IMMUNE FUNCTION AND DEVELOPMENT IN ALTRICIAL-DEVELOPING PASSERINE HOUSE SPARROWS (*PASSER DOMESTICUS*)

By

MARISA OLSON KING

A dissertation submitted in partial fulfillment of
the requirements for degree of

Doctor of Philosophy

WASHINGTON STATE UNIVERSITY
School of Biological Sciences

MAY 2010

`

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MARISA OLSON KING find it satisfactory and recommend that it be accepted.

Hubert G. Schwabl, Ph.D., Chair

Jeb P. Owen, Ph.D.

Derek J. McLean, Ph.D.

`

ACKNOWLEDGMENTS

I want to thank my husband, Matt King, for the endless support and patience he has provided during this long process. I would like to thank my committee members, both past and present (Hubert Schwabl, Jeb Owen, Derek McLean, Jerry Reeves, and Mike Webster), for their guidance and support. I would like to thank the Center for Reproductive Biology (CRB) at Washington State University (WSU) for providing the resources and training necessary for the completion of my degree. In particular, I would like to thank David and Jeanene de Avila, from the CRB Assay Core, for their guidance in the lab. I would like to thank Kyle Caires, Liang Yu Chen,and Nada Cummings, from the Animal Science Department at WSU, and my lab mates Jeremy Egbert, Jesko Partecke, Brian Schwartz, and Willow Lindsay for the laboratory help, comic relief and moral support they have provided over the years. I would like to thank the undergraduate students, Kelly Guevara and Vanessa Talbot, for their assistance in the lab and field. I would like to thank my funding sources, Achievement Rewards for College Scientists (ARCS) and the Environmental Protection Agency's Science to Achieve Results Fellowship (EPA STAR). I would like to thank Miikka Kiprusoff and his teammates from the Calgary Flames for providing the background noise throughout the writing process of my dissertation. Finally I would like to thank D.B.R., T.S.G., J.B.F., and W.T.H. for sticking by me through thick and thin.

`

IMMUNE FUNCTION AND DEVELOPMENT IN ALTRICIAL-DEVELOPING PASSERINE HOUSE SPARROWS (*PASSER DOMESTICUS*)

Abstract

by Marisa Olson King, Ph.D.
Washington State University
May 2010

Chair: Hubert G. Schwabl

ABSTRACT

Passerine birds are known to be ecologically, agriculturally, and environmentally relevant fixtures on six of the seven world continents and yet the immune systems for these altricial-developing species are poorly defined. We profiled the early humoral development of the adaptive immune system in an altricial-developing wild passerine species, the house sparrow (*Passer domestics*) and characterized the half-life of maternal antibodies in nestling plasma, the onset of *de novo* synthesis of endogenous antibodies by nestlings, and the timing of immunological independence, where nestlings rely entirely on their own antibodies for immunologic protection. We developed assays to measure both antigen-specific and total antibody concentration in the plasma of females, yolks, and nestlings and traced the transfer of maternal antibodies from females to nestlings through the yolk. Based on the short half-life of maternal antibodies, the rapid production of endogenous antibodies by nestlings and the relatively low transfer of maternal antibodies to nestlings, our findings suggest that 1) altricial-developing sparrows achieve immunologic independence much earlier than precocial chickens and 2)

`

maternal antibodies may not confer the immunologic protection or immune priming previously proposed in other passerine studies.

Maternal antibodies are believed to protect immunologically immature avian offspring during the critical period between hatch and the onset of endogenous antibody production. Yet, as aforementioned, house-sparrow specific patterns of immunity suggest that maternal antibodies may play a limited role in protecting and priming immunologically naïve young against environmental pathogens. We tested the ability of female house sparrows to influence the immunologic phenotype of their offspring's humoral immune system through the transfer of antigen-specific maternal antibodies and found no effect of maternal antibodies on nestling body condition or immunity. Our results suggest that maternal antibodies may be a neutral byproduct of the female immune system more so than a reflection of adaptive maternal investment. Further research needs to be conducted on other altricial passerines to determine if the results of our study are a species-specific phenomenon or if they can be applied to other avian species.

`

TABLE OF CONTENTS

Page

`

`

LIST OF TABLES

`

LIST OF FIGURES

`

Dedication

This dissertation is dedicated to the two and four-footed members of my family. You stood by me through every tantrum, triumph and burnout.

`

CHAPTER 1

Are Maternal Antibodies Really that Important? Patterns in the Immunologic Development of Altricial Passerine House Sparrows (*Passer domesticus*)

`

INTRODUCTION

The following manuscript was prepared for publication in the Public Library of Science (PLoS) ONE and adheres to their submission and publication guidelines.

Immune-mediated maternal effects are believed to play an integral role in the disease resistance of mammalian [1-4] and avian offspring [5-9]. Maternal antibodies passively immunize immunologically naïve young against virulent antigens and parasites that the offspring might encounter in its immediate developmental environment [3,4,7,10,11]. Passerine birds are known to be ecologically, agriculturally, and environmentally relevant fixtures on six of the seven world continents and yet the effects of maternal antibodies on offspring development are not well defined for these altricial-developing species [12]. Studies that have examined humoral-immunologic development in passerines often designed experiments based on information gleaned from the primary literature using the domestic chicken (*Gallus gallus domesticus*) as a model of humoral immunity. Although this model has generated a great breadth of knowledge on the physiology and function of the avian immune system, its applicability to passerine birds is dubious, as passerines express an altricial mode of development that differs dramatically from the precocial mode of development. Altricial birds hatch from the egg with immature physiological functions. For example, thermoregulation, motor control, endocrine function, and neural control are not fully developed at the time of hatch [12-14]. Therefore, one might also predict major differences in both immunologic development and the role that maternal antibodies play in conveying protection from antigens early in life.

`

In precocial chickens, maternal antibodies are transferred across the follicular epithelium into the yolk during oogenesis [12,15,16]. There are three classes of avian immunoglobulins (IgY, IgM and IgA). Of these, IgY is transferred at the highest concentration and is functionally homologous to mammalian IgG [17]. IgA and IgM are found predominantly in the egg white of chicken eggs, but have been detected in the yolk at low concentration [18,19]. Prior to hatch, maternal IgY is absorbed into embryonic circulation [16], where it confers passive immunity to immunologically immature hatchlings [4,6,7,10,11,17,20] . IgM is also absorbed into circulation, though at low concentrations (<1%) [17]. However, at present, there is no evidence supporting the uptake of IgA into nestling circulation from the yolk [18].

Both altricial and precocial species hatch with incompletely developed immune systems, making them vulnerable to pathogens in their environment [12,21-24]. In precocial chickens, maternal antibodies persist in the chick's circulation for 14-21 days after hatch and have a half-life of 3-7 days [6,25-27]. Chicks begin to produce their own endogenous antibodies 3-4 days after hatch and are not considered immunologically independent until all of the maternal antibodies have been catabolized from their system [12,17,27-30]. To our knowledge, the ontogeny and timing of immunologic independence in a passerine species remains unknown. This information is vital to the growing field of ecological immunology, which often focuses on passerine bird systems [8,31-33].

In this study we describe a developmental profile of humoral immunologic independence in the altricial house sparrow by measuring total and antigen specific maternal antibodies

`

in the yolk and nestling plasma as well as endogenous antibody synthesis by nestlings prior to fledge. We report the half-life of maternal antibodies, the time point at which nestlings synthesize their own antibodies and the time at which they become immunologically independent. Collectively, these results suggest that maternal antibodies may play a very limited role in immunologic development. Furthermore, we suggest that house sparrows may achieve immunologic independence at a much earlier stage of development than do precocial species such as the chicken.

MATERIALS AND METHODS

Ethics Statement

All animal capture, handling and experimental procedures described herein were approved by the Institutional Animal Care and Use Committee (protocol #2551) at Washington State University.

Animal Capture and Care

During December of 2005 and January of 2006, 35 breeding pairs of wild house sparrows were captured with mist nets from agricultural sites near Pullman, WA (46°44′N 117°10′W). The birds were housed in five separate outdoor aviaries (1 X 2 X 2.5 m) with seven breeding pairs per aviary. Each aviary was equipped with 10 nest boxes, perches, nesting material (grass hay and pillow stuffing), and *ad libitum* access to food and water. Individuals were banded with a numbered aluminum ring and two colored bands for individual identification. The birds were allowed to acclimate to their surroundings for 30 days before experimentation began.

`

Female Vaccination

Beginning in February, prior to egg laying, female house sparrows were placed on a vaccine schedule to stimulate antibody production. 20 females were injected subcutaneously with DNP-KLH in mCFA at a dose of 1 mg/kg of body mass. DNP-KLH is a novel, non-pathogenic antigen that females would not normally encounter in their environment. The detection of antibodies against DNP-KLH in the egg yolk and nestling plasma can, therefore, be assumed to be of maternal in origin. Modified Freund's adjuvant contains inactivated *Mycobacterium butyricum*, which acts as an immunostimulant for cell-mediated immunity. Individuals injected with this were expected to express increases in overall antibody titers regardless of antigen treatment. Two booster shots containing DNP-KLH in incomplete Freund's adjuvant (IFA), which lacks *M butyricum*, were injected at 28-day intervals. The remainder of the females (n=15) received comparable injections of PBS for both the primary and booster shots. Experimental and control females were distributed across the five aviaries. To monitor total and DNP-KLH-specific antibody concentrations females were bled a total of five times at 28-day intervals beginning directly before they received their primary injection. We collected pre- and post-vaccination blood samples by puncturing the brachial vein with a 28-gauge needle and collecting the blood into heparinized microcapillary tubes (~100 μL/bleed/individual). Blood samples were stored on ice for no more than two hours, whereupon plasma was separated from red blood cells by centrifuging samples at 9,000 rpm for 10 min and stored at -20°C until further analyses.

`

Egg Sampling

Nest boxes were monitored daily for eggs. Once a female initiated a clutch the nest box was videotaped to determine parental identity. The day that an egg was laid it was marked with a non-toxic marker to determine laying order. The second laid egg from each clutch was collected and stored at -20°C until further processing for antibody concentrations in yolk.

Antibody Extraction From Yolk

In preparation for assay, antibodies were extracted from the yolk using a modified chloroform-based method [17,52]. Briefly, the yolk was separated from the albumen, washed with deionized water (dH_2O) and weighed to the nearest 0.0001g. A volume of PBS equal to the mass of yolk was added to the yolk sample and vortexed. To that suspension, a volume of chloroform equal to the volume of the PBS +Yolk solution was added, vortexed and then centrifuged at 1,000 x g for 30 min at room temperature. After centrifugation, three distinct layers were observable: a lecithin layer on the bottom, a layer of emulsified yolk and chloroform in the middle, and a watery protein layer on the top, which contains antibodies. The clear, aqueous protein layer was removed and stored at -20°C until further analysis.

Nestling Sampling

In previous aviary studies with house sparrows we observed a high incidence of nestling mortality during the first breeding season after their capture. This was subsequently attributed to impaired parental care and infanticide by neighboring pairs. To ameliorate

`

this effect we fostered out aviary offspring to wild house sparrows breeding at a local field site at the University of Idaho's Sheep Center in Moscow, ID (46°43′N 116°59′W). Nest boxes had previously been mounted in three different barns in clusters of 20 and had been placed ~1 m apart from each another. To the entrance of each box we attached a mesh-wire cylinder that allowed sparrows to freely enter and exit, but limited the ability of predators to gain access to the box. House sparrows were observed to occupy this site at a high density and readily occupied the boxes. Nest box activity was monitored daily at this site and on the day an egg was laid by a wild sparrow it was removed and replaced with a wooden dummy egg. Prior to this study we observed that house sparrows incubate dummy eggs for upwards of 3 weeks, thus giving us ample time to foster out aviary nestlings to wild breeding pairs. To insure nestling survival, eggs were removed from the aviary nest boxes 1-2 days before their estimated hatch date and placed in an incubator until hatch. Within 3 hours after their hatch they were transported to an active nest box at the field site. No more than 4 nestlings were placed in any one box and nestlings were paired according to age to reduce sibling competition.

Blood samples were collected every three days beginning at hatch (day 0) and ending on day 15, for a total of 6 bleeds. Hatch day zero and day three nestlings were bled from the jugular vein, while day 6-15 blood samples were obtained from the brachial wing vein. No more than 20 μL of blood was taken at any one time.

`

Antibody Extraction and Purification

In preparation of producing secondary-antibodies specific to house sparrow-Immunoglobulins (HOSP-Ig) we extracted and purified antibodies from 30 house sparrow eggs following the protocol described by Hansen et al [53]. The recovered fraction of HOSP-Ig was separated from other water-soluble proteins in the yolk via thiophilic interaction chromatography as described in the manufacturers protocol (Clontech Laboratories, USA, #635616.). Per 3mL column, 10mL of extracted antibodies in sample buffer (50 mM sodium phosphate; 0.55 M sodium sulfate, pH 7.0) was loaded onto an equilibrated column (pH 7). The non-absorbed proteins were washed out using the equilibration buffer (50 mM sodium phosphate; 0.5 M sodium sulfate, pH 7.0) until the absorbance decreased to ~ 0.030 AU, whereupon the bound antibodies were collected by running elution buffer (20 mM sodium phosphate, 20% glycerol, pH 7.0) over the column. The collected volume was loaded into a dialyses cassette to remove residual salts and glycerol from the solution. Finally, the purified suspension was lyophilized and stored at -20°C until further use. The presence of HOSP-Ig was confirmed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% slab-gels and stained with Coomassie Blue R-250. All of the eggs used for immunoglobulin extraction were obtained from a separate population of house sparrows who were not subject to experimentation.

`

Secondary-Antibody Production

Polyclonal HOSP-Ig specific antibodies were generated in laboratory rats and rabbits for use in DNP-KLH specific and total antibody enzyme-linked immunosorbent assays (ELISA). The antiserum was generated against intact immunoglobulin molecules.

Rats

Three rats were vaccinated against purified HOSP-Ig. The lyophilized HOSP-Ig was redisolved in PBS (1 μg/μl) and emulsified with an equal volume of mCFA. The rats received one primary, subcutaneous injection of purified HOSP-Ig in mCFA at 50 μg of protein/100 μl emulsion and two booster shots containing HOSP-Ig in IFA at 4-week intervals. Rats were exsanguinated 4-weeks after the final booster shot.

Cross-reactivity between house sparrow antibodies and the rat antiserum was confirmed using western-blot analysis as described in Huber et al [54]. Briefly, purified antibodies were separated using SDS-PAGE and transferred on to a nitrocellulose membrane for immunoblotting. Chicken plasma diluted 1:10 in ddH2O was included on the gel to act as negative control. Filters were blocked with casein blocking buffer for 1h at room temperature and then washed three times in double deionized water (ddH2O). The blots were incubated for 1 hr at room temperature with rat-anti-house-sparrow-Ig (RarαHOSP-Ig) diluted to 1:1,000 in fluorescent treponemal antibody (FTA)-hemaaglutination buffer (pH 7.2) and then washed three times again with ddH2O. The blots were then incubated for another hour at room temperature with goat-anti-mouse plasma conjugated to horseradish peroxidase (GαM-hrp**)** (Bethyl Laboratories, Inc., Mongomery, TX) and

`

washed a final three times with ddH2O. The blots were analyzed using enhanced chemiluminesence (Figure S1).

Rabbits

Sigma-Genosys was commissioned to build antiplasma specific to purified HOSP-Ig in rabbits. We supplied the company with 3.21 mg of purified lyophilized HOSP-Ig protein. Two rabbits received one primary injection of HOSP-Ig in mCFA followed by five booster shots containing HOSP-Igs in IFA at 14-day intervals. Cross reactivity between purified HOSP-Ig and the rabbit antiplasma was confirmed via indirect ELISA by Sigma-Genosys.

DNP-KLH Specific Antibody ELISA

To detect DNP-KLH specific antibodies in female and nestling plasma as well as in the yolk we developed a DNP-KLH specific ELISA protocol. Prior to running the samples, the ELISA was optimized to determine the ideal concentration at which to dilute the capture-antigen, samples, secondary antibody, and detection antibody. The assay was validated via serially diluting pooled samples to show gradations in absorbance.

Immulon-4, 96-well plates were coated with 100 µL/well of DNP-KLH (0.1 mg/mL) in sodium bicarbonate coating buffer (0.05 M, pH 9.6) and incubated overnight in the cold room (4°C). Plates were washed three times with wash solution 200 µL/well (150mM NaCl, 0.01% Tween-20). Each plate contained, in duplicate, a blank, a NSB (measured binding between the capture antigen and the secondary and detection antibodies), and

`

DNP-KLH-positive and DNP-KLH-negative controls that were developed via pooled samples of plasma obtained from DNP-KLH vaccinated and unvaccinated female plasma, respectively. Positive and negative controls were serially diluted in sample buffer (FTA-hemmaglutination (pH 7.2), 10% casein) at concentrations of 1:500, 1:1000, and 1:2000 and were run in duplicate. Female plasma samples, nestling plasma samples and the chloroform extracted yolk fraction were diluted 1:1,000 in sample buffer and added, in duplicate, at a volume of 100 µL/well. Plates were incubated for 1 hr at 37°C on a rocker before being washed three times with wash solution. 100 µL of RatαHOSP-Ig diluted at 1:1000 in sample buffer was added to each well and the plates were again incubated for 1 hr at 37°C on a rocker before being washed three times. 100 µL/well of detection antibody, goat-anti-rat-horse-radish-peroxidase (GαR-hrp) (Invitrogen # A10549, Carlsbad, CA), was added to each wells at a dilution of 1:40,000 of antibody to sample buffer and incubated for 1 hr at 37°C on a rocker before being washed a final three times. Finally, 100 µL of peroxidase substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS: Sigma cat. A1888) and peroxide was added to each well and the plates were covered with tinfoil and allowed to develop for 1 hr at room temperature before being read on a spectrophotometer using a 405-nanometer wavelength filter to measure OD.

Plates were standardized to control for between plate variations in absorbance. Prior to standardization, the NSB OD was subtracted from the samples and control ODs for each plate. Per plate, the positive ratio was calculated by dividing the average absorbance of the positive control by the average absorbance of the highest positive control reading

`

observed amongst all of the plates, collectively. Final absorbance readings were calculated by dividing the average sample absorbance by the positive ratio for that plate.

Total Immunoglobulin ELISA

We developed a quantitative immunoglobulin ELISA to measure total Igs in the plasma and yolk. The protocol was modified from a commercial quantitative ELISA kit used to detect chicken IgY in plasma and yolk samples (Bethyl Laboratories, Montgomery, TX). To generate a standard curve we weighed out lyophilized HOSP-Ig and diluted it to1000 ng/mL in sample buffer. From this we made seven additional standards, which were serially diluted to range in value from 3.12-200 ng/ml. Antisera against HOSP-Ig were developed in both rabbits and rats, with the RabbitαHOSP-Ig acting as the capture antibody and RatαHOSP-Ig acting as the secondary antibody. Previous attempts to use only antibody from a single species for both the capture and secondary antibody showed reduced binding and poor repeatability. The dilutions reported for the capture antibody, secondary antibody, and detection antibody were all optimized to increase binding efficiency and decrease NSB prior to running the samples. Yolk and plasma samples were diluted based on predetermined working dilutions for adult plasma, yolk and nestlings of each sample age group nestling age (Table S1). Immunlon-4, 96 well plates were coated with RabbitαHOSP-Ig diluted 1:1000 in coating buffer and left to incubate overnight in the cold room (4°C). Each plate contained, in duplicate, a blank, a NSB (measured binding between the capture antibody and the secondary and detection antibody), a positive control, and 8 standards. All of the controls, standards and samples were added in duplicate at a volume of 100 μL/well. The plates were incubated for 1hr at

`

37°C on a rocker before being washed three times with wash solution. After washing, 100 µL of RatαHOSP-Ig diluted 1:1000 in sample buffer was added and the plates were again incubated for 1hr at 37°C on a rocker before being washed three additional times. 100 µL of the detection antibody, GαRat-hrp, was added to each well at a dilution of 1:40,000 and the plates were incubated one last time for 1hr at 37°C on a rocker before being washed a final three times. To each well, including the blank, 100 µL of ABTS developing reagent was added and the plates were covered with tinfoil and left to develop for 1hr. At the end of 1hr the plates were read on a spectrophotometer using a 405-nanometer wavelength filter to measure OD. The OD values were converted to ng/mL using softmax pro 4.8 (Molecular Devices Corp., Sunnyvale, CA). Samples were multiplied by their dilution factor and are presented as mg/mL. Yolk samples are presented as both mg/mL and total mg/yolk.

Biological Half-life of Maternal Antibodies in Nestlings

The biological half-life of maternal antibodies was determined using the following equation:

$t_{1/2} = t * \ln(2) \div \ln(N_o \div N_t)$ where,

t = time elapsed,

N_o = immunoglobulin concentration on hatch day 0, and

N_t= immunoglobulin concentration at time point t (3 days).

The DNP-KLH present in the yolk and nestling plasma was assumed to be maternal in origin, as nestlings were not exposed to the antigen naturally or experimentally. In nestling plasma the decline of maternal DNP-KLH was most pronounced between hatch

`

day 0 and day 3. The concentration of total antibodies in nestling plasma decreased in parallel with maternal DNP-KLH specific antibodies and was, therefore, assumed to be maternal in origin. We, therefore, used the total immunoglobulin concentrations on day 0 and day 3 to calculate the biological half-life of maternal antibodies.

Antibody Production in Nestlings

In nestling plasma, the total immunoglobulin concentration reached its nadir on day 3, post-hatch. Antibodies were determined to be endogenous in origin when an increase in antibody concentration was observed relative to the average concentration recorded on day 3. Any increase in total antibody concentration would have to come from the nestling.

Immunologic Independence

Immunologic independence of nestlings was defined as the time point at which maternal antibodies were no longer detectable in the plasma in conjunction with the production of endogenous antibodies. This was determined by solving for *t* in the following formula:

$$N_t = N_o(1/2)^{t / t1/2}$$ where,

t = the duration of time maternal antibodies remain detectable in nestling plasma

$t_{1/2}$ = half life of maternal antibodies,

N_o = immunoglobulin concentration on hatch day 0, and

N_t= the lowest concentration observed on day 3.

`

The lowest concentration of detectable antibodies on day 3 was selected as the value for N_t as it reflected lowest concentration of detectable maternal antibodies in nestling plasma.

Statistical Analyses

All statistical analyses were carried out using JMP 7.0.1 (SAS Institute Inc., Cary, NC, U.S.A.). Pseudo-replication was avoided by analyzing data obtained from only one nestling per female, which was chosen at random. Data were analyzed using Student's t-tests for pairwise comparisons of female and nestling plasma immunoglobulin concentration, as measured at specific time points. Linear least-squares models were used to analyze the effects of time on specific (DNP-KLH) and total immunoglobulin concentrations in nestlings and breeding females. Correlations between female specific (DNP-KLH) and total immunoglobulin levels relative to those found in the yolk and nestling plasma on hatch-day 0 were also determined via linear least-squares analyses. The percent transfer of female total Ig to nestlings on hatch-day 0 was determined by dividing the concentration in nestling plasma on hatch-day 0 by the plasma concentration of total Ig in female plasma on the bleed date closest to the nestling's hatch date and multiplying that value by 100. Data are reported as the mean ± SE, and differences were considered statistically significant at $P \leq 0.05$. All data are expressed as the mean ± SE.

RESULTS

Female Immunoglobulin Production in Response to Vaccination

`

Vaccination with dinitrophenol keyhole limpet hemocyanin (DNP-KLH) versus phosphate buffered saline (PBS) did not have a significant effect on circulating antibody levels in female plasma (ANOVA: $F_{5, 111} = 4.7602$, P=0.4586, $R^2= 0.1834$) and was removed as a model effect. Female plasma antibody levels increased more than two-fold over the course of the sample period after injection with modified complete Freund's adjuvant (mCFA) (ANOVA, $F_{4, 111}=5.8363$, P=0.0003, $R^2= 0.1791$; Figure 1: DNP-KHL-vaccination and control-injection combined). Female antibody concentrations were log-transformed to meet the assumption of normality. Pre-vaccination total Ig antibody titers ranged between 3.323 and 56.626 mg/mL with a mean of 23.096 ± 13.365mg/mL and a median of 19.445 mg/mL.

Female KLH-Specific Antibody Production in Response to Vaccination

In contrast to total immunoglobulin levels, plasma levels of antibodies specific to dinitrophenol keyhole limpet hemocyanin (DNP-KLH) increased over time in vaccinated females versus controls (ANOVA: $F_{9, 100}= 10.1280$, P=0.0001, $R^2= 0.4768$; Figure 2). We observed DNP-KLH-specific antibody levels increase seven-fold, with a peak between 2 and 3 months after vaccination. This confirms an antigen specific antibody response in females vaccinated against DNP-KLH.

Transfer of maternal antibodies to Nestlings

There was a positive relationship between DNP-KLH-specific antibodies in the female plasma relative to the yolk ($F_{1,5}=12.6544$, P=0.0163, $R^2=0.7168$; Figure S2, a) and plasma samples from hatch day 0 nestlings ($F_{1,8}=7.9484$, P=0.00225, $R^2=0.4984$; Figure

`

S2, b), which confirms the transfer of maternally derived antibodies to yolk and nestlings. There was no significant relationship between maternal total antibody levels and those in the yolk ($F_{1, 7}$= 0.1044, P=0.7597, R^2=0.0205; Figure S3, a), or nestling plasma on hatch day 0 ($F_{1, 7}$= 0.2396, P=0.6395, R^2=0.0331; Figure S3, b). The transfer of total immunoglobulin from female plasma to nestling plasma on hatch-day 0 was estimated to be 11.6 ± 1.7% (N=10; Table 1).

Nestling Antibody Profile

Total Antibodies

Female treatment did not have a significant effect on nestling antibody levels over time (t (68)= -1.34, P=0.1855) and was removed as a model effect from further analysis. Age had a significant effect on total immunoglobulin levels in nestling plasma (ANOVA: $F_{5, 63}$=7.1616, P < 0.0001, R^2= 0.3623; Figure 3). A comparison of means from each sampling time point revealed the lowest antibody titers occurred 3 days after hatch (1.0460 ± 1.2595; Table S2). Antibody titers decreased between Hatch day 0 and day 3 and increased, relative to day 3, beginning on day 6 and continuing through day 15 (Figure 1, Table S2). Antibody titers on day 15 were significantly greater than those recorded at the five other time points (Table S2).

Maternal DNP-KLH-Specific Antibodies

The detection of DNP-KLH specific antibodies in nestling plasma (as estimated by optic density (OD)) was highest on hatch day 0 (0.1034 ± 0.0192). This value was significantly greater than that in plasma from nestlings on day 0 that hatched from control females

`

lacking DNP-KLH-specific antibodies (0.0386 ± 0.01622; Table S3). However, DNP-KLH-specific antibody levels in nestling plasma from day 3 through day 15 were not significantly different from that in controls (Table S3). This indicates that DNP-KLH-specific maternally derived antibodies were undetectable by 3 days post-hatch and appeared to decrease rapidly with age (Figure 4). We did not detect a significant relationship of maternal DNP-KLH antibodies in nestling plasma over time (ANOVA: $F_{6, 40}$= 1.6945, P=0.1475, R^2= 0.202664),

Half-Life of Maternal Antibodies

The biological half-life of maternal antibodies in nestling plasma was calculated as 2.2 ± 0.25 days (N=17) with values ranging between 0.17 and 4.7 days. Defining 0.172 mg/mL as the minimum concentration of detectable maternal antibodies, post-hatch, maternal antibodies appear to persist in nestling plasma for up to 8.6 ± 0.5 days post-hatch.

Immunologic Independence

We defined immunologic independence as the time at which nestlings are capable of synthesizing antibodies and all maternal antibodies have been cleared from their plasma. In accordance with this definition, immunologic independence was estimated to occur between 8-10 days after hatch. This value is in accordance with the calculated half-life of 2.2 ± 0.25 days maternal antibodies and the detection of endogenously produced antibodies in nestling circulation beginning 3-6 days after hatch.

