	5	-95.92	202.38	2.50	0.29	0.20
	8. age of vaccination	4	-97.86	204.08	4.20	0.12	0.08
	5. early immune experience depending on antigen familiarity	6	-96.65	206.12	6.23	0.04	0.03
	9. null	3	-102.60	211.43	11.55	0.00	0.00
	6. juvenile challenge antigen	4	-103.30	215.08	15.20	0.00	0.00
	1. global	12	-95.65	218.52	18.64	0.00	0.00
	2. yolk derived antibodies and age of vaccination	8	-100.90	219.33	19.44	0.00	0.00
	3. yolk derived antibodies	7	-105.20	225.50	25.62	0.00	0.00

b)	model	K	log*L*	AICc	ΔAICc	*L*ΔAICc	AICc*w*
	7. early immune response/pleiotropy	4	-121.70	251.59	0.00	1.00	0.76
	4. yolk derived antibodies and early immune experience	5	-121.90	254.14	2.55	0.28	0.21
	5. early immune experience depending on antigen familiarity	6	-123.00	258.62	7.03	0.03	0.02
	9. null	3	-129.40	264.97	13.38	0.00	0.00
	6. juvenile challenge antigen	4	-129.30	266.99	15.40	0.00	0.00
	8. age of vaccination	4	-130.60	269.39	17.80	0.00	0.00
	3. yolk derived antibodies	7	-133.80	282.53	30.94	0.00	0.00
	1. global	12	-128.10	282.70	31.11	0.00	0.00

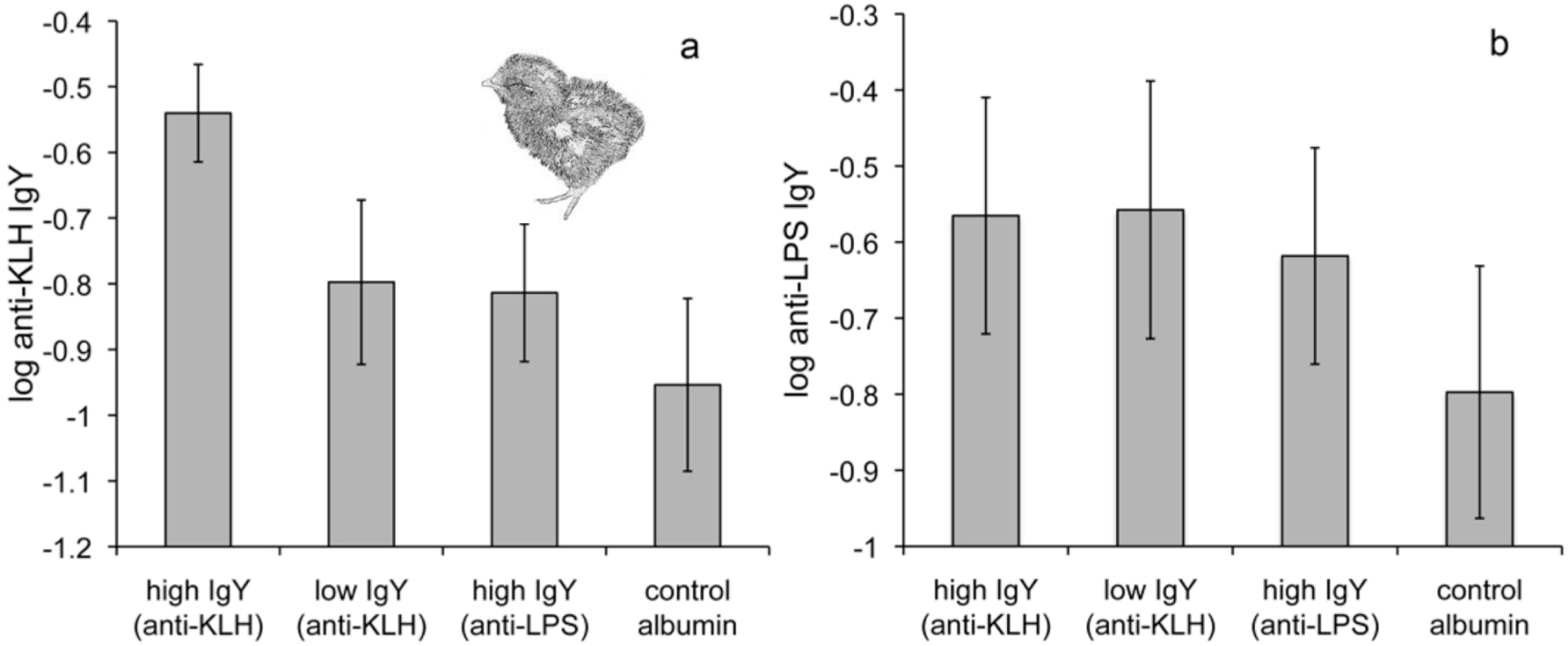

Figure 1. a) Neonatal anti-KLH IgY differed among yolk treatment groups (mean ± 95% CI), indicating that yolk sac IgY injections were incorporated into circulation. b) Neonatal anti-LPS IgY did not differ among treatment groups.

