Immune Capacity Development and Factors that Influence the Strength of Immune Response in Growing Altricial Birds

By

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ABSTRACT

The nestling period of altricial birds is a time of rapid body growth and development, yet there is a paucity of published studies examining the ontogeny of immune function and the impact of environmental perturbations, such as food restriction, during this time. The first study of this thesis examined the ontogeny of specific adaptive immune response in altricial zebra finches *Taeniopygia guttata,* using repeated vaccinations with non-infectious keyhole limpet hemocyanin (KLH) antigen. We found that capacity for adaptive antibody response developed after the first week post-hatch and that strength of adaptive antibody response significantly increased with age throughout the nestling period. However, we found that mature adult secondary antibody response level was not achieved in zebra finches prior to fledging (21d).

Because innate immune functions purportedly develop rapidly post-hatch, prior to adaptive functions, we then examined developmental patterns of constitutive innate and adaptive immune indices in house sparrows *Passer domesticus* (fledging age: 14-15d). Lysozyme activity significantly decreased with age, likely representing catabolism of maternal lysozyme from the egg albumen. Levels of circulating adaptive IgY, as well as innate agglutination and lysis, increased throughout the nestling period, but were significantly below levels found in fully-grown birds near fledging (12d). There were no significant differences between hatch-year birds (2-3 months) and adults in these measures, indicating that full maturation occurs post-fledging.

After examining the ontogeny of immune function throughout the nestling period, we examined how immune function is altered by food restriction and whether trade-offs occur between growth and development and immune function. The third study examined the phenomenon of compensatory growth in zebra finches following food restriction during the

nestling period. While compensatory growth allows birds to fledge at a suitable size and time, it may have persistent detrimental effects on the development of tissues and of complex systems such as the adaptive immune system. Compensatory body mass, but not tarsus and culmen, growth was observed in previously food restricted zebra finches. No impact on nestling adaptive antibody response to KLH was observed post-restriction. Reductions in tissue maturity, indexed by water content, were observed following compensatory growth, indicating a decoupling of chronological age and physiological age as a result of accelerated growth in food restricted birds.

While we saw no impact of food restriction on immune function after re-feeding, we were also interested in examining whether trade-offs occur between growth and immune function during food restriction. In the final study, we imposed food restriction on early and late-stage house sparrow nestlings and measured multiple indices of immune function, growth, and development. During food restriction, levels of the acute phase protein haptoglobin were reduced compared with controls. Food restriction did not significantly impact complement-mediated lysis or circulating IgY antibody levels. Food restriction resulted in significant reductions in alimentary organ, heart, and flight muscle masses. Reductions in muscle maturity (indexed by tissue water content and mean total citrate synthase enzyme activity) were also observed with early food restriction and were not recovered with re-feeding. Findings from this study suggest that immune function, like organ growth, appears to be flexible to resource supply during the nestling period, and that growth may be prioritized or relatively more costly than immune function during this time.

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INTRODUCTION

Background

Altricial passerines have short incubation times and nestlings hatch featherless, with eyes closed, and with limited mobility and thermoregulatory capacity (Starck and Ricklefs 1998). As a group, altricial passerines have the fastest growth rates of land animals (Case 1978, Arendt 1997) and they rely on parental delivery of food to the nest for up to several weeks before fledging (Starck and Ricklefs 1998). Through experimental studies of house sparrow (*Passer domesticus*) and zebra finch (*Taeniopygia guttata*) nestlings, this dissertation focuses on understudied aspects of growth and development in altricial birds: the ontogeny of immune function and its response to resource limitation. The first two chapters examine age-related changes in adaptive and innate immune function throughout the entire developmental period. The subsequent two chapters examine the response of immune measures to food restriction and re-feeding during the nestling period. Consequently, I begin with an overview of the avian immune system and previous studies of development of immune function relevant to the studies in this dissertation. I also introduce previous studies of food restriction and growth responses in altricial birds and a framework for examining trade-offs between growth and immune function in food-restricted nestlings.

The avian immune system

Birds are reservoir hosts for many pathogens that impact humans and livestock, and coordinated actions of the innate and adaptive arms of the avian immune system are required to respond to these pathogens. The innate immune system is a rapid, highly effective first line of defense. Innate immune components such as phagocytic cells and antimicrobial proteins

recognize classes of microbial molecules and bind, internalize, or destroy foreign invaders (Kindt et al. 2007). Innate immune cells such as dendritic cells and macrophages clear antigen by phagocytosis and also stimulate the adaptive immune system by presenting peptide fragments on MHCII molecules (Davison et al. 2008). Polyreactive natural antibodies act as a first line of defense by directly neutralizing bacteria and viruses and by agglutination and opsonization of particulate antigens (Ochsenbein and Zinkernagel 2000). Natural antibodies also play an important role in innate immunity by activating complement, a system of serum proteins that circulate as inactive precursors but become activated when exposed to foreign antigen. Complement activation can lead to cell lysis (Merchant et al. 2006), enhancement of phagocytosis of particulate antigens, and activation of inflammatory responses (Kindt et al. 2007). Constitutive antibacterial proteins are secreted by immune cells and digest cell wall and other bacterial components (Kindt et al. 2007, Millet et al. 2007, van de Crommenacker et al. 2010). Acute phase proteins, induced by inflammatory stimuli, bind foreign antigen, protect host tissues, and prevent damage through antioxidant action (Davison et al. 2008).

The avian adaptive immune system is a highly specific and diverse arm of defense (reviewed in Davison et al. 2008). Cell-mediated immune function is carried out by T-cells. T-helper cells contain the CD4 glycoprotein and an antigen-specific T-cell receptor on their surfaces. T-helper cells recognize foreign peptides presented on MHC-II molecules of antigen-presenting cells and activate other immune cells to respond to the foreign antigen (Kindt et al. 2007). T-cytotoxic cells contain the CD8 glycoprotein and the T-cell receptor on their surfaces, recognize self-peptides presented on MHC-I molecules (present on all nucleated cells), and function to destroy virally-infected cells and tumor cells (Kindt et al. 2007). Humoral immune function is mediated by B-cells that contain antigen-specific antibodies on their surfaces.

Activated B-cells secrete IgM antibodies in response to novel antigen exposure, then class-switch to IgY antibodies, the main isotype secreted in response to systemic infection (Davison et al. 2008). Memory B-cells stored after a primary response generate a rapid and robust IgY response upon secondary exposure to the specific antigen (Davison et al. 2008). In contrast to innate immune function, adaptive cell-mediated and humoral components improve recognition and interaction with antigens and retain long-term memory with repeated exposure (Davison et al. 2008).

A spectrum of development among bird species

Much of the investigation into avian innate and adaptive immune functions and disease has been performed in poultry because of their economic importance in food production. However, it has recently been noted that free-living birds can contribute to the spread of zoonotic diseases. For example, free-living birds play a primary role as amplifying hosts in the enzootic cycle of West Nile Virus (WNV). A study of experimental WNV infection in 25 bird species revealed that Passeriformes (perching birds or songbirds) were more reservoir-competent than species in other orders (Langevin et al. 2003). Recently it was proposed that young passerine birds in particular might play important roles as vectors for West Nile Virus, given that they are confined to nests in close proximity to others post-hatch and may therefore be vulnerable to virus-carrying mosquitoes (O'Brien et al. 2010).

Given that many free-living birds with potential as disease hosts have a different mode of development compared with domestic poultry, it has become increasingly important to examine whether differences in the development of immune function exist between domesticated and wild bird species. Domestic birds like chickens, ducks, and turkeys have a precocial mode of

development: they hatch covered with feathers and with eyes open, and are capable of mobility, independent feeding, and metabolic heat production soon after emerging from the egg (Starck and Ricklefs 1998). On the other hand, passerines are altricial birds: they have short incubation times, and nestlings hatch featherless, with eyes closed, and with limited mobility and thermoregulatory capacity (Starck and Ricklefs 1998). Altricial passerines, as a group, have the fastest growth rates of land animals (Case 1978, Arendt 1997) and they rely on parental delivery of food to the nest for up to several weeks before fledging (Starck and Ricklefs 1998). In altricial house sparrow nestlings, adult body mass and structural size are attained by 9 days post-hatch, prior to fledging from the nest at day 14-15 post-hatch (Killpack and Karasov 2012). Assimilation organs (gizzard, liver, pancreas, and intestine) account for ~18% of total body mass at 6 days post-hatch, and make energy from ingested food available to fuel rapid growth. Flight muscles and leg muscles, on the other hand, account for only 7% of total body mass at day 6 post-hatch, and rapidly increase to account for 17.5% of the body mass by 12 days post-hatch, just prior to fledging (Killpack and Karasov 2012). While it is clear from this study and others (Starck and Ricklefs 1998) that the nestling period is a time of rapid growth and development in altricial birds, there is a paucity of published studies regarding the development of immune function during this time.

Development of avian immune function

Development of the adaptive immune system of chickens has been well-documented, and is summarized below (reviewed in Davison et al. 2008). T-cell progenitors have been shown to colonize the chicken thymus in the middle third of incubation and T-cell receptor rearrangement (to generate antigen-specific molecules) occurs in the thymus during final third of incubation. T-

cells then migrate from the thymus to peripheral and intestinal epithelial tissues and the spleen near the end of incubation. The thymus involutes prior to sexual maturity, beginning at 3-6 months post hatch (Ciriaco et al. 2003). B-cells colonize the chicken bursa of Fabricius during the middle third of incubation. In the bursa, specific immunoglobulin (Ig) molecules are generated through the process of gene conversion, in which sequences on variable region gene segments are replaced with sequences from upstream pseudogenes. This process of gene conversion to generate unique, specific antibody molecules differs from the process in mammals, called Ig gene rearrangement. Avian IgM is the predominant B cell antigen receptor and first isotype to be expressed in development. IgM is detectable in the bursa of chickens during the final third of incubation and in the periphery several days before hatching. Rapid expansion of IgM-containing B-cells occurs in spleen, an important secondary lymphoid organ, during the first week post-hatch. Avian IgY, which shares homology with both mammalian IgG and IgE, is detectable in the bursa at hatch and in periphery at 4dph in chickens. Rapid expansion of IgY has been observed in the spleen of chickens after the first week post-hatch (Davison et al. 2008). The bursa involutes from 3-6 months post hatch in chickens, prior to sexual maturity (Ciriaco et al. 2003).

Development of adaptive immune function is hypothesized to occur during the early weeks post-hatch in all bird species (Klasing and Leshchinsky 1999, Davison et al. 2008), though few empirical studies in altricial birds have been performed until recently. Studies utilizing non-infectious vaccinations have been used to determine when birds are capable of mounting a specific primary antibody response (IgM response to the first injection of a given antigen) and secondary adaptive antibody response (memory IgY response to subsequent injections of the same antigen). Specific primary IgM response to vaccinating antigens was detectable as early as

3 days post-hatch in turkeys, kestrels and quails (McCorkle and Thaxton 1988, Smits and Bortolotti 2008, Staszewski and Siitari 2010). However, detection of primary antibody response in altricial pigeons and pied flycatchers (Koppenheffer and Robertson 1980, Grindstaff et al. 2006), as well as precocial turkeys (Suresh et al. 1993) was more reliable after the first week post-hatch. IgM levels have been shown to continually increase in the subsequent weeks post-hatch in altricial pigeons and macaws (Koppenheffer and Robertson 1980, Selvaraj and Pitchappan 1988, Lung et al. 1996).

Fewer studies have examined the ontogeny of secondary adaptive IgY response in altricial birds. A study in semi-altricial kestrels demonstrated that adaptive secondary antibody response was significantly higher at 7-9 days post-hatch compared to responses at 3-5 days post hatch, and that adult secondary response was four times higher than that of nestlings (Smits and Bortolotti 2008). Based on these data, it is likely that altricial birds continue to develop adaptive humoral immune function throughout the nestling period prior to fledging, though it is unclear when adult adaptive IgY levels are reached.

Because expansion of innate immune components does not require development of diverse receptor specificities or memory, it has been proposed that this arm of the immune system develops early post-hatch, prior to the adaptive immune system (Klasing and Leshchinsky 1999). However it is unclear whether innate immune components reach adult levels prior to fledging from the nest, after which altricial birds are exposed to a diverse range of environmental antigens. Natural antibody agglutination activity reached adult levels by fledging age (day 16 to 22 post-hatch) in free-living great tit nestlings, yet complement-mediated lysis was not detectable in nestlings prior to one year post-hatch (De Coster et al. 2010). Tree swallow nestlings had significantly lower natural antibody agglutination and complement lysis levels than

adults, indicating that full maturation of innate components continues after fledging (Palacios et al. 2009). However, studies of constitutive lysozyme levels in turkeys (Franciosini et al. 2009) and barn swallows (Saino et al. 2002) do not show age-related changes during the post-hatch period. More detailed and comprehensive study within species of the patterns of innate immune components in nestlings, fledglings, and adults will shed light on when these components reach maturity in altricial birds.

Food restriction and plasticity in growth of altricial birds

Nestling altricial birds rely solely on parental delivery of food to the nest for up to several weeks prior to fledging. Food shortage can occur during the development of altricial birds in the wild, due to fluctuating environmental conditions, inadequate parental delivery of food to the nest, or sibling competition for the delivered food within the nest (Bize et al. 2006, Losdat et al. 2010). Additionally, climate change is affecting the phenologies of breeding birds and their prey, causing asynchrony between the timing of nestling needs and the availability of the resources on which they depend (Thomas et al. 2001, Visser et al. 2005). The nestling period of altricial birds is an energetically demanding time period during which rapid anatomical growth and considerable maturation in physiology and biochemical digestive capacity occurs (Caviedes-Vidal and Karasov 2001). Altricial birds undergo more rapid growth and generally reach asymptotic size in a shorter period of time during the post-hatch period compared with precocial birds, which hatch with more mature tissues and thermoregulatory and locomotive abilities (Ricklefs, 1973; Ricklefs, 1979). Consequently, food shortage experienced during the energetically demanding nestling period may have a considerable impact on growth and development prior to fledging and on future survival and fitness of altricial birds in the wild.

Evolved plasticity in growth and developmental patterns allows birds to respond to fluctuating environmental conditions and to mitigate the impact of poor feeding conditions on chick survival. A growing bird's response to food shortage may depend on the magnitude, duration, and frequency of the shortage, as well as the timing of the shortage with respect to the course of the bird's growth (Schew and Ricklefs 1998). If growth and development cannot be maintained at the normal pace given the available food intake and energy stores, birds may slow growth and maturation, thereby extending the nestling period. Alternatively, they may preferentially allocate resources to body parts that are more critical to survival and future success at the expense of other less critical parts (Schew and Ricklefs 1998). A final strategy is to accelerate growth and/or development relative to age once food becomes freely available, termed compensatory growth, to achieve normal asymptotic size at the same time as well-fed birds (Schew and Ricklefs 1998). Studies of experimental food restriction during the nestling period have been used to examine this plasticity in growth and developmental patterns in altricial birds.