`

DISCUSSION

In this study we report that maternal antibodies persist for a relatively short period of time during early development in an altricial-developing wild passerine bird. Previous research on the role of immune-mediated maternal effects in wild passerines has assumed that the ontogeny of immunocompetence in altricial passerines follows the time course described for precocial-developing *Gallus* species. Our results suggest that the ontogeny of the adaptive immune system in altricial-developing birds deviates from that of the chicken model.

Half-life of Maternal Antibodies

In newly hatched chickens, maternal antibodies have a half-life of approximately 3 days [27]. For antigen specific antibodies, a longer half-life has been reported (5-7 days) [6,26,34]. In our study with altricial house sparrows, maternal antibodies in the plasma of nestlings have a biological half-life of 2.2 ± 0.25 days. This estimate is supported by the lack of detectable maternal DNP-KLH-specific antibodies in nestling plasma 3 days post-hatch (Figure 4). This half-life value is shorter than the 3 days reported for maternal West Nile Virus-specific antibodies in nestling house sparrows [31]. However, the assay used in that study measured antibody concentration qualitatively (% neutralizing activity) and samples were taken 1-7 days after hatch [31]. We have shown that house sparrow nestlings are capable of *de novo* synthesis of antibodies 3-6 days post-hatch, which, in regard to the study with West Nile Virus, suggests that the methods did not differentiate between maternal and endogenous antibodies when calculating the half-life. The sampling time of nestling plasma plays a critical role in determining the half-life

`

of maternal antibodies. For example, using plasma samples obtained from hatch day 0 and day 6 nestlings, the estimated half-life is 9.045 ± 0.65 days. This half-life is 4-times greater than the value we report (2.2 ± 0.25 days), which is based on the dynamics of antibody levels across the period of nestling development. Thus, the frequency and timing of plasma sampling can have a significant effect on the perceived changes in antibodies derived from the mother versus produced by the chick.

We estimated maternal antibodies to persist in nestling plasma up to 8 - 9 days post-hatch. In chickens, maternal antibodies have been reported to remain detectable for 14 - 21 days after hatch [12,17,35]. This difference, in conjunction with the shorter half-life we report, strongly indicates that maternal antibodies persist in nestlings of the altricial house sparrow for much less time than in the precocial chicken. The absence of maternal DNP-KLH-specific antibodies 3 days after hatch suggest that maternal antibodies are limited in their ability to confer passive immunity against specific antigens in nestlings. This brings into question the suggestion in the literature that maternal antibodies play a central role in conferring immunologic protection to developing altricial nestlings [11,31,36-40]. Our data suggest that antigen-specific maternal antibodies are absent a few days prior to nestlings producing their own antibodies.

The fraction of maternal antibodies transferred to nestling plasma on hatch day 0 was estimated as 11.6 ± 1.7%, which is about a 1/3 of what has been reported in chickens [17]. All females vaccinated against DNP-KLH tested positive for plasma DNP-KLH-specific antibodies and 85.7% of their eggs were positive for DNP-KLH-specific

`

antibodies. However, only 44.4% of the chicks that hatched from DNP-KLH positive females tested positive for DNP-KLH-specific antibodies in their plasma on hatch day 0. This suggests that the transmission of antigen-specific antibodies from mother to offspring is limited or incomplete. Given that only 11.6% of the mothers total antibody concentration can be detected in newly hatched chicks the concentration of antigen-specific antibodies can be assumed to be even lower. The rapid decline of DNP-KLH specific antibodies in nestling serum is not surprising, given the short half-life paired with incomplete transfer of antigen-specific maternal antibodies. These results also bring into question the conclusions drawn from studies examining the effects that maternal antibodies have on nestling immunocompetence. If antigen specific antibodies are not transferred to the nestling or are catabolized before nestlings are sampled there is no definitive link between maternal antibodies and immune defense of the chicks [8,32,37,38,41,42].

Nestling Antibody Production

In this study we demonstrate that nestlings begin to synthesize antibodies between 3 and 6 days after hatch. This is consistent with what has been reported for chickens, where *de novo* synthesis begins 3-4 days post-hatch [30,43-45]. Nestlings in our study showed a steep rise in plasma antibody levels, relative to day 3 titers, beginning on day 6 and continuing through the sampling period. By 15 days post-hatch nestling plasma antibody concentrations were well within the range observed for pre-vaccinated adult females (Range: 3.3231to 56.6262 mg/mL), suggesting that nestlings may be immunocompetent well before they fledge from the nest.

`

Nestling Immunologic Independence

We defined immunologic independence as the time at which maternal antibodies were no longer detectable in nestling plasma and when *de novo* synthesis of antibodies began. Thus, in house sparrows, immunologic independence occurred at approximately 8-10 days of age. Based on these criteria, altricial-developing house sparrows may achieve immunologic independence much earlier than precocial chickens. Furthermore, given the rapid decline of DNP-KLH specific maternal antibodies over the first 3 days post-hatch, it is not unreasonable to question if maternal antibodies protect chicks at an early age. Studies on both altricial [31,46] and precocial [35,47,48] avian species have reported that maternal antibodies may interfere with the offspring's ability to detect foreign antigen and, thus initiate an antigen-specific antibody response. It may be advantageous for both precocial and altricial species such as the house sparrow to achieve immunologic independence as early as possible given the high density of virulent antigens and parasites in their natural environment. Factors contributing to inter-specific variation in the timing of immunologic independence remain, as yet, undetermined.

Conclusions

The results of this study suggest that altricial-developing passerines deviate from the model of avian immunity that is based on the precocial chicken. This has major implications for research examining immune-mediated maternal effects in offspring of altricial birds. A more in depth analysis of the ontogeny of the immune system needs to be undertaken before proper experiments can be designed and executed on wild

`

passerines. Although maternal antibodies may protect nestlings during the period between hatch and endogenous antibody production, we have shown that there is a limited and incomplete transfer of novel antigen-specific maternal antibodies to nestlings, even when the mother is rigorously immunologically challenged. If, indeed, maternal antibodies have a limited protective effect for nestlings and do not confer much passive immunity, existing hypotheses need to be reexamined. Researchers might examine other pathways through which the mother may influence her offspring's immune defenses. It may be that genetic quality [9,17,49] and investment in parental care [41,50,51] play a more integral role in nestling immunologic development than immune-mediated maternal effects. Indeed, in chickens, it has been observed that some lines are more immunologically active than others and that these line differences are heritable [9,17].

Our results indicate that maternal antibodies may not confer the immunologic protection or immune priming previously reported in other studies of passerine birds. Further research on other passerine species is warranted to determine if the results of our study on house sparrows are revealing a species-specific pattern or if they are applicable to other altricial-developing passerine and non-passerine birds. It may also be of interest to examine both proximate and ultimate level questions regarding deviations observed in the immunologic development of altricial passerines in relation to metabolism and growth rate, species-specific immunoglobulin structure, rearing environment, domestication, disease prevalence, and trade-offs between condition dependent life history traits.

`

LITERATURE CITED

1. Lyche JL, Larsen HJS, Skaare JU, Tverdal A, Johansen GM, et al. (2006) Perinatal exposure to low doses of PCB 153 and PCB 126 affects maternal and neonatal immunity in goat kids. Journal of Toxicology and Environmental Health-Part a-Current Issues 69: 139-158.
2. Anderson RW (1995) On the maternal transmission of immunity - a molecular attention hypothesis. Biosystems 34: 87-105.
3. Beam AL, Lombard JE, Kopral CA, Garber LP, Winter AL, et al. (2009) Prevalence of failure of passive transfer of immunity in newborn heifer calves and associated management practices on US dairy operations. Journal of Dairy Science 92: 3973-3980.
4. Castinel A, Kittelberger R, Pomroy WE, Duignan PJ, Chilvers BL, et al. (2008) Humoral immune response to Klebsiella spp. in New Zealand sea lions (*Phocarctos hookeri*) and the passive transfer of immunity to pups. Journal of Wildlife Diseases 44: 8-15.
5. Chaffer M, Schwartsburd B, Heller ED (1997) Vaccination of turkey poults against pathogenic *Escherichia coli*. Avian Pathology 26: 377-390.
6. Fahey KJ, Crooks, J.K., and Fraser, R.A. (1987) Assessment by ELISA of passively acquired protection against infectious bursal disease virus in chickens Australian Veterinary Journal 64: 203-207.
7. Wang YW, Sunwoo H, Cherian G, Sim JS (2004) Maternal dietary ratio of linoleic acid to alpha-linolenic acid affects the passive immunity of hatching chicks. Poultry Science 83: 2039-2043.

`

8. Grindstaff JL, Hasselquist D, Nilsson JA, Sandell M, Smith HG, et al. (2006) Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proceedings of the Royal Society B-Biological Sciences 273: 2551-2557.

9. Abdel-Moneim AS, Abdel-Gawad MMA (2006) Genetic variations in maternal transfer and immune responsiveness to infectious bursal disease virus. Veterinary Microbiology 114: 16-24.

10. Gasparini J, McCoy KD, Staszewski V, Haussy C, Boulinier T (2006) Dynamics of anti-Borrelia antibodies in Blacklegged Kittiwake (Rissa tridactyla) chicks suggest a maternal educational effect. Canadian Journal of Zoology-Revue Canadienne De Zoologie 84: 623-627.

11. Grindstaff JL (2008) Maternal antibodies reduce costs of an immune response during development. Journal of Experimental Biology 211: 654-660.

12. Apanius V (1998) Ontogeny of immune function. In: Stark JM, Ricklefs RE, editors. Avian Growth and Development Oxford: Oxford University Press. pp. 203-222.

13. Stark JM, Ricklefs RE (1998) Patterns of Development: The Altricial-Precocial Spectrum. In: Stark JM, Ricklefs RE, editors. Avian Growth and Development Oxford: Oxford University Press. pp. 3-26.

14. Ar A, & Yom-Tov Y (1979) The Evolution of Parental Care in Birds. Evolution 32: 655-688.

15. Loeken MR, Roth TF (1983) Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. Immunology 49: 21-28.

`

16. Kowalczyk K, Daiss J, Halpern J, Roth TF (1985) Quantitation of maternal-fetal IgG transport in the chicken. Immunology 54: 755-762.

17. Hamal KR, Burgess SC, Pevzner IY, Erf GF (2006) Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poultry Science 85: 1364-1372.

18. Kaspers B, Schranner I, Losch U (1991) Distribution of immunoglobulins during embryogenesis in the chicken Journal of Veterinary Medicine Series a-Zentralblatt Fur Veterinarmedizin Reihe a-Physiology Pathology Clinical Medicine 38: 73-79.

19. Yamamoto H, H. Watanabe, G. Sato, and T. Mikami. (1975) Identification of immunoglobulins in chicken eggs and their antibody activity. Japanese Journal of Veterinary Research 23: 131-140.

20. Heller ED, Leitner G, Drabkin N, Melamed D (1990) Passive-immunization of chicks against *Eserichia-coli* Avian Pathology 19: 345-354.

21. Glick B (1983) The saga of the bursa of Fabricius. Bioscience 33: 187-191.

22. Glick B, Chang, T.S., and Jaap, R.G (1956) The bursa of Fabricius and antibody production in the domestic fowl. Poultry Science 35: 224-225.

23. Glick B (1983) Bursa of Fabricius. Avian Biology 7: 443-500.

24. Rose ME (1981) Lymphatic system. In: McLelland ASKJ, editor. Form and function in birds. London: Academic Press. pp. 341-384.

25. Darbyshire JH, Peters RW (1985) Humoral antibody-response and assessment of protection following primary vaccination of chicks with maternally derived

antibody against avian infectious-bronchitis virus. Research in Veterinary Science 38: 14-21.

26. Kaleta EF, Siegmann O, Lai KW, Aussum D (1977) Kinetics of NDV-specific antibodies in chickens.VI. Elimination of maternal and injected antibodies. Berl Munch Tierarztl Wochenschr 90: 131-134.

27. Patterson R (1962) The metabolism of serum proteins in the hen and chick and secretion of serum proteins by the ovary of the hen. Journal of General Physiology 45.

28. Lawrence EC, Arnaudbattandier F, Grayson J, Koski IR, Dooley NJ, et al. (1981) Ontogeny of humoral immune function in normal chickens - a comparison of immunoglobulin-secreting cells in bone-marrow, spleen, lungs and intestine. Clinical and Experimental Immunology 43: 450-457.

29. Leslie GA, and Martin, L.N. (1973) Modulation of immunoglobulin ontogeny in chicken - effect of purrified antibody specific for MU-chain on IgM, IgY, and IgA production. Journal of Immunology 110.

30. Leslie GA (1975) Ontogeny of chicken humoral immune mechanism. American Journal of Veterinary Research 36.

31. Nemeth NM, Oesterle PT, Bowen RA (2008) Passive immunity to West Nile virus provides limited protection in a common passerine species. American Journal of Tropical Medicine and Hygiene 79: 283-290.

32. Tschirren B, Siitari H, Saladin V, Richner H (2009) Transgenerational immunity in a bird-ectoparasite system: do maternally transferred antibodies affect parasite fecundity or the offspring's susceptibility to fleas? Ibis 151: 160-170.

`

33. Dhondt AA, Dhondt KV, McCleery BV (2008) Comparative infectiousness of three passerine bird species after experimental inoculation with Mycoplasma gallisepticum. Avian Pathology 37: 635-640.

34. Pendleton AR, Machamer CE (2006) Differential localization and turnover of infectious bronchitis virus 3b protein in mammalian versus avian cells. Virology 345: 337-345.

35. Tizard I (2002) The avian antibody response. Seminars in Avian and Exotic Pet Medicine 11: 2-14.

36. Hasselquist D, Nilsson JA (2009) Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Philosophical Transactions of the Royal Society B-Biological Sciences 364: 51-60.

37. Staszewski V, Gasparini J, McCoy KD, Tveraa T, Boulinier T (2007) Evidence of an interannual effect of maternal immunization on the immune response of juveniles in a long-lived colonial bird. Journal of Animal Ecology 76: 1215-1223.

38. Boulinier T, Staszewski V (2008) Maternal transfer of antibodies: raising immuno-ecology issues. Trends in Ecology & Evolution 23: 282-288.

39. Forsman AM, Vogel LA, Sakaluk SK, Johnson BG, Masters BS, et al. (2008) Female house wrens (Troglodytes aedon) increase the size, but not immunocompetence, of their offspring through extra-pair mating. Molecular Ecology 17: 3697-3706.

40. Fouchet D, Marchandeau S, Bahi-Jaber N, Pontier D (2007) The role of maternal antibodies in the emergence of severe disease as a result of fragmentation. Journal of the Royal Society Interface 4: 479-489.

`

41. Lozano GA, Ydenberg RC (2002) Transgenerational effects of maternal immune challenge in tree swallows (Tachycineta bicolor). Canadian Journal of Zoology-Revue Canadienne De Zoologie 80: 918-925.

42. Karell P, Kontiainen P, Pietiainen H, Siitari H, Brommer JE (2008) Maternal effects on offspring Igs and egg size in relation to natural and experimentally improved food supply. Functional Ecology 22: 682-690.

43. Leslie GA, Clem LW (1969) Phylogeny of immunoglobulin structure and function: III. Immunoglobulins of the chicken. Journal of Experimental Medicine 130: 1337-1352.

44. Goldammer A, Derfler K, Herkner K, Bradwell AR, Horl WH, et al. (2002) Influence of plasma immunoglobulin level on antibody synthesis. Blood 100: 353-355.

45. Martin LN, Leslie GA (1973) Ontogeny of IgA in normal and neonatally bursectomized chickens, wiht corroboarative data on IgY and IgM. Proceedings of the Royal Society for Experimental Biology and Medicine 143.

46. Gasparini J, Piault R, Bize P, Roulin A (2009) Pre-hatching maternal effects inhibit nestling humoral immune response in the tawny owl Strix aluco. Journal of Avian Biology 40: 271-278.

47. Abou Elazab MF, Fukushima Y, Horiuchi H, Matsuda H, Furusawa S (2009) Prolonged Suppression of Chick Humoral Immune Response by Antigen Specific Maternal Antibody. Journal of Veterinary Medical Science 71: 417-424.

48. Mondal SP, Naqi SA (2001) Maternal antibody to infectious bronchitis virus: its role in protection against infection and development of active immunity to vaccine. Veterinary Immunology and Immunopathology 79: 31-40.

`

49. Cichon M, Sendecka J, Gustafsson L (2006) Genetic and environmental variation in immune response of collared flycatcher nestlings. Journal of Evolutionary Biology 19: 1701-1706.

50. Moller AP, Haussy C (2007) Fitness consequences of variation in natural antibodies and complement in the Barn Swallow Hirundo rustica. Functional Ecology 21: 363-371.

51. Saino N, Calza S, Moller AP (1997) Immunocompetence of nestling barn swallows in relation to brood size and parental effort. Journal of Animal Ecology 66: 827-836.

52. Polson A (1990) Isolation of IgY from the yolks of eggs by a chloroform polyethylene-glycol procedure. Immunological Investigations 19: 253-258.

53. Hansen P, Scoble JA, Hanson B, Hoogenraad NJ (1998) Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. Journal of Immunological Methods 215: 1-7.

54. Huber SK, Owen JP, Koop JAH, King MO, Grant PR, et al. (2010) Ecoimmunity in Darwin's Finches: Invasive Parasites Trigger Acquired Immunity in the Medium Ground Finch (Geospiza fortis). PLOS One: In Press.

`

TABLES

Table 1. Total antibody levels in the mother's plasma, yolk and nestling plasma.

Female	
Plasma Ig (mg/mL)[1,2]	24.4545 ± 5.4043
Yolk (mg/mL)[1,3]	5.5884 ± 1.8404
Yolk (mg)[3,4]	6.6233 ± 2.3279
% Transfer[5]	11.5785 ± 1.7430
Nestling	
Plasma (mg/mL)[1]	
Day 0	3.8282 ± 1.2195
Day 3	1.0460 ± 1.2595
Day 6	2.3649 ± 1.4082
Day 9	5.5843 ± 1.5426
Day 12	5.2140 ± 1.8438
Day 15	12.6370 ± 1.6260

[1] For mother and nestling plasma, antibody levels are presented as means ± SE.
[2]Pre-vaccination antibody concentration in mother's plasma.
[3]Yolk concentrations derived from whole yolks that underwent chloroform extraction.
[4]Antibody weight/yolk as calculated from antibody concentration * egg mass.
[5]Percent tranfer of maternal antibod concentration from the mother to nestling.

`

FIGURE LEGENDS

Figure 1. Female immunoglobulin production in response to vaccination with modified complete (mCFA) and incomplete (IFA) Freund's adjuvant.

Mean ±SE plasma antibody concentrations (measured as mg/mL) in females vaccinated against DNP-KLH in mCFA (N=20) or PBS (N=15). Two booster shots containing DNP-KLH in IFA or PBS were given at 28-day intervals following the primary injection. A total of five bleeds were taken, 28-days apart, to measure total antibody concentration. Samples taken on day 0 reflect pre-vaccination total antibody titers. Samples were combined from both DNP-KLH and control treated females, as we found no effect of treatment on antibody concentration (P=0.4586).

Figure 2. Female DNP-KLH-specific antibody response of adult females vaccinated against DNP-KLH in modified (mCFA) and incomplete (IFA) Freund's adjuvant.

Mean ± SE of DNP-KLH-specific antibody levels (measured as optic density (OD)) in females vaccinated against DNP-KLH in mCFA (N=20) or PBS (N=15) on day 0. Two booster shots containing DNP-KLH in IFA or PBS were given at 28-day intervals following the primary injection. A total of five bleeds were taken, 28-days apart, to measure DNP-KLH-specific antibody levels. Samples taken on day 0 reflect pre-vaccination DNP-KLH specific antibody levels. Closed circles (●) represent DNP-KLH treated females and closed squares (■) represent PBS treated females.

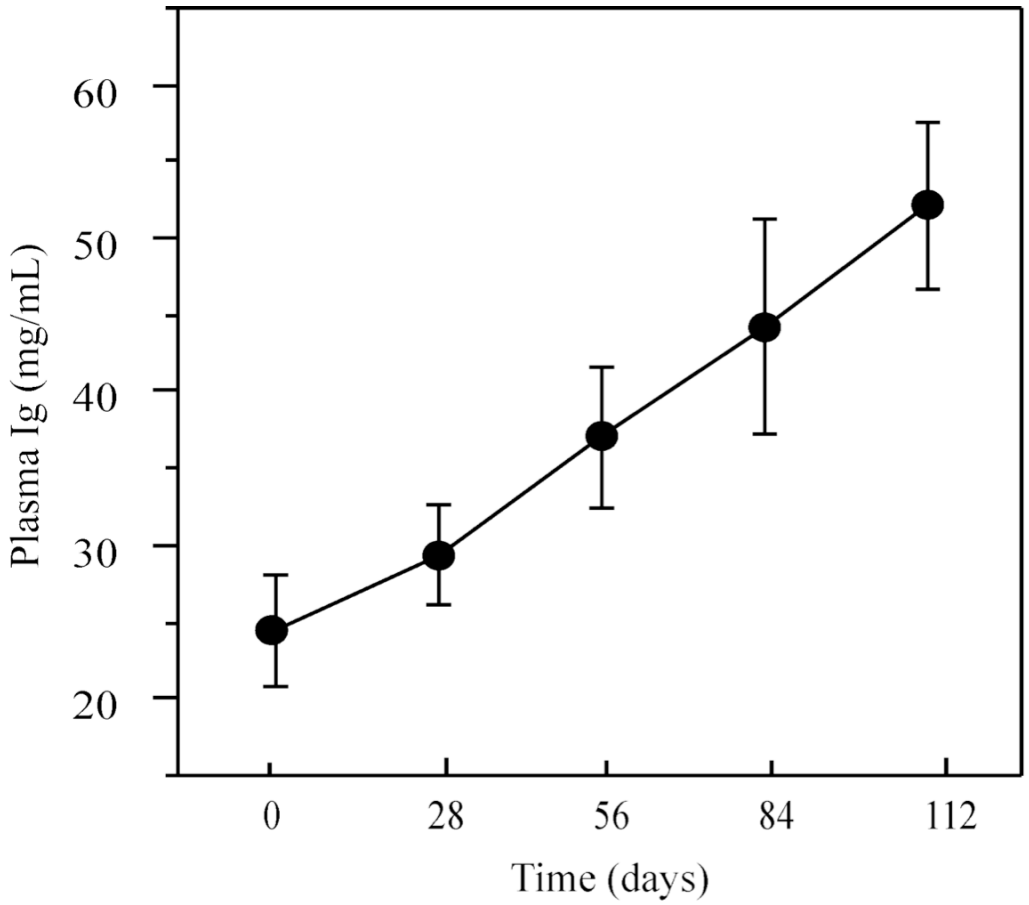
Figure 3. Total antibody concentration in nestlings at 0, 3, 6, 9, 12, and 15 days of age.

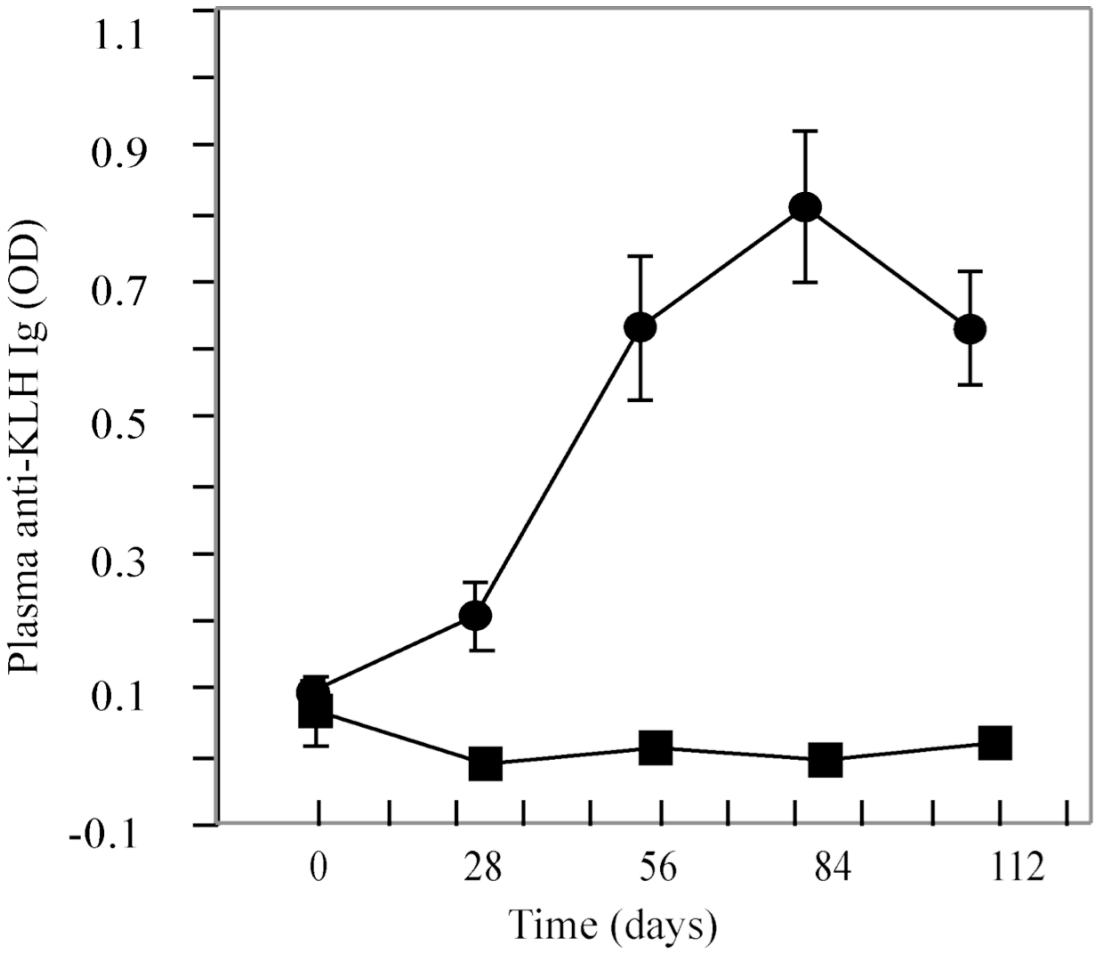
`

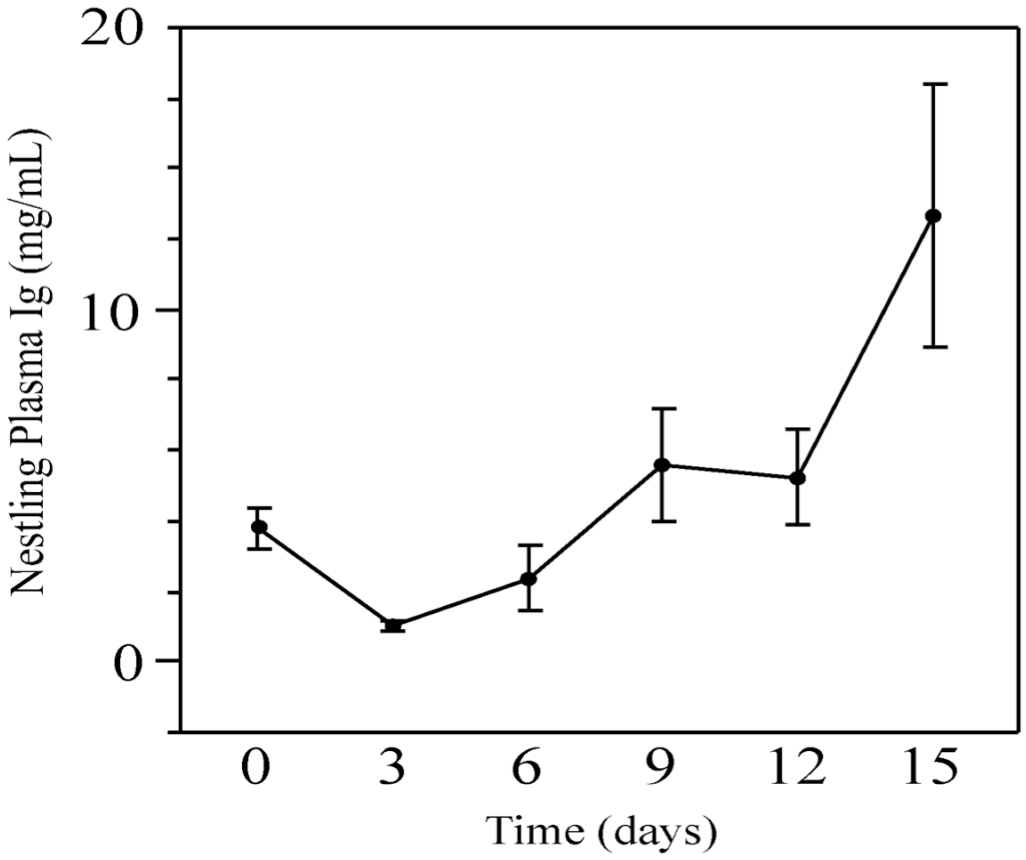
Mean ± SE plasma antibody concentration (measured as mg/ml) from nestlings sampled at 0, 3, 6, 9, 12, and 15 days of age. The antibodies in hatch day 0 nestlings were assumed to be maternal in origin, while those from nestlings 3-9 day old are assumed as being a mixture of endogenous and maternal antibodies. Antibody concentrations measured in 12 and 15 day old nestlings were determined to be endogenous in origin.