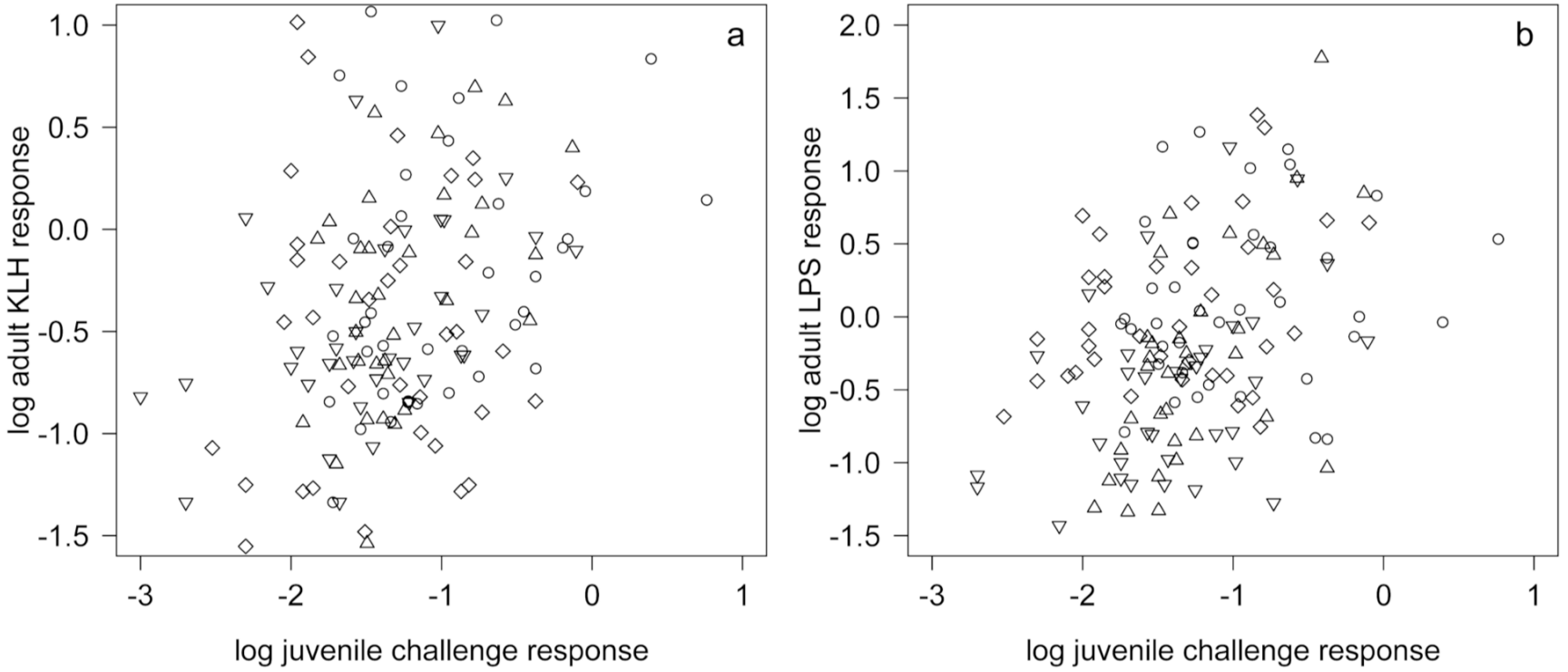

Figure 2. Juvenile challenge response had a positive effect on adult response to KLH (a) and LPS (b), regardless of the antigen in the first vaccine, and the order of subsequent vaccines. Circles are KLH-KLH-LPS, upward triangles are KLH-LPS-KLH, diamonds are LPS-KLH-LPS, and downward triangles are LPS-LPS-KLH.

EFFECTS OF SEASONAL TIMING, REPRODUCTIVE INVESTMENT, AND LAYING ORDER ON MATERNAL ANTIBODY ALLOCATION IN CLAY-COLORED ROBINS

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Abstract

Maternally derived IgY can defend neonates against infection, dampen the acute phase response to immune challenge, and enhance growth. Strategic deposition of yolk IgY has been proposed to ameliorate sibling competition and seasonal nutritional deficits. Many previous field studies on this topic have measured IgY in yolks, but never tested whether differences in yolk concentrations resulted in differences in plasma IgY of hatched chicks. We examine the effects of season, nest attempt number and laying/hatching order on maternally derived IgY levels in eggs and newly hatched chicks of clay-colored robins nesting in lowland Panama. We found that eggs from first nest attempts had higher yolk IgY levels than eggs from subsequent nest attempts, however, neonate plasma IgY did not differ by nest attempt, season, or hatching order. Our results suggest: 1) that IgY deposition may be resource limited, or that chicks from later nest attempts do not benefit from additional IgY, and 2) that studies measuring IgY in eggs or chicks only should be cautious in their interpretations, as big differences in yolk IgY may not appear in chick plasma titres.

Introduction

Mothers can influence offspring phenotype by provisioning nutrients, hormones, and defensive proteins in the egg (maternal effects *sensu* Mousseau and Fox 1998). Maternal effects can prepare offspring for the physical, nutritional and social environments into which they will be born, and can also allow mothers to fine-tune their investment in offspring of greater or lesser fitness value to themselves. Previous studies

of steroid hormones in avian eggs have found statistical effects of social environment, seasonal timing, laying order, and clutch size (Gil 2003, Groothuis et al. 2005). Where nutrients are limited, mothers can enhance offspring begging behavior and growth (e.g. Eising and Groothuis 2003) or adaptively decrease brood size (e.g. Reed and Vleck 2001, Sockman and Schwabl 2000) via the strategic deposition of androgens or corticosterone in eggs.

Defensive proteins, and specifically antibodies, have received relatively little attention in the ecological literature. Recent studies of wild birds have shown variability within and between clutches in yolk antibody levels (Pihlaja et al. 2006, Groothuis et al. 2006, Müller et al. 2004), but factors affecting yolk antibody allocation appear to vary among species. However, adaptive adjustment of yolk antibodies within and between clutches is physiologically possible and hypothetically advantageous. Provisioning of passive immunity could reduce pathogen infection and the need for a matured immune response, and thus allow the chick to allocate additional resources towards growth or other requirements (Grindstaff et al. 2003). Thus, maternal yolk antibodies benefit offspring during the early developmental period, particularly when pathogen pressure is high, or nutrition is poor. Where maternal antibodies are costly, mothers should increase the relative allocation to offspring of higher reproductive value and influence offspring phenotypes to match her capacity to feed and rear young more closely. We know little about the costs of maternal antibody allocation. There is little evidence of nutritional or energetic limitations on yolk antibody deposition in lab systems (Klasing 1998, Grindstaff et al. 2005), but nutrition appears to either limit yolk antibody deposition, or act as a signal of environmental quality, in field systems with experimental food supplementation (Hargitai et al. 2006, Pihlaja et al. 2006, Gasparini et al. 2007).

Alternatively, costs of maternal antibody provisioning may be largely borne by the offspring, through depressing effects of maternal antibodies on the development of immunity (Carlier et al. 1992, Staszewski et al. 2006, Hasselquist and Nilsson 2009).

Studies of maternally derived antibodies in wild birds frequently measure yolk antibodies in eggs or plasma antibodies of neonates to study patterns of maternal antibody allocation and their fitness consequences (see for example Saino et al. 2003, Morales et al. 2006, Hargitai et al. 2006, Gasparini et al. 2007). However, the assumption that yolk antibodies are directly related to neonate antibodies is seldom validated in wild systems. Limitations on neonatal antibody uptake could dampen variability in yolk antibody levels, or alternatively, small differences in yolk antibodies could be amplified by differences in neonate uptake or body size. Here we test the relationship between maternal antibody deposition in egg yolks to neonatal plasma, in the framework of several hypotheses for adaptive adjustment of maternally derived antibodies.