Organs of assimilation, such as the intestine and liver, process food to fuel growth and thus represent a large proportion of the total body mass at hatch and early in development when rapid body growth occurs (Starck 1996). Maintenance of assimilation organ size during food shortage can be energetically costly, given the high metabolic activity and cell turnover rates of these organs (Ricklefs et al. 1998). Studies have shown that growing birds down-regulate the size and function of some assimilation organs and also reduce body temperature and resting metabolic rates in response to energy limitations encountered during reductions in food intake (Kitaysky 1999, Konarzewski and Starck 2000, Burness et al. 2000, Brzek and Konarzewski 2001, Moe et al. 2004). Assimilation organ processing capacity is therefore relatively flexible and can be adjusted to meet changing energy demands and nutrient flow during growth.

Skeletal growth during food shortage appears to be less flexible than patterns of soft tissue growth. Food-restricted nestlings maintain skeletal growth despite reductions in the mass of the whole body and soft tissues (Lepczyk and Karasov 2000, Moe et al. 2004, Killpack and Karasov 2012). Long bone growth in endotherms is under strict endocrine control (Leach and Rosselot 1992) and may be constrained to a critical window during development given that levels of circulating growth hormones decline and growth-suppressing sex hormones increase with age (Scanes and Balthazart 1981, Schew et al. 1996). Therefore, if growth is not maintained when circulating levels of growth hormones are high, negative and permanent consequences for bird fitness and survival may result. Thus, prioritizing growth of skeletal structures during the growth period may prevent long-term negative effects of food shortage.

Ecological immunology and trade-offs between growth and immune function

While there is abundant evidence that trade-offs between organ and skeletal growth occur when energy is limited during the nestling period (cited above), it is unclear how developmental patterns of immune function are altered in response to food restriction in altricial birds. A central assumption in the field of ecological immunology is that development and use of the immune system may lead to trade-offs with other functions such as growth, development and reproduction, because these functions rely on a shared pool of limited resources in an animal (Sheldon and Verhulst 1996, Norris and Evans 2000). Thus, investment in immune defense may be optimized and traded off with other costly functions, which may reflect or influence species evolution, ecology, and life history (Sheldon and Verhulst 1996, Norris and Evans 2000). These trade-offs in investment can impact fitness of avian species. Reduced growth during the nestling period can have negative ecological consequences in the form of decreased competitive ability

within the nest, delayed fledging, and reduced recruitment and survival after fledging (Emlen and Wrege 1991, Emlen et al. 1991, Searcy et al. 2004, Mock et al. 2009, Miller 2011). Alternatively, reduced investment in immune responses may make birds more susceptible to infection and increase the likelihood that birds serve as vectors for zoonotic diseases (Saino et al. 1997, Moreno et al. 2005).

Different arms of the immune system are proposed to have different relative costs in terms of energy and immuno-pathology (summarized in Table I, Lee 2006). Constitutive innate immune functions have purportedly low costs to develop because they are non-specific, and low costs to use because they do not increase with inflammatory challenge. Induced innate immune responses, though nonspecific, may be costly to use because they involve systemic inflammation and synthesis of acute phase proteins in the liver. Adaptive humoral (antibody-mediated) and cell-mediated immune functions are purportedly costly to develop because they require generation of diverse receptor specificities and memory lymphocyte pools. Adaptive cell-mediated (but not humoral) immune function may also incur elevated costs to use because it involves systemic inflammatory response. Therefore, trade-offs between immune defense and life history components may depend on the timing and type of immune challenge measured.

Many ecological immunology studies in growing birds have correlated immune measures and growth parameters without directly manipulating food intake (Saino et al. 1997, Soler et al. 1999, Hoi-Leitner et al. 2001, Snoeijs et al. 2005, Martín-Vivaldi et al. 2006, Palacios et al. 2009, Forsman et al. 2010, Arriero et al. 2013). However, alterations in allocation patterns to immune function and other functions that share the same pool of resources may only be detectable when resources are limited (French et al. 2009). Therefore, in order to experimentally examine potential trade-offs among different arms of immune function and between immune

function and growth, these parameters must be examined in the context of experimental manipulation of food intake during the nestling period.

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CHAPTER 1

Ontogeny of adaptive antibody response to a model antigen in captive altricial zebra finches

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Abstract

Based on studies from the poultry literature, all birds are hypothesized to require at least 4 weeks to develop circulating mature B-cell lineages that express functionally different immunoglobulin specificities. However, many altricial passerines fledge at adult size less than four weeks after the start of embryonic development, and therefore may experience a period of susceptibility during the nestling and post-fledging periods. We present the first study, to our knowledge, to detail the age-related changes in adaptive antibody response in an altricial passerine. Using repeated vaccinations with non-infectious keyhole limpet hemocyanin (KLH) antigen, we studied the ontogeny of specific adaptive immune response in altricial zebra finches *Taeniopygia guttata*. Nestling zebra finches were first injected at 7 days (7d), 14 days (14d), or 21 days post-hatch (21d) with KLH-adjuvant emulsions, and boosted 7 days later. Adults were vaccinated in the same manner. Induced KLH-specific IgY antibodies were measured using ELISA. Comparisons within age groups revealed no significant increase in KLH-specific antibody levels between vaccination and boost in 7d birds, yet significant increases between vaccination and boost were observed in 14d, 21d, and adult groups. There was no significant difference among age groups in KLH antibody response to priming vaccination, yet KLH antibody response post-boost significantly increased with age among groups. Post-boost antibody response in all nestling age groups was significantly lower than in adults, indicating that mature adult secondary antibody response level was not achieved in zebra finches prior to fledging (21 days post-hatch in zebra finches). Findings from this study contribute fundamental knowledge to the fields of developmental immunology and ecological immunology and strengthen the utility of zebra finches as a model organism for future studies of immune ontogeny.

Introduction

The adaptive humoral (or 'antibody-mediated') immune system of birds comprises a highly protective, specific arm of defense that retains long-term memory of invading pathogens [1,2]. The model for avian humoral immune function proposes that production of adequate numbers of peripheral B-cell lineages that express functionally different immunoglobulin (Ig) specificities requires at least four to six weeks to develop in all birds [3]. This model is derived from studies of precocial galliformes, in which B-cell development begins embryonically during approximately the final week of egg incubation and is completed approximately three to six weeks after hatching, well before chickens reach adult size [2–4]. However, some altricial bird species, which have short incubation times and among the fastest growth rates of land animals [5,6], fledge at adult size less than four weeks after the start of embryonic development. Given the proposed model of humoral immune development, and evidence that maternal antibodies (Igs) from the egg are catabolized within two weeks post-hatch in altricial birds [7–9], it is likely that rapidly-developing altricial birds are immunologically immature and relatively vulnerable to infection throughout the nestling and early post-fledging periods.

Non-infectious antigen vaccinations and antigen-specific immune assays have been used to examine induced antibody responses in developing birds. Upon first exposure to an antigen, a primary antibody response predominated by IgM is generated, isotype class-switching occurs, and memory B-cells are stored. Upon repeated exposure to the same antigen, a rapid secondary, memory response occurs, predominantly in the form of IgY antibodies [1,2]. Capacity for specific primary antibody response was detectable in altricial pigeons, pied flycatchers, and American kestrels injected with foreign antigen during the first week post-hatch [10–12].

Primary response levels were been shown to increase and become more reliable after the first week post-hatch in altricial pigeons and macaws [10,13], which was in agreement with studies of response levels in precocial chickens and turkeys [14–16].

Fewer studies have examined the ontogeny of secondary, adaptive antibody responses in altricial birds. Pigeons initially injected with sheep red blood cells (SRBC) during the first 10 days post-hatch produced IgY antibodies when a second immunization was administered 2-3 weeks later, indicating that the capacity for class-switching from IgM to IgY and memory cell formation occurs within the first weeks of hatching. Secondary antibody responses to repeated injections of SRBC and the non-pathogenic conjugate dinitrophenol keyhole limpet hemocyanin (DNP-KLH) were studied in American kestrels using hemagglutination and ELISA assays, respectively [17]. Kestrels first injected at 10 days post-hatch and boosted with the same antigen 6 days later showed robust secondary antibody responses, though detection of responses to DNP-KLH using ELISA was more sensitive than the SRBC hemagglutination assay [17]. More detailed studies of age-related changes in adaptive secondary antibody response of kestrels found significantly higher secondary responses in birds first vaccinated with DNP-KLH at 7-9 days post-hatch compared with those vaccinated at 3-5 days post hatch, and that adult secondary response was four times higher than that of nestlings [12]. Based on these data, it is likely that altricial birds continue to develop adaptive immune function throughout the nestling period, though it is unclear whether nestlings and recent fledglings experience a period of susceptibility to infection before mature adaptive IgY levels are reached.

We present the first study, to our knowledge, to detail the ontogeny of specific adaptive antibody response in an altricial passerine, the zebra finch *Taeniopygia guttata*, throughout the entire nestling period. Studies of age-related changes in antibody response in zebra finches will

contribute knowledge to the fields of comparative immunology and ecological immunology. Additionally, the elaboration of zebra finches as a model is of interest given that their genome has been sequenced and organismal-level studies could provide fundamental knowledge for integrative studies of immune function at a variety of biological levels, including the molecular level. This work aims to address two research questions: i) when are zebra finches capable of mounting a detectable specific secondary antibody response post-hatch? and, ii) does magnitude of secondary adaptive antibody response reach adult levels prior to fledging (21 days post-hatch) in zebra finches? We vaccinated zebra finch nestlings and adults with keyhole limpet hemocyanin (KLH) and detected adaptive KLH-specific antibody response using ELISA. KLH is a model antigen in immunology studies and is a T-cell dependent antigen, providing insight into the integrated ontogeny of both B- and T-cell mediated responses in zebra finches [12,18]. We used guidelines from previous studies of immunological development in altricial birds [12,17] as well as a preliminary study using adult male zebra finches to ensure effective stimulation of the immune system of developing zebra finch nestlings and detection of an antibody response, if present.

Methods

Ethics Statement

All experimental procedures were approved by the University of Wisconsin, Madison Animal Care and Use Committee (permit no. 1370).

Birds

A breeding colony of adult zebra finches was maintained under standardized conditions of 14 h : 10 h L:D photoperiod (lights on 0600 h), 21-24 °C temperature, and 40-50% relative humidity. Birds were provided with a seed diet consisting of commercial seeds and Mazuri Small Bird Breeder food supplement, cuttlefish bone (calcium source), grit, and water *ad libitum*. Enrichment foods of green vegetables and egg food mixture (hard-boiled eggs with shells, dried Volkman Featherglow egg food, and vitamin and mineral supplement) were provided to birds three times per week. Breeding pairs of adult zebra finches were taken from our stock population and housed in individual breeding cages. Pairs were provided with nest boxes and nesting material at the time of pairing. Nests were monitored daily after the onset of egg-laying to determine the exact hatch date of each nestling.

Vaccine preparation

We conducted a preliminary study of adult male zebra finches to determine a combination of antigen (Keyhole limpet hemocyanin (KLH) or Bovine serum albumen), adjuvant (Complete Freund's Adjuvant (CFA), Montanide ISA720, or Titermax), and sampling time (7 or 9 days post-injection) that ensured effective stimulation of the immune system of zebra finch nestlings and detection of an antibody response, if present. Given the magnitude of the effect and variance, we determined that we had the most power to generate and detect responses using the combination of KLH antigen with CFA adjuvant (or Titermax adjuvant for boost), and that 7 days post-injection was a sufficient sampling time. Therefore, for the ontogeny study, vaccine formulations were prepared by diluting KLH antigen (Calbiochem #374817) in phosphate buffered saline at a dose of 2.0 μg antigen per g bird body mass. Diluted antigen was

emulsified with Complete Freund's Adjuvant (or Titermax adjuvant for boost) at a ratio of 50:50 diluted antigen to adjuvant, per manufacturer instructions. Vaccine emulsions were prepared using the vortex method and verified using the 'drop test' [19].

Antibody production in nestlings and adults

Upon hatching, zebra finch nestlings were assigned to one of three groups for initial sampling and vaccination: 7 days (7d), 14 days (14d), or 21 days (21d) post-hatch (n=13 for 7d and 14d groups, n=14 for 21d group). A baseline blood sample (≤70 μL) was taken from zebra finch nestlings and previously unvaccinated adults (n=14) for measurement of background absorbance signal on the KLH-specific ELISA. Birds were then injected in the pectoralis muscle with 50 μL KLH-CFA emulsion prepared at a dose of 2.0 μg antigen per g bird body mass. Nestlings at 7 days post-hatch, too small for baseline blood sampling by veinipuncture and subsequent vaccination, were euthanized and decapitated for collection of a baseline blood sample, and sibling 7-day nestlings were injected intramuscularly in the pectoralis with 25 μL KLH-CFA emulsion (2.0 μg antigen per g bird body mass). A blood sample was taken 7 days post-priming vaccination from injected birds in all age groups by puncturing the brachial vein with a 28-gauge needle and collecting the blood into heparinized microcapillary tubes (≤70 μL per bird). Nestlings and adults were then immediately boosted with 50 μL of the same dose of KLH (2.0 μg antigen per g bird body mass) emulsified with Titermax (1 week post-priming vaccination), and a post-boost sample was taken 7 days later (≤70 μL per bird). Blood samples were centrifuged and plasma was stored at -20°C until analysis of antibodies. Body mass and hematocrit were measured at the time of blood sampling. At the time of KLH injections, all birds were also injected with a commercial West Nile Virus (WNV) vaccine, for the purposes of

another study. We have no reason *a priori* to expect the WNV vaccine to interfere with measurement of KLH-specific antibody response in our ontogeny study. A pilot study comparison of adult zebra finches simultaneously injected with KLH and WNV (n=9) compared with those injected with KLH alone (n=11) showed no significant difference in post-vaccination ($p=0.77$) or post-boost ($p=0.50$) KLH antibody response (data not shown). If there were an effect of WNV on KLH-specific antibody response in our ontogeny study, we anticipate that it would be uniform across treatments, given that all birds injected with KLH were also always injected with WNV.

Specific IgY antibody detection using enzyme-linked immunosorbent assay (ELISA)

Adaptive immune function was measured using an ELISA for KLH-specific IgY antibodies. Flat-bottomed 96-well plates were coated with 50 µl/well of KLH antigen (Calbiochem #374807) diluted to 0.5 mg/ml of KLH antigen in carbonate-bicarbonate coating buffer. Plates were incubated overnight at 4 °C. Plates were washed four times with wash buffer (PBS+Tween) to remove unbound coating antigen, blocked with 100 µL/well of blocking buffer (5% nonfat milk+wash buffer) and incubated for 1 hour at 37 °C. Blocking buffer was discarded and 100 µl of zebra finch plasma samples (and positive and negative plate control samples), diluted 1:100 in blocking buffer, were added to the wells in duplicate and the plates were incubated for 1 hour at 37 °C. Plates were washed four times with wash buffer, 50 µl of horseradish peroxidase-conjugated goat-anti-bird IgG (=IgY) antibody (Bethyl Laboratories, Inc. #A140-110P) diluted 1:700 in wash buffer was added to each well, and the plates were incubated for 1 hour at 37 °C. Plates were washed four times with wash buffer, 100 µl of ABTS substrate was added to all wells, and the plates were incubated in the dark at room temperature for 10

minutes. Optical densities of the wells were read at 405 nm using an automated plate reader and average absorbance values of duplicate wells were recorded. All samples from a given bird were run on the same plate and samples from birds of different treatment groups were included on each plate. Average absorbance values for post-priming and post-boost samples were corrected for background absorbance values on KLH-specific ELISA by subtracting the average absorbance value of the pre-injection blood sample for each bird (or from a sibling bird, in the case of the 7d group).