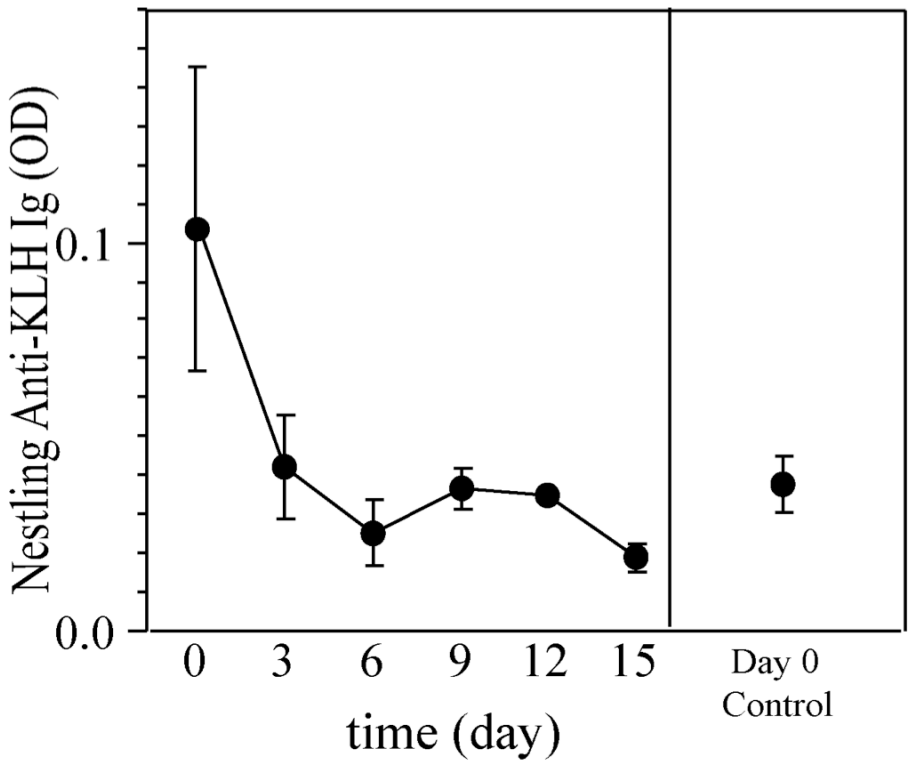
Figure 4. Maternal DNP-KLH-specific antibodies in nestlings sampled at 0, 3, 6, 9, 12, and 15 days of age.

Mean ± SE of maternal DNP-KLH-specific antibodies (measured as optic density (OD)) in nestling plasma at 0, 3, 6, 9, 12, and 15 days of age. DNP-KLH specific antibodies are purely maternal in origin, as nestlings were not exposed to this antigen. The OD recorded for hatch day 0 nestlings was significantly different from control values obtained from hatch day 0 nestlings hatched from control females at $P<0.05$. All other samples taken from 3-15 day old nestlings did not significantly differ from the control at $P< 0.05$.


60
50
40
30
20
Plasma Ig (mg/mL)
0
28
56
84
112
Time (days)


1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Plasma anti-KLH Ig (OD)
0
28
56
84
112
Time (days)


20
10
0
Nestling Plasma Ig (mg/mL)
0
3
6
9
12
15
Time (days)


Nestling Anti-KLH Ig (OD)
0.1
0.0
0
3
6
9
12
15
time (day)
Day 0
Control

`

APPENDIX

SUPPLEMENTARY TABLES AND FIGURES:

Table S1. Working dilutions used for plasma and yolk samples for the total Antibody ELISA.

Sample	Working Dilution
Maternal Plasma	1:300,000
Nestling Plasma	
Day 0	1:150,000
Day 3	1:25,000
Day 6	1:25,000
Day 9	1:25,000
Day 12	1:25,000
Day 15	1:25,000
Yolk[1]	1:25,000

[1]Yolk dilutions refer to the recovered fraction collected after yolk chloroform extractions were performed.

`

Table S2. P-values from Student's t-test comparing plasma antibody concentrations in nestlings at 0, 3, 6, 9, 12, and 15 days of age.[1]

Day	0	3	6	9	12	15
0	-----[2]					<0.0001
3		-----				<0.0001
6			-----			<0.0001
9		<0.0261		-----		<0.025
12					-----	<0.037
15						----

[1]Pairwise comparisons were considered significane at P<0.05. Only p-values less than 0.05 are presented in the table.
[2]Dashed lines represent time points that were not compared.

Table S3. P-values from Student's t-test comparing maternal DNP-KLH-specific antibody levels in nestling plasma at 0, 3, 6, 9, 12, and 15 days of age.[1]

Day	0	3	6	9	12	15	Control[3]
0	-----[2]	0.0398	0.0177				0.0138
3		-----					
6			-----				
9				-----			
12					-----		
15						-----	
Control[3]							-----

[1]Pairwise comparisons were considered significane at P<0.05. Only p-values less than 0.05 are presented in the table.
[2]Dashed lines represent time points that were not compared.
[3]Control plasma obtained from nestling on hatch day 0 that hatched from control females.

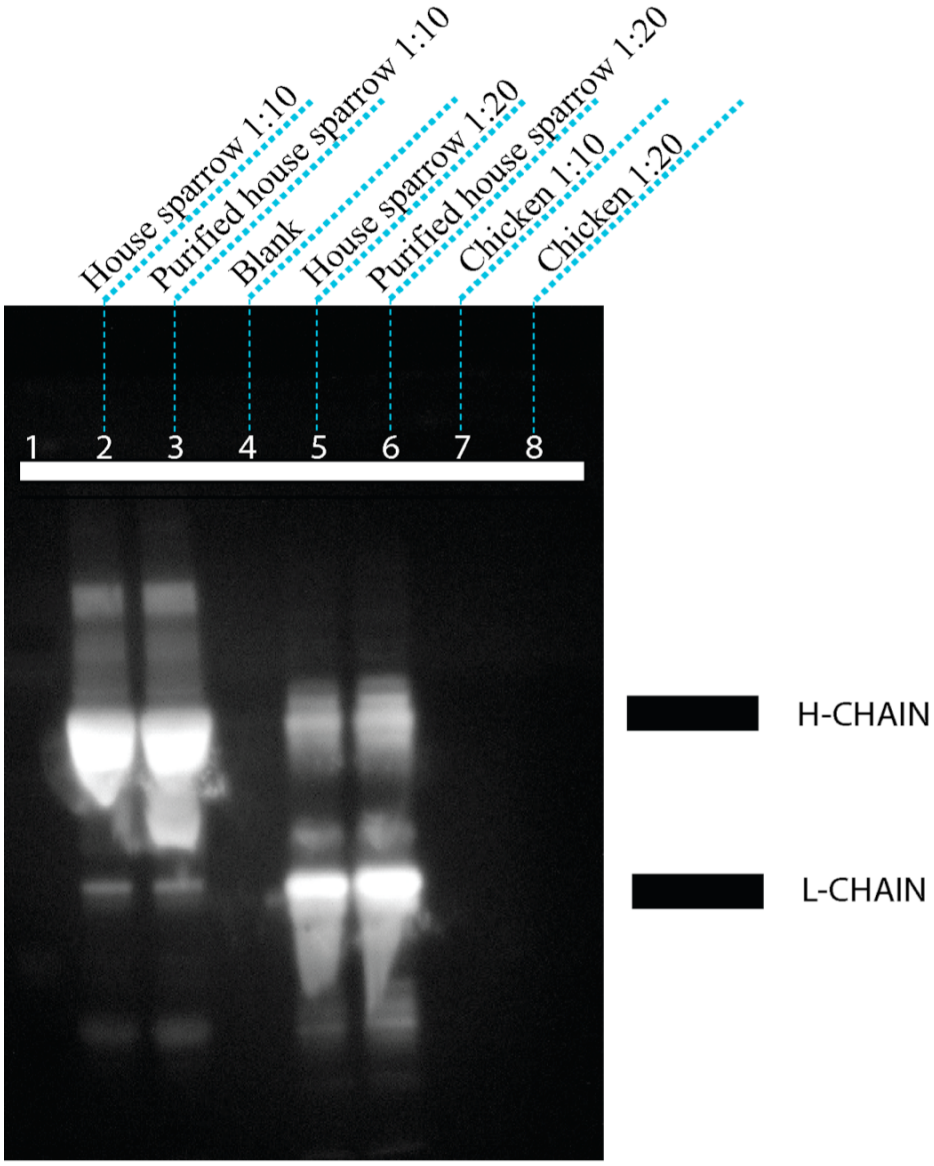
`

Figure S1. Western blot analyses of binding between HOSP-Ig and rat antiserum. Western blot of purified house sparrow immunoglobulins and chicken plasma. Purified house sparrow immunoglobulins were obtained from thiophilic resin chromatography. Lyophilized immunoglobulin recoveries were diluted in sample buffer at 1mg/mL. Both plasma and purified samples were loaded into columns at 1:10 and 1:20 dilutions. Images reflect cross reactivity between house sparrow immunoglobulins and the antiserum built in rats against purified Ig. Antiserum built in rats against house sparrow Ig did not cross react with chicken plasma.

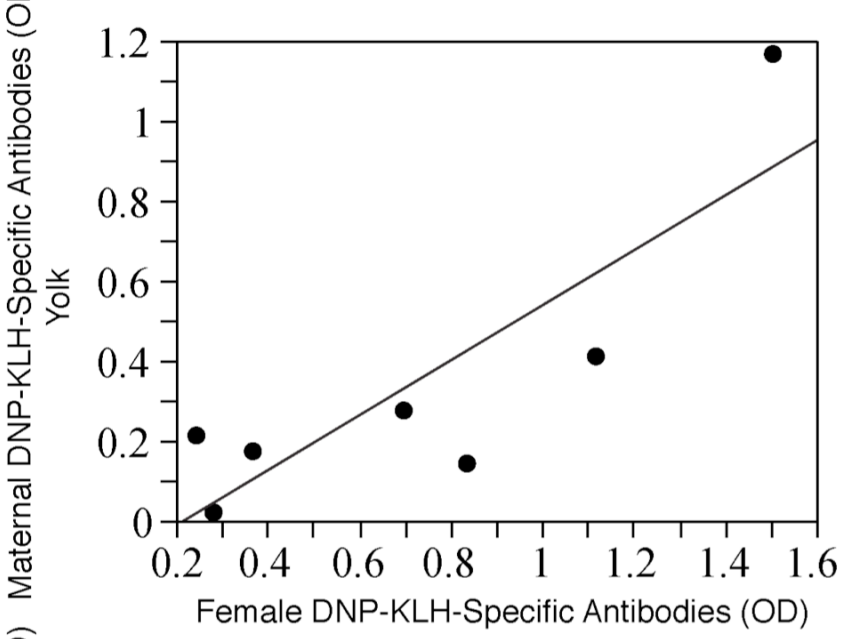
Figure S2. Correlations between DNP-KLH-specific antibodies in mothers relative to a) the yolk ($F_{1,8}$=7.9484, P=0.00225, R^2=0.4984) **and b) nestling plasma on hatch day 0.** ($F_{1,8}$=7.9484, P=0.00225, R^2=0.4984). Antibody levels are expressed as the mean ± SE of optic density (OD).

Figure S3. Correlations between total immunoglobulin concentration in mothers relative to a) the yolk ($F_{1,7}$= 0.1044, P=0.7597, R^2=0.0205) **and b) nestling plasma on hatch day 0** ($F_{1,7}$= 0.2396, P=0.6395, R^2=0.0331)**.** Antibody concentration is expressed as the mean ± SE (mg/mL).

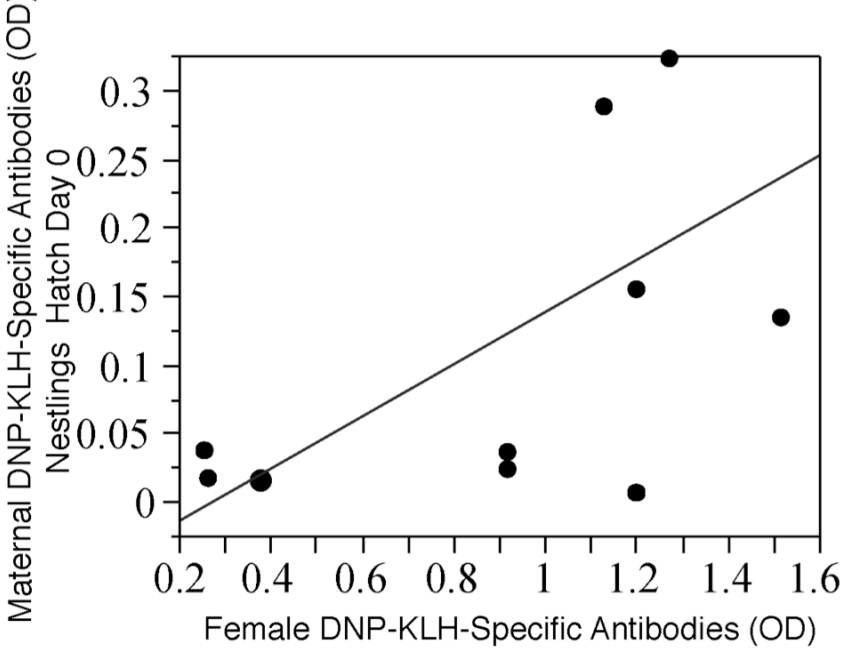
`


House sparrow 1:10
Purified house sparrow 1:10
Blank
House sparrow 1:20
Purified house sparrow 1:20
Chicken 1:10
Chicken 1:20
1
2
3
4
5
6
7
8
H-CHAIN
L-CHAIN

a.

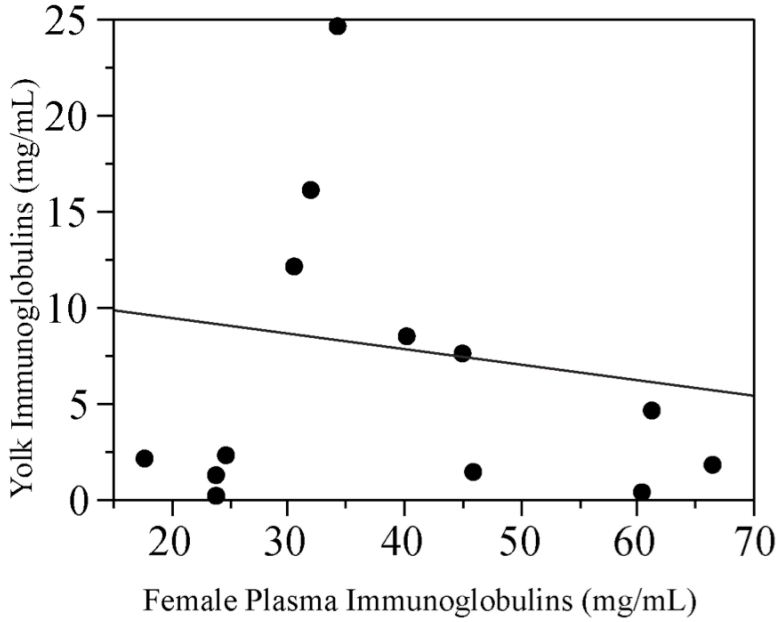

Maternal DNP-KLH-Specific Antibodies (OD)
Yolk
1.2
1
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Female DNP-KLH-Specific Antibodies (OD)

b.

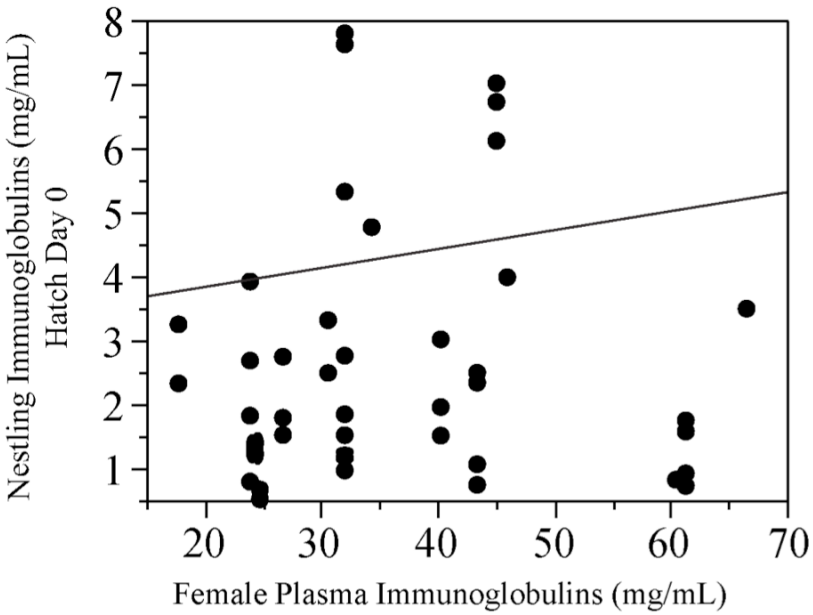

Maternal DNP-KLH-Specific Antibodies (OD)
Nestlings Hatch Day 0
0.3
0.25
0.2
0.15
0.1
0.05
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Female DNP-KLH-Specific Antibodies (OD)

`

a.


Yolk Immunoglobulins (mg/mL)
0
5
10
15
20
25
20
30
40
50
60
70
Female Plasma Immunoglobulins (mg/mL)

b.


Nestling Immunoglobulins (mg/mL)
Hatch Day 0
1
2
3
4
5
6
7
8
20
30
40
50
60
70
Female Plasma Immunoglobulins (mg/mL)

`

CHAPTER 2

Species-specific patterns of immunity predict the function of maternal antibodies in house sparrows

`

INTRODUCTION

The following manuscript was prepared for publication in the Journal of Animal Ecology and adheres to their submission and publication guidelines.

The primary function of the immune system is to protect organisms against virulent pathogens and parasites. Altricial and precocial developing birds hatch from the egg with incompletely developed immune systems (Rose 1981; Glick 1983a, b; Apanius 1998). At this stage of development they are highly vulnerable to environmental pathogens. The transfer of antigen-specific maternal antibodies from mothers to offspring is well documented (Loeken & Roth 1983; Kowalczyk et al. 1985; Beam et al. 2009), as are the protective qualities that maternal antibodies confer to immunologically naïve young (Fahey et al. 1987; Anderson 1995; Chaffer et al. 1997; Wang et al. 2004; Abdel-Moneim & Abdel-Gawad 2006; Lyche et al. 2006; Castinel et al. 2008). In birds, maternal antibodies are transferred to the chicks in a two-step process. First, antibodies are transferred across the follicular epithelium from the female circulation into the yolk during oogenesis. Second, the embryo absorbs maternal antibodies from the yolk into its circulation (Loeken & Roth 1983; Kowalczyk et al. 1985; Apanius 1998). Factors influencing the transfer of maternal antibodies include the mother's age, antigen-specific antibody titers, and time in reproductive cycle (Tizard 2002). Once in offspring circulation, maternal antibodies are rapidly catabolized (Patterson 1962; Kaleta et al. 1977; Darbyshire & Peters 1985; Fahey et al. 1987; Tizard 2002) and have a limited window of time in which to provide passive immunologic protection to chicks (Wang et al. 2004; Gasparini et al. 2006; Castinel et al. 2008; Beam et al. 2009).

`

It has been proposed that, in addition to providing passive immunity, maternal antibodies may shape the immunologic phenotype of offspring (Gasparini et al. 2006; Grindstaff et al. 2006; Reid et al. 2006). Maternal antibodies potentially reflect the antigenic make-up of the post-hatching environment (Mousseau & Fox 1998; Harrington et al. 2009; Wright et al. 2009). Studies on mice have reported that maternal antibodies enhance offspring immunity (Anderson 1995; Lemke et al. 2009) and the same has been proposed in a single wild bird species (Grindstaff et al. 2006). However, the predominant evidence reported for birds indicates that maternal antibodies suppress the initiation of offspring humoral immunity by interfering with antigen recognition and processing (Becky et al. 2004; Abou Elazab et al. 2009). In studies of galliforms, antigen-specific maternal antibodies prevent antigens from interacting with immature immune cells, which inhibits the formation of generational antigen tolerance (Ayal & David 2004), where the immune systems fails to process foreign antigen. The immunosuppressive effects of maternal antibodies have also been reported in wild birds (Staszewski et al. 2007; Gasparini et al. 2009). In these studies, it has been proposed that the binding of foreign antigen by maternal antibodies offsets the energetic costs associated with mounting an immune response during a critical period in offspring development (Boulinier & Staszewski 2008).

Recent studies on wild passerine species (songbirds) have questioned the effects of maternal antibodies on offspring fitness and immunologic development. These studies suggest that maternal antibodies may have no beneficial effects on offspring

`

development, immune response (Lozano & Ydenberg 2002; Tschirren et al. 2009; King et al. 2010), or parasite resistance (Tschirren et al. 2009). Further support for this argument has arisen from studies examining humoral immunologic development in passerines, where it has been reported that antigen-specific maternal antibodies are often absent from offspring circulation at the time of hatch (Lozano & Ydenberg 2002; Nemeth et al. 2008; King et al. 2010). Developmental studies also report that antigen-specific maternal antibodies in newly hatched chicks are frequently catabolized days before chicks are capable of producing their own antibodies (King et al. 2010). Collectively, this evidence suggests that maternal antibodies may be ineffectual in protecting offspring during the critical period between hatch and the onset of *de novo* synthesis of endogenous antibodies, when offspring are predicted to be most vulnerable to pathogens in their environment.

In this study, we challenged female and nestling house sparrows (*Passer domesticus*) with lipopolysaccharide (LPS) bacterial antigen to examine the effects of antigen-specific maternal antibodies on nestling humoral immunity. LPS is a potent, T-cell independent, immune stimulant that is encountered in the natural and aviary environments of free-living and captive birds. LPS containing, gram-negative bacteria, such as *Escherichia coli*, are also found in the natural micro-flora of the avian digestive track (Jang et al. 2007; Engberg et al. 2009; Ewers et al. 2009) and it has been detected in the fecal material of nestling house sparrows as young as 3-days old (King, Unpublished data). House sparrows are an ideal model to use in a study of the effects of antigen specific maternal antibodies on nestling humoral immune response, because an early humoral

`

immunologic profile has already been generated for this species. In this study, house sparrows have been proposed to exhibit species-specific patterns of humoral immunologic development that deviate from the classic chicken model of immunity (King et al. 2010). Relative to the chicken model, the half-life of maternal antibodies in nestling circulation is shorter (house sparrow: 2.2 ± 0.25 days; chicken: 3-7 days post-hatch) (Patterson 1962; Kaleta et al. 1977; Darbyshire & Peters 1985; Fahey et al. 1987; King et al. 2010), while the onset of *de novo* synthesis of endogenous antibodies is comparably similar (house sparrow: 3-6 days after hatch; chicken: 3-4 days after hatch) (Patterson 1962; Lawrence et al. 1981; Apanius 1998; Hamal et al. 2006; King et al. 2010). The timing of immunological independence, when nestlings rely entirely on their own antibodies for immune protection, is estimated to occur much sooner in house sparrows than chickens (house sparrow: 8-10 days after hatch; chicken: 14-21 days after hatch) (Patterson 1962; Kaleta et al. 1977; Darbyshire & Peters 1985; Fahey et al. 1987; King et al. 2010). Thus, we were in the position to design our study, the vaccination schedules, and the interpretation of the results within the context of house sparrow-specific patterns of maternal antibody transmission and humoral immune development.

MATERIALS AND METHODS

Animal Capture and Care

During the winters of 2006 and 2007 30 breeding pairs of wild house sparrows were captured with mist nets from agricultural sites near Pullman, WA (46°44′N 117°10′W). The birds were housed in five separate outdoor aviaries (1 X 2 X 2.5 m) with six breeding pairs per aviary. Each aviary was equipped with 10 nest boxes, perches, nesting material

`

(grass hay and pillow stuffing), and *ad libitum* access to food and water. In addition to seed, during the breeding and nestlings rearing stage, birds had access to a nutritional mash containing water, oatmeal, boiled eggs, ground eggshells, cooked ground beef, and Kaytee Exact Hand-Feeding Formula (Kaytee Products, Chilton, WI). Adults were banded with a numbered aluminum ring and two colored bands for individual identification. The birds were allowed to acclimate to their surroundings for 90 days before experimentation began.

Female vaccination

Beginning in March, nest boxes were monitored daily for egg laying activity. At the initiation of each clutch, nest boxes were videotaped to determine female identity. On the day after the last egg was laid from a female's first clutch, the female was captured and weighed on an electric balance to the nearest 0.01 g. Females were alternately assigned to a treatment of lipopolysaccharide (LPS: Sigma#L4005, serotype 055:B5) dissolved in phosphate buffered saline (PBS) at mg/mL or a control treatment of PBS alone. Females received an intraperitoneal injection of LPS at a dose of 1 mg/kg of body weight. The specific dose was derived from a study conducted on the similarly sized white-crowned sparrow (*Zonotrichia leucophrys gambelii*) (Owen-Ashley & Wingfield 2006). Control females were injected with a comparable volume of PBS. To minimize any negative effects of female capture and handling on parental care and reproductive output, after vaccination, female were no longer sampled and all measures of female immunity were assessed through the egg yolk. All but two females initiated a first clutch and received an injection.

`

Egg collection and processing

Nest boxes were monitored daily for eggs. Once a female initiated a clutch, the nest box was videotaped to determine parental identity. The day that an egg was laid it was marked with a non-toxic marker to determine laying order. For first laid clutches, all of the eggs were removed on the day they were laid and replaced with a wooden dummy egg. The wooden dummy eggs were then removed from the nest on the same day that the female was vaccinated to stimulate the initiation of a new clutch. For all clutches laid subsequent to female vaccination, only the second laid egg was collected and replaced with a wooden dummy egg. Eggs removed from the nest were placed in individually marked containers and stored at -20°C until further analysis.

Antibody extraction from yolks

Pre-vaccination LPS-specific antibody levels and total yolk antibody concentration were assessed from the second laid egg from a female's first clutch. Post-vaccination levels of LPS-specific and total antibody concentration were assessed from second laid eggs removed from clutches subsequent to female vaccination. Antibodies were extracted from the yolk using a modified chloroform-based method (Polson 1990; Hamal et al. 2006). Each yolk was separated from the albumen, washed with deionized water (dH_2O) and weighed to the nearest 0.0001g. A volume of PBS equal to the mass of yolk (mg/mL) was added to the yolk sample and vortexed. To that suspension, a volume of chloroform equal to two times the volume of PBS was added, vortexed and then centrifuged at 1,000 x g for 30 min at room temperature. After centrifugation, three distinct layers were

`

observed: a lecithin layer on the bottom, a layer of emulsified yolk and chloroform in the middle, and a watery protein layer on the top, which contains antibodies. The clear, aqueous protein layer was removed and stored at -20°C until further analysis.