We investigated whether clay-colored robins (*Turdus grayi*), a Neotropical lowland resident, varied yolk antibody levels within and between clutches in relation to time during the breeding season or position of the egg in the laying sequence. At our study site in Panama, clay-colored robins begin breeding during the dry season and continue well into the wet season (February to July), usually laying a clutch of 2-3 eggs. Incubation is initiated after the second egg is laid. We predicted that third eggs that experience more sibling competition will either have higher yolk IgY levels to help alleviate sibling competition (Pihlaja et al. 2006), or lower yolk IgY to provide for brood reduction in the event of food shortage (Müller et al. 2004). We also predicted that first clutches might be allocated more IgY, if resources limit IgY allocation and females have

less to expend on subsequent clutches. Also, clutches laid in the dry season, when food is more limiting, may have more IgY to help alleviate costs of immune response and direct resources towards growth. Alternatively, if pathogen pressure is higher during the wet season when there are more vectors and temperature and humidity environments are better suited to fungal and bacterial growth, mothers may deposit higher yolk IgY in the wet season compared to the dry season. We evaluated the predicted patterns using both yolk IgY and neonate plasma IgY measures in an AICc model selection framework (Burnham and Anderson 2002), which allows us to simultaneously compare among all these possible hypotheses.

Methods

We located nests of clay-colored robins in Gamboa, Panama, from 15 March – 30 June 2006, and 27 February – 25 May 2007. Nests were checked daily, and fresh eggs were collected on the day they were found and replaced with a dummy egg. Once clutches were complete, two days after the appearance of the last egg (this species usually lays daily), all the dummy eggs were collected and the female left undisturbed so that she might relay. All nests and nesting territories were rechecked every three days starting two weeks after egg collection, for activity that would indicate the bird intended to relay. Clay-colored robins frequently use the same nest several times in a breeding season. Collected eggs (42 eggs from 16 nests) were stored at 4°C for up to 24 hours until IgY extraction. All collections were under permit from Panamanian ANAM authorities, University of Missouri IACUC, and the Smithsonian Tropical Research Institute.

Nests located with complete clutches were checked every two days until the eggs pipped, after which they were checked daily. Nestlings were marked with coloured

markers to identify hatching order. After nestling yolk absorption was complete, usually about two days after hatch, we collected 30 uL of blood into heparinized microhematocrit tubes by brachial venipunture (77 chicks from 33 nests). Blood was centrifuged for 8 mins at 8000 rpm to separate plasma, and plasma and red blood cells were stored at -20°C until transfer to the laboratory at University of California Davis for analysis.

IgY extraction and measurement

Eggs were weighed whole, and then broken out, and the yolks separated and weighed to the nearest 0.001g. Shells were dried at 25°C for 48 hours (until constant weight) and weighed. For extraction, we aliquoted 0.1g of yolk into a 1.5mL Eppendorf tube and removed the lipid by vortexing with 400 uL of 3.5% PEG-6000 in 0.9% NaCl, incubating overnight at 4°C, and centrifuging for 30 mins at 8000g and 4°C. The yolk pellet was discarded, and the supernatant reserved for analysis.

Yolk IgY was quantified in 5 uL samples of the extract by SDS-PAGE on 5% gels run at 90 volts for 30 mins. Chicken IgY (purified polyclonal, Sigma-Aldrich, St Louis) standard dilutions were run simultaneously to generate a standard curve. Protein was stained with gel-code blue (commassie) stain for 1 hour, and washed in distilled water overnight. Gels were then photographed on a white light table, and the images were analyzed for band density using the gel tool in ImageJ (NIH). Integrated density values were converted to concentration, in mg/ml, using the chicken IgY standard curve, and the values multiplied by 5 to account for the dilution involved with the extraction method. Total egg IgY was then calculated by multiplying the yolk mass with the sample concentration.

Chick plasma samples were analysed by direct ELISA as in (Martinez et al. 2003) with the following modifications. We used 1% BSA in 0.05% PBS-tween for blocking buffer rather than 1% non-fat dry milk. We also used goat anti-bird IgY-HRP (Bethyl Laboratories) for our detection antibody, diluted 1:20 000 in 1% BSA. Samples were run against a chicken serum standard to provide an index of IgY concentration. All samples were run in duplicate and the results were averaged. Plasma was defrosted and 1 uL of each sample was incubated for 2 hours in 100uL volumes of carbonate-bicarbonate binding buffer inon Nunc maxi-sorp 96 well plates. Plates were washed 4 times with 0.05% PBS-tween, and then blocked with 1% BSA. Chick samples were incubated 1 hour at room temperature in anti-bird IgY detection antibody, then again washed four times with 0.05% PBS-tween. Horseradish peroxidase was developed with TMB (tetramethylbenzidine) PO substrate (Thermo Scientific, Rockford IL) and stopped with 1N sulfuric acid after 15 mins, and the plate read at 450 nm.

Statistical Analysis

All IgY data were log transformed for normality. We generated ten different statistical models (table 1) for hypotheses for yolk IgY allocation, and fit each model to the egg IgY data using the lme() function in the nlme package of R (v. 2.7.2). Our models included the parameters year (2006 or 2007), season (dry or wet), nest attempt number (first or subsequent), and laying order (1, 2 or 3). Each model contained nest ID as a random effect on intercept. We then selected the models with DAICc values less than 10, and considered to have some support (Burnham and Anderson 2002), to test these hypotheses on the chick plasma data set.

Results

We found evidence for an effect of nest attempt order (table 1), where first clutches had more IgY than subsequent clutches (table 2). There is a suggestion in the data that clutches laid in the dry season to have greater IgY than those laid in the wet season (fig. 1, table 2). There is also a suggestion of a laying order effect, where third eggs have higher IgY than first and second eggs, but this effect may interact with the effect of clutch laying order (fig. 2).

We tested all models from the egg analysis with ΔAICc values less than 10 on the chick plasma data set. The null model (differences correspond to nest identity only) was selected as the best model for chick plasma IgY concentration (AICcw > 0.99), so that none of the previously supported models are supported with the chick dataset.

Discussion

Our most striking finding is that the effects found in the egg data set do not appear in the chick data set when IgY is measured after yolk absorption. This is in contrast to a study on specific antibodies in kittiwakes (Gasparini et al. 2002). There are two possible interpretations of this result. First, it is possible that the small differences among eggs are lost during the transfer of antibodies from yolk to chicks because of dilution or receptor limitation, and there are no adaptive consequences of adjusting yolk antibody allocation. Second, it is possible that some minimum level of antibodies are taken into circulation of the neonate, and any excess is utilized and metabolized very early, during pipping and shortly after hatch. The loss of measurable differences in yolk antibody allocation at the chick phase suggests that we should be cautious in the interpretation of studies that only measure maternal antibodies at the egg or chick phase until we have a

better understanding of the utilization of antibodies during the very early neonatal period.

We found evidence that subsequent clutches had lower IgY than first clutches, independent of the season. This is consistent with a resource limitation hypothesis for maternal antibody allocation. Laboratory studies and calculations have found relatively little evidence for nutritional effects on yolk antibodies (Klasing 1998, Grindstaff et al. 2005). However, supplemental feeding experiments in the field appear to increase yolk antibody levels (Pihlaja et al. 2006, Moreno et al. 2008) but see (Gasparini et al. 2007). However, no previous work has looked at the possibility that costs of egg production and yolk antibody allocation might carry over to the next reproductive event. Egg production is generally thought to be costly and potentially to have lasting effects on future reproductive efforts (Williams 2005). Moreover, mothers in our study seldom laid three eggs in subsequent clutches (see n values in fig. 2), suggesting that resource limitation might be an important factor in resource allocation in this species.