Statistics

Results are given as means ± sem (N = number of individuals). All tests were carried out using R statistical package [20]. Assumptions of normality and homoscedasticity were examined prior to use of parametric tests and log-transformation (after adding a constant to the corrected antibody response values to make them positive and non-zero) was performed to meet normality assumptions. ANOVA was used to compare absorbance values in pre-vaccination samples, body mass, and hematocrit among age groups. Linear mixed models of repeated measures of specific antibody response were fit using maximum likelihood criteria (including individual bird and parental breeding pair as random effects), and chi-square-based tests were used to determine the significance of fixed and random effects. Non-significant variables were iteratively removed from the model. Post-hoc Tukey's contrasts distinguished differences among groups. In all tests, the significance level was set at $p<0.05$.

Results

Baseline absorbance values from pre-vaccination samples, which likely measure cross-reactivity of ELISA reagents with non-KLH specific antibodies in samples, significantly differed among age groups ($F_{3,44}$: 6.5, $p<0.001$). Adults (0.179±0.026) had significantly higher baseline absorbance values than all of the nestling groups. Nestlings at 7d (0.106±0.007), 14d (0.111-±0.016), and 21d (0.096±0.006) post-hatch did not significantly differ from each other in baseline absorbance values. To correct for differences among age groups, baseline absorbance values were subtracted from post-priming vaccination and post-boost antibody response values for each bird (or sibling bird in the case of the 7d group).

Repeated measures analysis of corrected KLH antibody response revealed a significant interaction of age and sampling time (Chi-square: 53.94, d.f. 3, $p<0.001$) (Figure 1). Comparisons within an age group revealed no significant increase in KLH-specific antibody levels between vaccination and boost in 7d birds ($p=0.992$), yet highly significant increases between vaccination and boost were observed in birds vaccinated at 14d ($p=0.005$) and 21d ($p<0.001$) and as adults ($p<0.001$).

There was no significant difference among age groups in corrected KLH antibody levels in response to priming vaccination (Figure 1), yet there were significant differences in corrected KLH antibody levels post-boost, with responses at 7d < 14d < 21d < adults (Figure 1). Post-boost antibody responses in all nestling age groups were significantly lower than in adults, indicating that mature adult secondary antibody response level was not achieved in zebra finches prior to fledging (Figure 1).

Hematocrit (Chi-square <0.001, d.f. 1, $p>0.99$) and body mass (Chi-square: <0.001, d.f. 1, p: 0.994) were not significant factors in predicting KLH-specific antibody response. Parental

breeding pair was a significant random effect for corrected antibody response (Chi-square: 5.42, d.f. 1, p: 0.020). Vaccination did not significantly impact body mass or hematocrit in growing birds, as birds in different vaccination groups showed no significant differences in body mass or hematocrit measures at a given blood sampling age (data not shown).

Discussion

We present the first study, to our knowledge, to detail the age-related changes in adaptive antibody response in an altricial passerine. Adaptive antibody response was not detectable in birds first injected at 7 days post-hatch, as there was a lack of significant increase in KLH-specific IgY between priming injection and boost. Significantly elevated levels of KLH IgY were detected post-boost in 14d, 21d and adult age groups, indicating capacity for adaptive antibody response. Lack of significant increase in 7d birds under our sampling regimen may be a result of an incompletely developed cell system, resulting in a more prolonged process of Ig class-switching in younger birds. Turkey poults injected with *Brucella abortus* antigen on the day of hatch reached peak antibody response at 15 days post-injection (dpi), whereas the specific antibody response of birds injected at 3 weeks post-hatch peaked sooner at 7dpi [15], indicating that the number and the functional competence of the B-cells likely increased with age. Detailed study of lymphocyte subpopulations and of the kinetics of antibody response over a range of times post-injection would shed light on the time course of Ig class switching and of the response of specific memory B-cell clones in altricial birds at different ages post-hatch.

In agreement with a previous study in American kestrels, we found that magnitude of adaptive antibody response gradually increases with age, yet is significantly lower than mature adult levels throughout the nestling period. We found that adaptive antibody response in zebra

finches did not reach adult levels prior to fledging (21 days post-hatch in zebra finches), when free-living birds leave the nest and experience a more diverse antigenic environment.

Given that nestling birds have limited adaptive immune defenses, they may rely on other immune defenses for resistance. The lack of mature functional antibody response may be partly compensated by maternal provisioning of IgY via the egg yolk, which is absorbed into nestling circulation during development. However, studies of altricial passerine nestlings have shown that maternally derived antibodies are catabolized from circulation within two weeks post-hatch [7–9], which, according to our study, overlaps with the time that nestlings have immature endogenous humoral immune response capacity. Lack of mature adaptive immune function in growing nestlings may be compensated by endogenous innate immune defenses because their development does not require generation of diverse receptor specificities or memory cell lines [21,22]. However, a recent study of tree swallows throughout the nestling period demonstrated that the bactericidal ability of whole blood was at sub-adult level at fledging age [23]. Levels of other innate immune measures, such as natural antibodies and complement, have also been shown to be at immature levels near fledging age in altricial passerines [24,25]. Alternatively, nestlings may rely more on tolerance of infection while immune system components develop. Given that birds early post-hatch are overwhelmed with large quantities of novel foreign antigen and have high energetic demands of growth and development, it may be energetically cheaper to tolerate infection and limit damage rather than invest in immune defenses [26]. Given these previous studies of altricial passerines, this study provides another instance of immunological immaturity and is consistent with the hypothesis that passerine birds are particularly susceptible throughout the nestling period and immediately after fledging. More integrated studies of age-related changes in both passive and active components of immune resistance as well as tolerance

will continue to shed light on the development of effective immune defense in growing altricial birds.

Knowledge of the ontogeny of adaptive immune defense in altricial passerines could have important implications for disease ecology. Passerines play an important role in the spread of zoonotic diseases in the wild, and nestling passerines in particular may be important hosts for vector-borne diseases given their undeveloped defenses and confinement to nests in close proximity to others, making them vulnerable to virus-carrying insects [27,28]. Because detection of active viral infections can be difficult [28], wild birds are often caught and screened for virus-specific antibodies to provide evidence of a past infection. Knowledge of the ontogeny of antibody response can contribute to understanding of patterns of natural viremia in free-living nestlings and to effectively evaluate nestling exposure to zoonotic diseases.

A number of studies have examined the link between body mass and immune response in young birds, to examine proposed trade-offs associated with growth and immune processes competing for shared resources [29]. Adaptive humoral defenses are proposed to incur low costs of use because they are associated with anti-inflammatory cytokines [1,30]. However, development of the adaptive humoral components may be relatively costly, given that generation and rapid proliferation of B-cell lineages with diverse, functional receptor specificities is confined to the energetically demanding developmental period of most vertebrates [30]. Reduced body mass growth was observed in young antigen-challenged precocial quail [31] yet vaccination did not interfere with growth in challenged altricial American kestrels [12] or the zebra finches in our study. Because the developing birds in our study were provided with *ad libitum* food, it is difficult to evaluate the relative costs of immunity. A study of skin-swelling response to phytohemagglutinin (PHA) in captive sand martins (*Riparia riparia)* demonstrated

that the magnitude of PHA response was positively correlated with body mass growth when animals were fed *ad libitum* and negatively correlated with body mass growth when food was restricted 40% for three days [32], highlighting the importance of considering energy intake when evaluating trade-offs in growth and immune response. Future studies of the ontogeny of immune function in birds should examine the relationship between growth and antibody response in the context of a variety of ecologically relevant feeding scenarios to examine the biological costs of developing and mounting specific antibody responses and the sustained impact of environmental perturbations on immune defense and fitness in free-living animals.

Lastly, we found that parental breeding pair was a significant predictor of antibody response, perhaps indicating a genetic component to the magnitude of antibody response. Genetic-based constraints have been posited to influence the strength of immune response [33] and likely contribute to variability in antibody response to diverse antigens in growing birds [14,16,33]. Future experiments should include use of cross-fostering designs or examination of genes involved in recognition (including highly polymorphic MHC loci) to more deliberately examine the contribution of genetic constraints to variability in immune response during development in altricial birds. Additionally, comparative studies of diverse antigens (e.g. live infection versus inert antigens, T-cell dependent versus independent antigens) could contribute knowledge to variability in immune ontogeny in growing birds.

Conclusions

We found that altricial zebra finch nestlings hatch with limited capacity for endogenous adaptive antibody defense and that capacity gradually increases throughout the nestling period.

We found that magnitude of adaptive antibody response of all nestling age groups was significantly lower than that of adults, indicating that completion of immunological development occurs post-fledging in zebra finches. This study serves as a framework for future studies investigating development of immune resistance and tolerance, as well as physiological and fitness consequences of antigen exposure and infection during the energetically demanding nestling period of altricial birds.

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Figure 1.

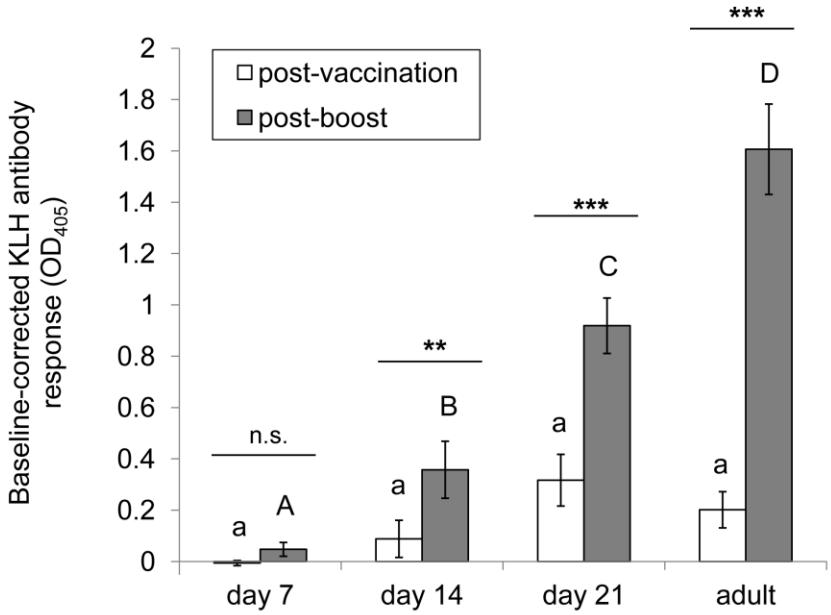

Figure 1. KLH-specific antibody response in vaccinated zebra finch nestlings and adults. Data are means ± s.e.m. (n=13 for 7d and 14d groups, n=14 for 21d and adult groups). Post-priming vaccination (unfilled bars) and post-boost (filled bars) absorbance values were corrected for absorbance values of baseline blood samples on KLH-specific ELISA. Post-vaccination values among age groups that share the same lowercase letter and post-boost values among age groups that share the same uppercase letter are not significantly different as determined by Tukey's *post hoc* tests. Significant differences between post-vaccination and post-boost values within an age group (as determined by Tukey's *post hoc* tests) are denoted by asterisks.

CHAPTER 2

Ontogenetic patterns of constitutive immune parameters in altricial house sparrows

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Abstract

Innate immune functions are proposed to develop rapidly post-hatch in altricial nestlings, compared with adaptive immune defenses that require development of receptor specificity and memory. Studies of ontogenetic changes in altricial birds have been few until relatively recently and often do not encompass the entire developmental period. We examined the patterns of development in constitutive innate and adaptive immune indices in house sparrows nestlings (3, 6, 9, and 12 days (d) post-hatch), hatch-year birds, and adults. Lysozyme activity significantly decreased with age, likely representing catabolism of maternal investment of lysozyme in the egg albumen. Levels of total IgY (indexing adaptive immune function), as well as agglutination and lysis (indexing innate immune function), increased throughout the nestling period, but were significantly below levels found in fully-grown birds at the time of fledging. There were no significant differences between hatch-year birds and adults in these measures, indicating that rapid, full maturation occurs early in the post-fledging period. In combination with previous studies, these data highlight the importance of sampling fledglings to assess full immune ontogeny and suggest that fledgling birds may be more vulnerable to infection than adults.

Introduction

Young animals in general (Firth et al. 2005, Levy 2007), and altricial nestlings in particular (Klasing and Leshchinsky 1999, Palacios et al. 2009), purportedly rely proportionally more on innate immune defenses while the specificity and memory associated with the adaptive immune system develop. Indeed, induced specific adaptive antibody responses were immature (i.e., at levels significantly reduced compared with adults) in nestling kestrels (Smits and Bortolotti 2008) and recently were shown to be immature at the time of fledging in zebra finches (Killpack and Karasov 2012b). Measures of total circulating immunoglobulins in house sparrows also conform to this pattern of significant immaturity near the time of fledging (King et al. 2010).

Constitutive innate immune functions, such as neutralization and agglutination by natural antibodies, and lysis by complement and antibacterial proteins (Ochsenbein and Zinkernagel 2000, Merchant et al. 2006, Millet et al. 2007), may reach mature levels more rapidly post-hatch compared with adaptive immune functions. Constitutive innate defenses do not require previous antigen exposure and their use purportedly incurs relatively low costs in terms of energy expenditure and behavioral changes (Lee 2006, Millet et al. 2007). However, studies conducted in free-living altricial passerines, which have short incubation times and that, as a group, have the fastest growth rates of land animals (Case 1978, Arendt 1997), show contrasting results regarding the time course of maturation of constitutive innate immune components. A study comparing nestling and adult tree swallows (*Tachycineta bicolor;* fledging age ~20d) found mixed support for the hypothesis that innate components develop prior to adaptive components (Palacios et al. 2009). No increase in natural antibody agglutination or complement-mediated lysis activity was observed throughout the nestling period and levels in late-stage tree swallow nestlings were significantly lower than in adults (Palacios et al. 2009). Bacterial killing capacity

of whole blood was also significantly lower in late-stage tree swallow nestlings compared with adults (Stambaugh et al. 2011). In 12-day old barn swallow nestlings (*Hirundo rustica;* fledging age ~20d), levels of the antibacterial protein lysozyme (Saino et al. 2002) and of complement-mediated lysis (Møller and Haussy 2007) were similar to those in adults, yet 12-day nestlings had significantly lower agglutination scores compared with adults (Møller and Haussy 2007). In great tits (*Parus major;* fledging age ~20d) late-stage nestlings had mature agglutination activity, yet complement-mediated lysis activity was completely undetectable until after fledging (De Coster et al. 2010).

While the studies cited all used the same assay to simultaneously measure agglutination and lysis (Matson et al. 2005), their use of different volumes and concentrations of red blood cell antigen may have led to differences in patterns of innate immune capacity observed in nestlings. Observed differences in patterns among innate immune measures could also be explained by differences in the life history strategies and local disease pressure among those birds (Klasing and Leshchinsky 1999, Lee 2006, Lee et al. 2008), which reinforces the need for comparative studies of immune function in altricial bird species. Additionally, immune function of introduced, invasive species is suggested to differ from species in their native habitats (Lee et al. 2005, 2006), and the ontogeny of immune function might also be expected to differ in invasive versus native species.