Nestling measurements

Nest boxes were monitored daily to determine hatch date. On hatch day 0 nestlings were individually marked using a non-toxic marker. On day 9, nestlings were banded with a numbered aluminum ring and two colored leg bands for individual identification.

Blood sampling and nestling vaccination

To determine the effects of maternal treatment on nestling humoral immunity we selected two nestlings from each clutch, laid subsequent to female vaccination, to receive an injection of either LPS or PBS. The average number of nestlings per clutch, 12 days after hatch, was 1.7 ± 0.9234. In instances where more than two nestlings were present in the nest, the two heaviest were randomly assigned to a treatment group and the remaining nestlings were alternately assigned to either LPS or PBS treatments as well. However, we found no effect of nestling mass on antibody production ($F_{1,17}$=0.4655, P=0.5092), so only the two heaviest nestlings were included in the analyses. Nestlings received a standard 25 μL intraperitoneal injection of either LPS (mg/mL) or PBS. We collected pre- and post-vaccination blood samples by puncturing the brachial vein with a 28-gauge needle and collecting the blood into heparinized microcapillary tubes (~10-20 μL). Post vaccination samples were taken 7 days after nestlings were injected with either LPS or PBS (day 19). Blood samples were stored on ice for no more than two hours, whereupon

`

plasma was separated from red blood cells by centrifuging samples at 9,000 rpm for 10 min. Plasma samples were stored at -20°C until further analysis.

LPS-specific antibody ELISA

To detect LPS-specific antibodies in yolk and nestling plasma, we developed a LPS-specific ELISA protocol. Prior to running the samples, the ELISA was optimized to determine the ideal concentration at which to dilute the capture-antigen, samples, secondary antibody, and detection antibody. The assay was validated via serially diluting pooled samples to show gradations in absorbance.

Immulon-4, 96-well plates were coated with 100 μL/well of LPS (5 μg/mL) in sodium bicarbonate coating buffer (0.05 M, pH 9.6) and incubated overnight in the cold room (4°C). After incubating overnight, plates were washed three times with 200 μL/well of wash solution (150mM NaCl, 0.01% Tween-20). Each plate contained, in duplicate, a blank (contained no capture antigen, sample, secondary antibody or detection antibody), a well measuring non-specific binding (NSB; measured binding between the capture antigen and the secondary and detection antibodies), and two LPS-positive controls serially diluted 1:100, 1:500, and 1:1000 in sample buffer (FTA-hemmaglutination (pH 7.2), 10% casein). A negative control was not used because house sparrows encounter LPS in both their natural and aviary environments making it impossible to find seronegative LPS samples. Nestling plasma and chloroform extracted yolk samples were diluted 1:500 in sample buffer and added at a volume of 100 μL/well (duplicate wells per sample). Plates were incubated for 1 hr at 37°C on a rocker before being washed three

`

times with 200 µL/well of wash solution. Rat-anti-house sparrow-antibodies (RatαHOSP-Ab), that had previously been shown to cross-react with house sparrow immunoglobulins (King et al. 2010), was diluted 1:1000 in sample buffer and added at 100 µL/well to all but the blank wells. Plates were again incubated for 1 hr at 37°C on a rocker before being washed three times. 100 µL/well of detection antibody, goat-anti-rat-horse-radish-peroxidase (GαR-hrp; Invitrogen # A10549, Carlsbad, CA), was added to all but the blank wells at a dilution of 1:40,000 of antibody to sample buffer and incubated for 1 hr at 37°C on a rocker before being washed a final three times. Finally, 100 µL of peroxidase substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS: Sigma cat. A1888) and peroxide was added to each well and the plates were covered with tinfoil and allowed to develop for 1 hr at room temperature before being read on a spectrophotometer using a 405-nanometer wavelength filter to measure optic density (OD).

Plates were standardized to control for between plate variations in absorbance. Prior to standardization, the OD of the NSB was subtracted from the samples and positive controls. Per plate, the positive ratio was calculated by dividing the average absorbance of the positive control by the average absorbance of the highest positive control reading observed amongst all of the plates, collectively. Final absorbance readings were calculated by dividing the average sample absorbance by the positive ratio for that plate.

`

Total Antibody ELISA

A quantitative antibody ELISA described by King et al. (2010) was used to measure total antibody concentration in plasma and yolk. In brief, this assay uses a standard curve generated from known concentration of purified house sparrow antibodies to convert OD values into ng/mL units. The assay was sensitive to plasma and yolk immunoglobulins in the range of 3.12-1000 ng/mL. Samples were diluted 1:25,000 in sample buffer in accordance with the optimal dilution values reported by King et al. (2010) for yolk and nestling plasma samples. Each plate contained, in duplicate, a blank, a NSB, eight house sparrow-antibody standards, four positive controls made from pooled nestling plasma, and one negative control made from pooled chicken serum. Immulon-4, 96-well plates were coated with 100 µL/well of Rabbit-anti-house sparrow-antibodies diluted 1:1000 in sodium bicarbonate coating buffer. Plates were incubated overnight at 4°C before being washed three-times with 200 µL/well of wash solution. Samples were added, in duplicate at 100 µL/well, incubated for 1hr at 37°C on a rocker and then washed three times with wash solution. After washing, 100 µL of RatαHOSP-Ab diluted 1:1000 in sample buffer was added and the plates were again incubated for 1hr at 37°C on a rocker before being washed three additional times. 100 µL of the detection antibody, GαRat-hrp, was added to each well at a dilution of 1:40,000 and the plates were incubated one last time for 1hr at 37°C on a rocker before being washed a final three times. To each well, including the blank, 100 µL of ABTS developing reagent was added and the plates were covered with tinfoil and left to develop for 1hr. At the end of 1hr the plates were read on a spectrophotometer using a 405-nanometer wavelength filter to measure OD. The OD values were converted to ng/mL using softmax pro 4.8 (Molecular Devices Corp.,

`

Sunnyvale, CA). Samples were multiplied by their dilution factor and are presented as mg/mL.

Statistics

Linear least-squares models were built to examine how nestling immune function is affected by female treatment, nestling treatment, pre-vaccination antibody levels, yolk LPS-specific antibody levels, and the delay between vaccination date and the initiation of the second laid clutch on nestling immunity. Quantitative differences between treatment groups were analyzed using Student's t-test. P-values less than 0.05 were considered significant. All data were checked for normality of residuals and homogeny of variance. Analyses were carried out using JMP 7.0.1 (SAS Institute Inc.,Cary, NC, U.S.A.). Data are expressed as the mean ± SE.

Ethics statement

All of the experiments and procedures described herein were approved by the Institutional Animal Care and Use Committee (protocol #2551) at Washington State University.

RESULTS

Female humoral immune response

Female humoral immune response to LPS challenge was assessed by comparing pre-and post-vaccination LPS-specific and total antibody levels in the yolks of eggs laid before and after vaccination. The data was examined by evaluating the effects of female treatment, pre-vaccination antibody levels (LPS-specific and total), and the delay

`

between female vaccination date and the initiation of a new clutch on post-vaccination antibody levels (LPS-specific and total). We found no effect of time delay from vaccination to laying on female-derived LPS-specific ($F_{1,21}$=3.0148, P=0.1030) or total antibody levels in the egg yolks ($F_{1,23}$=0.0269, P=0.8716) and removed it as a model effect. Pre- and post-vaccination LPS-specific (pre-LPS) yolk antibody levels were positively correlated with each other ($F_{1,21}$=5.7086, P=0.0274). Female treatment ($F_{1,21}$=0.5553, P=0.4653) and the interaction of female treatment with pre-LPS antibody levels ($F_{1,21}$=1.7405, P=0.2028) had no significant effect on post-LPS antibody levels (Table 1, top panel). However, a subsequent analysis examining the effects of treatment on the degree of change between pre- and post-LPS yolk antibody levels (Post- LPS antibodies – Pre-LPS-antibodies) yielded a significant treatment effect (ANOVA: $F_{1,21}$=6.5039, P=0.0186, R^2=0.2365) with the increase in LPS antibody levels in yolks of LPS treated females (0.0844 ± 0.0266) being greater than the change in the yolks of PBS treated females (- 0.0095 ± 0.0255) (fig. 1). However, for total antibody concentration, we did not detect a significant effect of female treatment or pre-vaccination total yolk antibody (pre-Ab) concentration on post-vaccination total yolk antibody (post-Ab) concentration (Two-way ANOVA: $F_{3,21}$=1.3131, P=0.2965, R^2=0.1580; log-transformed for normality).

Effects of maternal and nestling vaccination on nestling antibody production

Female treatment and the delay between female vaccination and nestling sampling did not have a significant stimulatory or inhibitory effect on LPS-specific or total antibody levels in 12-day old nestlings, the time just before their vaccination (Table 1). LPS-

`

specific and total antibody levels in nestlings 7-days after their vaccination were also unaffected by female treatment (LPS: $F_{1, 16}$=0.0920, P=0.7678; total Ab: $F_{1, 16}$=0.0021, P=0.9643; Table 2, upper panel) and the delay time between female vaccination and nestling vaccination (LPS: $F_{1, 15}$=3.3697, P=0.0996; log-transformed for normality; total Ab: $F_{1,14}$=1.6226, P=0.2385; Table 2, middle panel) and so these variables were removed as model effects. Further analysis also showed no effect of yolk post-LPS antibody levels ($F_{1,14}$=0.7089, P=0.4243) or yolk total post-Ab levels ($F_{1,14}$=0.5146, P=0.4935) on nestling LPS-specific or total antibody immune responsiveness, respectively (Table 2, lower panel).

Independent of female treatment and the delay time between female vaccination and nestling sampling, nestling post-LPS antibody levels were significantly affected by the interaction between nestling treatment and their pre-LPS vaccination antibody levels ($F_{1,16}$=5.9229, P=0.0289; fig. 2a, Table 3). LPS treated nestlings had higher overall LPS antibody levels compared to PBS treated nestlings (LPS: 0.0866 ± 0.0112; PBS: 0.0657 ± 0.0109; fig. 3a). Nestling total pre-vaccination Ab concentrations were positively correlated with total post-Ab concentrations in 19-day old nestlings 7-days after vaccination ($F_{1,16}$=8.2952, P=0.0121), but there was no effect of nestling treatment ($F_{1,16}$=0.6397, P=0.4372) or the interaction of nestling treatment with nestling total pre-Ab concentrations on post-Ab concentration ($F_{1,16}$=0.0947, P=7628) (fig. 2b, 3b, Table 3).

`

DISCUSSION

The role that maternal antibodies play in the immunologic protection and development of offspring has been questioned in recent studies of wild birds (Lozano & Ydenberg 2002; Tschirren et al. 2009; King et al. 2010). Here, we demonstrate that, while both adult and nestling house sparrows are capable of mounting a specific humoral immune defense against LPS bacterial antigen, LPS-specific maternal antibodies do not appear to have an effect on nestling humoral immunity.

Maternal antibodies have been proposed to have a stimulatory, an inhibitory, or neutral effect on offspring immune-responsiveness. In the stimulatory model of immune priming, maternal antibodies are assumed to interact with components of the offspring's immune system to enhance antigen-specific antibody production (Anderson 1995; Grindstaff et al. 2006; Lemke et al. 2009). Under this model, LPS treated nestlings hatched from LPS treated females are expected to show increased LPS-specific antibody titers. In contrast, the inhibitory model predicts that maternal antibodies will suppress offspring immune function by preventing foreign antigen from interacting with immune cells (Becky et al. 2004; Staszewski et al. 2007; Abou Elazab et al. 2009). Under this model, LPS treated nestlings hatched from LPS treated females are predicted to be less immune-reactive than LPS treated nestlings hatched from PBS treated females. In our study, female treatment had no effect on nestling humoral immunity and we found no clear patterns suggesting that maternal antibodies have either a stimulatory or inhibitory effect on offspring specific (LPS) or non-specific (total antibody concentration) antibody levels. One confounding variable in testing for patterns of immune-mediated maternal

`

effects is the large natural variation in LPS-specific antibody levels that we measured in the yolks of both unvaccinated and vaccinated females. While LPS treated females showed a significant change in yolk LPS-specific antibody levels, their average post-vaccination yolk LPS-specific levels was not significantly different from PBS treated females. However, subsequent analyses showed no effect of yolk post-LPS antibody levels on nestling immune-responsiveness.

At the time of immune challenge (12-days post-hatch), LPS-specific antibodies were present in 90% of nestlings. At this stage of development maternal antibodies are predicted to be absent or below the range of detection (King et al. 2010), so the presence of antibodies in this age group is likely reflected the synthesis of LPS-specific antibodies by nestlings in response to their exposure to LPS in the nest. As aforementioned, LPS is a component of the gram-negative bacteria that colonize the digestive tracks of avian species (Jang et al. 2007; Engberg et al. 2009; Ewers et al. 2009). It is reasonable to assume that fecal material in the aviary and nest boxes may be a source of LPS exposure for both adults and nestlings. House sparrows are capable of antibody production 3-6 days after they hatch and appear to rely entirely on their own antibodies as early as 8-10 days after hatch (King et al. 2010). At the time of challenge, the early interaction of maternal antibodies with the offspring's immune system is expected to have an effect (stimulatory or inhibitory) on offspring humoral immunity (Becky et al. 2004; Gasparini et al. 2006; Grindstaff et al. 2006; Reid et al. 2006; Abou Elazab et al. 2009). However, in house sparrows, species-specific patterns of immunity may interfere with the transfer of antigen-specific maternal antibodies from females to hatchlings (Nemeth et al. 2008;

`

King et al. 2010). In house sparrows, the transfer of antigen-specific antibodies to the yolk and nestling plasma has been reported to be limited or incomplete (Nemeth et al. 2008; King et al. 2010). Thus, the ability of females to exert an effect on their offspring's humoral immune response, via maternal antibody transmission, may be ineffectual in this species.

House sparrows appear to express a pattern of humoral immune development that deviates from the classic chicken model of immunity (King et al. 2010). In house sparrows, antigen-specific antibodies from seropositive females are frequently absent from nestling circulation on the day of hatch (Nemeth et al. 2008; King et al. 2010). For maternal antibodies to have either a stimulatory or inhibitory effect on nestling immune function, the transfer of antigen-specific maternal antibodies from females to their offspring via egg yolk is required. While we clearly demonstrate that females are immune-reactive to LPS challenge and that LPS-specific antibodies are present in the yolk, we did not examine the transfer of LPS-specific antibodies from yolk to nestling circulation by measuring plasma levels of antibody in nestlings on hatch day 0. If LPS-specific maternal antibodies failed to transfer into nestlings or if they were catabolized prior to natural or experimental exposure to LPS, it is possible that female treatment may have little to no effect on nestling immunity. Independent of female treatment, nestlings challenged with LPS had significantly higher antibody levels than PBS treated nestlings 7-days after challenge, suggesting that individuals of this age group (12-days old) are capable of mounting an antigen-specific immune response, independent of maternal antibodies.

`

Collectively, the results of this study suggest that maternal antibodies are an unlikely mechanism for either immune priming or suppression in nestling house sparrows. Still, females may be able to influence the immunologic phenotype of their offspring through other pathways. Maternal carotenoids (Fitze et al. 2007; Berthouly et al. 2008; De Neve et al. 2008), yolk androgens (Groothuis et al. 2005; Muller et al. 2005; Pitala et al. 2009; Sandell et al. 2009), nutritional reserve in the yolk (Moreno et al. 2008), and investment in parental care (Lozano & Ydenberg 2002) have all been shown to have effects on nestling immunity. It is pertinent that patterns in immunologic development be examined in other passerine species before broad conclusions can be made regarding the effects of maternal antibodies on nestling immunologic development and fitness. Whether our results are species- and/or antigen-specific or if they apply to other passerine species warrants further research. Clearly, such studies would benefit from a deeper knowledge of the uptake of maternal antibodies from yolk to embryo and the dynamics of immune development in birds other than galliforms.

`

LITERATURE CITED

Abdel-Moneim, A. S. & Abdel-Gawad, M. M. A. (2006) Genetic variations in maternal transfer and immune responsiveness to infectious bursal disease virus. *Veterinary Microbiology,* 114**,** 16-24.

Abou Elazab, M. F., Fukushima, Y., Horiuchi, H., Matsuda, H. & Furusawa, S. (2009) Prolonged Suppression of Chick Humoral Immune Response by Antigen Specific Maternal Antibody. *Journal of Veterinary Medical Science,* 71**,** 417-424.

Anderson, R. W. (1995) On the maternal transmission of immunity - a molecular attention hypothesis. *Biosystems,* 34**,** 87-105.

Apanius, V. (1998) Ontogeny of immune function. *Avian Growth and Development* (eds J. M. Stark & R. E. Ricklefs), pp. 203-222. Oxford University Press, Oxford.

Ayal, K. & David, S. A., F. (2004) Maternal antibodies block induction of oral tolerance in newly hatched chicks. *Vaccine,* 22**,** 493-502.

Beam, A. L., Lombard, J. E., Kopral, C. A., Garber, L. P., Winter, A. L., Hicks, J. A. & Schlater, J. L. (2009) Prevalence of failure of passive transfer of immunity in newborn heifer calves and associated management practices on US dairy operations. *Journal of Dairy Science,* 92**,** 3973-3980.

Becky, A., Claude, L. & Stuart, M. (2004) Neonatal adaptive immunity comes of age. *Nature Rev. Immunol.,* 4**,** 553-564.

Berthouly, A., Helfenstein, F., Tanner, M. & Richner, H. (2008) Sex-related effects of maternal egg investment on offspring in relation to carotenoid availability in the great tit. *Journal of Animal Ecology,* 77**,** 74-82.

`

Boulinier, T. & Staszewski, V. (2008) Maternal transfer of antibodies: raising immuno-ecology issues. *Trends in Ecology & Evolution,* 23**,** 282-288.

Castinel, A., Kittelberger, R., Pomroy, W. E., Duignan, P. J., Chilvers, B. L. & Wilkinson, I. S. (2008) Humoral immune response to Klebsiella spp. in New Zealand sea lions (*Phocarctos hookeri*) and the passive transfer of immunity to pups. *Journal of Wildlife Diseases,* 44**,** 8-15.

Chaffer, M., Schwartsburd, B. & Heller, E. D. (1997) Vaccination of turkey poults against pathogenic *Escherichia coli*. *Avian Pathology,* 26**,** 377-390.

Darbyshire, J. H. & Peters, R. W. (1985) Humoral antibody-response and assessment of protection following primary vaccination of chicks with maternally derived antibody against avian infectious-bronchitis virus. *Research in Veterinary Science,* 38**,** 14-21.

De Neve, L., Fargallo, J. A., Vergara, P., Lemus, J. A., Jaren-Galan, M. & Luaces, I. (2008) Effects of maternal carotenoid availability in relation to sex, parasite infection and health status of nestling kestrels (Falco tinnunculus). *Journal of Experimental Biology,* 211.

Engberg, R. M., Hammershoj, M., Johansen, N. F., Abousekken, M. S., Steenfeldt, S. & Jensen, B. B. (2009) Fermented feed for laying hens: effects on egg production, egg quality, plumage condition and composition and activity of the intestinal microflora. *British Poultry Science,* 50**,** 228-239.

Ewers, C., Antao, E. M., Diehl, I., Philipp, H. C. & Wieler, L. H. (2009) Intestine and Environment of the Chicken as Reservoirs for Extraintestinal Pathogenic

`

Escherichia coli Strains with Zoonotic Potential. *Applied and Environmental Microbiology,* 75**,** 184-192.

Fahey, K. J., Crooks, J. K. & Fraser, R. A. (1987) Assessment by ELISA of passively acquired protection against infectious bursal disease virus in chickens. *Australian Veterinary Journal,* 64**,** 203-207.

Fitze, P. S., Tschirren, B., Gasparini, J. & Richner, H. (2007) Carotenoid-based plumage colors and immune function: Is there a trade-off for rare carotenoids? *American Naturalist,* 169**,** S137-S144.

Gasparini, J., Bize, P., Piault, R., Wakamatsu, K., Blount, J. D., Ducrest, A. L. & Roulin, A. (2009) Strength and cost of an induced immune response are associated with a heritable melanin-based colour trait in female tawny owls. *Journal of Animal Ecology,* 78**,** 608-616.

Gasparini, J., McCoy, K. D., Staszewski, V., Haussy, C. & Boulinier, T. (2006) Dynamics of anti-Borrelia antibodies in Blacklegged Kittiwake (Rissa tridactyla) chicks suggest a maternal educational effect. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* 84**,** 623-627.

Glick, B. (1983a) Bursa of Fabricius. *Avian Biology,* 7**,** 443-500.

Glick, B. (1983b) The saga of the bursa of Fabricius. *Bioscience,* 33**,** 187-191.

Grindstaff, J. L., Hasselquist, D., Nilsson, J. A., Sandell, M., Smith, H. G. & Stjernman, M. (2006) Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. *Proceedings of the Royal Society B-Biological Sciences,* 273**,** 2551-2557.

`

Groothuis, T. G. G., Eising, C. M., Dijkstra, C. & Muller, W. (2005) Balancing between costs and benefits of maternal hormone deposition in avian eggs. *Biology Letters,* 1**,** 78-81.

Hamal, K. R., Burgess, S. C., Pevzner, I. Y. & Erf, G. F. (2006) Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poultry Science,* 85**,** 1364-1372.

Harrington, D., El Din, H. M., Guy, J., Robinson, K. & Sparagano, O. (2009) Characterization of the immune response of domestic fowl following immunization with proteins extracted from Dermanyssus gallinae. *Veterinary Parasitology,* 160**,** 285-294.

Jang, I. S., Ko, Y. H., Kang, S. Y. & Lee, C. Y. (2007) Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Animal Feed Science and Technology,* 134**,** 304-315.

Kaleta, E. F., Siegmann, O., Lai, K. W. & Aussum, D. (1977) Kinetics of NDV-specific antibodies in chickens.VI. Elimination of maternal and injected antibodies. *Berliner und Munchener Tierarztliche Wochenschrift,* 90**,** 131-134.

King, M. O., Owen, J. P. & Schwabl, H. G. (2010) Are Maternal Antibodies Really that Important? Patterns in the Devlopment of Humoral Immunity in Altricial Passerine House Sparrows (Passer domesticus). *PLoS ONE,* 5**,** e9639.

Kowalczyk, K., Daiss, J., Halpern, J. & Roth, T. F. (1985) Quantitation of maternal-fetal IgG transport in the chicken. *Immunology,* 54**,** 755-762.

`

Lawrence, E. C., Arnaudbattandier, F., Grayson, J., Koski, I. R., Dooley, N. J., Muchmore, A. V. & Blaese, R. M. (1981) Ontogeny of humoral immune function in normal chickens - a comparison of immunoglobulin-secreting cells in bone-marrow, spleen, lungs and intestine. *Clinical and Experimental Immunology,* 43**,** 450-457.

Lemke, H., Tanasa, R. I., Trad, A. & Lange, H. (2009) Benefits and burden of the maternally-mediated immunological imprinting. *Autoimmunity Reviews,* 8**,** 394-399.

Loeken, M. R. & Roth, T. F. (1983) Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. *Immunology,* 49**,** 21-28.

Lozano, G. A. & Ydenberg, R. C. (2002) Transgenerational effects of maternal immune challenge in tree swallows (Tachycineta bicolor). *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* 80**,** 918-925.

Lyche, J. L., Larsen, H. J. S., Skaare, J. U., Tverdal, A., Johansen, G. M. & Ropstad, E. (2006) Perinatal exposure to low doses of PCB 153 and PCB 126 affects maternal and neonatal immunity in goat kids. *Journal of Toxicology and Environmental Health-Part a-Current Issues,* 69**,** 139-158.

Moreno, J., Lobato, E., Morales, J., Merino, S., Martinez-De La Puente, J. & Tomas, G. (2008) Pre-laying nutrition mediates maternal effects on offspring immune capacity and growth in the pied flycatcher. *Oecologia,* 156**,** 727-735.

Mousseau, T. A. & Fox, C. W. (1998) *Maternal effects as adaptations,* Oxford University Press., New York, NY.

`

Muller, W., Groothuis, T. G. G., Kasprzik, A., Dijkstra, C., Alatalo, R. V. & Siitari, H. (2005) Prenatal androgen exposure modulates cellular and humoral immune function of black-headed gull chicks. *Proceedings of the Royal Society B-Biological Sciences,* 272**,** 1971-1977.

Nemeth, N. M., Oesterle, P. T. & Bowen, R. A. (2008) Passive immunity to West Nile virus provides limited protection in a common passerine species. *American Journal of Tropical Medicine and Hygiene,* 79**,** 283-290.

Owen-Ashley, N. T. & Wingfield, J. C. (2006) Acute phase responses in passerine birds: Characterization and life-history variation. *Journal of Ornithology,* 147**,** 61-61.

Patterson, R., J. S. Younger, W. O. Weigle, and F. J. Dixon. (1962) The metabolism of serum proteins in the hen and chick and secretion of serum proteins by the ovary of the hen. *Journal of General Physiology,* 45**,** 501–513.

Pitala, N., Ruuskanen, S., Laaksonen, T., Doligez, B., Tschirren, B. & Gustafsson, L. (2009) The effects of experimentally manipulated yolk androgens on growth and immune function of male and female nestling collared flycatchers Ficedula albicollis. *Journal of Avian Biology,* 40**,** 225-230.

Polson, A. (1990) Isolation of IgY from the yolks of eggs by a chloroform polyethylene-glycol procedure. *Immunological Investigations,* 19**,** 253-258.

Reid, J. M., Arcese, P., Keller, L. F. & Hasselquist, D. (2006) Long-term maternal effect on offspring immune response in song sparrows Melospiza melodia. *Biology Letters,* 2**,** 573-576.

Rose, M. E. (1981) Lymphatic system. *Form and function in birds* (ed A. S. K. J. McLelland), pp. 341-384. Academic Press, London.

`

Sandell, M. I., Tobler, M. & Hasselquist, D. (2009) Yolk androgens and the development of avian immunity: an experiment in jackdaws (Corvus monedula). *Journal of Experimental Biology,* 212**,** 815-822.

Staszewski, V., Gasparini, J., McCoy, K. D., Tveraa, T. & Boulinier, T. (2007) Evidence of an interannual effect of maternal immunization on the immune response of juveniles in a long-lived colonial bird. *Journal of Animal Ecology,* 76**,** 1215-1223.

Tizard, I. (2002) The avian antibody response. *Seminars in Avian and Exotic Pet Medicine,* 11**,** 2-14.

Tschirren, B., Siitari, H., Saladin, V. & Richner, H. (2009) Transgenerational immunity in a bird-ectoparasite system: do maternally transferred antibodies affect parasite fecundity or the offspring's susceptibility to fleas? *Ibis,* 151**,** 160-170.

Wang, Y. W., Sunwoo, H., Cherian, G. & Sim, J. S. (2004) Maternal dietary ratio of linoleic acid to alpha-linolenic acid affects the passive immunity of hatching chicks. *Poultry Science,* 83**,** 2039-2043.

Wright, H. W., Bartley, K., Nisbet, A. J., McDevitt, R. M., Sparks, N. H. C., Brocklehurst, S. & Huntley, J. F. (2009) The testing of antibodies raised against poultry red mite antigens in an in vitro feeding assay; preliminary screen for vaccine candidates. *Experimental and Applied Acarology,* 48**,** 81-91.