Yolk steroid hormones have formerly been investigated as a means by which mothers can alter offspring phenotype to better prepare them for the nutritional environment (Groothuis and Schwabl 2002, Verboven et al. 2003, Gasparini et al. 2007). Yolk antibodies may also influence the allocation and utilization of energetic and nutritional resources of neonates (Grindstaff et al. 2003, Boulinier and Staszewski 2008). Previous work has shown that maternal antibodies can help to shunt resources from immune response to growth (Grindstaff 2008); even in the absence of a specific immune challenge they can increase growth of neonates (Gallizzi et al. 2008) and so they might be used to modify the offspring utilization of nutritional resources. We found evidence of a seasonal effect on yolk antibody allocation, suggesting that mothers

might influence the utilization of resources during the more food-limited dry season. This indicates that the effect of maternally derived antibodies on growth may actually be more important than the effect on immune defense, because the wet season is generally thought to have higher pathogen pressure than the dry season.

There is also weak evidence in the data that mothers deposit higher concentrations of IgY into third eggs in their subsequent reproductive attempts, however sample sizes are very small. Yolk steroids have been shown to mitigate the disadvantages of hatching last in a brood (Eising and Groothuis 2003), and recent studies of yolk antibodies suggest a similar potential role in alleviation of sibling competition (Pihlaja et al. 2006, Hargitai et al. 2006). In clay-colored robins the third egg usually hatches the day after the first two, and the third chick is smaller than its two older siblings (personal observation and personal communication from D. Robinson and S. Austin). Deposition of more antibodies in the third egg may give the third chick an additional possible advantage over its larger siblings.

None of these effects appear in the chick plasma antibody measurements. Overall, we found evidence consistent with effects of resource limitation on yolk antibody deposition, and strategic increases in yolk antibodies to compensate for nutritional limitation of growth in nestlings. It is not clear whether the absence of these effects on neonate plasma IgY suggest that adaptive explanations for differences in egg yolk antibodies are premature. Yolk antibody allocation could be an important maternal effect, allowing mothers to make plastic, non-genetic alterations to offspring phenotype and thus better equip them for the early rearing environment.

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Table 1. AICc values for the models tested with the egg data set for IgY allocation. All models included nest ID as a random effect on intercept (n = 16 nests with 39 eggs).

model	k	log*L*	AICc	ΔAICc	*L*ΔAICc	AICc*w*
nest attempt	4	-26.2946	61.7657	0.0000	1.0000	0.5261
season	4	-27.0730	63.3224	1.5567	0.4592	0.2416
null	3	-29.0277	64.7411	2.9754	0.2259	0.1188
nest attempt, order, nest attempt x order	8	-22.7408	66.2817	4.5160	0.1046	0.0550
year	4	-28.9616	67.0996	5.3340	0.0695	0.0365
order	5	-28.5169	68.8520	7.0864	0.0289	0.0152
season, nest attempt, order, season x order, nest attempt x order	11	-20.1778	72.1334	10.3677	0.0056	0.0029
year, order, year x order	8	-26.0971	72.9941	11.2284	0.0036	0.0019
season, order, season x order	8	-26.1524	73.1048	11.3391	0.0034	0.0018
year, season, nest attempt, order, year x order, season x order, nest attempt x order	14	-18.3342	82.1685	20.4028	0.0000	0.0000

Table 2. AICc weights and weighted parameter estimates across all models predicting egg yolk IgY. The nest attempt parameter is the difference between subsequent and first clutches, season is the difference between wet and dry season, order(2) is the difference between the second and first eggs laid, order(3) is the difference between the third and first eggs laid, year is the difference between 2007 and 2006 egg collections.

Parameter	AICc*w*	*w*estimate	±95%CI
Intercept	1.0000	0.4779	1.0550
nest attempt	0.5841	-0.8298	0.6480
season	0.2463	-0.2735	0.3697
order(2)	0.0769	0.0129	0.0401
order(3)	0.0769	0.0349	0.0758
nest attempt x order(2)	0.0580	-0.0405	0.0861
nest attempt x order(3)	0.0580	0.0548	0.1147
year	0.0385	-0.0170	0.0589
season x order(2)	0.0048	-0.0004	0.0037
season x order(3)	0.0048	-0.0047	0.0102
year x order(2)	0.0019	-0.0004	0.0020
year x order(3)	0.0019	0.0025	0.0053