Light would be shed on the maturation of immune functions by more systematically measuring levels of innate and adaptive immunity across the period of rapid nestling growth and through adulthood in individual species of birds. In the present study, we examined the ontogeny of constitutive immune parameters in free-living altricial house sparrows *Passer domesticus*, an introduced invasive species in the United States. To understand patterns of ontogeny through the

full developmental period (fledging age ~14d), we sampled nestlings throughout nearly the whole nestling period (3, 6, 9, and 12 days post-hatch) as well as hatch-year birds capable of sustained flight and known to have fledged during the current reproductive season (2-3months) and adult birds (1+ year). We examined the maturation of three distinct components of constitutive innate immune defense using separate assays of natural antibody-mediated agglutination, complement-mediated lysis, and antibacterial lysozyme levels. We also measured total circulating IgY levels throughout development as an index of adaptive immune function and for comparison with timing of innate immune development. We optimized our assays for detecting immune responses in nestling birds, which allowed for better determination of whether previous findings of lack of signal are a methodological artifact or indicate biologically real changes in immune function (Palacios et al. 2009, De Coster et al. 2010). Also, because previous studies on altricial nestlings have shown that some components of immunity are at sub-adult levels at fledging age (Møller and Haussy 2007, Palacios et al. 2009, King et al. 2010, De Coster et al. 2010, Stambaugh et al. 2011), incorporating measurements of hatch-year birds provided information on continued immunological development post-fledging. In addition, we examined the influence of structural size, body condition, and date of hatch on immune function development during life in the nest, and the influence of condition, sex, and age on immune function post-fledging.

Methods

Monitoring, measuring, and sampling of house sparrows

House sparrow nest sites, both wooden nest boxes and natural nests, were located in dairy barns on the University of Wisconsin-Madison campus during the summer of 2011. From mid-

May to mid-July we visited the known active nesting sites daily during a specified time window to ensure accurate and consistent aging of nestlings. At each visit, we counted eggs and recorded the date that a new nestling was found (recorded as 'day 0' of age for that nestling). Nestlings were individually marked with non-toxic colored permanent markers and returned to the nest so that each bird could be distinguished from its nest mates at a later date (average brood size=4 nestlings). Nestling body mass (±0.01g) and tarsus length (to the nearest 0.1 mm with digital calipers) were measured daily beginning at day 3 post-hatch. Natural parents raised nestlings until 3, 6, 9, or 12 days post-hatch, at which time nestlings were collected for analysis of immune measures. Birds collected were from 24 different broods among 18 nest sites and we systematically distributed nestlings to minimize biases of nest and hatch order on the sampling age groups. At sampling age, we weighed, euthanized with CO_2, and then decapitated nestlings for blood collection. Euthanasia was required for collection of sufficient plasma volumes from birds (particularly those in younger age groups) for use in the three separate immune assays. Blood samples were centrifuged, and plasma aliquots were stored at -20°C for later use in agglutination, lysozyme and ELISA assays and at -80°C for use in hemolysis assays. We took final tarsus measurements after blood sampling.

Free-living hatch-year and adult birds were also collected during the fall of 2011 for comparison of innate immune measures. Age and sex of birds were reliably determined through inspecting skull ossification and plumage, respectively (Pyle 1997). We restricted our hatch-year samples to individuals with ossification scores of 1 (scale: 0-6) (Pyle 1997). After collection in mist nets, we weighed, euthanized with CO_2, and collected blood from the hatch-year and adult birds. Plasma was stored and tarsus measurements were taken as they were for the nestlings.

Hemagglutination assay

We modified the previously described hemolysis-hemagglutination assay (Matson et al. 2005) to optimize detection of natural antibody agglutination activity (if present) in young nestlings. We heat inactivated-complement in the plasma samples (30 minutes at 56°C to inactivate complement; Matson et al. 2005) to allow for accurate scorer recording of agglutination activity alone. Briefly, 25µL of 0.01 M phosphate buffered saline (PBS, Sigma#P3744, St. Louis, MO) was pipetted into columns 2-12 of a round-bottom 96-well plate (Corning Costar#3795, Corning, NY), washed once with PBS. Then, 25µL of heat-inactivated pooled chicken plasma standard (positive control) were pipetted into columns 1 and 2 of row 1, and 25µL of heat-inactivated house sparrow plasma samples were pipetted into columns 1 and 2 of the remaining rows of the plate. Using a multi-channel pipette, samples from column 2 were serially diluted (1:2) through column 11 (allowing for undiluted plasma wells in column 1 and PBS-only negative control wells in column 12). Then 2µL of a 2% rabbit blood suspension were added to all wells. The plates were gently shaken, covered with an acetate plate sealer (Thermo #3501, Milford, MA) and incubated in a 37 °C water bath for 90 min. Plates were then removed from the water bath and tilted at a 60-degree angle for 20 minutes at room temperature to enhance the visualization of agglutination. Plates were scanned at 200 dpi using the positive transparency setting of a flatbed scanner (HP Scanjet 3970). Agglutination scores for each sample were rated as the last well in a dilution series exhibiting agglutination.

Hemolysis assay

When measuring lysis activity using the hemolysis-hemagglutination assay (Matson et al. 2005), scores for adult house sparrows in our pilot studies were low (unpublished data) and

scores for nestlings in previously published studies were either zero (De Coster et al. 2010) or showed no age-related changes from 4-20 days old (Palacios et al. 2009). Therefore we chose to modify a separate *in vitro* assay that employs spectrophotometric methods for detection of complement-mediated lysis activity in house sparrows in our study (Merchant et al. 2006). Twenty microliters of plasma sample were diluted in 60µL of PBS in microcentrifuge tubes and 80µL of 2% rabbit red blood cell solution were added to each tube. Negative control tubes were prepared using 20µl of heat-inactivated plasma (30 minutes at 56°C to inactivate complement) and positive control tubes were prepared using heat-inactivated plasma as well as 2% rabbit red blood cell solution vortexed with Triton-X 100 detergent (to lyse all red blood cells). Tubes were incubated in a 37°C water bath for 30 minutes, then centrifuged for 2 minutes at 2000 x g to pellet any unlysed red blood cells. Then, 40µl of supernatant from each tube were added in triplicate to a round-bottom 96-well plate, and absorbance (or optical density, OD) was read at 540nm using an automated microplate reader. Hemolysis measures are reported as (OD of sample)-(OD of negative control).

Lysozyme assay

We modified a previously described procedure to measure the antimicrobial activity of plasma lysozyme (Millet et al. 2007). Briefly, 0.01 g of *Micrococcus lysodeikticus* and 20 ml of liquefied 1% sterilized agarose were added to a sterile 50 ml conical tube, quickly vortexed, and placed in a 55°C water bath. Serial dilutions of lysozyme standards were made using sterile 0.1 M sodium phosphate buffer, and 7µl of each dilution, as well as blanks (buffer only), were added in triplicate to a 96-well plate to serve as plate controls. Seven microliters of each plasma sample were added in duplicate to the plate. Next, 150µl of bacterial-agar solution were added to all

wells, the plate was incubated at 37°C for 16-20 hrs, and absorbance was recorded at 555 nm using an automated microplate reader. Lysozyme activity was reported as (OD of blank)-(OD of sample).

Total IgY Enzyme-linked immunosorbent assay (ELISA)

We modified a previously described direct ELISA to determine total IgY in house sparrows (Fassbinder-Orth et al. 2013). A flat-bottomed Immulon-4 96-well plate (Dynex Technologies) was coated in triplicate with 100 µl per well of house sparrow plasma samples (and positive and negative plate control samples), diluted to 1:100 in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6). The plate was incubated overnight at 37˚ C. The coating solution was removed, 200 µl of blocking buffer (PBS with 5% non-fat dry milk, 0.05% Tween) were added to each well, and incubated at room temperature for 30 minutes. The plate was washed four times with wash buffer (PBS with 0.05% Tween) using an automated plate washer. Fifty µl of the detecting horseradish peroxidase- conjugated goat anti-bird IgG (=IgY) (Bethyl Laboratories, Inc. #A140-110P) were added at 1:1000 in blocking buffer, incubated at 37˚ C for 1 hour, and washed. One hundred µl of ABTS substrate were added to each well, the plates were incubated for 5 minutes, and the reaction was stopped with 100 µl of 1% SDS. Optical densities of the wells were read at 405 nm using an automated plate reader and average absorbance values of triplicate wells were recorded. Total IgY measure was reported as (OD of sample)-(OD of negative control).

Analyses

All tests were carried out using R statistical package (R Development Core Team 2008).

ANOVA and Tukey's post-hoc analyses were used to compare body and immune measures across all age groups (analyzed as a categorical variable because the precise age in days of the hatch-year and adult birds was unknown).

To assess the effects of individual variation on constitutive immune parameters during life in the nest (3, 6, 9, 12 d post-hatch), we fit linear mixed models using maximum likelihood criteria, with structural size, body condition, and hatch date as continuous fixed effects. Nest was included as a random, categorical variable to control for inter-sibling consistency in immunological responses. Single-term deletion Chi-square-based tests were then used to determine the significance of individual fixed effect predictors. Structural size was calculated as the cube of tarsus length, which was used to create an index of body volume (Killpack and Karasov 2012a). Body condition was reported as the residuals from the linear regression of body mass on tarsus length ($F_{1,82}$=1006, $p<0.001$, R^2=0.92, linearity confirmed by even distribution of residuals after log-transformation of the data (Schulte-Hostedde et al. 2005)). All interactions between predictor variables in the mixed models were non-significant and removed from the models before testing for the main effects of structural size, condition, and hatch date on immune measures during life in the nest. There were no significant correlations among the predictor variables in the mixed models (tarsus cubed, body condition, and hatch date), though tarsus-cubed was found to be significantly correlated with nestling age (r=0.91, Holm-adjusted $p<0.001$). Thus, a significant effect of structural size on immune function is reflective of age effects. Inclusion of structural size in the mixed models allowed for determination of additional influences of condition and hatch date on innate function when developmental size (age) was taken into account.

To assess the effects of individual variation in condition, developmental age and sex on

constitutive immune functions in post-fledging birds (hatch-year and adults), we fit linear fixed effects models and using single term deletion Chi-square-based tests. All interactions between predictor variables in the models were non-significant and removed from the models before testing for the main effects.

Constants were added to the corrected hemolysis and lysozyme values to make them positive and non-zero prior to log-transformation in order to meet assumptions of normality and homogeneity of variance required for analyses. In all tests the significance level was set at $p<0.05$ and $0.05<p< 0.10$ was considered to indicate a non-significant trend. Results are given as means ± 1 SEM.

Results

Maturation of body and constitutive immune measures

Body measures significantly increased with age, though timing of maturation differed between traits (Fig 1). Body mass significantly increased through 9 days post-hatch, at which time body mass did not significantly differ from hatch-year or adult birds (Fig 1). Birds at 12-days post-hatch had significantly lower body mass compared with hatch-year and adult birds. Tarsus length significantly increased with age until reaching mature adult length by 9 days post hatch (Fig. 1).

Constitutive total IgY levels significantly increased with age ($F_{5,70}=31.8$, $p<0.001$), yet total IgY levels were significantly below mature adult levels at the end of the nestling period (12d) (Fig 2). Total IgY increased to mature levels following fledging, as levels in hatch-year birds did not significantly differ from adults (Fig 2).

Innate immune activities significantly differed with age, yet the patterns of maturation varied by the functions measured (Fig 3). Hemagglutination activity did not significantly differ throughout the nestling period, but increased to mature adult levels after fledging, as levels in hatch-year birds did not significantly differ from adults ($F_{5,76}$=22.9, p<0.001) (Fig 3a). Hemolysis activity significantly increased throughout the nestling period but mature adult levels were not reached until after fledging ($F_{5,67}$=20.7, p<0.001) (Fig 3b). Hemagglutination and hemolysis were measured in separate assays, yet hemolysis scores were significantly, positively correlated with average agglutination scores (r= 0.69; $F_{1,69}$= 63.85, p<0.001).

In contrast to the other two innate immune measures, lysozyme activity was highest in younger nestlings and significantly decreased with nestling age ($F_{5,77}$=8.3, p<0.001) (Fig 3c). There was a non-significant trend for higher lysozyme score in 9d (p=0.053) and 12d nestlings (p=0.085) compared with hatch-year birds.

Factors explaining individual variation in constitutive immune measures

Life in the nest

Tarsus-cubed, an index of structural size, was significantly, positively associated with constitutive total IgY levels (Chi Square: 26.66, d.f.=1, p<0.001) and hemolysis activity (Chi Square: 19.89, d.f.=1, p<0.001) and marginally associated with hemagglutination activity (Chi Square: 3.78, d.f.=1, p=0.052), as expected given age-related trends. Hatch date was significantly, negatively related to hemagglutination activity (Chi Square: 4.25, d.f.=1, p=0.039) and hemolysis activity (Chi Square: 6.42, d.f.=1, p=0.011) among nestling age groups, yet did not account for significant variation in constitutive total IgY levels (Chi Square: 0.423, d.f.=1,

p=0.516). Increased body condition had a significant, negative effect on total IgY levels (Chi Square: 4.78, d.f.=1, p=0.029) during life in the nest, but did not significantly explain variation in hemagglutination activity (Chi Square: 0.07, d.f.=1, p=0.795) or hemolysis activity of nestlings (Chi Square: 0.27, d.f.=1, p=0.603). Individual variation in lysozyme activity during life in the nest was negatively, but not significantly related to tarsus-cubed (Chi Square: 3.29, d.f.=1, p=0.070). Hatch date (Chi Square: 0.854, d.f.=1, p=0.355) and body condition (Chi Square: 0.56, d.f.=1, p=0.456) did not significantly impact lysozyme activity during life in the nest.

Post-fledging

Among post-fledging birds (hatch year and adult), constitutive total IgY levels were significantly, positively related to age (Chi Square: 5.487, d.f.=1, p=0.019), but did not significantly differ with body condition (Chi Square: 1.262, d.f.=1, p=0.261) or sex (Chi Square: 0.002, d.f.=1, p=0.963). Hemagglutination activity post-fledging was significantly, positively related to body condition (Chi Square: 9.96, d.f.=1, p=0.002) and marginally, positively related to age (Chi Square: 3.58, d.f.=1, p=0.059). Sex did not significantly impact hemagglutination activity (Chi Square: 1.44, d.f.=1, p=0.230). Hemolysis activity post-fledging was not significantly influenced by age (Chi Square: 0.023, d.f.=1, p=0.881) or condition (Chi Square: 1.55, d.f.=1, p=0.214) , but was related to sex, with male birds having significantly higher hemolysis activity than females (Chi Square: 7.77, d.f.=1, p=0.005). Finally, lysozyme activity post-fledging was not significantly impacted by age (Chi Square: 1.57, d.f.=1, p=0.211), condition (Chi Square: 2.26, d.f.=1, p=0.133) , or sex (Chi Square: 0.015, d.f.=1, p=0.903).