`

TABLES

Table 1. Effects of female treatment on LPS-specific and total antibody levels measured in the yolk and pre-vaccination nestling plasma.

dependent variable	yolk post-LPS antibody levels			yolk post-Ab levels		
	d.f.	*F*	*p*	*d.f.*	*F*	*p*
female treatment	1, 21	0.5553	0.4653	1, 23	0.9866	0.3319
yolk pre-LPS	1, 21	5.7086	0.0274**	1, 23	3.2703	0.0849
female treatment by yolk pre-LPS	1, 21	1.7405	0.2028	1, 23	3.5588	0.0731
	nestling pre-LPS antibody levels			nestling pre-Ab levels		
	d.f.	*F*	*p*	*d.f.*	*F*	*p*
female treatment	1, 12	0.0607	0.8092	1, 12	0.2047	0.6584
delay	1, 12	0.8623	0.3700	1, 12	0.0066	0.9367
female treatment by delay	1, 12	0.8315	0.3784	1, 12	0.0755	0.7878

** $p < 0.05$

`

Table 2. Effects of female treatment, the delay between female vaccination and nestling vaccination, and yolk LPS-specific and total antibody levels on nestling immunity

dependent variable	nestling post-LPS antibody levels			nestling post-Ab levels		
	d.f.	*F*	*p*	*d.f.*	*F*	*p*
nestling treatment	1, 16	1.0896	0.3211	1, 16	0.8269	0.3846
female treatment	1, 16	0.0920	0.7678	1, 16	0.0021	0.9643
pre-LPS/total-Ab	1, 16	0.6498	0.4389	1, 16	3.9693	0.0743
nestling treatment by female treatment	1, 16	1.6838	0.2236	1, 16	1.2437	0.2908
nestling treatment by pre-LPS/total-Ab	1, 16	5.0276	0.0488**	1, 16	0.9820	0.3451
female treatment by pre-LPS/total-Ab	1, 16	0.0090	0.9264	1, 16	0.0234	0.8814
nestling treatment by female treatment by pre-LPS/total-Ab	1, 16	0.0039	0.9512	1, 16	0.0247	0.8782
	nestling post-LPS antibody levels			nestling post-Ab levels		
	d.f.	*F*	*p*	*d.f.*	*F*	*p*
nestling treatment	1, 15	0.6106	0.4546	1, 14	1.9874	0.1963
delay	1, 15	3.3697	0.0996	1, 14	1.6226	0.2385
pre-LPS/total-Ab	1, 15	4.9179	0.0538	1, 14	1.8013	0.2164
nestling treatment by delay	1, 15	0.5804	0.4657	1, 14	2.0448	0.1902
nestling treatment by pre-LPS/total-Ab	1, 15	6.2092	0.0343**	1, 14	2.6686	0.1410
delay by pre-LPS/total-Ab	1, 15	5.0683	0.0509	1, 14	2.3821	0.1613
nestling treatment by delay by pre-LPS/total-Ab	1, 15	3.1705	0.1087	1, 14	2.3375	0.1648
	nestling post-LPS antibody levels*			nestling post-Ab levels		
	d.f.	*F*	*p*	*d.f.*	*F*	*p*
nestling treatment	1, 14	1.8797	0.2076	1, 14	1.4510	0.2628
pre-LPS/total-Ab	1, 14	0.1285	0.7293	1, 14	2.1672	0.1792
yolk post-LPS/total-Ab	1, 14	0.7089	0.4243	1, 14	0.5146	0.4935
nestling treatment by pre-LPS/total-Ab	1, 14	0.1308	0.7269	1, 14	1.3582	0.2774
nestling treatment by yolk post-LPS/total-Ab	1, 14	0.0141	0.9085	1, 14	0.0557	0.8193
yolk post-LPS/total-Ab by pre-LPS/total-Ab	1, 14	0.0664	0.8031	1, 14	4.1302	0.0765
nestling treatment by pre-LPS/total-Ab by yolk post-LPS/total-Ab	1, 14	0.187(	0.6769	1, 14	0.0789	0.7859

* Nestling post-LPS to post-Ab levels log-transformed for normality.

** $p < 0.05$

`

Table 3. Effects of nestling treatment and pre-vaccination LPS-specific and total antibody levels on nestling immunity.

dependent variable	nestling post-LPS antibody levels			nestling post-Ab levels		
		F	*p*	*d.f.*	*F*	*p*
nestling treatment	1, 16	1.7791	0.2036	1, 16	8.2952	0.0121
pre-LPS/pre-Ab	1, 16	0.9250	0.3525	1, 16	0.6397	0.4372
nestling treatment by pre-LPS/pre-Ab	1, 16	5.9229	0.0289**	1, 16	0.0947	0.7628

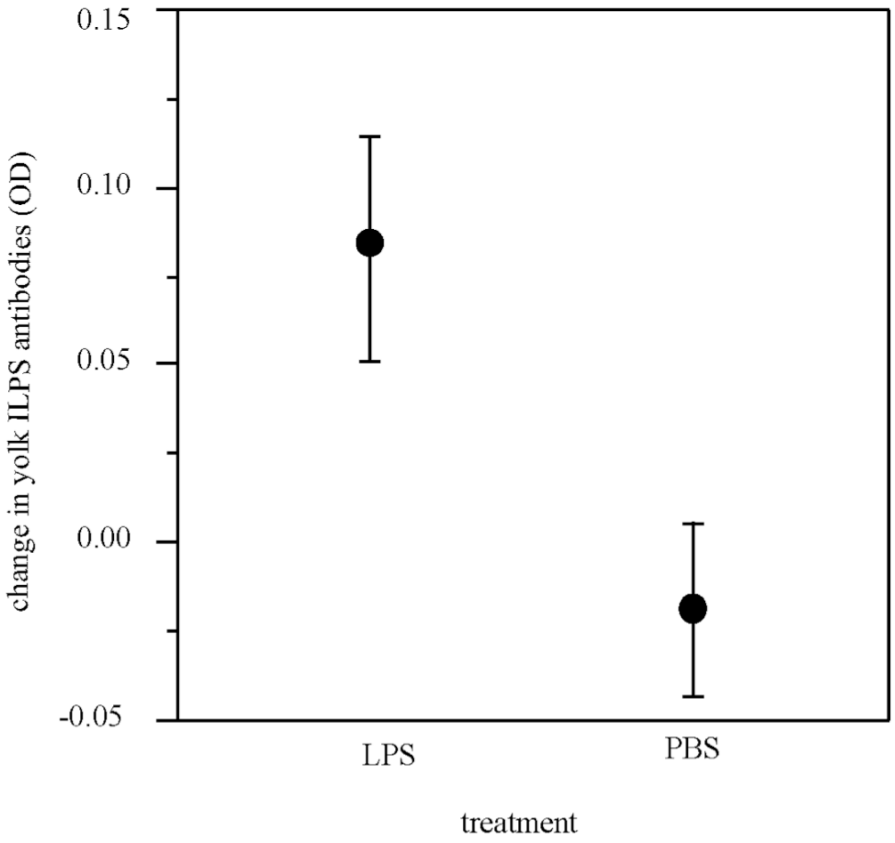
** $p < 0.05$

FIGURE LEGENDS

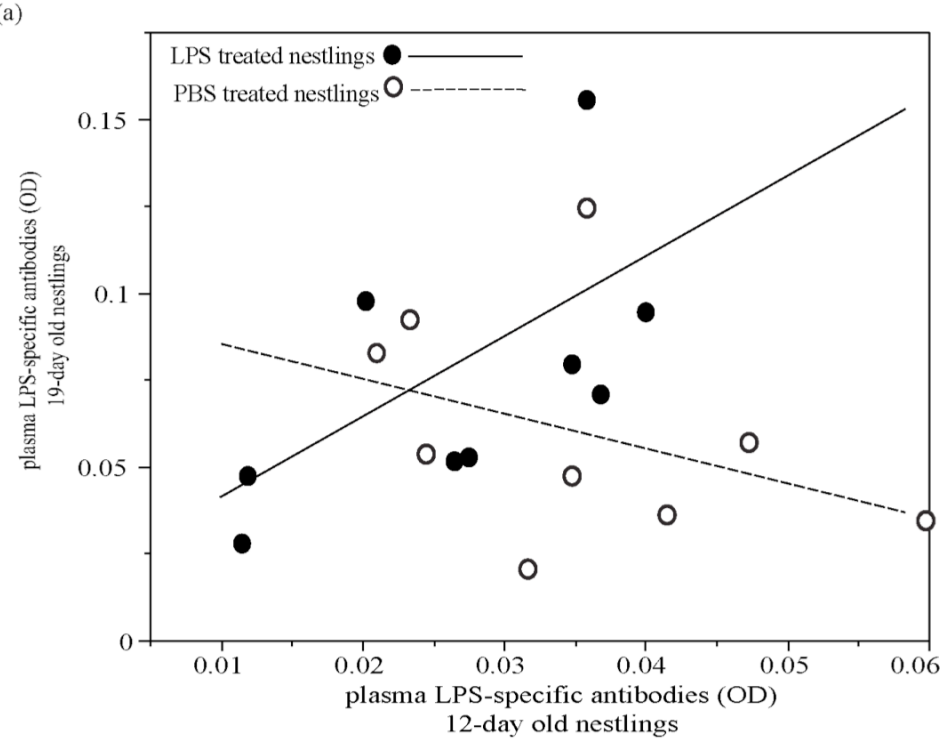
Figure 1. Effect of female vaccination against LPS on the change in pre- and post-vaccination LPS-specific antibody levels in second laid eggs collected from the first (pre-vaccination) and second (post-vaccination) laid clutch. Change in antibody levels were determined by subtracting pre-vaccination antibody titers from post-vaccination antibody titers. The data is presented as the mean ± SE, with LPS-specific antibody levels expressed as optic density (OD).

Figure 2. The interaction of nestling treatment and pre-vaccination (a) LPS-specific and (b) total antibody concentration on (a) post-vaccination LPS-specific antibody levels and (b) post-vaccination total antibody concentration. LPS-specific antibody levels are expressed as optic density (OD) and total antibody concentration as mg/mL. Pre-vaccination plasma samples came form 12-day old nestlings and post-vaccination plasma samples from 19-day old nestlings.

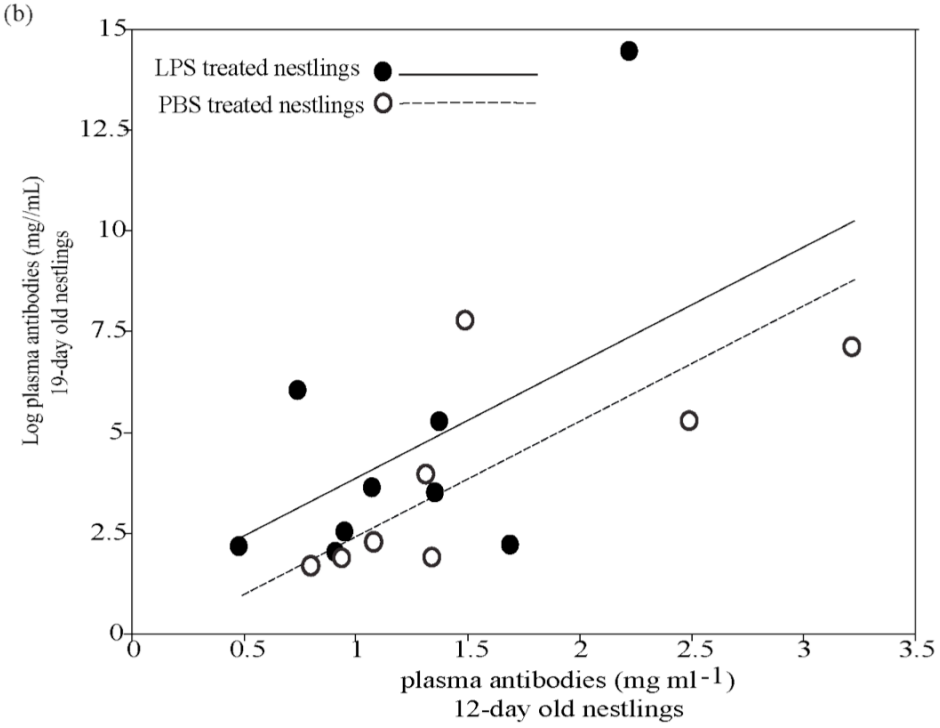
Figure 3. Effect of nestling vaccination with LPS on (a) plasma LPS-specific antibody levels and (b) total plasma antibody concentration. Pre-vaccination plasma samples came form 12-day old nestlings and post-vaccination plasma samples from 19-day old nestlings. The data is presented as the mean ± SE, with LPS-specific antibody levels are expressed as optic density (OD) and total antibody concentration as mg/mL.


0.15
0.10
0.05
0.00
-0.05
change in yolk ILPS antibodies (OD)
LPS
PBS
treatment

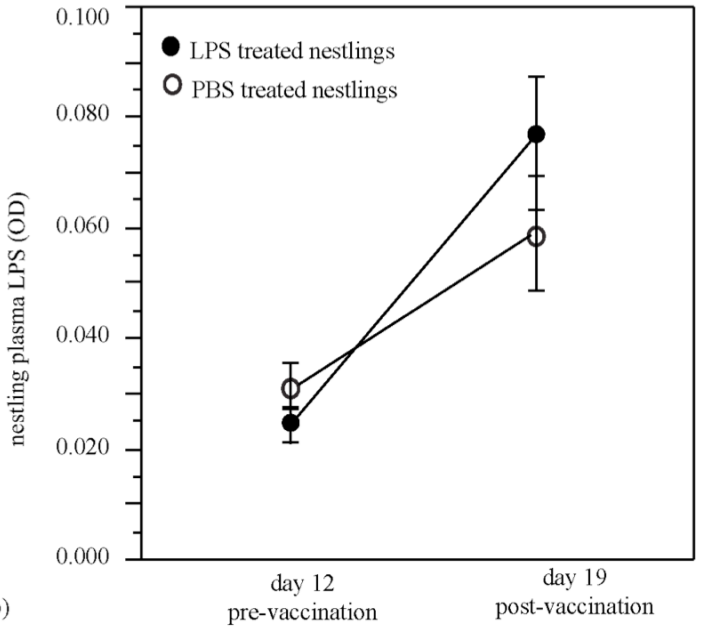
(a)


LPS treated nestlings
PBS treated nestlings
plasma LPS-specific antibodies (OD)
19-day old nestlings
0.15
0.1
0.05
0
0.01
0.02
0.03
0.04
0.05
0.06
plasma LPS-specific antibodies (OD)
12-day old nestlings

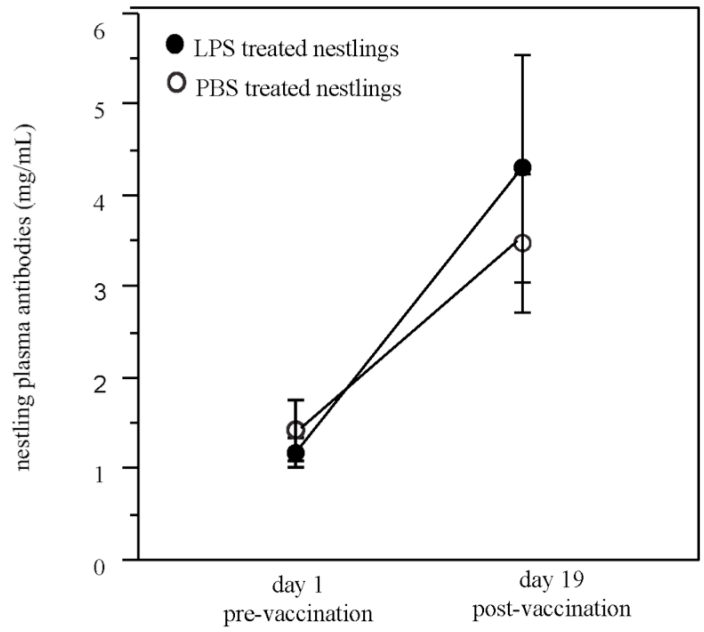
(b)


LPS treated nestlings
PBS treated nestlings
Log plasma antibodies (mg//mL)
19-day old nestlings
15
12.5
10
7.5
5
2.5
0
0
0.5
1
1.5
2
2.5
3
3.5
plasma antibodies (mg ml-1)
12-day old nestlings

(a)


0.100
0.080
0.060
0.040
0.020
0.000
nestling plasma LPS (OD)
LPS treated nestlings
PBS treated nestlings
day 12
pre-vaccination
day 19
post-vaccination

(b)


6
5
4
3
2
1
0
nestling plasma antibodies (mg/mL)
LPS treated nestlings
PBS treated nestlings
day 1
pre-vaccination
day 19
post-vaccination

CHAPTER 3

Injecting the mite into ecological immunology: Measuring the adaptive immune response of house sparrows (Passer domesticus) challenged with hematophagous mites.

INTRODUCTION

The following manuscript was prepared for publication in the AUK and adheres to their submission and publication guidelines.

Hematophagous ectoparasites impose negative fitness consequences on their avian hosts (Huber 2008; Mullens et al. 2009). Parasite infestation reduces host survivorship (Moller et al. 2003; Moller et al. 2004), reproductive success (Richner and Heeb 1995), growth (Ricklefs 1992), and predator avoidance (Moreno et al. 1999). In addition to the direct effects of ectoparasites on host condition (e.g. blood loss and tissue damage), parasite-induced host immune defenses may create an indirect cost, due to the trade-offs in energy and resources between the immune system and other life history traits (e.g. growth and reproduction) (Johnsen and Zuk 1999; Owen et al. *in review*). Such trade-offs are expected to be acute during the breeding season, when reproduction and sexual signaling compete with the immune system for energy (Moreno et al. 1999; Owen-Ashley et al. 2006). To our knowledge, there are no published studies that examine physiological trade-offs in a passerine species involving a specific, adaptive immune response against a hematophagous ectoparasite.

Avian species utilize various behavioral and physiological defenses against parasitism. Host immune function (Johnsen and Zuk 1999; Arkle et al. 2008), body temperature, skin thickness and grooming behavior (Elliot et al. 2003) are all factors that impair a parasite's ability to successfully establish itself and feed upon a prospective host. However, both altricial and precocial avian offspring hatch with incomplete immune systems, making

them vulnerable to pathogens (Apanius 1998). For young birds, immunologic protection is, in part, conferred via maternally derived antibodies, which act as the primary mode of defense for hatchlings against parasites during the early post-hatching stage of development (Apanius 1998; Mondal and Naqi 2001; Rahman and Strathmann 2002; Saino et al. 2002; Hamal et al. 2006; Harrington et al. 2009). The transfer of antigen-specific maternal antibodies from the mother to her offspring is well documented (Loeken and Roth 1983; Kowalczyk et al. 1985; Hamal et al. 2006; Harrington et al. 2009), as are the protective properties that maternal antibodies provide against pathogens in both chicken (Abdel-Moneim and Abdel-Gawad 2006; Hamal et al. 2006; Arkle et al. 2008; Harrington et al. 2009; Wright et al. 2009) and passerine species (Apanius 1998; Gasparini et al. 2002; Grindstaff et al. 2006; Tschirren et al. 2009).

During the breeding season, the reproductive and immune systems compete for energetic resources (Lochmiller and Deerenberg 2000; Bonneaud et al. 2003; Muller et al. 2005). In the absence of parasitic pressure, females are expected to invest more of their energetic resources into reproduction (eggs and nestling provisioning) to maximize reproductive output (Tinbergen and Boerlijst 1990; Linden et al. 1992; Both et al. 1999). Nestling mass (Tinbergen and Boerlijst 1990; Linden et al. 1992) and size (e.g. tarsus length) are positively correlated with fledging success (Christe et al. 1998; Forsman et al. 2008), as well as with subsequent survival to breeding age (Tinbergen and Boerlijst 1990; Tinbergen and Daan 1990; Moreno et al. 2005). The investment of maternal resources is further reflected in egg size, which is a predictor of nestling body condition and fledging success (Schifferli 1980; Ojanen 1983; Lochmiller and Deerenberg 2000; Christians

2002; Groothuis et al. 2005).

In chickens, ectoparasite infestations of hens, or experimental injection of parasite antigens, cause parasite-specific antibodies to be transferred into the yolk (Arkle et al. 2008; Harrington et al. 2009; Wright et al. 2009). The presence of parasite-specific antibodies in adults and hatchlings is associated with reduced parasite feeding and fecundity (Dusbabek et al. 1989; Harrington et al. 2009; Wright et al. 2009). In the passerine Great tit (*Parus major*), Tschirren and Richner (2006) examined the costs and benefits of optimal investment in immune function in nestlings. They found that somatic growth was blunted in nestlings that received artificial challenges to the immune system in the absence of parasites. In contrast, investment in the immune system, under parasite pressure, resulted in increased growth for immune stimulated nestlings. Collectively, these studies suggest that, in the presence of parasites, investment in immune function by both the female and the nestling may increase nestling fledging and survival.

Over the course of the breeding season, adult and nestling passerines are exposed to an array of ectoparasites, including hematophagous mites (Stoehr et al. 2000; Szabo et al. 2008). Robust research has been conducted examining the direct consequences of mite infestation on host fitness and immunologic function in chickens (Kirkwood 1985; Minnifield et al. 1993; Arkle et al. 2008; Harrington et al. 2009; Mullens et al. 2009; Owen et al. 2009; Wright et al. 2009). However, to our knowledge there are no studies that profile a specific adaptive immune response to hematophagous ectoparasites in a passerine species, in the context of reproduction. Most studies have used novel,

experimental reagents to challenge the host's immune system and examine trade-offs with condition-dependent life history traits (Westneat et al. 2003; Lindstrom et al. 2004; Westneat et al. 2004; Carleton 2008). Such indirect measurements of immunity may be misleading, in that they do not necessarily reflect the costs and benefits of mounting an immune response to natural parasites (de Bellocq et al. 2006a; de Bellocq et al. 2006b; de Bellocq et al. 2007).

We measured the humoral immune responses of nestling and adult house sparrows (*Passer domesticus*) to specific antigens derived from hematophagous mites occurring naturally in the population of this passerine bird. We also examined relationships between mite specific antibodies and nestling mass and body condition. Our objectives were to *i*) determine whether or not adult house sparrows mount an immune response against northern fowl mite (*Ornithonyssus sylviarum*) antigen; *ii*) determine whether or not anti-mite specific antibodies are transferred to the nestlings through the yolk; *iii*) determine whether or not hematophagous-mite infestation has an effect on nestling body condition; *iv*) determine whether or not the presence of anti-mite specific antibodies correlates with nestling body condition; and *v*) determine whether or not mite infestation and the presence of anti-mite specific antibodies is correlated with reduced female investment (egg mass).

MATERIALS AND METHODS

Ethics Statement

All of the experiments and procedures described herein were approved by the Institutional Animal Care and Use Committee (protocol #2551) at Washington State University.

Aviary Study.–In the winter of 2008, 20 breeding pairs of wild house sparrows were captured from sites near Pullman, WA (46°44′N 117°10′W). The birds were housed in five separate outdoor aviaries (1 X 2 X 2.5 m) with 2 breeding pairs per aviary. Each aviary was equipped with 10 nest boxes, perches, nesting material (grass hay and pillow stuffing), and *ad libitum* access to food and water. Individuals were banded with a numbered aluminum ring and two colored bands for individual identification. The sparrows were allowed to acclimate to their surroundings for 60 days before experimentation began.

Northern fowl mite-specific antigen.–Northern fowl mites (NFM), *Ornithonyssus sylviarum*, are a broadly distributed hematophagous parasite that has been found on diverse species of birds of temperate regions, including *P. domesticus* (Knee and Proctor 2007). NFM were acquired from a 7-year old colony maintained at the University of California, Riverside. Mite antigens were extracted by placing whole mites into a centrifuge tube and macerating them with a clean pestle in 1.0 mL of phosphate buffered saline (PBS) and 1 mM ethylenediaminetetraacetic acid (EDTA). The macerated sample was centrifuged at 20.2 x g for 10min, before the supernatant was collected, passed

through a 2 μm syringe filter and stored at -20°C. The collected fraction contained 0.80 mg mL^{-1} of mite proteins. The protein concentration was estimated using the Warburg-Christian method, which is a direct spectrophotometric method that uses a correction factor calculated from the absorbance of the sample read at 260 nm and 280 nm wavelength.

Vaccination and sampling.– In each of the five aviaries, females (N=20) and males (N=20) were randomly assigned to one of two treatment groups: PBS or NFM-antigen treatment (20 birds/group, with an equal sex ratio between groups). A pre-vaccination blood sample was taken from the brachial wing vein by puncturing the vein with a 28-gauge needle and collecting the blood with a heparinized microcapillary tube (~100 μL/bleed/individual). Blood samples were stored on ice for no more than two hours, whereupon plasma was separated from the red blood cells by centrifuging samples at 9,000 rpm for 10 min and stored at -20°C until analyses.

Immediately following the bleed, individuals were injected subcutaneously into the pectoralis muscle with either 30 μL of NFM-antigen or 30 μL of PBS. NFM-antigen was administered at 1 mg kg^{-1} body weight. A volume for both treatment groups was selected based on the average mass of adults housed in the aviary (28 g ± 1.2). Second and third injections were given in one week intervals 7 respectively and 14 days later. A second blood sample was taken 28 days after the final injection. All blood samples were processed using the same protocol described above.

Secondary-antibody production.– A secondary antibody used to detect house sparrow immunoglobulins was produced following the published protocol described by Huber et al (2010). Briefly, house sparrow immunoglobulin Y (HOSP-IgY) was isolated from house sparrow yolks using thiophilic interaction chromatography (Hansen et al. 1998). Rats were vaccinated against purified HOSP-IgY and later exsanguinated to collect rat-anti-house sparrow-IgY (RαHOSP-IgY). Cross-reactivity between the rat antiserum and HOSP-IgY was validated via western blot analyses.

NFM-specific antibody ELISA.– To detect the presence of house sparrow–anti-NFM immunoglobulins (HOSPαNFM-IgY) an enzyme-linked immunosorbent assay (ELISA) was developed. Prior to running the samples, the ELISA was optimized to determine the ideal concentration at which to dilute the capture-antigen, samples, secondary antibody and detection antibody. The assay was validated via serially diluting pooled samples to show gradations in absorbance. Briefly, pooled house sparrow plasma samples were diluted 1:100, 1:500, 1:1000, 1:2000, and 1:5000 in sample buffer and run on an ELISA plate in triplicate. Gradations in absorbance were observed with the more concentrated solutions generating higher absorbance values over the lower samples in succession (1:100 >1:500 >1:1000 > 1:2000 > 1:5000).

Immulon-4, 96-well plates were coated with 100 μL/well of NFM-antigen extract diluted 1:1,000 in coating buffer (0.05 M, pH 9.6) and incubated overnight at 4°C. Plates were washed three times with 200 μL/well of wash solution (150mM NaCl, 0.01% Tween-20). Each plate contained, in duplicate, a blank, a NSB (non-specific binding; measured

binding between the capture antigen and the secondary and detection antibodies), and two positive controls made from pooled plasma samples obtained from vaccinated adult house sparrows. Positive samples were serially diluted in sample buffer (FTA-hemmaglutination (pH 7.2), 10% casein) to concentrations of 1:500, 1:1000, and 1:2000 and were run in duplicate. A negative control was not used, as all the individuals used in this study had pre-existing antibodies built against some components of the NFM-extract. Plasma samples were diluted 1:1,000 in sample buffer (10% casein blocking buffer in FTA-hemmaglutination buffer; pH 7.2) and added in duplicate at 100 µL/well. Plates were then incubated for 1 hr at 37°C on a rocker before being washed three times with wash solution. 100 µL/well of RαHOSP-IgY was added to each well at a concentration of 1:1,000 of antibody to sample buffer and incubated for 1 hr at 37°C on a rocker before being washed three times. 100 µL/well of detection antibody, goat-anti-rat-horse-radish-peroxidase (GαR-hrp) (Invitrogen # A10549, Carlsbad, CA), was then added to each well at a dilution of 1:20,000 of antibody to sample buffer and incubated for 1 hr at 37°C on a rocker before being washed a final three times. Finally, 100 µL of peroxidase substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS: Sigma cat. A1888) and peroxide was added to each well and the plates were covered with tinfoil and allowed to develop for 1 hr at room temperature before being read on a spectrophotometer using a 405-nanometer wavelength filter to measure optic density (OD).

Plates were standardized to control for between plate variations in absorbance. Prior to standardization, the OD of the NSB was subtracted from the OD of each samples and

control on each plate. Per plate, the positive ratio was calculated by dividing the average absorbance of the positive control by the average absorbance of the highest positive control reading observed amongst all of the plates, collectively. Final absorbance readings were calculated by dividing the average sample absorbance by the positive ratio for that plate.

Mite-specific antibodies in wild house sparrow populations.–To explore evidence of mite specific antibodies in a wild population of house sparrows, samples and data were utilized from an unrelated, 2005 pilot study where birds were known to be infested or non-infested with hematophagous mites.