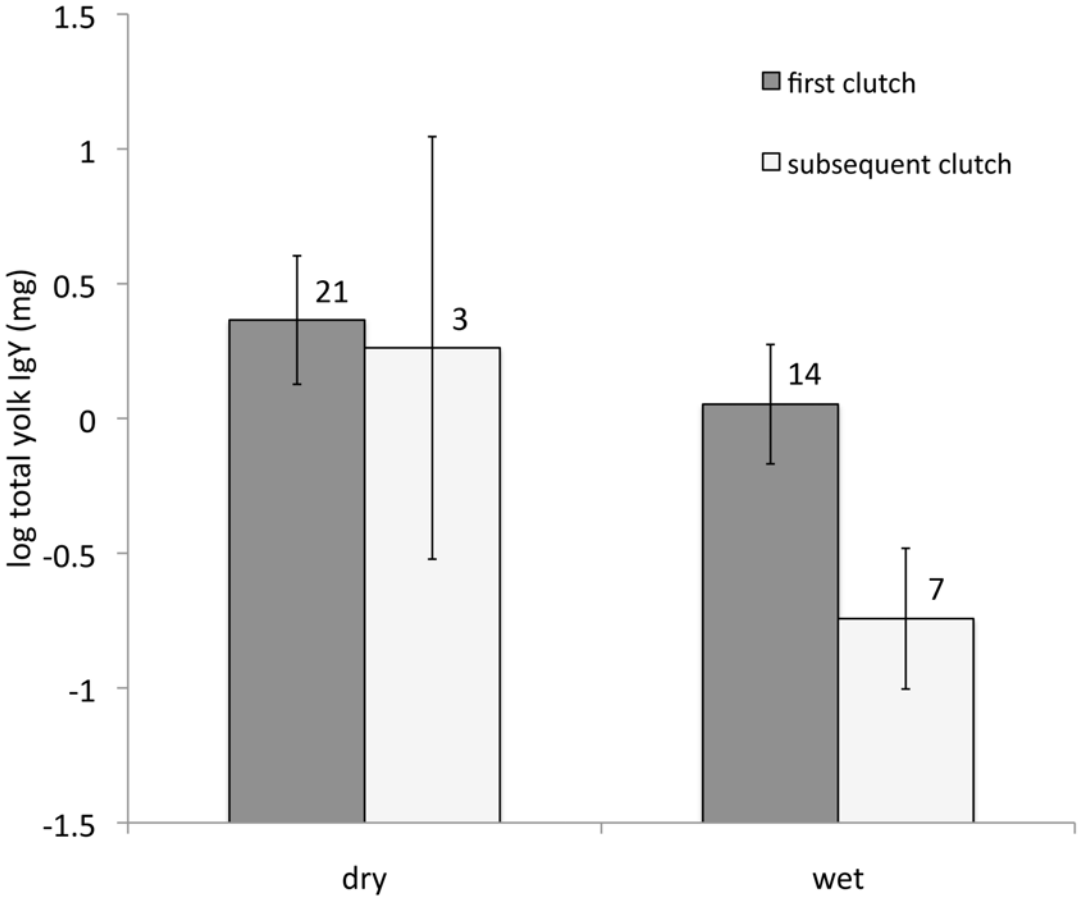

Figure 1. Egg yolk IgY (mg) in first and subsequent clutches in the dry and wet seasons.

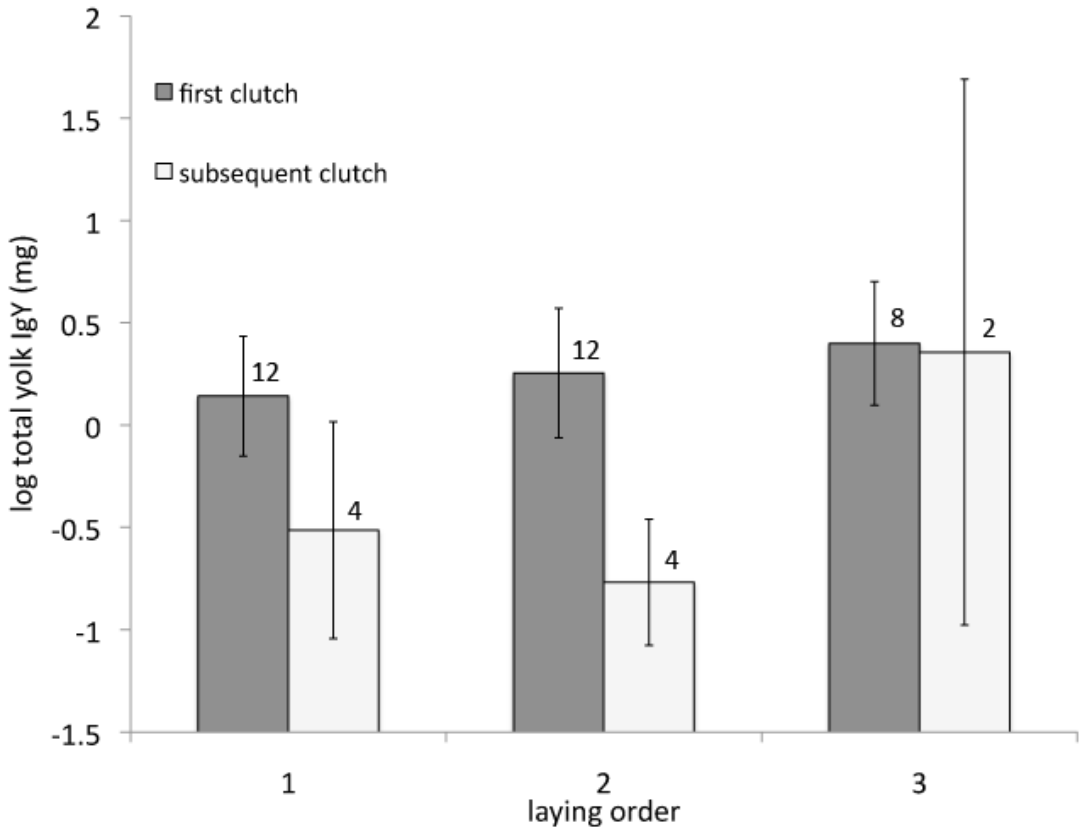

Figure 2. Egg yolk IgY in first and subsequent clutches for each egg in the clutch. The error for third eggs in subsequent clutches is very large because there was seldom three eggs in renest attempts.

MATERNAL ANTIBODIES, GROWTH, AND EARLY IMMUNE DEVELOPMENT IN CLAY-COLORED ROBINS

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Manuscript in preparation.

Introduction

Besides macronutrients for growth, the avian egg contains numerous other compounds utilized by the embryo and neonate, including vitamins, carotenoids, hormones, and defensive proteins, including yolk antibodies. The primary benefit of yolk antibodies is passive immunity to pathogens, reducing infection rates and dampening offspring immune responses that would divert resources away from growth (Grindstaff, Brodie & Ketterson, 2003). Moreover, maternal antibodies may have a priming effect on immune development (Grindstaff, Hasselquist, Nilsson *et al.*, 2006, Lemke & Lange, 1999), or conversely may interfere with immune development by reducing antigen exposure during B-cell clonal selection (Lemke *et al.*, 1999, Carlier & Truyens, 1995).

Neonatal plasma IgY derives from antibodies deposited in the egg yolk from maternal circulation (Apanius, 1998). IgY is actively transported from mother to yolk at the follicle membrane (Loeken & Roth, 1983) and is taken up by the embryo very late in development and continuing until completion of yolk absorption in the chick (Tressler & Roth, 1987). The chicks undeveloped immune system does not produce its own antibodies until B-cells mature several days after hatch (Apanius, 1998). Endogenously produced IgM appears in circulation first at about 4/20 days of age in the chicken/mallard, and IgY appears several days later (Liu & Higgins, 1990, Kaspers, Schranner & Losch, 1991).

Recent interest in ecological immunology has led to the study of maternal effects on immunity, but so far we have little information on relationships between maternally derived yolk antibodies, and offspring outcomes in terms of growth, immune

development, and survival. What information we do have focuses on domestic poultry and a limited number of wild species. Maternal antibodies are related to growth in Japanese quail (*Coturnix japonica,* Grindstaff, 2008), tree swallows (*Tachycineta bicolor,* Lozano & Ydenberg, 2002), and influence juvenile immunoglobulin production in magpies (*Pica pica,* Pihlaja, Siitari & Alatalo, 2006), and juvenile immune response in pied flycatchers (*Ficedula hypoleuca,* Grindstaff *et al.,* 2006), and song sparrows (*Melospiza melodia,* Reid, Arcese, Keller *et al.*, 2006). Moreover, ecological factors such as food limitation can influence juvenile immunocompetence (Gasparini, Roulin, Gill *et al.*, 2006). Maternally derived antibodies could have important effects on growth and immune development in wild populations, and should also correlate with pathogen pressure experienced by nesting hens and their offspring.