Discussion

Maturation of constitutive immune measures

Our results cast doubt on the validity of the commonly invoked hypothesis that nestlings rely proportionally more on constitutive innate defenses during early life while the specificity and memory associated with adaptive immunity develops (Klasing and Leshchinsky 1999, Lee 2006). It is difficult to experimentally test which arm makes a proportionally greater contribution to immune protection during ontogeny. One can, however, compare functional measures during ontogeny to those in adults. A test for differential development rates among simultaneously-measured innate and adaptive functions showed mixed support for the hypothesis, with some adaptive immune measures in tree swallow nestlings reaching mature levels prior to innate measures (Palacios et al. 2009). We further examined the ontogeny of constitutive adaptive and innate immune functions in free-living nestling (3, 6, 9, and 12 days post-hatch), hatch-year (2-3 months), and adult (1+ year) house sparrows. We found that total circulating IgY levels (an index of adaptive antibody function) gradually increase throughout the nestling period, as observed in other studies of circulating antibody levels in early and late-stage nestlings (Koppenheffer and Robertson 1980, Pihlaja et al. 2006, Grindstaff et al. 2006, King et al. 2010). Circulating IgY levels in the house sparrows in our study were significantly below mature adult levels near the time of fledging (12d), in agreement with findings of circulating and induced antibody levels in growing passerines in comparison with adults (King et al. 2010, Killpack and Karasov 2012b, respectively). These data support the hypothesis that adaptive antibody responses develop slowly during the nestling period, perhaps reflecting the time required to generate peripheral lymphocyte populations with diverse, functional receptor specificities (Klasing and Leshchinsky 1999, Lee 2006).

We found that measured innate immune components also develop slowly post-hatch and are immature near fledging age (12d). Similar to results found in tree swallow nestlings (Palacios et al. 2009), hemagglutination scores did not significantly increase with age throughout the nestling period. In contrast to studies in tree swallow and great tit nestlings (Palacios et al. 2009, De Coster et al. 2010), we did detect significant age-related increases in hemolysis activity throughout the nestling period; however hemolysis levels were still immature at 12d. Unlike previous studies, we measured natural antibody hemagglutination and complement-mediated lysis activities in separate functional *in vitro* assays that were optimized to detect levels of activity in nestling plasma. Lysis activity is functionally related to agglutination activity by the classical pathway of complement activation. By this pathway, antigen-bound antibody molecules (often IgM and natural antibodies) initiate a cascade of complement binding and hydrolysis that leads to, among other effects, lysis of the antigen cell (Kindt et al. 2007). In our separate *in vitro assays* we found a significant correlation between hemagglutination and hemolysis activities among individuals, reinforcing the notion of this functional relationship (though complement activation via other pathways may also be involved) (Kindt et al. 2007). Because the separate *in vitro* hemolysis assay uses objective spectrophotometric measures of activity, rather than subjective scoring, it may be a more appropriate assay to detect ontogenetic and interspecific differences in innate immune activity in future ecological immunology studies.

In combination with previous findings that constitutive innate immune measures are immature in passerine species near fledging age (Møller and Haussy 2007, Palacios et al. 2009, De Coster et al. 2010, Stambaugh et al. 2011), the present study begs the question of how much these nascent innate defenses can be relied upon during the nestling period while adaptive defenses develop. Therefore, we propose a testable prediction that nestlings and fledglings are

more vulnerable to infection than adults, based upon the mounting evidence of immature innate and adaptive immune functions in birds at this age. Future experimental studies should incorporate live infection models to examine the protection provided by nascent innate and adaptive immune defenses and should extend study measures to examine the roles that passively transferred immune components, tolerance, and other physiological factors play in the fitness consequences of infection in physiologically immature altricial birds.

In contrast to the other immune measures, lysozyme activity in nestling circulation declined with age. This pattern of decreasing plasma lysozyme, also observed in growing precocial prairie chickens (Meier et al. 2012), likely represents decay of maternal investment of lysozyme in the egg albumen. Rapid decrease could be attributed to high rates of metabolism and possibly dilution of maternal lysozyme in the increasing blood volume of rapidly growing birds, as has been proposed with decay of maternal antibodies in house sparrow nestlings (Nemeth et al. 2008). While maternal allocation of lysozyme may provide passive immune protection in nestlings, lysozyme may not be a good indicator of the development of endogenous, innate immunity in these growing birds.

Variation in constitutive immune measures in nestlings

In addition to age-related changes throughout ontogeny, we examined the impact of individual variation in structural size (a continuous variable that correlated with age), body condition, and hatch date on immune function during life in the nest. We found a negative relationship between hemagglutination and hemolysis measures with hatch date, indicating that nestlings hatching later in the breeding season may have reduced innate immune defenses. Late-season chicks may be from second or third broods and of lower quality compared with those

from earlier hatched broods. Reduced antioxidant capacity has been observed in passerines from late-season broods (Norte et al. 2009, Costantini et al. 2010, Serra et al. 2012), which may reflect seasonal changes in diet quality, parental phenotype, or parental reproductive investment (discussed in Serra et al. 2012).

Our index of nestling condition (residuals from the regression of body mass on tarsus length) was significantly, negatively related to total IgY levels. This is in agreement with patterns in late hatch-order 12d barn swallows, which had reduced body condition and increased IgY concentrations compared with earlier hatched brood mates (Saino et al. 2001). In contrast, hemagglutination or hemolysis activities were not significantly related to body condition in nestlings, in agreement a study in tree swallow nestlings (Palacios et al. 2009). This may reflect the independence of constitutive innate immune components from environmental variation (Matson et al. 2005) or the purportedly low energetic costs to develop and use constitutive innate immune components in comparison with inducible or specific immune components (Klasing and Leshchinsky 1999, Lee 2006). Alternatively, meaningfully relating condition to immune response in growing birds may be inappropriate. The condition index may reflect developmental patterns rather than nestling quality, and during growth the immune system is not fully developed and cannot respond maximally, even if body condition is high (Martin et al. 2006). Studies using controlled food restriction or manipulation of diet nutrient composition throughout the nestling period are needed to more accurately determine the resource-dependence of innate immune development and function and the potential energetic trade-offs between growth and immune function in young altricial birds (Brzek and Konarzewski 2007, Hasselquist and Nilsson 2012).

Immune development and variation post-fledging

While we found immune parameters were significantly below mature adult level near the end of the nestling period (12d), we found no significant differences in immune measures between hatch-year birds and adults. These data suggest that maturation of nestling constitutive adaptive and innate immune parameters occurs within several weeks post-fledging. Continued immunological development in fledglings occurs coincident with exposure to increased diversity in the antigenic environment, which may present increased infection risk, but may also stimulate development of more robust constitutive immune measures in comparison with nestlings (Parejo and Silva 2009, De Coster et al. 2010). When examining individual variation in immune measures among the post-fledging groups of birds, we found that hemagglutination activity was positively related to body condition and that hemolysis activity was significantly reduced in female birds compared with males. Reduced hemolysis activity in adult females may indicate tradeoffs experienced by reproductive females recovering from increased energy demands of breeding, as has been observed in other studies of immune function in reproductive passerines (De Coster et al. 2010). However, this does not explain reduced hemolysis activity in hatch-year females compared with males, given that those females fledged in 2011 and were unlikely to have bred that summer. Total IgY levels, but not constitutive innate measures, were significantly, positively related to age, which may indicate that adaptive immune measures continue maturation longer than innate measures. These associations may serve as hypotheses to stimulate future, detailed studies of factors that contribute to variation in immune responses in this understudied age class of juvenile birds in comparison with adults.

In summary, we showed that, despite reaching adult size, near-fledgling birds have levels of constitutive adaptive and innate immune functions that are significantly lower than those in

fully grown birds. We showed that maturation of constitutive immune parameters occurs within several weeks post-fledging, as measures in hatch-year birds did not significantly differ from adults. Future studies should continue to focus on development during the post-fledging period to provide a more complete picture of immune ontogeny and that factors that influence it, and to examine the vulnerability of fledglings to infection compared with adults.

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Figure Legends:

Figure 1. Body mass and tarsus growth. Data are means ± SEM and sample sizes are indicated in parentheses below each age group (Error bars are smaller than the size of the symbols). Within a body measure, values that share the same letter are not significantly different as determined by Tukey's *post hoc* tests.

Figure 2. Ontogeny of total circulating IgY antibodies. Data are means ± SEM and sample sizes for each immune measure are indicated in parentheses below each age group. Values that share the same letter are not significantly different as determined by Tukey's *post hoc* tests.

Figure 3. Ontogeny of agglutination (A), lysis (B), and lysozyme (C) activities. Data are means ± SEM and sample sizes for each immune measure are indicated in parentheses below each age group. Within an immune measure, values that share the same letter are not significantly different as determined by Tukey's *post hoc* tests.

Figure 1.

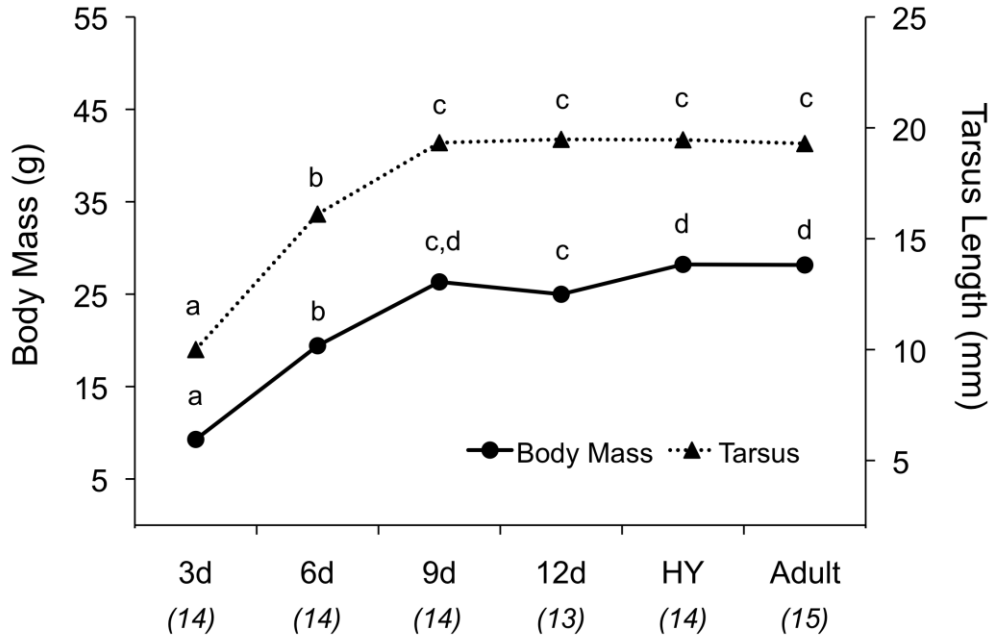

Body Mass (g)
Tarsus Length (mm)
55
45
35
25
15
5
25
20
15
10
5
a
b
c,d
c
d
d
a
b
c
c
c
c
Body Mass
Tarsus
3d
(14)
6d
(14)
9d
(14)
12d
(13)
HY
(14)
Adult
(15)

Figure 2.

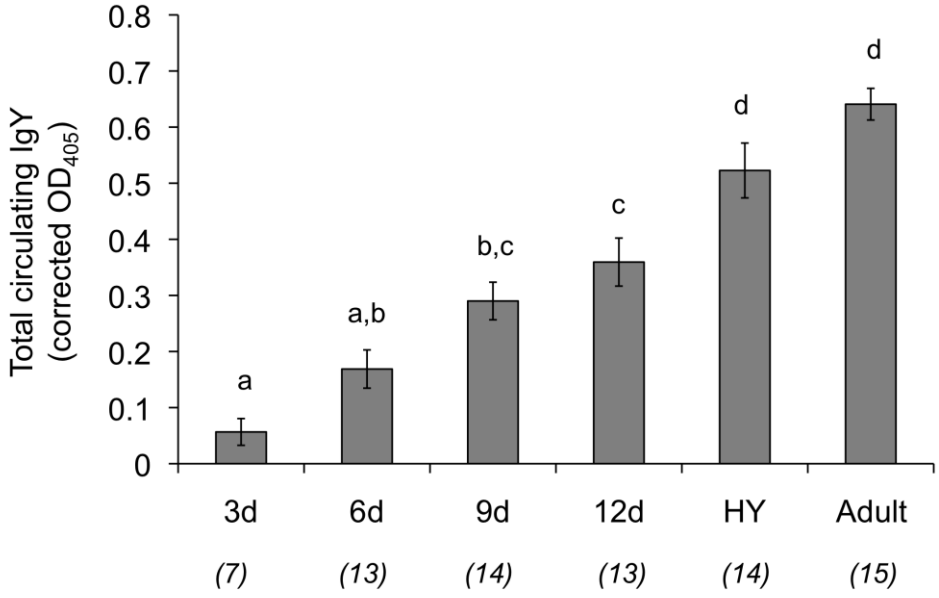
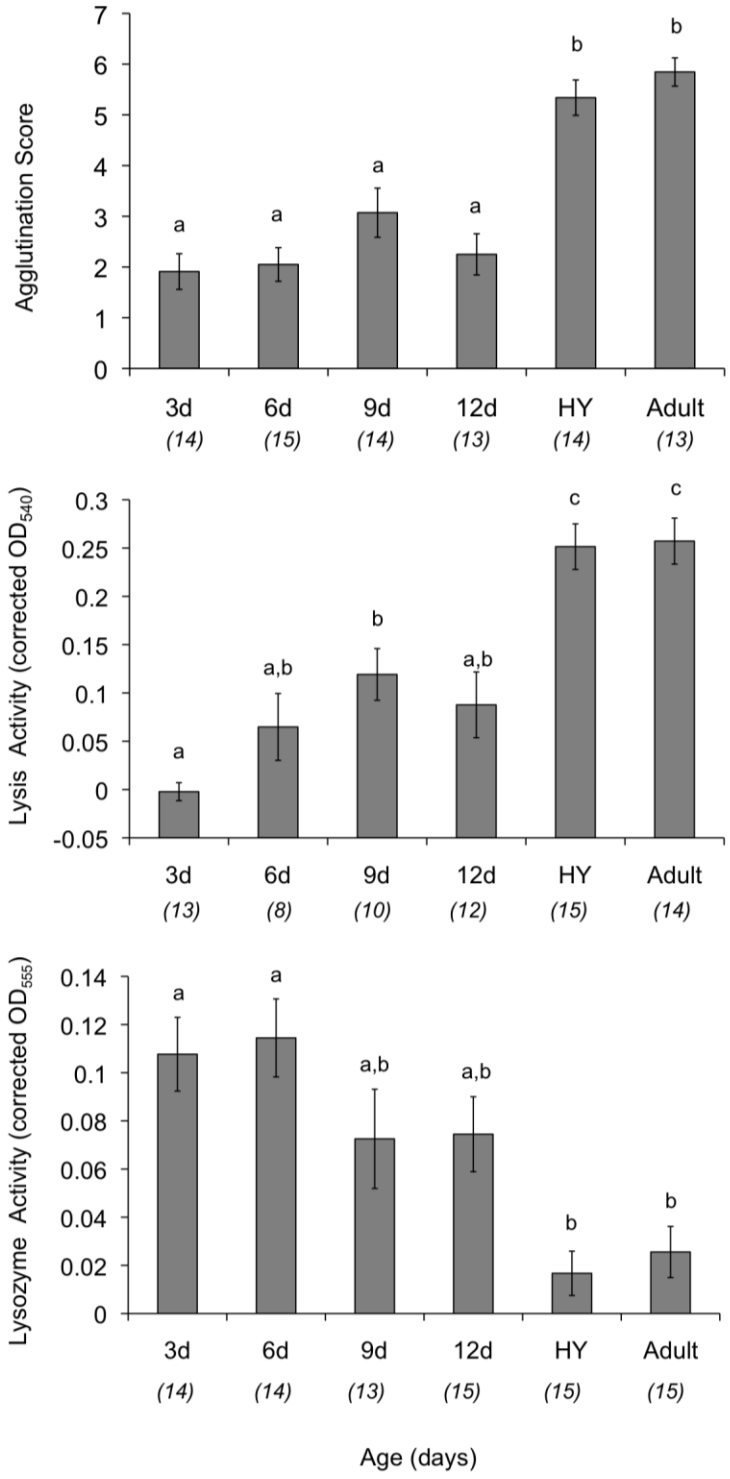

Total circulating IgY
(corrected OD405)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a,b
b,c
c
d
d
3d
6d
9d
12d
HY
Adult
(7)
(13)
(14)
(13)
(14)
(15)

Figure 3.