Field site.–In the spring of 2005, 60 nest boxes were hung at the University of Idaho's Sheep Center in Moscow, ID (46°43′N 116°59′W). Nest boxes were mounted in three different barns in clusters of 20 and were placed ~1 m apart from each another. To reduce nest predation a mesh-wire cylinder was attached to the entrance of each box. This allowed sparrows to freely enter and exit, but limited the ability of predators to gain access to the interior of the box. House sparrows were observed to occupy this site at a high density and readily occupied the boxes.

Yolk sampling.– Nest boxes were monitored daily for house sparrow eggs. On the day that an egg was laid it was marked with a non-toxic marker to determine laying order. Egg mass was measured on an electronic balance to the nearest 0.01 g. A yolk biopsy sample (< 0.02 mg) was obtained following the methods described by Schwabl (1993).

In some instances, the second laid egg was collected and replaced with a wooden dummy egg, in lieu of sampling an entire clutch. Subsequent analyses showed that yolk biopsies had no effect on hatching success (ANOVA $F_{1, 43}=0.7533$, P=0.3904) with hatching defined as the number of nestlings that hatched from a clutch. After sampling, the eggs were returned to their respective nest boxes. Yolk samples were stored on ice for no more than 2 hours before they were transported to the laboratory for further processing. In the laboratory, yolks from intact eggs were collected and the membrane was washed with ddH_2O. Both whole yolk and yolk biopsy samples were weighed to the nearest 0.001 g and an equal volume of ddH_2O was added to the sample. The yolk in ddH_2O sample was vortexed and stored at -20°C.

Antibody extraction from the yolk.– For future antibody-assays, immunoglobulins were extracted from yolk biopsy and whole egg samples using a modified chloroform-based protocol (Polson 1990; Hamal et al. 2006). Briefly, an equal volume of phosphate buffered solution (PBS) was added to the yolk + ddH_2O emulsion and vortexed vigorously. A volume of chloroform equal to the volume of the PBS, yolk and ddH_2O homogenate was added and vortexed to produce a thick emulsion. Samples were then centrifuged at 1,000 x g for 30 min at room temperature. In the centrifuge tube, three distinct layers were observable: a lecithin layer on the bottom, a layer of emulsified yolk and chloroform in the middle, and a watery protein layer on to the top, which contains antibodies. The clear, aqueous uppermost layer was removed and stored at -20°C until analysis.

Nestling measurements.– At the time of expected hatching nest boxes were monitored twice daily to determine hatch order and match hatchling to egg. On hatch day zero, nestlings were individually marked according to the egg they hatched from using a non-toxic permanent marker. On day nine nestlings were banded with a numbered aluminum ring and three colored leg bands for individual identification. Nestlings were weighed and measured every three days beginning on hatch day (day 0) and ending on post hatch day 12. Body mass was measured to the nearest 0.01 g using an electronic balance. The right tarsus was measured to the nearest 0.01 mm using an electronic caliper. For day zero and day three the right wing chord was measured using electronic calipers to the nearest 0.01 mm. On day 6, 9 and 12 the wing chord was measured using a wing chord ruler that measured to the nearest 0.5 mm. Body condition scores were calculated for 3 and 12-day-old nestlings by dividing mass by tarsus length.

Blood samples.–Blood samples were collected every three days beginning on hatch day zero and ending on day 12. Hatch day zero and day three nestlings were bled from the jugular vein, while day 6, 9 and 12 nestlings were bled from the brachial wing vein. No more than 20 μL of blood was taken at any one time. We sampled nestlings by puncturing the vein with a 28-gauge needle and collecting the blood with a heparinized microcapillary tube. Blood samples were processed in the same manner as described above.

Ectoparasite sampling and identification.–Individual ectoparasite loads were quantified on day 12 using the dust ruffling protocol described by (Walther and Clayton 1997).

Briefly, we dusted Johnson's Rid-Mite Insect Powder (Johnson's Veterinary Products Ltd., Sutton Coldfield, West Midlands, United Kingdom), a pyrethrin based commercial insecticide, onto nestlings and massaged the powder into their feathers. After 3 minutes, each feather tract was ruffled over a large white cloth, used as a collecting surface, and the ectoparasites that fell onto the cloth were counted and an average value for the entire clutch was calculated to account for variation in ectoparasite load between siblings. Although both mite and insect specimens were present in the nest and on individuals, only mite species were included in the count.

Mite-specific antigen.– Mites collected from the nests belonged to the genus *Dermanyssus*. They were collected from whole nests after placing nests into sealed plastic bags for 1-week at ambient temperature. Mites in the nesting material migrated out of the nest and were aspirated into collection tubes. Prior to antigen extraction, ectoparasites were identified to the genus level (Krantz and Walter 2009). Mite antigens were extracted using the aforementioned protocol for NFM-antigen. The collected fraction contained 0.80 mg mL^{-1} of mite antigen in PBS.

Mite-specific antibody ELISA.– House sparrow-anti-mite-IgY (HOSPαMite-IgY) was measured with a mite-specific ELISA. The plate was optimized to enhance binding efficiency and validated prior to sample analyses using the methods described above for the NFM ELISA. Samples were assayed via a modified protocol of the aforementioned NFM-specific antibody ELISA methods. Briefly, plates were coated with 100 µL/well of 5µg/mL mite-antigen extract in coating buffer, incubated overnight in the cold room, and

washed three times. Plasma samples were diluted 1:1,000 and yolk-extract 1:100 in sample buffer and added in duplicate at 100 µL/well. Plates were then incubated for 1 hr at 37°C on a rocker and washed three times. RαHOSP-IgY, diluted 1:1,000 in sample buffer was added to each well, incubated for 1 hr at 37°C on a rocker and washed three times. The detection antibody, GαR-hrp, was diluted 1:40,000 in sample buffer, incubated for 1 hr at 37°C on a rocker and washed a final three times. Finally, plates were developed using ABTS and measurements (OD) were standardized using the methods described above.

Statistical Analyses.– All statistical analyses were carried out using JMP 7.0.1 (SAS Institute Inc., Cary, NC, U.S.A.). For the aviary study, Student's t-test was used to compare mite-specific antibody levels between NFM and PBS treated groups and between mite positive and mite negative nestlings. Data were reported as the mean ± SE, and the differences were considered significant at $P \leq 0.05$.

Aviary study.–A two-way ANCOVA was used to analyze the antibody response of adult house sparrows vaccinated against NFM-antigen extract. Adults were treated as the experimental unit and the average absorbance of plasma collected from pre-vaccination bleeds (B1) was a covariate with sex and treatment as main factors.

Field study.–Body condition (mass/tarsus), mite specific antibody levels and egg mass were compared using one-way ANOVA, with the nestling as the experimental unit and presence or absence of ectoparasites in the nest as the main factor. Linear least squares

were used to determine the effects of mite-specific antibody levels and egg mass on body condition on day 3 and 12. Correlations between mite-specific antibody titers in yolk and nestling plasma on hatch day zero were analyzed by linear least-squares, with day 0 antibody levels being log-transformed to conform to normality assumptions.

To reduce instances of pseudo-replication and to maximize sample size, only the heaviest nestling, out of a multi-nestling clutch, and single nestlings, from broods with only one nestling, were used in the analyses. In boxes that were used multiple times, only the first laid clutch was included in the analyses, as we did not monitor nest boxes for individual female use. A one-way-ANOVA comparison of the body condition of heaviest-nestlings from broods with several nestlings and single-nestlings in a brood from first clutches did not yield a significant difference in 3-day-old nestlings (ANOVA: $F_{1, 20} = 0.3258$, $P = 0.5742$) or 12-day old nestlings (ANOVA: $F_{1, 20} = 1.4857$, $P = 0.2364$) and were, therefore, combined for analyses.

We combined results from both yolk biopsied and whole yolk samples after finding no significant effect of yolk biopsy on nestling body condition (day3: ANOVA $F_{1,19} = 1.8293$, $P = 0.1921$; day 12: ANOVA $F_{1,19} = 1.5214$, $P = 0.2325$), or mite-specific antibody levels in nestling plasma on hatch day 0 (ANOVA: $F_{1, 13} = 0.7953$, $P = 0.3887$). We also found no effect of egg lay order on mite specific antibodies in the yolk (ANOVA: $F_{1, 13} = 0.0208$, $P = 0.8877$), which validated our combining whole yolks collected from second laid eggs with yolk biopsies taken from eggs laid on different days.

RESULTS

Immune response of adult house sparrows to vaccination against NFM-antigen.–Sex did not have a significant effect on antibody levels ($F_{1,38}$=1.8312, P = 0.1855) and was removed as a model effect from the analyses. In a two-way ANOVA both treatment ($F_{1,38}$=6.0199, P=0.0191) and pre-vaccination anti-NFM antibody levels ($F=_{1,38}$=11.4136, P=0.0018) independently had a significant main effect on post-vaccination anti-NFM antibody levels (Figure 1). However, we did not observe a significant interaction between treatment and pre-vaccination anti-NFM antibody ($F=_{1, 38}$=2.1806, P=0.1485). Adults immunized against NFM-antigen had significantly higher plasma anti-NFM antibody levels (0.2463 ± 0.0250) compared with PBS treated individuals (0.1461 ± 0.0278) (Student's t-test. P<0.05).

Transfer of maternally derived mite-specific antibodies from the yolk to nestlings.–A positive trend, though not statistically significant, was detected between yolk anti-mite antibodies and plasma anti-mite antibody levels in nestlings on hatch day 0 ($F_{1,10}$ = 1.9402, P = 0.1938, R^2 = 0.1625; Fig. 2a). A subsequent analysis revealed low statistical power (1-β = 0.2431), increasing the likelihood of making a type II error. Though not statistically significant, the detection of mite-specific antibodies in the yolk and nestling plasma on hatch day 0, by itself, confirms the transfer of maternal mite-specific antibodies from females to nestlings through the yolk.

Effects of mite load on nestling body condition and immunity.– Absence or presence of mites had no effect on nestling body condition in 3-day or 12 day old nestlings (one-way

ANOVA; day 3: $F_{1,18} = 0.2648$, P =0 .6131, R^2=0.0145; Table 1; Fig. 3a; day 12: $F_{1,18} = 1.6488$, P = 0.2154. R^2=0.0839; Table 1; Fig. 3b). However, the average body mass for both age groups was greater in mite negative than mite positive nestlings (Table 1). We found no significant effect of mite presence or absence on mite-specific antibody levels in the yolk ($F_{1,15} = 2.1600$, P=0.1623, R^2=0.1259) or plasma of nestlings on hatch day 0 ($F_{1,11} = 1.3830$, P = 0.2644, R^2=0.1117; Table 1). However, mite presence had a significant effect on mite-antibody levels in the plasma of 12-day old nestlings ($F_{1,12} = 7.2595$, P = 0.0195, R^2=0.3769; Table 1; Fig. 4).

Effect of mite-specific antibodies on nestling body condition and immunity.–The levels of mite-specific antibodies in the egg yolk were negatively correlated with body condition in 12-day old ($F_{1,16} = 4.8789$, P = 0.0421, $R^2 = 0.2337$; Fig. 5b), but not 3-day old ($F_{1,16} = 1.6195$, P = 0.2213; $R^2 = 0.0471$; Fig. 5a) nestlings. However, a subsequent analysis controlling for egg mass reduced the significance level for effects of mite-specific antibodies in the yolk on body condition of 12-day old nestlings ($F_{1,16}$=3.9984, P=0.0653). There was no significant relationship detected between mite-specific IgY in the yolk and antibodies in plasma of 12-day old nestlings ($F_{1, 9} = 2.5329$, P = 0.1460, $R^2 = 0.2196$; Fig 2b).

Effects of egg mass on nestling body condition and immunity.–Egg mass had a significant effect on the body condition of 12-day old ($F_{1,19} = 13.5448$, P = 0.0016, $R^2 = 0.4162$; Fig. 6b), but not 3-day old nestlings ($F_{1,19} = 0.5495$, P = 0.4676, $R^2 = 0.0281$; Fig 6a). Egg mass did not have an effect on mite-specific antibodies in the yolk ($F_{1,16} = 0.8173$, P =

0.3794, $R^2 = 0.0486$) or plasma of 12-day old nestlings ($F_{1,11} = 0.0829$, $P = 0.7788$, $R^2 = 0.0075$). The absence or presence of mites in the nest did not have a significant effect on eggs mass ($F_{1,15} = 0.6721$, $P = 0.4230$; Table 1).

DISCUSSION

Immune response to vaccination against NFM-antigen in adult house sparrows.– In this study we found substantial variation in NFM-antigen levels in plasma among unvaccinated and vaccinated adult sparrows (pre-vaccination) and demonstrated a humoral immune response against NFM-antigen of adult house sparrows (Fig. 1). The large variation in NFM-specific antibody levels observed in pre-vaccinated individuals most likely reflected individual exposure to similar ectoparasites in the wild and, potentially, the aviary. Sparrows were injected with mite-antigen acquired from the homogenization of whole mite specimens. It is reasonable to expect that components of this mixture are common across mite species (Lee et al. 2002). Furthermore, the parasite exposure history of individual sparrows in the wild may have contributed to the variation observed, as temporal and spatial variations in exposure to ectoparasites has been shown to have an impact on parasite-specific antibody levels in wild birds (Huber et al. 2010).

The antibody response of sparrows to NFM antigen is consistent with results for chickens, where individuals have been shown to mount an immune response against parasite-specific antigen either through vaccination (Minnifield et al. 1993; Arkle et al. 2008; Harrington et al. 2009; Wright et al. 2009) or through direct contact with the parasite itself (Minnifield et al. 1993). Parasite-specific antibodies have also been

detected in Darwin's finches (*Geospiza fortis*) on the Galápagos Islands, where adults tested positive for antibodies against two-introduced parasites, avian pox virus (*Poxvirus avium*) and nest flies (*Philornis downsi*) (Huber et al. 2010). In avian species, the presence of parasite specific antibodies has been shown to increase parasite mortality (Arkle et al. 2008), decrease parasite fecundity (Tschirren et al. 2007), and decrease parasite-feeding rate on the host (Wright et al. 2009). However, in wild passerines parasite-specific immunologic defenses need to be examined in greater detail before inferences can be made regarding the effects of parasitism on host and parasite fitness.

Transfer of maternally derived mite-specific antibodies from the yolk to nestlings on hatch day 0.– The presence of antibodies raised by females against naturally occurring hematophagous mites was verified in the yolk and plasma of nestlings. Though a statistically significant relationship between maternal antibodies in the yolk and those in nestlings on hatch day 0 was not observed (Fig. 2), the detection of antibodies in both the yolk and nestling plasma confirms that mite-specific antibodies produced by the female are transferred to yolk and embryo and nestling.

The positive correlation between mite-specific antibodies in yolk and those in the plasma of 12-day old nestlings (Fig. 4) do not necessarily reflect a quantitative transmission of maternal antibodies to nestlings. We demonstrated that maternal antibodies were not detectable in nestling circulation 8-10 days after hatch and nestlings were capable of producing their own antibodies 3-6 days after hatch (King et al. *in review*). Thus, the correlation between antibody levels in yolk and plasma of 12-day old nestlings most

likely reflected similar exposure levels of both the female and her nestlings to mites in the nest. This interpretation is further supported by the observation that average mite-specific antibody levels were higher in the yolk and plasma of 12-day old nestlings in mite-positive than mite-negative nests (Table 1; Fig. 3). Although protective qualities of maternal antibodies for offspring is well documented in mammals (Castinel et al. 2008; Beam et al. 2009) and gallus species (Fahey et al. 1987; Chaffer et al. 1997; Abdel-Moneim and Abdel-Gawad 2006), the effects of maternal antibodies on passive-immunologic protection and humoral development in wild birds needs to be examined in greater detail, before any potential benefits or developmental effects can be postulated.

Effects of mite load on nestling body condition and immunity.– The presence of mites in the nest did not have a significant effect on nestling body condition in 3-day old (Fig. 4a) or 12-day old nestlings (Fig. 4b). However, in both instances, the mean body condition of nestlings from mite-positive nests was lower than that of nestlings from mite-negative nests (Table 1). Blood-feeding mites can have a significant effect on nestling quality and fitness (Berggren 2005; Heylen et al. 2009). Several studies have reported a positive relationship between maternal antibody transmission and chick growth (Groothuis et al. 2006; Grindstaff 2008; Moreno et al. 2008). However, negative effects of parasitism can be ameliorated by parental care, whereby increased feeding rate and nest attentiveness allow nestlings to thrive despite parasite infestation (Lozano and Ydenberg 2002; Moreno-Rueda 2004; Gallizzi et al. 2008).

Effects of mite-specific antibodies on nestling body condition and immunity.– The presence of mite-specific antibodies in the yolk did not have a significant effect on nestling body condition in 3-day and 12-day old nestlings (Fig. 5a, b), though in mite-positive nests, both age groups had lower body condition than in mite-negative nests (Table 1). As aforementioned, the costs associated with mounting an immune response could be ameliorated by increased parental care (Lozano and Ydenberg 2002; Moreno-Rueda 2004; Gallizzi et al. 2008). Alternatively, the energetic costs associated with mounting an immune response by nestlings may be relatively inexpensive compared with the gallus model of immunity.

Effects of egg mass on nestling body condition and immunity.– Egg mass is a strong predictor of offspring body mass at fledge (Christians 2002; Karell et al. 2008). Consistent with this, egg mass was positively correlated with the body condition of 12-day old (Fig. 6b), though not 3-day old (Fig. 6a) nestlings. However, we did not detect a significant relationship between egg mass and mite-specific antibodies in the yolk. Nor did we find a significant relationship between egg mass and absence or presence of mites (Table 1). Other maternal resources deposited in the yolk may account for body condition. For example, maternal carotenoids (De Neve et al. 2008), hormones (Schwabl 1993; Groothuis et al. 2006), and nutritional reserves in the yolk (Moreno et al. 2008) all contribute to nestling quality and survivorship.

Conclusions.– It has been asserted that hematophagous ectoparasites impose negative fitness consequences on passerine hosts. We examined parasite-specific immunologic

responses to mites in a wild passerine and demonstrated that house sparrows *i)* are capable of mounting a humoral immune response against a hematophagous ectoparasite; and *ii)* mite-specific antibodies are transferred to nestlings through the yolk. We did not, however, find a strong relationship between mite-specific antibody levels and nestling body condition. This observation raises questions about the fitness consequences of maternal antibody transmission to the egg. Future studies examining parasite-specific immune responses to ectoparasites should examine parasite density and exposure time when assessing immunologic costs and benefits. Furthermore, birds are exposed to various environmental pathogens and parasites in their natural environment, so it may be of interest to examine the effects that multiple immunologic stressors have on adult and nestling fitness.

LITERATURE CITED

Abdel-Moneim, A. S., and M. M. A. Abdel-Gawad. 2006. Genetic variations in maternal transfer and immune responsiveness to infectious bursal disease virus. Veterinary Microbiology 114:16-24.

Apanius, V. 1998. Ontogeny of immune function. Pages 203-222 in Avian Growth and Development (J. M. Stark, and R. E. Ricklefs, Eds.). Oxford University Press, Oxford.

Arkle, S., D. Harrington, P. Kaiser, L. Rothwell, C. De Luna, D. George, J. Guy, and O. A. E. Sparagano. 2008. Immunological Control of the Poultry Red Mite The Use of Whole Mite Antigens as a Vaccine Candidate. Pages 36-40 in Animal Biodiversity and Emerging Diseases: Prediction and Prevention, vol. 1149 (O. A. E. Sparagano, J. C. Maillard, and J. V. Figueroa, Eds.).

Beam, A. L., J. E. Lombard, C. A. Kopral, L. P. Garber, A. L. Winter, J. A. Hicks, and J. L. Schlater. 2009. Prevalence of failure of passive transfer of immunity in newborn heifer calves and associated management practices on US dairy operations. Journal of Dairy Science 92:3973-3980.

Berggren, A. 2005. Effect of the blood-sucking mite Ornithonyssus bursa on chick growth and fledging age in the North Island robin. New Zealand Journal of Ecology 29:243-250.

Bonneaud, C., J. Mazuc, G. Gonzalez, C. Haussy, O. Chastel, B. Faivre, and G. Sorci. 2003. Assessing the cost of mounting an immune response. American Naturalist 161:367-379.

Both, C., M. E. Visser, and N. Verboven. 1999. Density-dependent recruitment rates in great tits: the importance of being heavier. Proceedings of the Royal Society of London Series B-Biological Sciences 266:465-469.

Carleton, R. E. 2008. Ectoparasites affect hemoglobin and percentages of immature erythrocytes but not hematocrit in nestling Eastern Bluebirds. Wilson Journal of Ornithology 120:565-568.

Castinel, A., R. Kittelberger, W. E. Pomroy, P. J. Duignan, B. L. Chilvers, and I. S. Wilkinson. 2008. Humoral immune response to Klebsiella spp. in New Zealand sea lions (Phocarctos hookeri) and the passive transfer of immunity to pups. Journal of Wildlife Diseases 44:8-15.

Chaffer, M., B. Schwartsburd, and E. D. Heller. 1997. Vaccination of turkey poults against pathogenic Escherichia coli. Avian Pathology 26:377-390.

Christe, P., A. P. Moller, and F. de Lope. 1998. Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. Oikos 83:175-179.

Christians, J. K. 2002. Avian egg size: variation within species and inflexibility within individuals. Biological Reviews 77:1-26.

de Bellocq, J. G., B. R. Krasnov, I. S. Khokhlova, L. Ghazaryan, and B. Pinshow. 2006a. Immunocompetence and flea parasitism of a desert rodent. Functional Ecology 20:637-646.

de Bellocq, J. G., B. R. Krasnov, I. S. Khokhlova, and B. Pinshow. 2006b. Temporal dynamics of a T-cell mediated immune response in desert rodents. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 145:554-559.

de Bellocq, J. G., A. Porcherie, C. Moulia, and S. Morand. 2007. Immunocompetence does not correlate with resistance to helminth parasites in house mouse subspecies and their hybrids. Parasitology Research 100:321-328.

De Neve, L., J. A. Fargallo, P. Vergara, J. A. Lemus, M. Jaren-Galan, and I. Luaces. 2008. Effects of maternal carotenoid availability in relation to sex, parasite infection and health status of nestling kestrels (Falco tinnunculus). Journal of Experimental Biology 211.

Dusbabek, F., S. Lukes, V. Matha, and L. Grubhoffer. 1989. Antibody-mediated response of pigeons to argus plonicus larval feeding and characterization of larval antigen. Folia Parasitologica 36:83-92.

Elliot, S. L., F. R. Adler, and M. W. Sabelis. 2003. How virulent should a parasite be to its vector? Ecology 84:2568-2574.

Fahey, K. J., J. K. Crooks, and R. A. Fraser. 1987. Assessment by ELISA of passively acquired protection against infectious bursal disease virus in chickens. Australian Veterinary Journal 64:203-207.

Forsman, A. M., L. A. Vogel, S. K. Sakaluk, B. G. Johnson, B. S. Masters, L. S. Johnson, and C. F. Thompson. 2008. Female house wrens (Troglodytes aedon) increase the size, but not immunocompetence, of their offspring through extra-pair mating. Molecular Ecology 17:3697-3706.

Gallizzi, K., O. Alloitteau, E. Harrang, and H. Richner. 2008. Fleas, parental care, and transgenerational effects on tick load in the great tit. Behavioral Ecology 19:1225-1234.

Gasparini, J., K. D. McCoy, T. Tveraa, and T. Boulinier. 2002. Related concentrations of specific immunoglobulins against the Lyme disease agent Borrelia burgdorferi sensu lato in eggs, young and adults of the kittiwake (Rissa tridactyla). Ecology Letters 5:519-524.

Grindstaff, J. L. 2008. Maternal antibodies reduce costs of an immune response during development. Journal of Experimental Biology 211:654-660.

Grindstaff, J. L., D. Hasselquist, J. A. Nilsson, M. Sandell, H. G. Smith, and M. Stjernman. 2006. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proceedings of the Royal Society B-Biological Sciences 273:2551-2557.

Groothuis, T. G. G., C. M. Eising, J. D. Blount, P. Surai, V. Apanius, C. Dijstra, and W. Muller. 2006. Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment. Journal Compilations 19:1304-1313.

Groothuis, T. G. G., C. M. Eising, C. Dijkstra, and W. Muller. 2005. Balancing between costs and benefits of maternal hormone deposition in avian eggs. Biology Letters 1:78-81.

Hamal, K. R., S. C. Burgess, I. Y. Pevzner, and G. F. Erf. 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poultry Science 85:1364-1372.

Hansen, P., J. A. Scoble, B. Hanson, and N. J. Hoogenraad. 1998. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. Journal of Immunological Methods 215:1-7.

Harrington, D., H. M. El Din, J. Guy, K. Robinson, and O. Sparagano. 2009. Characterization of the immune response of domestic fowl following immunization with proteins extracted from Dermanyssus gallinae. Veterinary Parasitology 160:285-294.

Heylen, D., F. Adriaensen, T. Dauwe, M. Eens, and E. Matthysen. 2009. Offspring quality and tick infestation load in brood rearing great tits Parus major. Oikos 118:1499-1506.

Huber, S. K. 2008. Effects of the introduced parasite Philornis downsi on nestling growth and mortality in the medium ground finch (Geospiza fortis). Biological Conservation 141:601-609.

Huber, S. K., J. P. Owen, J. A. H. Koop, M. O. King, P. R. Grant, B. R. Grant, and D. H. Clayton. 2010. Ecoimmunity in Darwin's Finches: Invasive Parasites Trigger Acquired Immunity in the Medium Ground Finch (Geospiza fortis). PLoS ONE 5:e8605.

Johnsen, T. S., and M. Zuk. 1999. Parasites and tradeoffs in the immune response of female red jungle fowl. Oikos 86:487-492.

Karell, P., P. Kontiainen, H. Pietiainen, H. Siitari, and J. E. Brommer. 2008. Maternal effects on offspring Igs and egg size in relation to natural and experimentally improved food supply. Functional Ecology 22:682-690.

King, M. O., J. P. Owen, and H. Schwabl. in review. Are Maternal Antibodies Really that Important? Patterns in the Devlopment of Humoral Immunity in Altricial Passerine House Sparrows (Passer domesticus). PLoS ONE.

Kirkwood, A. C. 1985. Some observations on the biology and control of the sheep scab mite psoroptes ovis (hering) in Britain. Veterinary Parasitology 18:269-279.

Knee, W., and H. Proctor. 2007. Host records for Ornithonyssus sylviarum (Mesostigmata : Macronyssidae) from birds of North America (Canada, United States, and Mexico). Journal of Medical Entomology 44:709-713.

Kowalczyk, K., J. Daiss, J. Halpern, and T. F. Roth. 1985. Quantitation of maternal-fetal IgG transport in the chicken. Immunology 54:755-762.

Krantz, G. W., and D. E. Walter. 2009. A Manual of Acarology, 3rd ed. Texas Tech Univ. Press, Lubbock.

Lee, S., J. Kin, and C. Jee. 2002. Immune effects on the somatic antigens against Dermanyssus gallinae and Dermatophagoides pteronyssinus in chicken. Korean Journal of Veterinary Research 44:253-260.

Linden, M., L. Gustafsson, and T. Part. 1992. Selection on fledging mass in the collared flycatcher and the great tit. Ecology 73:336-343.

Lindstrom, K. M., J. Foufopoulos, H. Parn, and M. Wikelski. 2004. Immunological investments reflect parasite abundance in island populations of Darwin's finches. Proceedings of the Royal Society of London Series B-Biological Sciences 271:1513-1519.

Lochmiller, R. L., and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87-98.

Loeken, M. R., and T. F. Roth. 1983. Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. Immunology 49:21-28.

Lozano, G. A., and R. C. Ydenberg. 2002. Transgenerational effects of maternal immune challenge in tree swallows (Tachycineta bicolor). Canadian Journal of Zoology-Revue Canadienne De Zoologie 80:918-925.

Minnifield, N. M., J. Carroll, K. Young, and D. K. Hayes. 1993. Antibody development against northern fowl mites (Acari macronyssidae) in chickens. Journal of Medical Entomology 30:360-367.