Mothers could modify yolk antibody allocation to manipulate offspring phenotypes, increasing offspring growth rates, altering immune response allocation, or advantaging late-hatching offspring allowing them to catch up to earlier hatched siblings (Grindstaff *et al.*, 2003, Boulinier & Staszewski, 2008, Hasselquist & Nilsson, 2009). Also, maternal antibodies could modify early immune development (Lemke, Tanasa, Trad *et al.*, 2009), which could be measured in titres of IgM and natural antibodies. We investigated the relationship between maternally derived IgY titres shortly after hatching, and growth and endogenous IgM production of nestlings in the clay-colored robin (*Turdus grayi*). We have previously found differences in the amount of yolk IgY deposited among eggs laid in the dry versus wet season, and moderate hints of differences among laying order within a clutch. It is not clear how these differences might translate into differences in nestling development.

Clay-colored robins in our study site begin nesting in the middle of the dry season, when nest predation is supposed to be lower (Morton, 1971), but food resources, including fruit and insects, are not very abundant. Most individuals have multiple nesting attempts during the breeding season, which extends several months into the wet season, sometimes rearing two or more broods. This species typically lays a clutch of 2 or 3 eggs on subsequent days. The first two chicks hatch nearly synchronously and the third chick hatches about 1 day later (pers. obs., S. Austin pers. comm.). Chicks fledge between 11 and15 days of age. Robin chicks in our study site commonly disappear before fledge due to predation (W.D. Robinson and T.R. Robinson, unpublished data), and also have been observed to suffer bot fly parasitism, ticks, and body mites (pers obs). Starvation, particularly of the third chick, is common (Morton, 1971).

Methods

We located nests of clay-colored robins in Gamboa, Panama, between 27 February – 25 May 2007. Nests found with complete clutches were checked every other day, and nests found at the laying stage were checked daily starting 13 days after the first egg was laid. Chicks were weighed and measured on the day that they were found, and a 50 uL blood sample was collected into heparainized microhematocrit tubes by brachial venipuncture after the chick had achieved 6 g mass, which was always within the first 48 hours post-hatch, and around the time that yolk absorption was completed. Chicks were weighed and measured at 5 to 6 days of age, and weighed, measured and bled again (50-100uL) at 10 days of age. Blood samples were kept on ice in the field, and centrifuged as soon as possible. Plasma was stored at –20C, and red blood cells stored at 4C in lysis buffer. Chicks surviving past 10 days of age were assumed to fledge unless predation or

starvation was known to have occurred. All collections were under permit from Panamanian ANAM authorities, University of Missouri IACUC, and the Smithsonian Tropical Research Institute.

In the lab, plasma samples were analysed for total IgY and total IgM in a direct ELISA based on Martinez, Tomas, Merino *et al.*, (2003). Briefly, plasma samples were diluted 1:100 in carbonate-bicarbonate binding buffer and 100 uL of each sample dilution were loaded in duplicate into wells of 96 well Nunc maxisorp ELISA plates. Samples were incubated at room temperature for 2 hours in the plates, and plates were washed 4x with 0.05% PBS-tween. Plates were blocked for 1 hour at room temperature with 1% BSA in PBS-tween. Again, plates were washed 4x with 0.05% PBS-tween, and wells were incubated 1 hour in 1:20 000 dilutions of goat anti-bird IgY-HRP antibodies (Bethyl Laboratories, Montgomery TX, neonate samples) or goat anti-chicken IgM-HRP antibodies (10 day samples). Plates were again washed 4x with 0.05% PBS-tween before colorimetric detection by TMB PO substrate, and stopped after 15 mins with 1N sulfuric acid. Plates were read at 450 nm and a standard curve was estimated from known dilutions of chicken IgY and IgM.

Chick red blood cells were used for genetic sexing in the laboratory at the University of Missouri – St Louis. We extracted DNA with ammonium acetate and an ethanol precipitation, and amplified the CHD-Z sex-linked genes with the P2, P8 primer pair by polymerase chain reaction as in Griffiths, Double, Orr *et al.*, (1998). PCR product was separated on 3% agarose gels run for 60 mins at 90 volts, and visually scored as having one (males) or two bands (females).

Data were analysed in an AICc model selection framework. We looked at the relationship between growth and endogenous IgM of chicks with the season of hatch,

position in the brood, maternally derived IgY titres, and sex. The candidate model set (table 1) contained 9 models, including a saturated model and a null model. Following rules of thumb discussed in Burnham and Anderson (2002), we used multimodel inference to estimate effect sizes for models sets in which the top model had an AICc weight less than 0.95. All analysis was done using R (v. 2.7.2).

Results

Growth

The top model for chick growth using AICc model selection was the saturated model (table 1). The parameter estimates for two interaction terms did not bound zero; season x IgY and chick ID x season (table 2). During the wet season, growth among all chicks was not related to hatching order, maternal IgY, or sex (table 3). During the dry season, chicks with higher neonatal IgY titres had slower growth rates, but this relationship varied with hatching order, third hatched chicks growing slowest and second hatched chicks having the strongest relationship with IgY titres (table 3, fig 1). In the top model, the variance component for nest identification (random effect) was 40%. The generalized R^2 (Nagelkerke 1991) was 72%.

IgM production

There is weak evidence that chick IgM titres at 10 days of age were lower in the dry season compared to the wet season (table 1, 3), and that there was a relationship between neonatal IgY titres and IgM titres that depended on what season the chick hatched in (table 1, fig 2). All of the parameter estimates bounded zero so the evidence is weak, though the confidence limits around the season effect do not substantially

bound zero (table 2). In the top model, the variance component for nest identification was 75%. The generalized R^2 was 48%.

Discussion

Robin chicks had low but measureable levels of IgY as neonates, and substantial levels of endogenously produced IgM by 10 days of age. Only about 60% of nestlings survived to fledging age, and so the sample size for measures of endogenous IgM production is low. Nest identification accounted for a large part of the explained variance in the top models, suggesting that parental effects, and possibly nest location, have a strong influence on both growth and endogenous IgM production of chicks.

Growth rates were consistently high during the wet season, but decline with hatching order during the dry season. Third chicks in particular suffered low growth rates in the dry season. This third chick disadvantage may be exacerbated by the fact that first and second chicks hatch on the same day, and third chicks hatch a day later (pers. obs.). Clay-colored robins are omnivores that feed their chicks primarily fruit and insects, both of which are more abundant in the wet season (Morton, 1971). The limited number of three chick broods in the dry season (2 out of 8 broods, compared to 12 out of 24 in the wet season), could be an adaptive adjustment of clutch size when food is potentially limiting.