Agglutination Score
7
6
5
4
3
2
1
0
a
a
a
a
b
b
3d (14)
6d (15)
9d (14)
12d (13)
HY (14)
Adult (13)
Lysis Activity (corrected OD540)
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
a
a,b
b
a,b
c
c
3d (13)
6d (8)
9d (10)
12d (12)
HY (15)
Adult (14)
Lysozyme Activity (corrected OD555)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
a,b
a,b
b
b
3d (14)
6d (14)
9d (13)
12d (15)
HY (15)
Adult (15)
Age (days)

CHAPTER 3

Adaptive immune function and body composition in Zebra Finches following compensatory growth

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ABSTRACT.

Compensatory strategies in birds have evolved that minimize the effects of poor rearing conditions on future survival and fitness. Nestlings may accelerate growth and/or development relative to age, termed compensatory growth, to achieve normal asymptotic size at the same time as well-fed birds. While compensatory growth allows birds to fledge at a suitable size and time, it may have persistent detrimental effects on the development of complex systems, such as the adaptive immune system. The current study examines compensatory growth following short term food restriction during the nestling period and tests for post-restriction effects on adaptive immune function and body composition in captive, altricial Zebra Finches, *Taeniopygia guttata*. Compensatory body mass, but not tarsus and culmen, growth was observed in previously food restricted birds. No impact on nestling adaptive antibody response to a model antigen post-restriction was observed, despite food restriction and compensatory growth occurring during the time of development of this arm of the immune system. Reductions in tissue maturity, indexed by tissue water content, were observed following compensatory growth, indicating a decoupling of chronological age and physiological age as a result of accelerated growth in food-restricted birds. These data provide evidence of capacity for rapid, compensatory body mass growth in nestling Zebra Finches and suggest that the energetic costs of body growth and development are relatively larger than costs of development of the humoral immune system.

INTRODUCTION

PERIODS OF FOOD restriction can be a common occurrence for growing altricial birds, which hatch in a relatively undeveloped state and rely on parental care and delivery of food during the nestling period. Compensatory strategies have evolved that minimize the effects of poor rearing conditions on future survival and fitness. Birds experiencing slowed growth and/or development resulting from a period of resource limitation may extend the growth and developmental period to achieve asymptotic size (Schew and Ricklefs 1998). Alternatively, once food becomes freely available, they may accelerate growth and/or development relative to age, termed compensatory growth, to achieve normal asymptotic size at the same time as well-fed birds (Schew and Ricklefs 1998).

While compensatory growth allows birds to fledge at a suitable size and time, it may have persistent detrimental effects (Metcalfe and Monaghan 2001). In Zebra Finches, *Taeniopygia guttata,* early resource limitation during the nestling period has been associated with reduced post-fledging survival (DeKogel 1997) and reduced lifespan (Birkhead et al. 1999), despite adult birds attaining similar size to well-fed controls. Decreased flight performance was observed in adult Zebra Finches following compensatory growth (Criscuolo et al. 2011), perhaps reflecting reduced tissue differentiation, which has been observed in Japanese quail artificially selected for rapid growth (Shea et al. 1995). Compensatory growth has also resulted in reduced cognitive function (Fisher et al. 2006), increased susceptibility to oxidative damage (Alonso-Alvarez et al. 2007), and elevated adult resting metabolic rates (Criscuolo et al. 2008) in Zebra Finches. These phenotypic effects may be manifestations of oxidative stress or altered development of complex

tissue and organ systems resulting from accelerated growth (Alonso-Alvarez et al. 2007, Criscuolo et al. 2008).

Compensatory growth may alter another complex system, the immune system, if resources are allocated to rapid growth at the expense of the development of immune cells and receptors during the nestling period (Norris and Evans 2000). Artificial selection for rapid growth has been linked to decreased immune function and increased pathogen susceptibility in a variety of taxa including mice, salmon, turkeys, and chickens (Bayyari et al. 1997, Bradford and Famula 1984, van der Most et al. 2011, Smoker 1986). However, the effect of early food restriction and compensatory growth on adaptive immune function in Zebra Finches and other non-domesticated passerines has not been extensively studied. Development of adaptive humoral components in birds is purportedly costly because it requires generation and proliferation of B-cell lineages with diverse, functional antibody specificities (Lee 2006). Because Zebra Finches develop capacity for adaptive antibody response during the early weeks post-hatch (Killpack and Karasov 2012b), food restriction and compensatory growth during that time may reduce the ability to mount diverse functional antibody responses later in life.

The current study examines compensatory growth and tests for effects on adaptive immune function development and body composition in captive, altricial Zebra Finches, *Taeniopygia guttata*. While compensatory growth in Zebra Finches has been previously reported (Alonso-Alvarez et al. 2007, Criscuolo et al. 2008, 2011; Fisher et al. 2006, Krause and Naguib 2011), those studies examined the impact of inadequate feeding conditions (e.g. enlarged brood sizes, low quality diets) lasting the majority or entirety of the nestling period, and measured compensatory growth responses over a 15-day or longer period post-fledging following return to adequate feeding conditions. Because Zebra Finches grow rapidly to adult size during the first

two weeks of the approximately 21- day nestling period (Brzek et al. 2010), we were interested in examining capacity of food-restricted nestlings to rapidly "catch-up" to control size during the nestling period. Therefore, we imposed a restriction of seed quantity for 72 h during the nestling period (6d-9d post-hatch). We then used two criteria to evaluate compensatory growth upon return to *ad libitum* feeding: accelerated growth rate in the previously food-restricted birds compared with controls in the first 72 h of re-feeding (9d-12d post-hatch), and whether food-restricted birds reached the same asymptotic size at the same time as control-fed birds. We chose two criteria because there is no uniform or even consensus protocol in the literature for demonstrating compensatory growth (cf., Alonso-Alvarez et al. 2007, Bize et al. 2006, Lepczyk and Karasov 2000), and both of these criteria correspond well operationally to the concept. We demonstrated compensatory growth in body mass, but not skeletal structures, and examined whether trade-offs in humoral immune development and tissue maturation, indexed by decreasing tissue water content (Choi et al. 1993, Marsh and Wickler 1982) are evident following restriction and compensatory growth. Hence, this is the first study in a rapidly growing altricial bird to test for compensatory growth entirely during the nestling period and for an associated effect on immunity during this period when they are still developing adult-level capacity for adaptive antibody production.

METHODS

Experimental design.–All experimental procedures were approved by the University of Wisconsin-Madison animal care and use committee (permit no. 1421). Pairs of adult Zebra Finches were taken from our stock population and housed in individual breeding cages. Birds

were maintained under standardized conditions of 14 h:10 h L:D photoperiod (lights on 0600 h), 21-24 °C temperature, and 40-50% relative humidity. Breeding pairs were provided with nesting material and a nest box. Prior to their chicks hatching, pairs were provided with a seed diet consisting (by mass) of 70% commercial seeds and 30% Mazuri Small Bird Breeder food supplement (hereafter: seed mix), cuttlefish bone (calcium source), grit, and water *ad libitum*. Enrichment foods of green vegetables and egg food mixture (hard-boiled eggs with shells, dried Volkman Featherglow egg food, and vitamin and mineral supplement) were provided to breeding pairs three times per week. There was no food restriction during the egg laying period.

Upon hatching of the first chick, enrichment feeding ceased, and cage groups (composed of two parents and their brood) were randomly assigned to either control or food-restricted experimental feeding treatment. Broods were culled to have a maximum of three nestlings, though some pairs successfully hatched only one or two nestlings. There was no significant difference in brood size between control (2.54 ± 0.2 nestlings) and food restricted groups (2.17 ± 0.2 nestlings) (P=0.19; t-test). If pairs had multiple broods, experimental treatment assignments were alternated. Cages were visited every day of the experiment between 1400 and 1600 h CST. Because Zebra Finches hatch asynchronously, average ages of nestlings in a brood were used to determine timing of imposed experimental treatment. Control and food restricted groups were fed *ad libitum* seed mix until the average age of nestlings in the brood was 6 days post-hatch (experiment days -6 to 0). There was no significant difference in nestling age at the start of the food restriction (experiment day 0) between control (5.9 ± 0.1 days) and food restricted (6.0 ± 0.1 days) groups ($P > 0.8$). Beginning on experiment day 0 and ending on experiment day 3 (72 h duration), food restricted groups were given 70% of *ad libitum* seed mix. The imposed food restriction level of 70% of *ad libitum* was calculated based on the percentage of seed mix that

was edible food (i.e. not seed hulls) and the average daily food consumption in the three days prior to experiment day 0. Food-restricted groups were returned to *ad libitum* feeding on experiment day 3 and fed *ad libitum* until the feeding experiment ended on experiment day 16. Control groups were fed *ad libitum* for the duration of the experiment. A total of 33 nestlings in 13 broods of 9 parental pairs comprised the control treatment and 26 nestlings in 12 broods of 9 parental pairs comprised the food restriction treatment.

Daily measurement of nestlings, adults, and food consumption.–Body mass (±0.01 g) and tarsus and culmen lengths (±0.10 mm using digital calipers) of nestlings were measured during daily visits (1400 – 1600 h) from experiment days -6 to 16. Body masses of male and female adults in each pair were also measured daily during the entire experiment. After these body measurements, all seeds were collected from each cage, fecal matter was removed, and daily food consumption for the cage was calculated (seed mass provided from the previous day minus orts (i.e., remaining seed mass)).

Immune system challenge.–A subset of nestlings was used to test the effect of food restriction on immune function after re-feeding (control $n=17$, food restricted $n=13$). Nestlings on experiment day 11 (average brood age: 17 days post-hatch) were injected with 50 µl of lipopolysaccharide (LPS) dissolved in phosphate buffered saline (PBS) at the concentration of 1 µg LPS per g body mass (Millet et al. 2007) and attempts were made to collect blood samples 16 hours post-injection (on experiment day 12) to measure inducible innate immune responses to LPS. However, we could not collect sufficient blood volumes to assess inducible innate immune responses, so no further analysis was run on these samples. To test antibody production, a

component of adaptive immunity, on experiment day 13 (average brood age: 19 days post-hatch), nestlings were injected intramuscularly in the pectoralis muscle with a 50 µl primary injection of 25 µl keyhole limpet hemocyanin (KLH) antigen diluted in PBS at a dose of 1.0 µg antigen per g body mass emulsified with 25 µl Titermax adjuvant (modified from (Killpack and Karasov 2012b). A blood sample was taken 7 days post-primary injection (experiment day 20, average brood age 26 days post-hatch), by puncturing the brachial vein with a 28-gauge needle and collecting the blood into heparinized microcapillary tubes. Following the primary blood sample, nestlings were boosted with the same dose of KLH emulsified with Titermax adjuvant, and a post-boost blood sample was taken 7 days later (experiment day 27, average brood age 33 days post-hatch). Blood samples were centrifuged and the plasma was stored at -80 °C until ELISA analysis of KLH-specific antibodies was performed. After the post-boost blood sample was taken, all nestlings were euthanized with CO_2 and carcasses were frozen for later use in body composition analysis.

Body composition analysis.–The intestines of all nestling carcasses were removed for use in a separate study. The wet carcass mass (without intestine) was measured and carcasses were individually wrapped in pre-weighed, labeled filter paper. Carcass packets (carcass + filter paper) were dried to constant mass in a 65 ˚C drying oven and dry masses were recorded and water mass (g wet tissue-g dry tissue) was calculated. The skulls were then cracked (to allow ether extraction to penetrate the skull cavity). Lipids were extracted from the carcass packets using diethyl ether in a modified Soxhlet side-arm extractor. Lean dry mass, lipid mass extracted (g dry tissue-g lean dry tissue), and indices of tissue water content (g water/g lean dry tissue) and tissue lipid content (g lipid extracted/g lean dry tissue) were recorded.

Enzyme-linked immunosorbent assay (ELISA) for KLH-specific IgY antibodies.–Adaptive immune function was measured using an ELISA for KLH-specific antibodies (Killpack and Karasov 2012b). Flat-bottomed 96-well plates were coated with 50 µl/well KLH antigen (diluted to 0.5 mg/ml of KLH antigen in carbonate-bicarbonate coating buffer). Plates were incubated overnight at 4 °C. Plates were washed four times with wash buffer (PBS+Tween) to remove unbound coating antigen, blocked with 100 µL/well of blocking buffer (5% nonfat milk+wash buffer) and incubated for 1 hour at 37 °C. Blocking buffer was discarded and 100 µl of Zebra Finch plasma samples (and positive and negative control samples), diluted 1:100 in blocking buffer, were added to the wells in duplicate and the plates were incubated for 1 hour at 37 °C. Plates were washed four times with wash buffer, 50 µl of HRP-conjugated goat-anti-wild bird IgG (=IgY) antibody (Bethyl Laboratories, Inc.) diluted 1:700 in wash buffer was added to each well, and the plates were incubated for 1 hour at 37 °C. Plates were washed four times with wash buffer, 100 µl of ABTS substrate was added to all wells, and the plates were incubated in the dark at room temperature for 15 minutes. One hundred microliters of 1% SDS solution was added to all wells and the optical densities of the wells were read at 405 nm using an automated plate reader and average absorbance values of duplicate wells were recorded.

Statistical analysis.–Results are given as means ± SE (n=number of individuals). All tests were carried out using R statistical package (R Development Core Team 2008), except for nonlinear curve-fitting to growth models, which was performed in SYSTAT (Wilkinson 2000). Parametric tests were used when data were normal or could be transformed to an approximately normal

distribution. In all tests, the significance level was set at $P<0.05$, and $0.05<P<0.10$ was considered to indicate a trend.

For analysis of repeated measures of food consumption and adult body masses, linear mixed models were fit using maximum likelihood criteria, with day, treatment, and their interaction as fixed effects. Random effects of pair ID and brood ID were used in analysis of food consumption and individual bird ID (adult male or adult female) was added as a random effect in analyses of repeated daily body mass measurements. Chi-square-based tests were used to determine the significance of fixed effects and post-hoc analyses were performed with simultaneous tests for general linear hypotheses, using defined contrasts of treatment differences on a given experiment day.