Moller, A. P., F. De Lope, and N. Saino. 2004. Parasitism, immunity, and arrival date in a migratory bird, the barn swallow. Ecology 85:206-219.

Moller, A. P., J. Erritzoe, and N. Saino. 2003. Seasonal changes in immune response and parasite impact on hosts. American Naturalist 161:657-671.

Mondal, S. P., and S. A. Naqi. 2001. Maternal antibody to infectious bronchitis virus: its role in protection against infection and development of active immunity to vaccine. Veterinary Immunology and Immunopathology 79:31-40.

Moreno, J., E. Lobato, J. Morales, S. Merino, J. Martinez-De La Puente, and G. Tomas. 2008. Pre-laying nutrition mediates maternal effects on offspring immune capacity and growth in the pied flycatcher. Oecologia 156:727-735.

Moreno, J., S. Merino, J. Potti, A. de Leon, and R. Rodriguez. 1999. Maternal energy expenditure does not change with flight costs or food availability in the pied flycatcher (Ficedula hypoleuca): costs and benefits for nestlings. Behavioral Ecology and Sociobiology 46:244-251.

Moreno, J., J. Morales, E. Lobato, S. Merino, G. Tomas, and J. Martinez-de la Puente. 2005. Evidence for the signaling function of egg color in the pied flycatcher Ficedula hypoleuca. Behavioral Ecology 16:931-937.

Moreno-Rueda, G. 2004. Reduced parental effort in relation to laying date in house sparrows (Passer domesticus): a study under controlled conditions. Behavioural Processes 67:295-302.

Mullens, B. A., J. P. Owen, D. R. Kuney, C. E. Szijj, and K. A. Klingler. 2009. Temporal changes in distribution, prevalence and intensity of northern fowl mite (Ornithonyssus sylviarum) parasitism in commercial caged laying hens, with a comprehensive economic analysis of parasite impact. Veterinary Parasitology 160:116-133.

Muller, W., T. G. G. Groothuis, A. Kasprzik, C. Dijkstra, R. V. Alatalo, and H. Siitari. 2005. Prenatal androgen exposure modulates cellular and humoral immune function of black-headed gull chicks. Proceedings of the Royal Society B-Biological Sciences 272:1971-1977.

Ojanen, M. 1983. Egg development and the related nutrient reserve depletion in the pied flycatcher, Ficedula hypoleuca. Annales Zoologici Fennici 20:293-299.

Owen, J. P., M. E. Delany, C. J. Cardona, A. A. Bickford, and B. A. Mullens. 2009. Host inflammatory response governs fitness in an avian ectoparasite, the northern fowl mite (Ornithonyssus sylviarum). International Journal for Parasitology 39:789-799.

Owen, J. P., A. C. Nelson, and D. H. Clayton. in review. Ecoimmunity of bird-ectoparasite systems. Trends in Parasitology.

Owen-Ashley, N. T., M. Turner, T. P. Hahn, and J. C. Wingfield. 2006. Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in

captive and free-living white-crowned sparrows (Zonotrichia leucophrys gambelii). Hormones and Behavior 49:15-29.

Polson, A. 1990. Isolation of IgY from the yolks of eggs by a chloroform polyethylene-glycol procedure. Immunological Investigations 19:253-258.

Rahman, Y. J., and R. R. Strathmann. 2002. Does scarcity of sites for egg deposition limit reproductive success? Integrative and Comparative Biology 42:1298-1298.

Richner, H., and P. Heeb. 1995. Are clutch and brood size patterns in birds shaped by ectoparasites. Oikos 73:435-441.

Ricklefs, R. E. 1992. Embryonic-development period and the prevalence of avian blood parasites. Proceedings of the National Academy of Sciences of the United States of America 89:4722-4725.

Saino, N., R. P. Ferrari, M. Romano, R. Ambrosini, and A. P. Moller. 2002. Ectoparasites and reproductive trade-offs in the barn swallow (Hirundo rustica). Oecologia 133:139-145.

Schifferli, L. 1980. Juvenile house sparrows parasitizing nestling nestling house sparrows. British Birds 73:189-190.

Schwabl, H. 1993. Yolk as a source of maternal testosterone for developing birds. Proceedings of the National Academy of Sciences of the United States of America 90:11446-11450.

Stoehr, A. M., P. M. Nolan, G. E. Hill, and K. J. McGraw. 2000. Nest mites (Pellonyssus reedi) and the reproductive biology of the house finch (Carpodacus mexicanus). Canadian Journal of Zoology-Revue Canadienne De Zoologie 78:2126-2133.

Szabo, K., A. Szalmas, A. Liker, and Z. Barta. 2008. Adaptive host-abandonment of ectoparasites before fledging? Within-brood distribution of nest mites in house sparrow broods. Journal of Parasitology 94:1038-1043.

Tinbergen, J. M., and M. C. Boerlijst. 1990. Nestling weight and survival in individual great tits (Parus major). Journal of Animal Ecology 59:1113-1127.

Tinbergen, J. M., and S. Daan. 1990. Family-planning in the great tit (Parus major) - Optimal cluctch size as integration of parent and offspring fitness. Behaviour 114:161-190.

Tschirren, B., L. L. Bischoff, V. Saladin, and H. Richner. 2007. Host condition and host immunity affect parasite fitness in a bird-ectoparasite system. Functional Ecology 21:372-378.

Tschirren, B., H. Siitari, V. Saladin, and H. Richner. 2009. Transgenerational immunity in a bird-ectoparasite system: do maternally transferred antibodies affect parasite fecundity or the offspring's susceptibility to fleas? Ibis 151:160-170.

Walther, B. A., and D. H. Clayton. 1997. Dust-ruffling: A simple method for quantifying ectoparasite loads of live birds. Journal of Field Ornithology 68:509-518.

Westneat, D. F., D. Hasselquist, and J. C. Wingfield. 2003. Tests of association between the humoral immune response of red-winged blackbirds (Agelaius phoeniceus) and male plumage, testosterone, or reproductive success. Behavioral Ecology and Sociobiology 53:315-323.

Westneat, D. F., J. Weiskittle, R. Edenfield, T. B. Kinnard, and J. P. Poston. 2004. Correlates of cell-mediated immunity in nestling house sparrows. Oecologia 141:17-23.

Wright, H. W., K. Bartley, A. J. Nisbet, R. M. McDevitt, N. H. C. Sparks, S. Brocklehurst, and J. F. Huntley. 2009. The testing of antibodies raised against poultry red mite antigens in an in vitro feeding assay; preliminary screen for vaccine candidates. Experimental and Applied Acarology 48:81-91.

TABLES

Table 1. Body condition, mite-specific antibodies and egg mass in relation to the presence or absence of mites.

			HOSPαMite-IgY			
	Body Condition day 3[2]	**Body Condition day 12[2]**	**Yolk IgY[3]**	**NSTL IgY day 3[4]**	**NSTL IgY day 12[4]**	**Egg mass (g)**
			mean ± SE			
Mite (N)[1]	1.150 ± 0.064	1.150 ± 0.036	0.095 ± 0.015	0.150 ± 0.132	0.096 ± 0.031	2.77 ± 0.078
Mite (Y)[1]	1.105 ± 0.052	1.437 ± 0.034	0.153 ± 0.031	0.255 ± 0.18	0.277 ± 0.035	2.69 ± 0.183

[1] Absence (N) or presence (Y) of mites recovered from 12-day old nestlings.

[2] Body condition as measured as mass (g) divided by tarsus in 3-day and 12-day old nestlings.

[3] Maternal HOSPαMite-IgY levels in the yolk as expressed by optic density (OD)

[4] HOSPαMite-IgY in nestling plasma in 3-day and 12-day old chicks as expressed by OD

All values are expressed as the mean ± SE.

FIGURE LEGENDS

Figure 1. Standard least squares regression of post-vaccination levels of HOSPaNFM-IgY in plasma of male and female adult house sparrows injected with either NFM or PBS with treatment as the main factor and pre-vaccination serum levels of HOSPaNFM-IgY as the covariate (ANCOVA: $F_{2, 37} = 8.2509$, $P = 0.0011$, $R^2 = 0.3084$). Antibody concentration is expressed as optic density (OD). NFM treated individuals are represented by grey circles and solid lines and control treated individuals are represented by black circles and dashed lines.

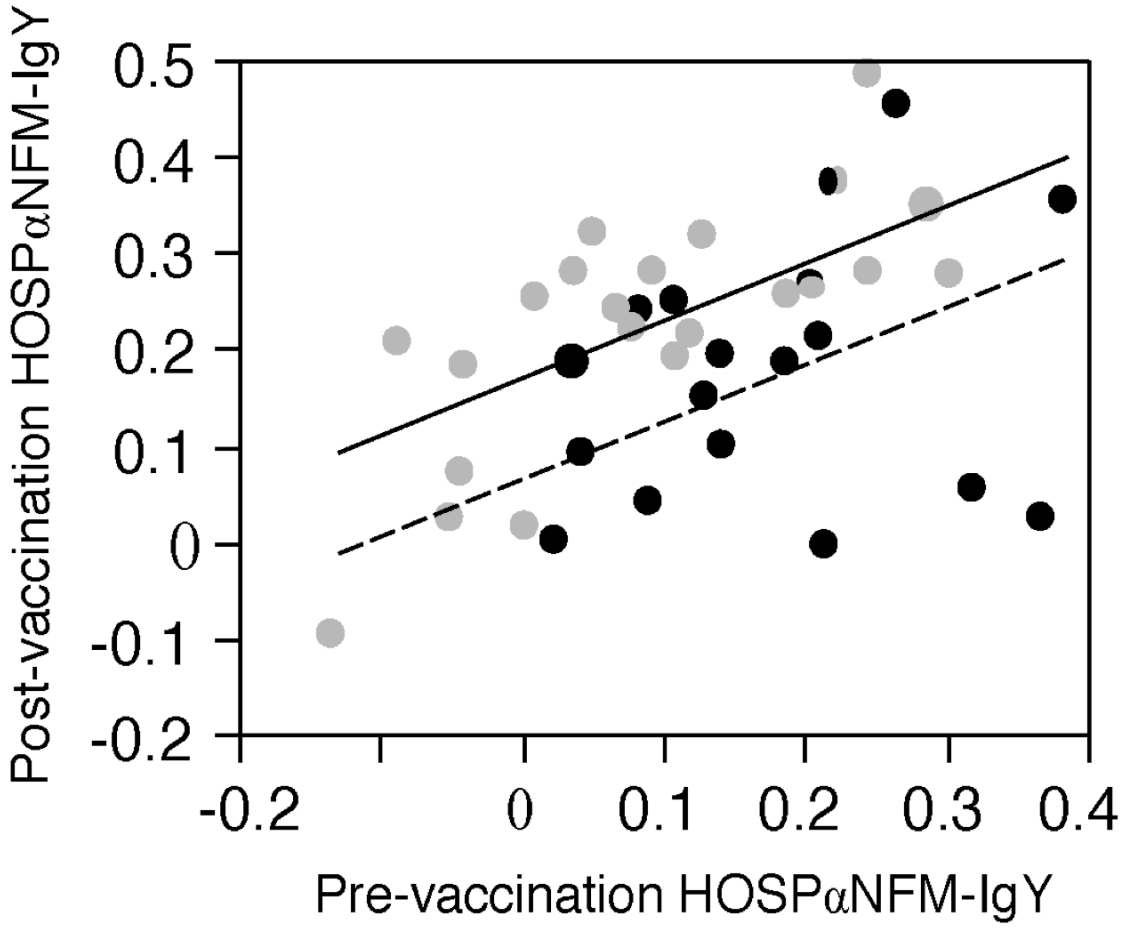
Figure 2. Correlation between the concentrations of mite specific antibodies in the yolk and nestling plasma on a) hatch day 0 ($F_{1,10} = 1.9402$; $P = 0.1938$; $R^2 = 0.1625$) and b) 12-day old nestlings (NSTL) ($F_{1, 9} = 2.5329$, $P = 0.1460$, $R^2 = 0.2196$). Antibody levels are expressed as optic density (OD).

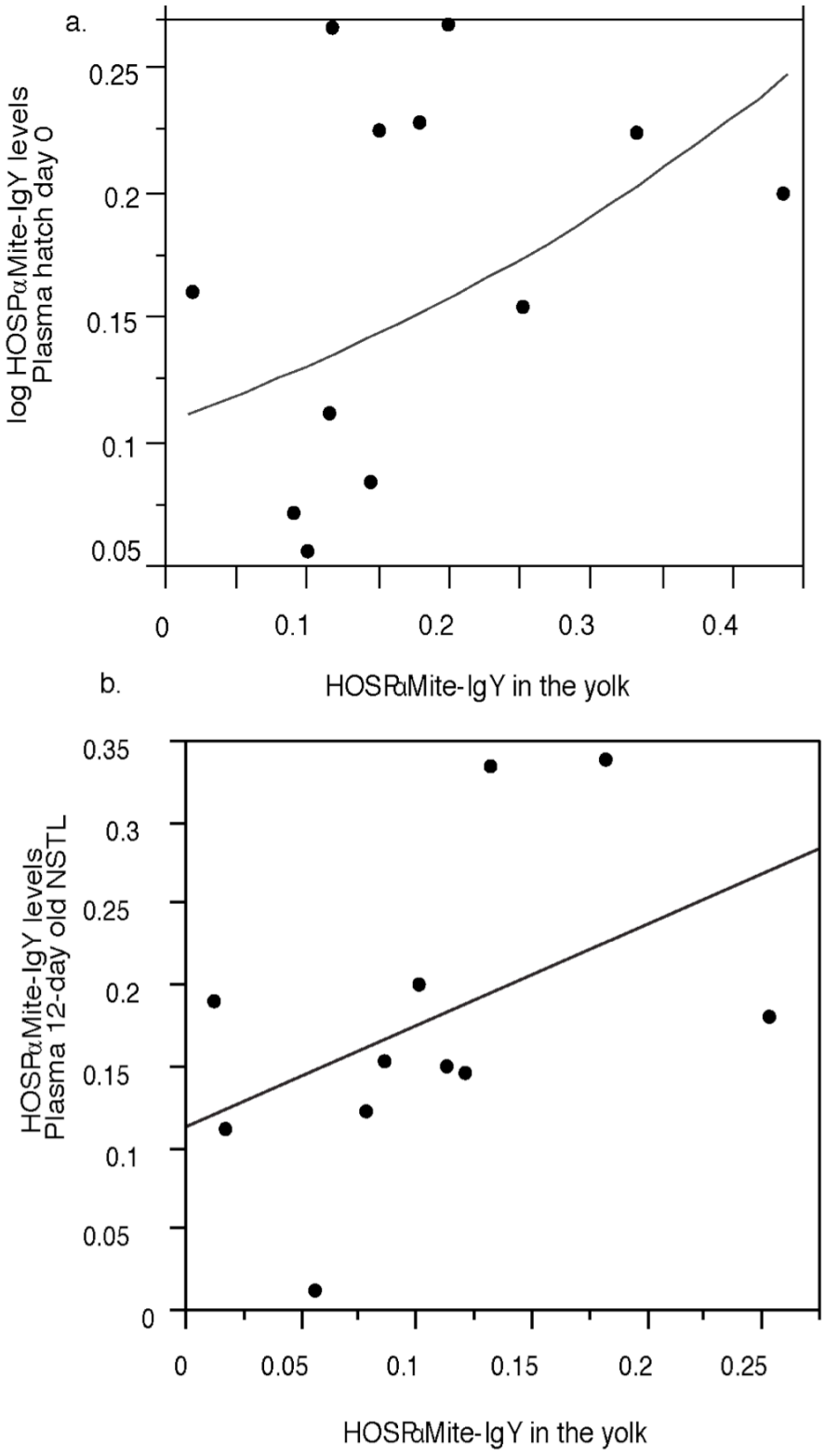
Figure 3. Box plots comparing nestling body condition for a) 3-day old nestlings ($F_{1,18} = 0.2648$, $P = 0.6131$) and b) 12-day old nestlings ($F_{1,18} = 1.6488$, $P = 0.2154$) from mite positive (Y) and mite negative (N). Values are expressed as the mean ± SE of the optic density (OD).

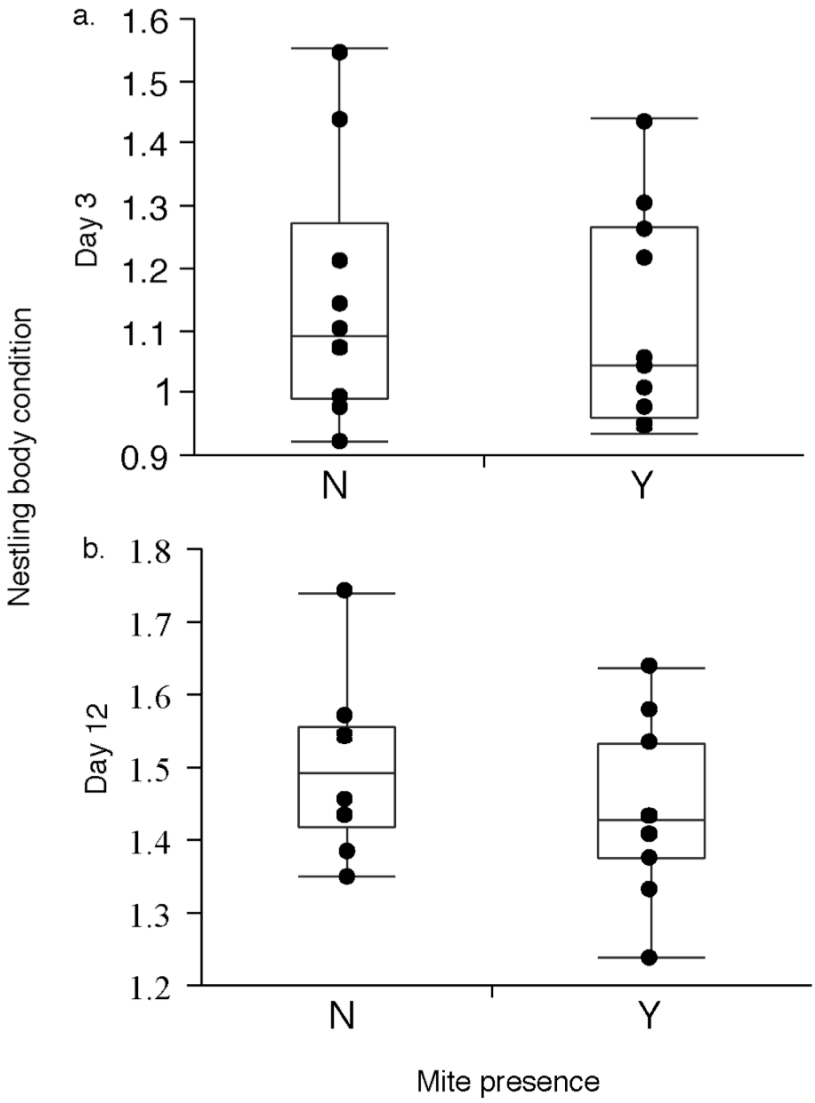
Figure 4. Box plots comparing HOSPαMite-IgY in 12-day old nestlings from mite positive (Y) and mite negative (N) nests ($F_{1,12} = 7.2595$, $P = 0.0195$). Values are expressed as the mean ± SE of the optic density (OD). Relationships were considered significant at ($P<0.05$).

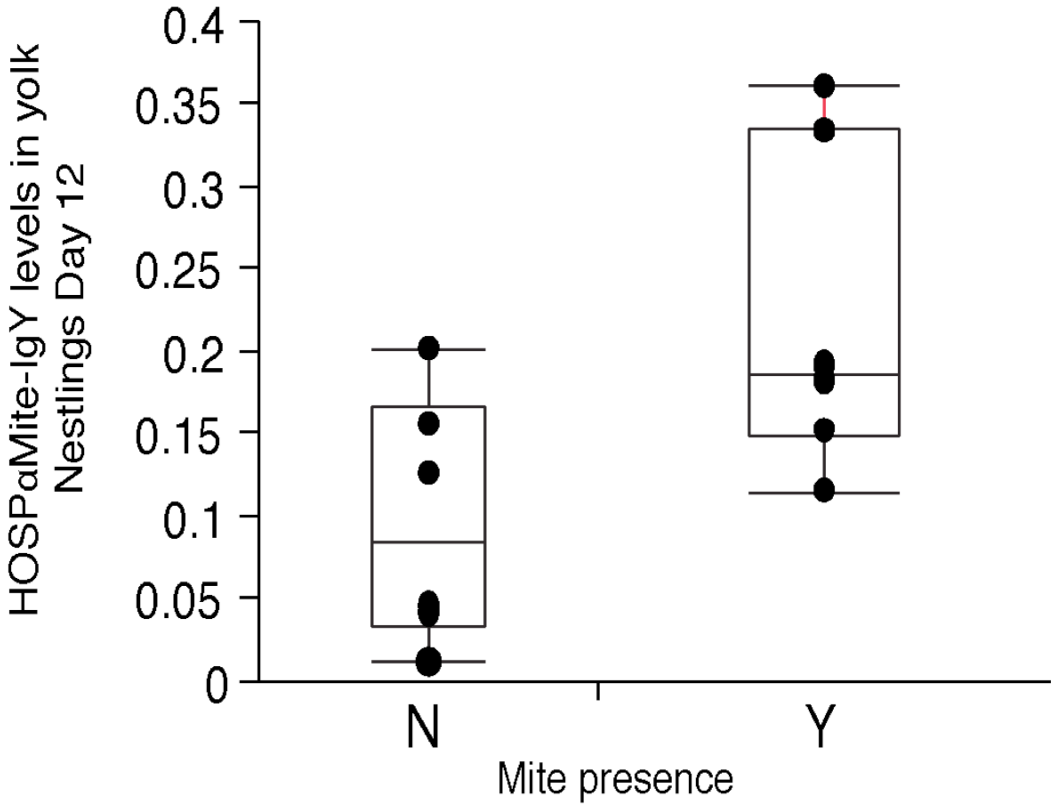
Figure 5. Correlation between the body condition for a) 3-day old nestlings ($F_{1,16}$ = 1.6195, P = 0.2213, R^2 = 0.0471) and b) 12-day old nestlings ($F_{1,16}$ = 4.8789, P = 0.0421, R^2 = 0.2337) in relation to maternal HOSPαMite-IgY specific antibodies in the yolk. Antibody levels are expressed as optic density (OD).

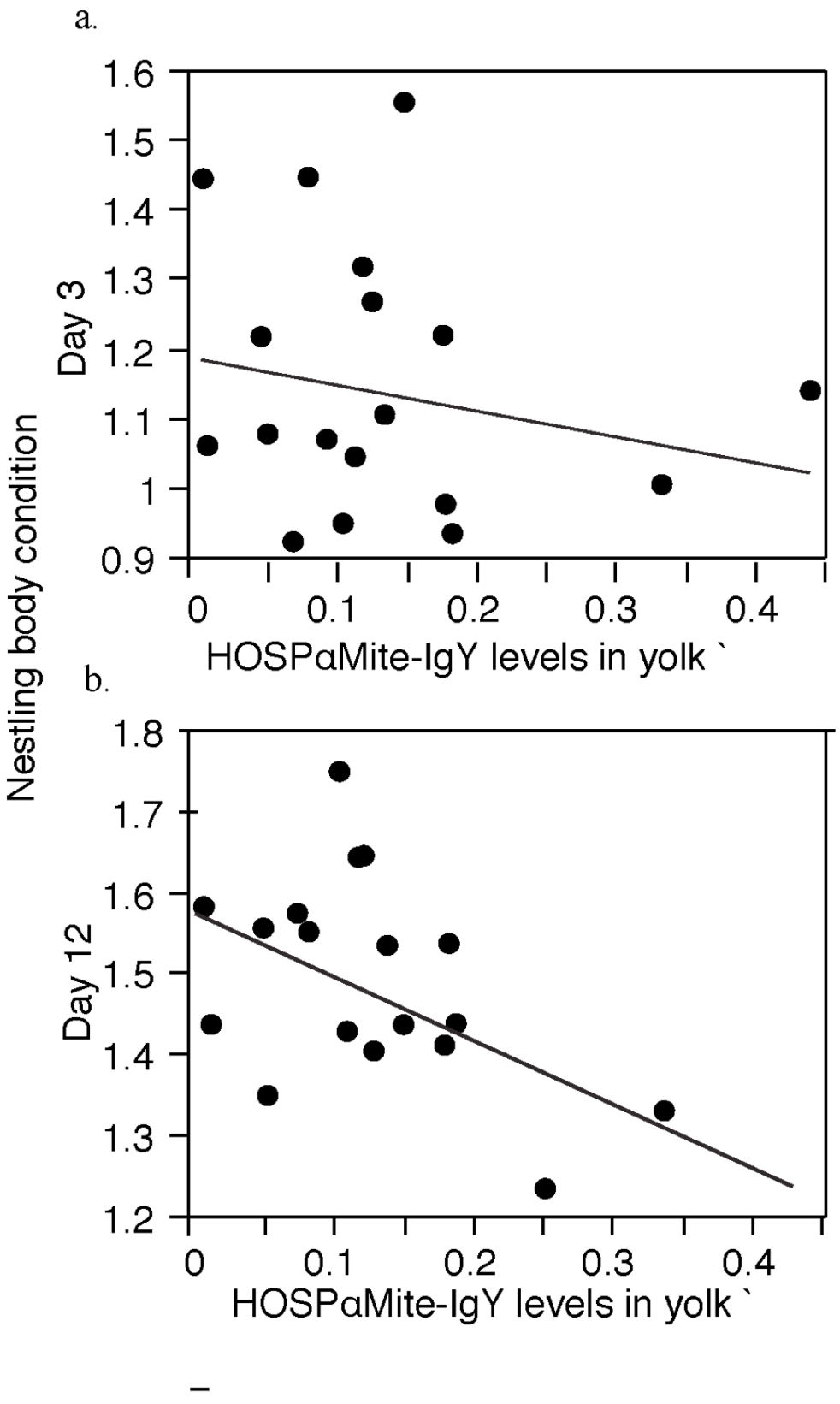
Figure 6. Correlation between the body condition for a) 3-day old nestlings ($F_{1,19}$ = 0.5495, P = 0.4676, R^2 = 0.0281) and b) 12-day old nestlings($F_{1,19}$ = 13.5448, P = 0.0016, R^2 = 0.4162) in relation egg mass. Antibody levels are expressed as optic density (OD).