Maternally derived IgY levels are likely a good indicator of pathogen pressure because mothers undergoing an immune response will put higher levels of IgY into egg yolks (Tizard, 2002). Moreover, neonatal IgY levels are negatively related to growth rates, suggesting that immune challenges early in life are directing nutritional resources

away from growth. Depression of growth rates is commonly seen in laboratory studies of immune challenged poultry (Klasing & Johnstone, 1991).

Endogenous IgM production in chicks was slightly lower in the dry compared to the wet season. This pattern could arise if either the pathogen pressure is higher in the wet season, inciting earlier and stronger immune responses, or if nutrition is less limiting of immune development and response in the wet season. Endogenous IgM production is an indicator of B-cell development (Tizard, 2002), and it is likely that most of the IgM measured is natural antibodies that are not necessarily secreted in response to a specific immune insult. This aspect of immunity develops post-hatch and depends in large part on exposure to antigens from the environment (Apanius, 1998).

Clay-colored robin adults have high blood bactericidal activity (Millet, Bennett, Lee *et al.*, 2007, Matson, Tieleman & Klasing, 2006), and high natural antibodies (Lee, Wikelski, Robinson *et al.*, 2008), and deposit high levels of IgY into their egg yolks (Addison et al., ms) relative to other neotropical passerines measured. This, combined with the apparent rapid endogenous production of IgM in neonates suggests an immune syndrome designed to rapidly, and likely non-specifically, identify and remove pathogens via innate immunity. Thus, the clay-colored robin immune syndrome is consistent with a fast-pace life history strategy (Lee, 2006).

The lack of clear predictors for IgM production is probably due to strong selection on, and early canalization of this trait. Parasites and pathogens appear to play an important role in life history evolution (eg Martin, Møller, Merino *et al.*, 2001, Tella, Scheuerlein & Ricklefs, 2002, Tieleman, Williams, Ricklefs *et al.*, 2005), and the timing and prioritization of immune development is probably fairly inflexible within species. There is virtually no information on immune development in wild passerine species, and

it would be useful to document measures of cell-mediated and humoral immunity in nestlings of a variety of wild species to test hypotheses about the evolution of immunity and the role of pathogens in life history physiology.

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Table 1. AICc evaluation of models for (a) chick growth (g/day, n = 26 nests with 72 chicks) and (b) endogenous IgM production at 10 days of age (mg/mL [IgM] in plasma, n = 18 nests with 45 chicks) for clay-colored robin chicks. The parameters included in the models are: chickID (hatching order of the chick), season (whether hatched in the dry or wet season), sex, IgY (plasma maternally derived IgY levels measured ~ 2 days of age, in mg/mL), and second order interaction terms for season, IgY and chickID.

a) growth	model	k	log*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*
	chickID, season, sex, IgY, season x IgY, chickID x IgY, chickID x season	13	-62.338	156.953	0.000	1.000	0.996
	season, IgY, season x IgY	6	-77.424	168.140	11.187	0.004	0.004
	chickID, season, chickID x season	8	-85.329	188.943	31.991	0.000	0.000
	chickID, IgY, chickID x IgY	8	-87.589	193.464	36.511	0.000	0.000
	season	4	-94.227	197.050	40.097	0.000	0.000
	IgY	4	-99.825	208.247	51.295	0.000	0.000
	null	3	-108.285	222.922	65.970	0.000	0.000
	sex	4	-107.825	224.247	67.295	0.000	0.000
	chickID	5	-108.371	227.651	70.699	0.000	0.000

b) IgM	model	k	log*L*	AICc	ΔAICc	*L*ΔAICc	AICc*W*
	season, IgY, season x IgY	6	-8.186	30.582	0.000	1.000	0.868
	chickID, IgY, chickID x IgY	8	-7.284	34.568	3.986	0.136	0.118
	IgY	4	-15.575	40.150	9.568	0.008	0.007
	chickID, season, sex, IgY, season x IgY, chickID x IgY, chickID x season	13	-1.393	40.527	9.945	0.007	0.006
	season	4	-20.843	50.686	20.104	0.000	0.000
	null	3	-22.825	52.235	21.652	0.000	0.000
	sex	4	-23.363	55.726	25.143	0.000	0.000
	chickID	5	-23.598	58.734	28.152	0.000	0.000
	chickID, season, chickID x season	8	-20.979	61.957	31.375	0.000	0.000

Table 2. Parameter estimates for effects on plasma [IgM] (weighted across all models using AICc*W*), and chick growth (estimated from the saturated model which had an AICc*W* > 0.99) models. Effect size estimates that do not bound zero are bolded.

	plasma [IgM]			growth	
parameter	AICc*W*	*W*estimate	± 95% CI	estimate	± 95% CI
intercept	1.000	1.221	±1.224	5.175	±2.235
IgY	1.000	-50.463	±119.693	-255.740	±257.567
season(wet)	0.874	0.804	±1.085	-1.049	±2.289
season(wet) x IgY	0.874	-33.949	±120.655	**282.718**	**±268.066**
chickID(2)	0.124	0.030	±0.106	-0.342	±1.682
chickID (3)	0.124	0.011	±0.147	-1.431	±2.647
chickID(2) x IgY	0.124	-1.468	±12.041	-20.223	±170.215
chickID(3) x IgY	0.124	0.220	±16.744	-96.301	±215.928
sex(m)	0.006	0.000	±0.002	-0.106	±0.372
chickID(2) x season(wet)	0.006	0.002	±0.005	0.574	±0.890
chickID(3) x season(wet)	0.006	0.001	±0.007	**1.965**	**±1.759**

Table 3. Growth, IgM, and IgY means ± 95% CI (n) for clay-colored robin chicks by hatching order and season.

	dry season						wet season					
	1st		2nd		3rd		1st		2nd		3rd	
growth (g/d)	3.153 ± 0.784 (8)		2.301 ± 0.931 (7)		0.860 ± 0.216 (2)		4.232 ± 0.329 (24)		4.237 ± 0.329 (23)		3.903 ± 0.444 (12)	
IgM (mg/mL)	1.076 ± 0.423 (4)		0.938 ± 0.471 (3)		0.368 (1)		1.076 ± 0.169 (17)		1.288 ± 0.244 (16)		1.191 ± 0.127 (7)	
IgY (mg/mL)	0.008 ± 0.001 (8)		0.009 ± 0.002 (7)		0.010 ± 0.002 (2)		0.008 ± 0.001 (24)		0.007 ± 0.001 (22)		0.009 ± 0.001 (12)	