We used two criteria to evaluate compensatory growth in food-restricted Zebra Finches: accelerated growth rates following restriction, and same asymptotic size achieved at the same time as control-fed birds. Growth rates (g/d or mm/d) were calculated for 72 h prior to food restriction (Pre), 72 h during food restriction (Restriction), and 72 h following food restriction (Post). Linear mixed models were fit for each 72 h period using maximum likelihood criteria, with treatment as a fixed effect and pair ID and brood ID as random effects. Chi-square-based tests were used to determine whether there were significant treatment differences for that period. We also examined treatment effects on growth rates with size at the start of each period included as a covariate in the mixed models. To test for asymptotic size and the time when it was reached, we performed nonlinear curve fitting of control data using the Richards equation to fit body mass data (Richards 1959); shape parameter value: 2.5) and the logistic growth equation for tarsus and culmen data. Both equations have been used to describe growth in Passeriform species (Lepczyk and Karasov 2000, Ricklefs 1968) and we chose equations based on least squares criteria. We

calculated the average asymptotic size (g or mm) and 95% confidence interval from the models of all control birds. We then determined the day that control and restricted birds reached the modeled asymptotic size and used a t-test to compare the mean day asymptotic size was reached between treatment groups.

For analysis of treatment differences in nestling body composition, chi-square-based tests were performed on linear mixed models fit using maximum likelihood. Wilcoxon rank sum non-parametric tests were used to examine treatment differences in post-primary injection and post-boost KLH-specific antibody responses. Paired t-tests were used to compare post-primary injection and post-boost KLH antibody responses within each treatment group.

RESULTS

Food consumption and adult body mass.–Food consumption did not significantly differ between treatment groups except during the three-day food restriction period (day x treatment interaction $\chi 2=102$, $P<0.001$, daily contrasts of food consumption on experiment days 1, 2, 3 $P<0.001$). Seed consumption in the food-restricted groups (6.25±0.28 g/day) averaged 54% of control group consumption (11.55±0.39 g/day) during the three-day period. Adults lost mass during the restriction period and regained and maintained mass thereafter, but the patterns differed by sex (Fig. 1). Males had significantly lower body masses compared with females throughout the experimental period ($\chi 2=5.2$, $P=0.022$) regardless of treatment and day (i.e. there were no significant sex interactions with day or treatment in adult body mass). Analysis of sex-specific day x treatment interactions in adult males revealed a trend for reduced body mass during restriction on experiment day 2 ($P=0.076$) and a significant reduction in body mass by 7% on

experiment day 3 (P=0.001) (Fig. 1a). A trend for increased body mass in food-restricted males compared with controls was observed following restriction on experiment day 11 (P=0.059) (Fig. 1a). Adult females showed a trend for reduced body mass during restriction on experiment day 2 (P=0.057) and had significantly reduced body mass by 5% on experiment day 3 (P=0.01) compared with females in control cages (Fig. 1b). Food-restricted adult females had higher body mass compared with controls following restriction, on experiment days 5 (P=0.061, trend), 6 (P=0.082, trend), 9 (P=0.038), 10 (P=0.087, trend), and 11 (P=0.044) (Fig. 1b).

Compensatory growth in nestlings.–Both criteria for compensatory growth in body mass were observed in food-restricted nestlings. Body mass growth rates did not significantly differ between treatments pre-restriction (χ2=0.001, P=0.975), were significantly lower in food-restricted birds during restriction (χ2=33, P<0.001), and were significantly higher in food-restricted birds post-restriction (χ2=9.7, P=0.002) (Fig. 2a).

Mean modeled asymptotic body mass, calculated using Richards equation fit to individual control bird growth curves, was 12.09g (95% confidence interval: 11.80g, 12.39g). Experiment day when asymptotic size was reached did not significantly differ between control (9.8 ± 0.75d) and food restricted birds (11.8 ± 1.2d) (P=0.149) and experimental body masses reached the modeled asymptotic size prior to fledging age (21 days post-hatch or experiment day 15) (Fig. 2b).

In contrast to body mass growth, the dual criteria for compensatory tarsus growth were not met in food-restricted nestlings. No accelerated tarsus growth was observed following food restriction. Tarsus growth rates did not significantly differ between treatments during pre-restriction (χ2=3.63, P=0.06), were significantly lower in food-restricted birds during restriction

($\chi 2=8.99$, $P=0.003$), and did not significantly differ between treatments post-restriction ($\chi 2=0.22$, $P=0.14$) (Fig. 3a).

Mean asymptotic tarsus size, calculated using the logistic equation fit to individual control bird growth curves, was 14.76mm (95% confidence interval: 14.49mm, 15.02mm). The majority of control (19/26 nestlings) and food-restricted (13/21 nestlings) reached this asymptotic tarsus length prior to the end of our experimental period and prior to fledging. Among those birds, there was no significant difference in day when asymptotic length was reached (control 9.1 ± 0.72d vs. food restricted 9.5 ± 0.67d; $P=0.725$) (Fig. 3b). Some of the birds that did not reach the modeled asymptotic tarsus length by the end of the experimental period were in the immune subset, and their tarsus sizes were compared at the time of euthanization. At experiment day 27 (average age: 33 days post-hatch), there were no significant differences between the subsets of control (13.47 ± 0.12mm, $n=6$) and food restricted birds (13.22 ± 0.21mm, $n=6$) in tarsus length ($P=0.268$) (Fig. 3b).

No compensatory culmen growth was observed in Zebra Finch nestlings. Culmen growth rates did not significantly differ between treatment groups pre-restriction ($\chi 2=0.01$, $P=0.91$), were significantly lower in food-restricted birds during restriction ($\chi 2=0.492$, $P=0.027$), and continued to be significantly lower in food-restricted birds post-restriction ($\chi 2=3.96$, $P=0.047$) (Fig. 4a).

Mean asymptotic culmen size, calculated using the logistic equation fit to individual control birds, was 8.93mm (95% confidence interval: 8.30mm, 9.56mm). The majority of control (18/26 nestlings) and food-restricted (18/21 nestlings) did not reach this asymptotic culmen length prior to the end of our experimental period and prior to fledging. Some of the birds that did not reach the modeled asymptotic culmen length by the end of the experimental period were

in the immune subset, and their culmen sizes were compared at the time of euthanization. When measured at experiment day 27, there were no significant differences between the subsets of control (8.85 ± 0.12mm, n =12) and food restricted birds (8.78 ± 0.09mm, n =13) in culmen length (P=0.613) (Fig. 4b).

Nestling immune function and body composition following compensatory growth.–A subset of birds from the compensatory growth experiment (control n=17 and food restricted n=13) were analyzed for adaptive immune function and body compostion following re-feeding. KLH-specific antibody response of birds in both treatments significantly increased between the sampling following primary injection (on experiment day 13) and that following booster injection (on experiment day 20) (P=0.003) (Fig. 5), providing evidence of significant secondary IgY antibody response to booster in both control and food-restricted groups. However, there was no significant difference between control and food-restricted birds in KLH-specific primary antibody response 7 days post-primary injection (P=0.812) or secondary antibody response 7 days post-booster injection (P=0.95) (Fig 5). There was no significant correlation between post-booster KLH response and growth rate during the three days post-restriction within the food restricted group (r=0.45, t_{11}=1.68, P=0.122) or the control group (r=0.18, t_{15}=0.69, P=0.503).

There were no significant differences in lean dry mass between control (2.89±0.13 g) and food-restricted (2.82±0.09 g) nestlings ($\chi 2$=0.331, P = 0.565) at the end of the experimental period. Lean body water content (g water per g lean dry mass) was significantly higher in food-restricted nestlings (2.59±0.02 g) compared with controls (2.46±0.02 g) ($\chi 2$=12.70, P < 0.001). Body lipid content (g lipid per g lean dry mass) did not significantly differ between food-restricted nestlings (0.42±0.02 g) and controls (0.39±0.02 g) ($\chi 2$=1.17, P=0.280).

DISCUSSION

Rapid compensatory body mass growth in nestlings.–We demonstrated rapid compensatory body mass growth in nestling Zebra Finches following a 72 h period of food restriction. We observed accelerated growth rates in food restricted birds in the 72 h following restriction and the same asymptotic body mass was reached at the same experimental day as controls. Food-restricted birds did not significantly differ from controls in lean-dry body mass at the end of the experiment, providing additional evidence that the compensatory growth in body mass likely represented functional lean tissue mass gain rather than increased lipid stores. This capacity to accelerate body mass growth lends support to the theory that normal body growth under typical feeding conditions is regulated at an optimized, rather than maximized, rate (Metcalfe and Monaghan 2001). While compensatory growth in Zebra Finches has been previously reported (Alonso-Alvarez et al. 2007, Criscuolo et al. 2008, 2011; Fisher et al. 2006, Krause and Naguib 2011), those studies imposed feeding stress throughout the majority or entirety of the nestling period, and measured compensatory growth responses over a long period (15d or more) post-fledging following return to adequate feeding conditions. This is the first study, to our knowledge, to show capacity for catch-up growth in young Zebra Finch nestlings within a few days following slowed growth early in the nestling period. Previous studies examining compensatory body mass growth after short-term food restriction in altricial nestlings have presented mixed results. Laboratory studies using hand-feeding to impose short-term food restriction in house sparrow (Lepczyk and Karasov 2000), song thrush (Konarzewski et al. 1996), and bank swallow (Brzek and Konarzewski 2001) nestlings did not find evidence of compensatory growth in body mass upon re-feeding of the birds. In contrast, free-living altricial

alpine swifts and collared flycatchers (Bize et al. 2006, Hegyi and Török 2007) experiencing short-term food restriction early in the nestling period demonstrated accelerated rates of body mass growth upon return to improved feeding conditions. Models utilizing parents to feed nestlings, whether in the wild or in the laboratory (the current study), may therefore be better experimental models for comparative studies of capacity for nestling compensatory growth and the influence of the timing and magnitude of the food restriction and refeeding on nestling growth patterns.

No rapid compensatory growth in skeletal structures.–In contrast to body mass growth, we observed near-parallel, rather than accelerated, skeletal growth in Zebra Finch nestlings during the immediate re-feeding period. There was no significant increase in growth rate in food-restricted birds in the 72h following restriction, though control and food restricted birds reached the same asymptotic size at the same time. Previous studies of skeletal growth following food restriction showed that tarsus length reductions in collared flycatchers were not recovered immediately prior to fledging at 12 days post-hatch (Hegyi and Török 2007) and sternum size reductions in alpine swifts persisted and were not recovered until 30 days after restriction, demonstrating slow growth to asymptotic sternum size (Bize et al. 2006). Lack of accelerated skeletal growth may reflect the strong endocrine control of long bone growth (Leach and Rosselot 1992) which may impose a critical rate and time window for structural growth. Decreased structural size may have negative implications for fledging time (Miller 2010, Searcy et al. 2004), social rank (Richner et al. 1989) and recruitment (Emlen and Wrege 1991); thus it is advantageous for structural size to be achieved prior to fledging. Because restriction occurred early in the nestling period, food-restricted Zebra Finches appeared to reach control tarsus length

prior to fledging without significantly accelerating growth rates (P=0.14; Fig. 3a). However, if food restriction had occurred later in the nestling period, control size may not be have been reached prior to fledging. Future studies should examine the impact of the timing and magnitude of food restriction during the nestling period on patterns of subsequent growth of body mass and skeletal structures (Bize et al. 2006).

Reduced culmen growth rates in food-restricted Zebra Finches compared with controls persisted following in the 72h after return to *ad libitum* feeding. Food-restricted birds in the immune subset did not have significantly shorter average culmen lengths compared with controls on experiment day 27 (average age 33 days post-hatch), indicating slow recovery of reduced culmen length during the weeks post-fledging. It is possible that compensatory body mass growth acceleration post-restriction comes at the cost of a reduction in culmen growth rates. Apparent trade-offs between body mass and culmen growth may be related to relatively slow growth to asymptotic size of the culmen and skull compared with other skeletal components. In a study of growth in red-winged blackbirds, the head was shown to have a relatively low post-natal growth constant compared to other body components such as the wings and digestive organs (Ricklefs 1967). In marbled murrelets, bill growth continued to increase significantly after nest departure, and bill length was positively influenced by quality of post-fledging (at-sea) diet (Janssen et al. 2011).

No effect of compensatory growth on development of adaptive immune capacity during the nestling period.–Given evidence that many animals are capable of growing at faster rates than they generally display, it is possible that the benefits of always maximizing growth rate may be outweighed by other physiological and/or ecological costs (Metcalfe and Monaghan 2001).

Compensatory growth in Zebra Finches has been associated with reduced cognitive ability (Fisher et al. 2006), reduced flight performance (Criscuolo et al. 2011), increased susceptibility to oxidative damage (Alonso-Alvarez et al. 2007) and elevated adult resting metabolic rates (Criscuolo et al. 2008). However, to our knowledge this is the first study to examine the effects of accelerated, compensatory growth on adaptive humoral immune development in Zebra Finches, or rapidly growing altricial birds in general.

Components of the immune system are purportedly energetically and nutritionally costly to develop and use (Lee 2006). Reductions in innate and cell-mediated immune responses have been observed during food restriction in nestling passerines (Birkhead et al. 1999, Brzek and Konarzewski 2007, Saino et al. 1997, Snoeijs et al. 2005). Additionally, in free-living nestlings (Arriero et al. 2013, Brommer 2004, Mauck et al. 2005, Soler et al. 2003) and in food-restricted nestlings in the laboratory (Brzek and Konarzewski 2007), an inverse relationship between the rate of body growth and the rate of immune development has been observed, suggesting a trade-off between investment in growth and investment in immune response. Development of adaptive humoral components is purportedly costly because it requires generation and proliferation of B-cell lineages with diverse, functional antibody specificities (Lee 2006). Thus, we imposed food restriction and measured compensatory growth when humoral immune system development was underway (Killpack and Karasov 2012b) and measured responses after re-feeding. Food restriction and accelerated growth did not alter development of the humoral immune system or capacity for functional memory antibody response in our current study. Previously-restricted Zebra Finches mounted functional secondary antibody responses to a booster KLH injection and the magnitude of the response did not significantly differ from controls. Additionally, neither the previously-restricted birds nor the control birds showed a significant correlation between post-

restriction growth rate and the adaptive antibody response level, reinforcing the absence of a tradeoff between growth and development of adaptive immune capacity. Together these data suggest that energetic costs of growth are relatively larger than costs of development of the humoral immune system (Hasselquist and Nilsson 2012, van der Most et al. 2011) and that tradeoffs in adaptive antibody response may not be expected following rapid growth.