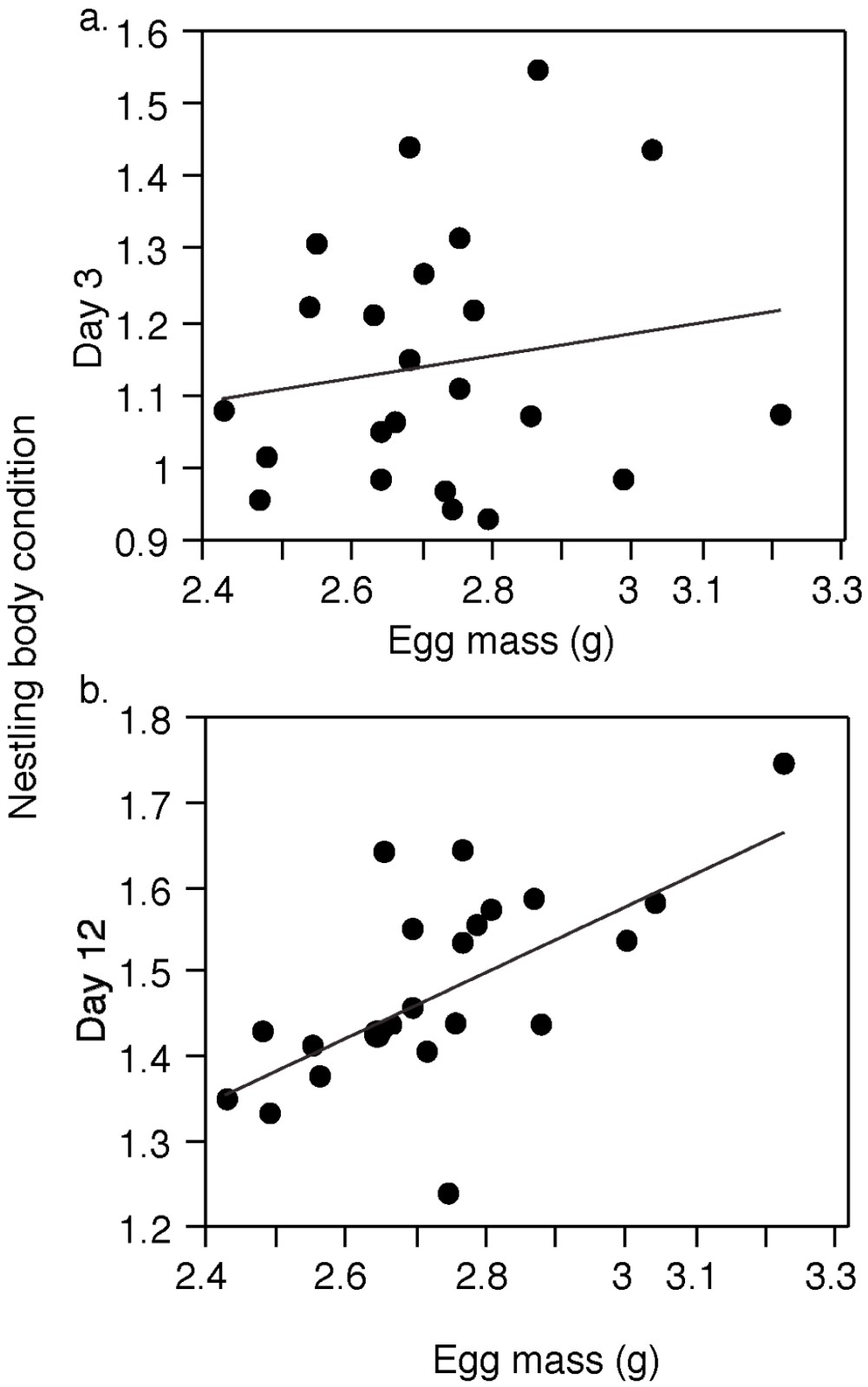

Post-vaccination HOSPαNFM-IgY
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.2
0
0.1
0.2
0.3
0.4
Pre-vaccination HOSPαNFM-IgY


a.
log HOSPαMite-IgY levels
Plasma hatch day 0
0.25
0.2
0.15
0.1
0.05
0
0.1
0.2
0.3
0.4
HOSPαMite-IgY in the yolk
b.
HOSPαMite-IgY levels
Plasma 12-day old NSTL
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.05
0.1
0.15
0.2
0.25
HOSPαMite-IgY in the yolk


a.
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
Day 3
N
Y
b.
1.8
1.7
1.6
1.5
1.4
1.3
1.2
Day 12
N
Y
Nestling body condition
Mite presence


HOSPαMite-IgY levels in yolk
Nestlings Day 12
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
N
Y
Mite presence


a.
Nestling body condition
Day 3
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0
0.1
0.2
0.3
0.4
HOSPαMite-IgY levels in yolk `
b.
Day 12
1.8
1.7
1.6
1.5
1.4
1.3
1.2
0
0.1
0.2
0.3
0.4
HOSPαMite-IgY levels in yolk `


a.
Day 3
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
2.4
2.6
2.8
3
3.1
3.3
Egg mass (g)
b.
Day 12
1.8
1.7
1.6
1.5
1.4
1.3
1.2
2.4
2.6
2.8
3
3.1
3.3
Egg mass (g)
Nestling body condition

CHAPTER 4

The applicability of using antiserum built against purified house sparrow immunoglobulins to detect antibodies in wild birds

INTRODUCTION

The following manuscript was prepared for publication in the Journal of Avian Biology and adheres to their submission and publication guidelines.

The avian humoral immune system has become a popular area of study for evolutionary biologists and ecological immunologist examining immune-mediated maternal effects (Apanius 1998,BoulinierandStaszewski 2008,SmitsandBortolotti 2008,Gasparini et al. 2009) and trade-offs between the immune system and other condition-dependent life-history traits (LochmillerandDeerenberg 2000,Saino et al. 2002,TschirrenandRichner 2006,KoppandMedzhitov 2009). Unfortunately, a major limitation in designing experiments on wild birds is that most commercially available antibodies and assay kits are designed for the domestic chicken (Gallus gallus domesticus). Although there have been several advances in studying innate immune components in wild birds (Matson et al. 2005,Cohen et al. 2008), examining the adaptive humoral immune response is limited to species that either: 1) cross-react with anti-chicken immunoglobulins (Martinez et al. 2003,Pihlaja et al. 2006,Arriero 2009), or 2) those where it is feasible to produce antiserum against species-specific immunoglobulins (Hasselquist et al. 1999,Ilmonen et al. 2002,SmitsandBortolotti 2008).

Birds produce three classes of immunoglobulins, IgA, IgM and IgY. Immunoglobulin Y is the predominant antibody in the egg yolk and is functionally equivalent to mammalian IgG (LeslieandClem 1969). IgA and IgM are located primarily in the egg white (Hamal et al. 2006), but have been found at low concentrations in the egg yolk (Kaspers et al.

1991,Hamal et al. 2006). Several studies have demonstrated different methods of extracting yolk immunoglobulins from the egg (Polson 1990,Hansen et al. 1998). The extracted, purified antibodies can be used to create antiserum against species-specific immunoglobulins that are used to detect antigen-specific or total immunoglobulin levels in the plasma (Huber et al. 2010) and yolk (King et al. 2010) Unfortunately, for many bird species it is not always feasible to collect the volume of yolk necessary to make a species-specific antiserum. For example, in the Darwin's finch (Geospiza fortis) sampling of plasma and yolk is tightly regulated, making it impossible to collect enough samples to extract antibodies for use in vaccination. Huber et al. (2010) demonstrated that rat-anti-house-sparrow IgY (RαHOSP-Ig) cross-reacted with Darwin's finch plasma and used this antiserum to detect parasite-specific antibodies in finch populations on the Galapagos Islands.

House sparrows (*Passer domesticus*) are invasive to North and South America and there are no restrictions on the collection of eggs for use in antibody extraction and purification. They breed easily in captivity, their nests are highly accessible, and they lay multiple 3-6 egg clutches during the breeding season (LowtherandCink 1992) making them an ideal candidate to use in experimental immunologic studies. House sparrow plasma does not cross-react with antiserum built against chicken IgY and vise versa (Huber et al. 2010,King et al. 2010). The inability of anti-chicken-IgY to detect house sparrow immunoglobulins most likely reflects species-specific variation in epitope composition on the immunoglobulin itself. House sparrows are more closely related to other wild passerines than are chickens (Barker et al. 2004), which may increase the

likelihood that antiserum built against HOSP-Ig will cross-react with the plasma of other passerine species.

In this study, we demonstrate the applicability of using antiserum built against house sparrow immunglobulins to detect antibodies in the plasma of multiple free-living and captive passerine species.

MATERIALS AND METHODS

Subjects and samples

Plasma samples were obtained from 21 species of both captive and free-living adult birds from North America, South America and Australia. Captive species were broiler chicken (n=2) and zebra finch (*Taeniopygia guttata*, n=3), housed in Pullman, WA (46°44′N 117°10′W). Free-living species included house sparrow (n=5), European starling (*Sturnus vulgaris*, n=3), sampled in Moscow, Id (46°43′N 116°59′W); prothonotary warbler (*Protonotaria citrea*, n=5), sampled in the Tensas River National Wildlife Refuge in Tallulah, LA (32°24′33″N 91°11′29″W); golden-faced tyrannulet (*Zimmerius chrysops*, n=5), slaty antwren (*Myrmotherula schisticolor*, n=5), three-striped warbler (*Basileuterus tristriatus*, n=5), grey-breasted wood-wren (*Henicorhina leucophrys*, n=5), plain antvireo (*Dysithamnus mentalis*, n=5), slaty-backed nightingale-thrush (*Catharus fuscater*, n=5), olive-striped flycatcher (*Mionectes olivaceus*, n=5), chestnut-capped brush-finch (*Buarremon brunneinucha*, n=5), slate-throated redstart (*Myioborus miniatus*, n=5), black-hooded thrush (*Turdus olivater*, n=5), ochre-breasted brush-finch (*Atlapetes semirufus*, n=5), andean solitaire (*Myadestes ralloides*, n=5), orange-billed

nightingale-thrush (*Catharus aurantiirostris*, n=5), yellow-legged thrush (*Platycichla flavipes*, n=5), all samples in Yacambú National Park, Venezuela (9°40’N 69°32’W); Darwin’s finch (n=5) sampled in the Galápagos Islands (°40'N-1°36'S, 89°16'-92°01'W); and the red-backed fairy-wren (*Malurus melanocephalus*, n=3) sampled near Herberton, Queensland, Australia (145°23’E, 17°23’S).

Secondary Antibody

House sparrow immunoglobulins were purified from whole yolks using the protocols described by Huber et al. (2010) and King et al. (2010). Two-species, rats and rabbits, were independently vaccinated against whole, purified house sparrow IgY (HOSP-Ig) to create a polyclonal antibody against HOSP-Ig (King et al. 2010).

Plasma Preparation

In preparation for running the assay, samples for each species were pooled, vortexed and diluted 1:100 in sample buffer (FTA-hemmaglutination buffer (pH 7.2), 10% casein). From the 1:100 solutions each sample was serially diluted 1:1000, 1:2000, 1:5000, 1:10,000, 1:15,000, 1:20,000, 1:25,000 and 1:50,000 in sample buffer. House sparrow samples were additionally diluted 1:150,000, 1:300,000, 1:450,000 and 1:600,000. King et al. (2010) previously reported that, for their total immunoglobulin assay, the optimal dilution of adult house sparrow plasma was 1:300,000.

Measure of Cross-Reactivity

Cross-reactivity between was analyzed using a modified enzyme-linked immunosorbent assay (ELISA) created, validated and optimized for house sparrow yolk and plasma by King et al. (2010). In brief, 96-well plates were coated with 100 µL of rabbit-anti-HOSP-Ig serum diluted 1:1,000 in sodium bicarbonate coating buffer (0.05 M, pH 9.6) and incubated for 12-15hrs at 4°C. Each plate contained the following samples 1) "blank" wells that were not coated and to which only developing solutions are added), 2) "non-specific binding" (NSB) wells, which measured binding between the capture antibody and secondary and/or detection antibody, 3) positive control wells made from pooled house sparrow plasma diluted 1:10,000 in sample buffer, 4) negative control wells made from pooled chicken plasma diluted 1:1000 in sample buffer, and 5) eight HOSP-Ig standards (1000, 200, 100, 50, 25, 12.5, 6.25, and 3.12 ng/mL) made from purified, lyophilized HOSP-Ig. All samples were analyzed in duplicate. Between each step plates were washed three times with 200 µL/well of wash solution (150mM NaCl, 0.01% Tween-20). Coated plates were washed and, in duplicate, 100 µL from each of the eight dilutions per species (12 for house sparrows) were added to the plate. The plates were incubated for 1hr at 37°C on a rocker and washed again. 100 µL Rat-anti-house-sparrow-IgY (RatαHOSP-Ig), diluted 1:1000 in sample buffer, was added to all but the blank wells, incubated for 1hr at 37°C on a rocker and washed. 100 µL of the detection antibody, goat-anti-rat-hrp (GαR-hrp: Invitrogen # A10549, Carlsbad, CA), was added to each well, except the blank, at a dilution of 1:40,000 and the plates were incubated one last time for 1hr at 37°C on a rocker before being washed one final time. To each well, including the blank, 100 µL of peroxidase substrate developing reagent (2,2'-azino-bis(3-

ethylbenzthiazoline-6-sulphonic acid) (ABTS: Sigma cat. A1888) was added and the plates were covered with tinfoil and left to develop for 1hr in the dark. At the end of 1hr the plates were read on a spectrophotometer using a 405-nanometer wavelength filter to measure optic density (OD).

Assay Validation

We used dilutional parallelism to validate the assay for species that cross-reacted with anti-HOSP-Ig. The ODs of serially diluted plasma samples were graphed as a function of their dilution factor. A curve for each species was generated and compared with the reference curve made from serially diluted pooled, house sparrow plasma. Chicken plasma does not cross-react with anti-HOSP-Ig (Huber et al. 2010,King et al. 2010) and acted as a negative control.

We further validated the assay by examining intra-assay variation, intra-assay dilutional variation, and inter-assay variation. Intra-assay variation was determined by calculating the coefficient of variation (%CV: (SD/grand mean)*100) for the three positive controls within each plate. Intra-assay dilutional variation was determined by calculating the %CV ((Sample SD/Sample mean) *100)) for each positive control and sample assayed in duplicate per plate. Finally, inter-assay variation was determined by calculating % CV of positive controls between each plate. For assay validation, intra and inter-assay %CVs ≥ 10-15 were considered an unacceptable source of experimental error. Species-specific assay validation was confirmed when dilutional curves ran parallel to the reference house sparrow plasma curve in conjunction with low intra-assay dilutional variation (%CV ≤

10-15).

All data are presented as mean ± SD. All analyses were carried out using JMP 7.0.1 (SAS Institute Inc., Cary, NC, U.S.A.) and Microsoft Excel for Windows (Microsoft, Redmond, USA).

Ethical note

All blood collection protocols for birds sampled in the United States, Venezuela, and Australia were approved by the Institutional Animal Care and Use Committee (protocol no. 2551, 3357 and 3067) at Washington State University. The sample collection protocols for the Galapagos Islands were approved by the Institutional Animal Care and Use Committee (protocol no. 07-08004) at the University of Utah. Additional approval for samples collected in Australia were obtained from the James Cook University Animal Ethics Review Committee (approval no. A1004) and the Queensland Government Environmental Protection Agency.

RESULTS

Parallelism

All of the species that cross-reacted showed strong dilutional parallelism with the reference house sparrow plasma curve (Fig. 1). Individuals that showed weak or no binding did not run parallel to the house sparrow curve and their OD values per dilution were consistent with what was reported for the broiler chicken (Fig. 2).

Assay variation

The average intra-assay %CV was 1.350 ± 0.848. Inter-assay %CV = 14.751. Intra-assay dilutional %CVs were all ≤ 10-15 for species that cross-reacted with anti-HOSP-Ig.

DISCUSSION

The results of this assay demonstrate the applicability of using antiserum built against HOSP-Ig to detect antibodies in other wild passerine species. Nine of the 21 species, including house sparrows, cross-reacted with antiserum built against HOSP-Ig. The species examined in this study belonged to 12 different avian families and of these families 9 showed strong cross-reactivity with the antiserum. Of the species that cross-reacted with anti-HOSP-Ig, all showed strong parallelism and low intra-assay dilutional variation (%CV ≤ 10-15). The assay itself had low intra and inter-assay variation, demonstrating the reproducibility of these results.

As previously mentioned, house sparrow immunoglobulins do not cross-react with antiserum built against chicken IgY (Huber et al. 2010, King et al., 2010). The inability for chicken-IgY specific antiserum to detect house sparrow immunoglobulins most likely reflects differences in the molecular make-up of the epitopes located on the surface of the immunoglobulins. Variation in the number of epitopes conserved across species may interfere with the sensitivity of this ELISA, making use of the assay with some species difficult. However, considering the assay is sensitive to a wide range of antibody titers and the reasonably high taxonomic diversity that the assay was cross-reactive with, this approach provides a valuable tool to avian ecologists. For example, both Huber et al.

(2010) and King et al. (2010) have independently demonstrated the applicability using anti-HOSP-Ig as a secondary antibody to detect antigen-specific antibody responses in the Darwin's finch and house sparrow, respectively. In the Darwin's finch study the assay was sensitive enough to detect spatial and temporal variation in the antibody response of populations differentially exposed to two species of invasive parasites, whereas for house sparrows it was used to detect age-related variation in the presence of antigen-specific maternal antibodies in yolk and nestling plasma.

The use of anti-HOSP-Ig serum in wild passerine studies has the potential to greatly enhance the ability of researchers to assess immune function and quality in a broad spectrum of avian species, including protected birds, where the production of species-specific antisera is not feasible do to regulations in sample collection. In this study, we demonstrate that purified HOSP-Ig cross-reacts with multiple bird species. Assessment of avian immune function may prove important, especially given the expanding use of avian models in the field of ecological immunology and the recognition that avian species can act as vectors and reservoirs of zoonotic disease (Sahin et al. 2001,Kent et al. 2003,Dubska et al. 2009).

LITERATURE CITED

Apanius, V. 1998. Ontogeny of immune function, Avian Growth and Development (eds. J. M. Stark & R. E. Ricklefs), pp. 203-222. Oxford University Press, Oxford.

Arriero, E. 2009. Rearing environment effects on immune defence in blue tit Cyanistes caeruleus nestlings. – Oecologia. 159: 697-704

Barker, F. K., Cibois, A., Schikler, P., Feinstein, J.andCracraft, J. 2004. Phylogeny and diversification of the largest avian radiation. – Proceedings of the National Academy of Sciences of the United States of America. 101: 11040-11045

Boulinier, T.andStaszewski, V. 2008. Maternal transfer of antibodies: raising immuno-ecology issues. – Trends in Ecology & Evolution. 23: 282-288

Cohen, A., Klasing, K.andRicklefs, R. 2008. Measuring circulating antioxidants in wild birds (vol 147, pg 110, 2007). – Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 149: 540-540

Dubska, L., Literak, I., Kocianova, E., Taragelova, V.andSychra, O. 2009. Differential Role of Passerine Birds in Distribution of Borrelia Spirochetes, Based on Data from Ticks Collected from Birds during the Postbreeding Migration Period in Central Europe. – Appl. Environ. Microbiol. 75: 596-602

Gasparini, J., Piault, R., Bize, P.andRoulin, A. 2009. Pre-hatching maternal effects inhibit nestling humoral immune response in the tawny owl Strix aluco. – Journal of Avian Biology. 40: 271-278

Hamal, K. R., Burgess, S. C., Pevzner, I. Y.andErf, G. F. 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. – Poult. Sci. 85: 1364-1372

Hansen, P., Scoble, J. A., Hanson, B.andHoogenraad, N. J. 1998. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. – Journal of Immunological Methods. 215: 1-7

Hasselquist, D., Marsh, J. A., Sherman, P. W.andWingfield, J. C. 1999. Is avian humoral immunocompetence suppressed by testosterone? – Behavioral Ecology and Sociobiology. 45: 167-175

Huber, S. K., Owen, J. P., Koop, J. A. H., King, M. O., Grant, P. R., Grant, B. R.andClayton, D. H. 2010. Ecoimmunity in Darwin's Finches: Invasive Parasites Trigger Acquired Immunity in the Medium Ground Finch (Geospiza fortis). – PLoS ONE. 5: e8605

Ilmonen, P., Taarna, T.andHasselquist, D. 2002. Are incubation costs in female pied flycatchers expressed in humoral immune responsiveness or breeding success? – Oecologia. 130: 199-204

Kaspers, B., Schranner, I.andLosch, U. 1991. Distribution of immunoglobulins during embryogenesis in the chicken –Journal of Veterinary Medicine Series a-Zentralblatt Fur Veterinarmedizin Reihe a-Physiology Pathology Clinical Medicine. 38: 73-79

Kent, R. J., Lacer, L. D.andMeisch, M. 2003. Initiating Arbovirus Surveillance in Arkansas. – J. of Medical Entomology. 40: 223-229

King, M. O., Owen, J. P.andSchwabl, H. G. 2010. Are Maternal Antibodies Really that Important? Patterns in the Devlopment of Humoral Immunity in Altricial Passerine House Sparrows (Passer domesticus). – PLoS ONE. 5: e9639

Kopp, E. B.andMedzhitov, R. 2009. Infection and inflammation in somatic maintenance, growth and longevity. – Evolutionary Applications. 2: 132-141

Leslie, G. A.andClem, L. W. 1969. Phylogeny of immunoglobulin structure and function: III. Immunoglobulins of the chicken. – Journal of Experimental Medicine. 130: 1337-1352

Lochmiller, R. L.andDeerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? – Oikos. 88: 87-98

Lowther, P. E.andCink, C. L. 1992. House Sparrow, Passer domesticus. Birds of North America. – Birds N. Am. 12: 1-19

Martinez, J., Tomas, G., Merino, S., Arriero, E.andMoreno, J. 2003. Detection of serum immunoglobulins in wild birds by direct ELISA: a methodological study to validate the technique in different species using antichicken antibodies. – Functional Ecology. 17: 700-706

Matson, K. D., Ricklefs, R. E.andKlasing, K. C. 2005. A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. – Developmental and Comparative Immunology. 29: 275-286

Pihlaja, M., Siitari, H.andAlatalo, R. V. 2006. Maternal antibodies in a wild altricial bird: effects on offspring immunity, growth and survival. – Journal of Animal Ecology. 75: 1154-1164

Polson, A. 1990. Isolation of IgY from the yolks of eggs by a chloroform polyethylene-glycol procedure. – Immunol. Investig. 19: 253-258

Sahin, O., Zhang, Q. J., Meitzler, J. C., Harr, B. S., Morishita, T. Y.andMohan, R. 2001. Prevalence, antigenic specificity, and bactericidal activity of poultry anti-Campylobacter maternal antibodies. – Appl. Environ. Microbiol. 67: 3951-3957

Saino, N., Ferrari, R. P., Romano, M., Ambrosini, R.andMoller, A. P. 2002. Ectoparasites and reproductive trade-offs in the barn swallow (Hirundo rustica). – Oecologia. 133: 139-145

Smits, J. E. G.andBortolotti, G. R. 2008. Immunological development in nestling American kestrels Falco sparverius: Post-hatching ontogeny of the antibody response. – Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology. 151: 711-716

Tschirren, B.andRichner, H. 2006. Parasites shape the optimal investment in immunity. – Proceedings of the Royal Society B-Biological Sciences. 273: 1773-1777

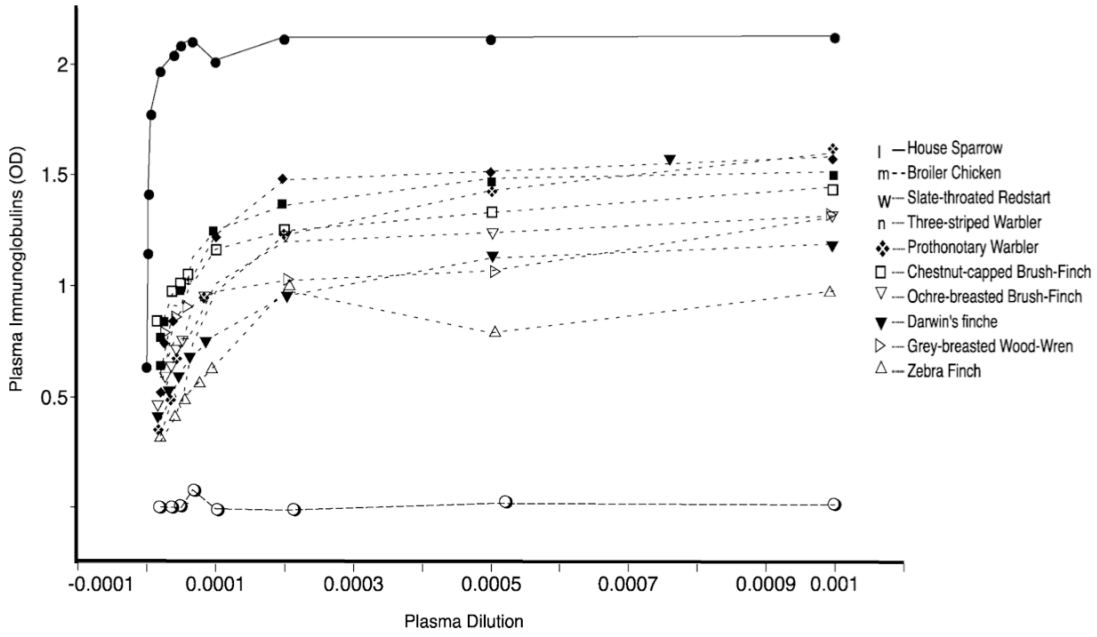
FIGURE LEGENDS

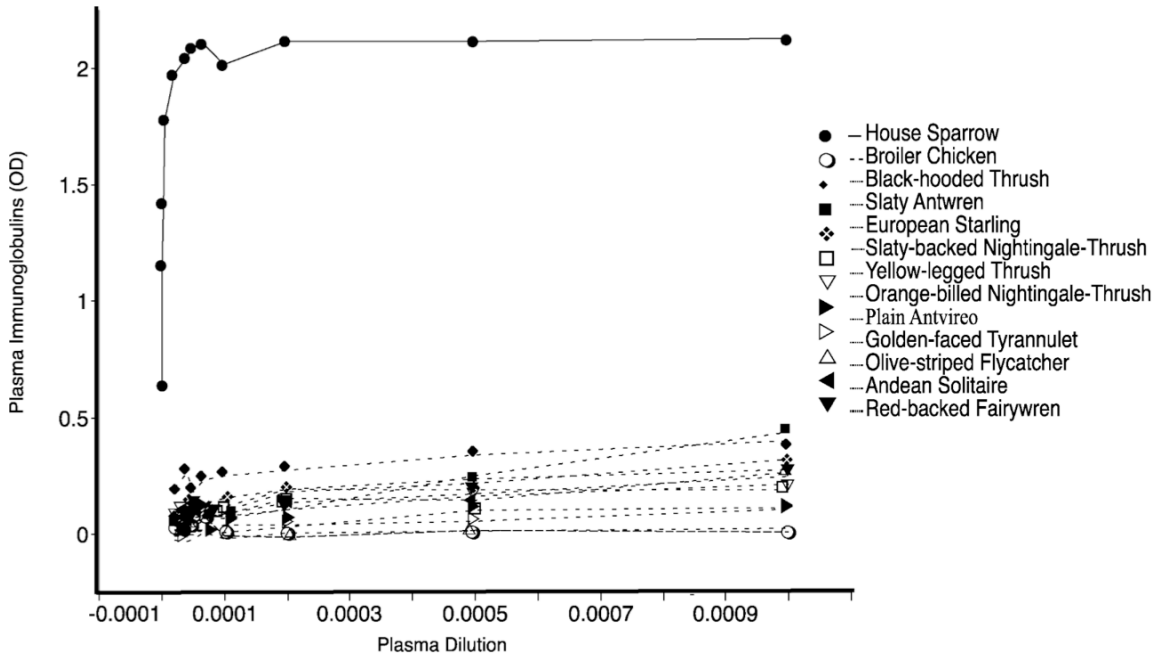
Figure 1.

Test of dilutional parallelism in curves generated from the serially diluted plasma samples of species that tested positive for cross-reactivity with RαHOSP-Ig. Dilution curves were compared with the reference curve generated from serially diluted house sparrow plasma. Chicken plasma served as a negative control. Antibody levels are presented as optic density (OD).

Figure 2.

Test of dilutional parallelism in curves generated from the serially diluted plasma samples of species that showed weak to no cross-reactivity with RαHOSP-Ig. Dilution curves were compared with the reference curve generated from serially diluted house sparrow plasma. Chicken plasma served as a negative control. Antibody levels are presented as optic density (OD)


Plasma Immunoglobulins (OD)
2
1.5
1
0.5
-0.0001
0.0001
0.0003
0.0005
0.0007
0.0009
0.001
Plasma Dilution
l — House Sparrow
m - - Broiler Chicken
w — Slate-throated Redstart
n — Three-striped Warbler
Prothonotary Warbler
Chestnut-capped Brush-Finch
Ochre-breasted Brush-Finch
Darwin's finche
Grey-breasted Wood-Wren
Zebra Finch


Plasma Immunoglobulins (OD)
2
1.5
1
0.5
0
-0.0001
0.0001
0.0003
0.0005
0.0007
0.0009
Plasma Dilution
House Sparrow
Broiler Chicken
Black-hooded Thrush
Slaty Antwren
European Starling
Slaty-backed Nightingale-Thrush
Yellow-legged Thrush
Orange-billed Nightingale-Thrush
Plain Antvireo
Golden-faced Tyrannulet
Olive-striped Flycatcher
Andean Solitaire
Red-backed Fairywren