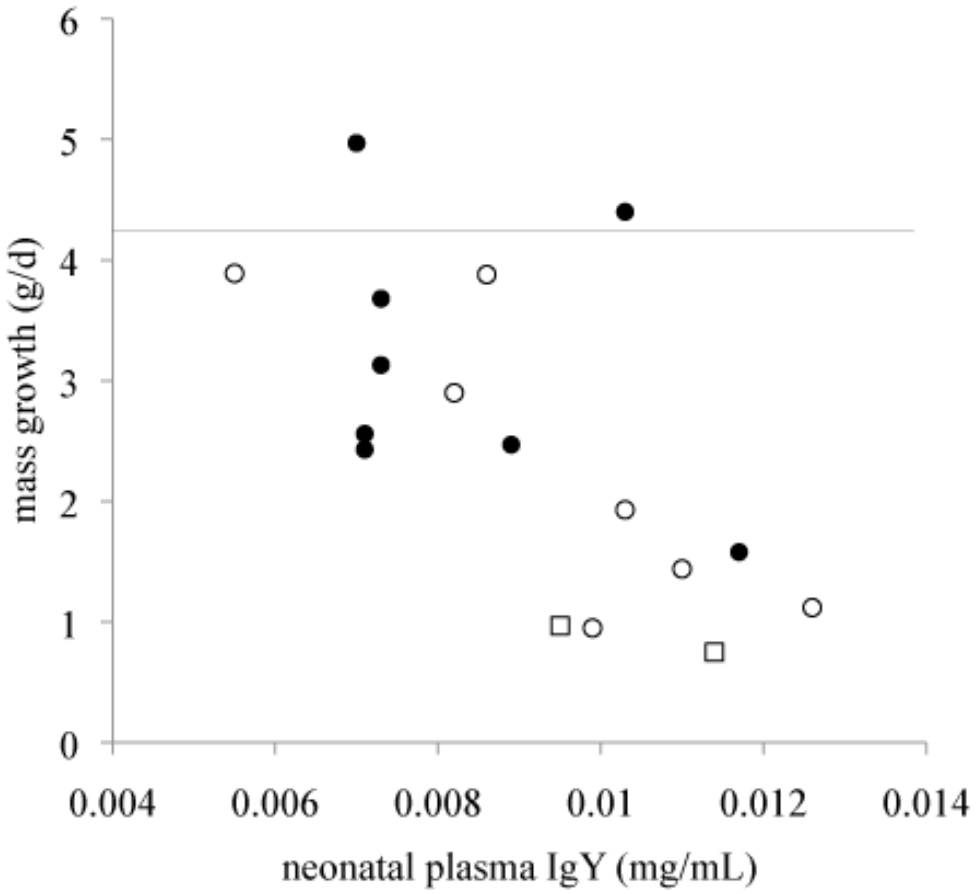

Figure 1. There is a negative relationship between maternally derived IgY and chick growth in the dry season. Third hatched chicks (open squares) generally grow slowest, and have the highest circulating concentrations of maternally derived IgY, while first hatched chicks (filled circles) generally grow fastest and have the lowest concentrations of maternally derived IgY. Chicks hatched during the wet season grow 4.17±0.20 g/day (grey line) irrespective of position in the brood or maternally derived IgY levels.

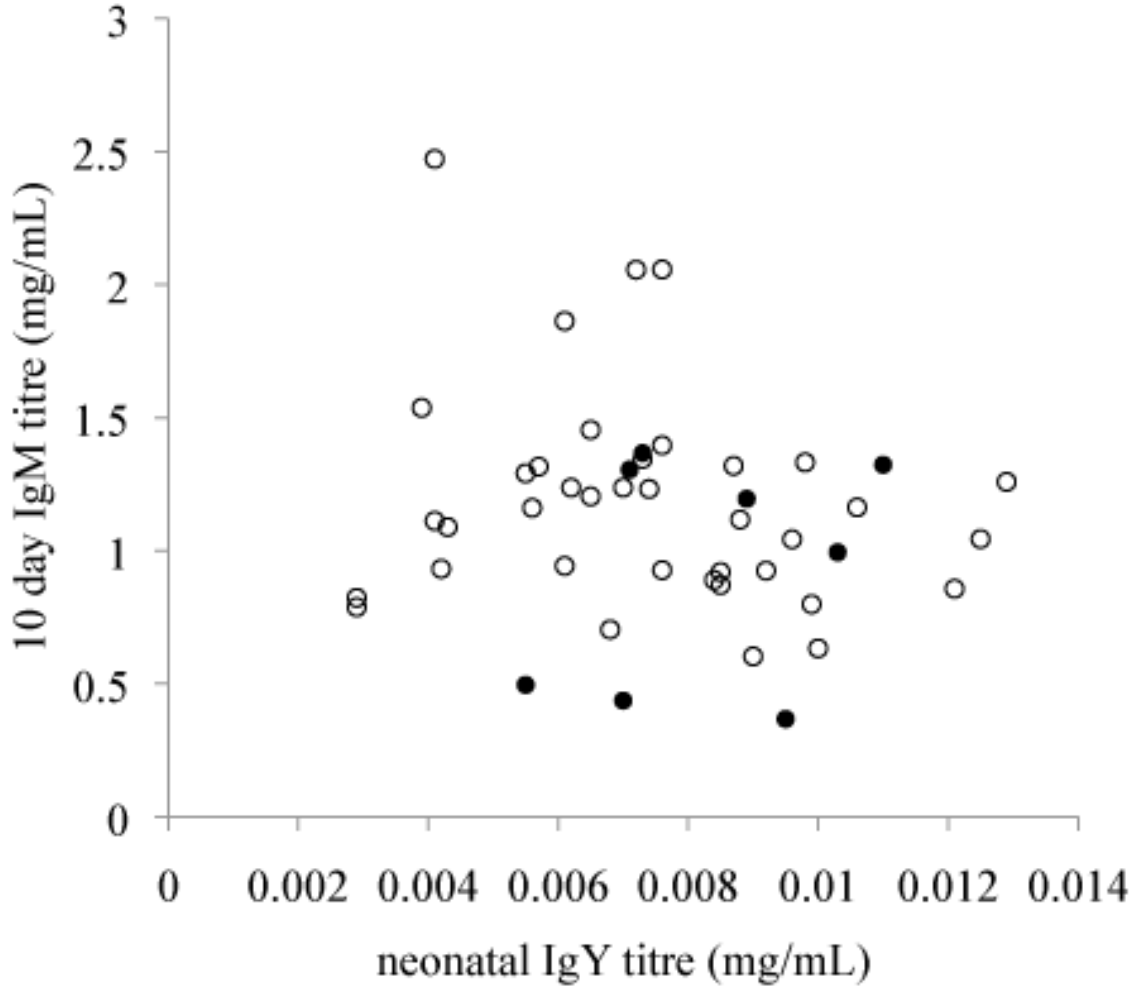

Figure 2. Plasma IgM titre (mg/mL) of chicks hatched in the dry (filled circles) versus wet (open circles) seasons.