Mounting inflammatory immune responses is considered to be relatively costly in terms of energy investment and immunopathology (Lee 2006). Thus, if lasting energy deficits occur in birds that experience compensatory growth, we may expect to see lasting reductions in innate immune responses to an inflammatory challenge. In growing chickens, anti-inflammatory immune components (IL-4 cytokine expression and IgY antibody concentration) were not significantly altered during food restriction yet pro-inflammatory immune components (IL-6 cytokine and inducible nitric oxide synthase) were significantly reduced. However, despite reductions in pro-inflammatory components in birds during restriction, measures were generally not significantly lower than controls after 20 days of re-feeding (Jang et al. 2009). Given lack of adequate sample volumes, we were unable to measure inflammatory innate immune responses to LPS injection. However, because investment in immune response appears to be flexible to immediate environmental resource availability, we may not expect to see reductions in inflammatory innate immune response in Zebra Finches following compensatory growth. Previously food-restricted birds showed no energy deficits in terms of body mass or lipid content and had access to *ad libitum* food at the time of LPS injection. Future studies should compare innate and adaptive immune responses in growing birds both during and following food restriction to separately examine impacts of restriction alone and restriction followed by catch-up growth (Metcalfe and Monaghan 2001) to further examine trade-offs in immune function.

Evidence of altered body composition following compensatory growth.–Though development of adaptive humoral immune function was not altered as a result of accelerated body mass growth, we did see a significant impact on tissue maturation. Tissue maturity is commonly indexed by lean tissue water content, because reductions in muscle water content occur with age and coincide with increasing lean content in birds (Ricklefs and Webb 1985). In previous studies of altricial and semi-altricial birds, no significant increases in muscle water content were observed during food restriction (Burness et al. 2000, Dahdul and Horn 2003, Killpack and Karasov 2012a, Konarzewski et al. 1996, Takenaka et al. 2005). In contrast, following compensatory growth Zebra Finches in our study had significantly higher lean tissue water content, and thus decreased tissue maturity, compared with controls. Therefore it appears that a tradeoff between growth and development occurred as result of accelerated growth during the nestling period, resulting in a decoupling of chronological age and physiological age in food-restricted birds. Acquisition of functional maturity through cellular differentiation could limit the proportion of tissue capable of cell proliferation and growth, and this antagonism may constrain postnatal growth and development (Ricklefs 1979). For example, exponential growth rate of muscles has been negatively correlated with indices of glycolytic activity, electric potential, and contractile protein content in birds (Ricklefs et al. 1994). Delayed tissue differentiation resulting from compensatory growth could have negative effects on maintenance metabolism and thermogenesis as well as flight performance (which has been observed in Zebra Finches following compensatory growth (Criscuolo et al. 2011)) in adults. Food-restricted Zebra Finches in our study did not differ from controls in body lipid content, in agreement with another study of birds re-fed after food restriction (Brzek and Konarzewski 2004). These patterns suggest that

while developmental maturation of the tissues may be suspended or delayed as a result of compensatory growth, ability to recover depleted fat stores resulting from food restriction is more flexible.

Adult responses to food restriction and re-feeding.–We demonstrated interesting findings with respect to body masses of adults feeding nestlings during the experimental period. In control adults, body mass appeared to decline more rapidly during the days immediately following nestling hatch, than during the time of rapid nestling growth (and high feeding demands). This observation is in line with the flight efficiency hypothesis recently supported by a study in female tree swallows (Boyle et al. 2012). This hypothesis proposes that the proactive response of mass gain prior to incubation and nestling hatch reflects parental investment in current reproduction and self-maintenance, and that mass is facultatively lost during incubation and following nestling hatch to increase flight efficiency and reduce foraging costs (Boyle et al. 2012). Interestingly, adult females conserved their body mass slightly more than adult males when food restriction was imposed and food restricted females also demonstrated more significant increases in body mass above controls upon return to *ad libitum* feeding in comparison with males. These facultative responses to poor rearing conditions in females may indicate increased investment in female survival and in future reproduction. More deliberate experiments regarding parental responses to food restriction during chick rearing should be performed to further explore these observations.

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FIGURE LEGENDS

FIGURE 1. Body masses of control (filled) and food-restricted (unfilled) parent adult male (left) and adult female (right) Zebra Finches. Data are means ± SE. Food restriction (denoted by white boxes on x-axis) occurred from experiment day 1 (average brood age is 6 days post-hatch) through experiment day 3. Significant daily contrasts between treatment groups are discussed in the text.

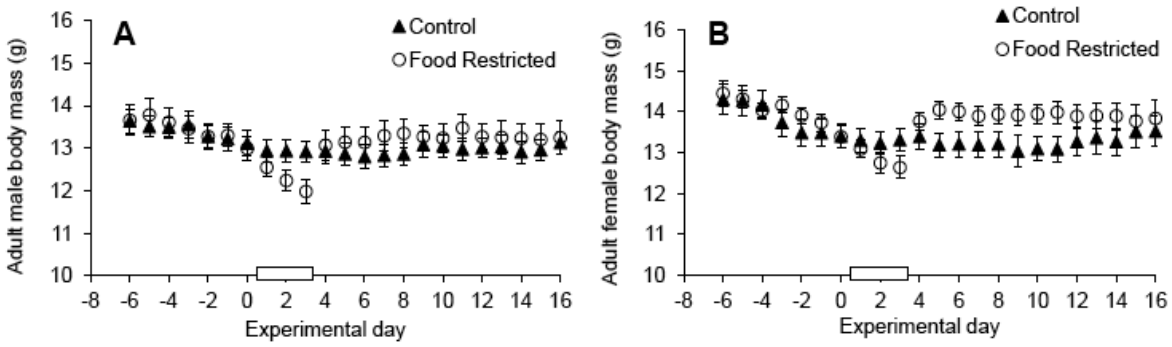
FIGURE 2. (A) Body mass growth rates of control (filled) and food-restricted (unfilled) Zebra Finch nestlings during the 72 hours pre-restriction (Pre; experimental days -2, -1, 0), during restriction (Restriction; experimental days 1, 2, 3), and post-restriction (Post; experimental days 4, 5, 6). Asterisks indicate experiment days when significant differences in growth rates were observed between control (filled) and food-restricted (unfilled) groups. (B) Body masses of control (filled) and food-restricted (unfilled) Zebra Finch nestlings. Food restriction (denoted by white box on x-axis) occurred from experiment day 1 (average brood age=6 days post-hatch) through experiment day 3. Data are means ± SE.

FIGURE 3. (A) Tarsus growth rates of control (filled) and food-restricted (unfilled) Zebra Finch nestlings during the 72 hours pre-restriction (Pre; experimental days -2, -1, 0), during restriction (Restriction; experimental days 1, 2, 3), and post-restriction (Post; experimental days 4, 5, 6). Asterisks indicate experiment days when significant differences in growth rates were observed between control (filled) and food-restricted (unfilled) groups. (B) Tarsus lengths of control (filled) and food-restricted (unfilled) Zebra Finch nestlings. Food restriction (denoted by white box on x-axis) occurred from experiment day 1 (average brood age=6 days post-hatch) through experiment day 3. Data are means ± SE.

FIGURE 4. (A) Culmen growth rates of control (filled) and food-restricted (unfilled) Zebra Finch nestlings during the 72 hours pre-restriction (Pre; experimental days -2, -1, 0), during restriction (Restriction; experimental days 1, 2, 3), and post-restriction (Post; experimental days 4, 5, 6). Asterisks indicate experiment days when significant differences in growth rates were observed between control (filled) and food-restricted (unfilled) groups. (B) Culmen lengths of control (filled) and food-restricted (unfilled) Zebra Finch nestlings. Food restriction (denoted by white box on x-axis) occurred from experiment day 1 (average brood age=6 days post-hatch) through experiment day 3. Data are means ± SE.

FIGURE 5. KLH-specific antibody response of control (filled) and food-restricted (unfilled) Zebra Finch nestlings. Data are means ± SE. Asterisk indicates significant difference between post-vaccination and post-boost antibody response levels. No significant treatment effect was observed between control and food-restricted groups.

FIGURE 1.


A
Control
Food Restricted
Adult male body mass (g)
16
15
14
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12
11
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-8 -6 -4 -2 0 2 4 6 8 10 12 14 16
Experimental day
B
Control
Food Restricted
Adult female body mass (g)
16
15
14
13
12
11
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-8 -6 -4 -2 0 2 4 6 8 10 12 14 16
Experimental day

FIGURE 2.

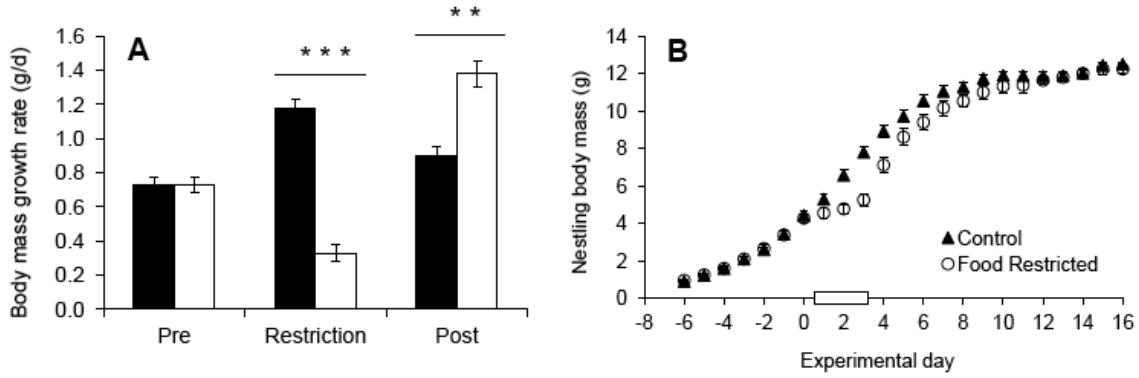


FIGURE 3.

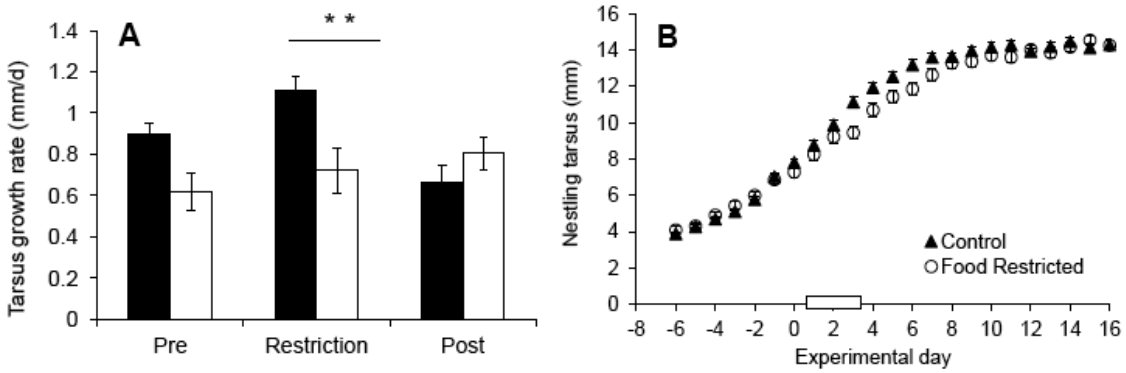


FIGURE 4.

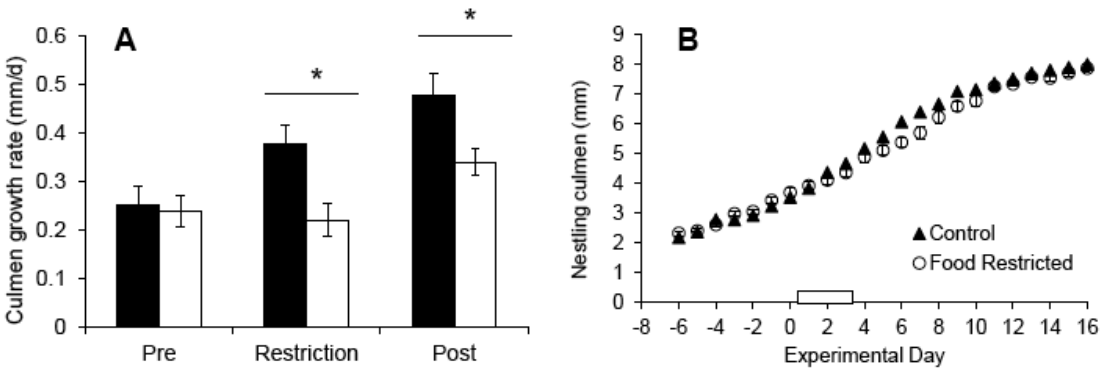
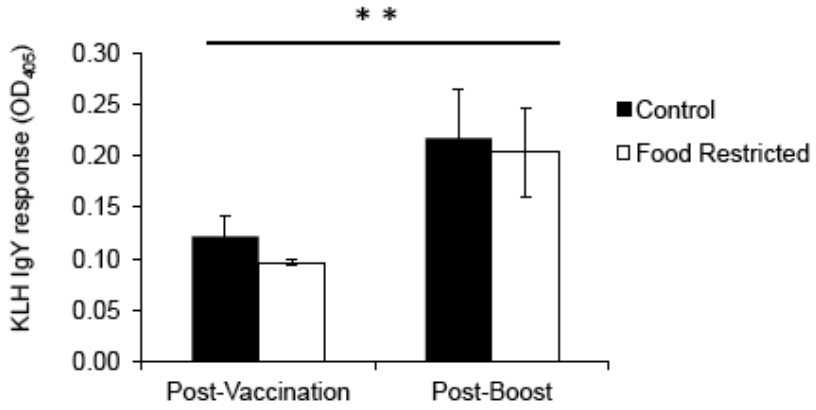


FIGURE 5.


* *
KLH IgY response (OD405)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Post-Vaccination
Post-Boost
Control
Food Restricted

CHAPTER 4

Immune function and growth in response to food restriction in altricial house sparrow nestlings.

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Abstract

If resources are limiting during the nestling period of altricial birds, then trade-offs may occur between immune defense and life history components such as growth and development. We tested for such trade-offs in food-restricted house sparrows, *Passer domesticus,* both early and late during the nestling period. In particular, we tested that immune function would be more reduced in defenses considered costly to use and develop (the induced acute phase response and adaptive humoral response, respectively), compared with one considered less costly (constitutive complement-mediated lysis). We compared trade-offs involving immune response with trade-offs in other life history traits by examining structural growth, organ growth and development, and body temperature in response to restriction. We also examined the long-term effects of early food restriction on birds refed and tested late in the nestling period. As predicted, levels of acute phase protein haptoglobin (Hp), an inducible component of the innate immune system, were reduced in early and late food restricted birds. Early food restriction had no long-term impact on Hp response. Food restriction did not significantly impact complement-mediated lysis, a constitutive component of immune function, yet there was some evidence that early food restriction and immune challenge may have caused increased reactive oxidative species leading to increased lysis. Circulating IgY antibody levels were not significantly altered with food restriction. Masses of the alimentary organs and heart were significantly reduced in both early and late food restricted birds, yet reductions resulting from early food restriction were reversible in refed birds. Declines in lean flight muscle mass and maturity (indexed by tissue water content and mean total citrate synthase enzyme activity) were observed with early food restriction, and refed birds had persistent reductions in muscle size and maturity. Findings from this study suggest that immune function, like organ growth, appears to be flexible to resource supply during

the nestling period, and early food restriction during the nestling period does not permanently stunt immune the functions we measured.

Introduction

Altricial nestlings may experience food restriction in nature as a result of sibling competition (Losdat et al. 2010), mismatch between breeding time and environmental food availability (Thomas et al. 2001, Visser et al. 2005), or temporary reductions in parental food delivery due to inclement weather (Bize et al. 2006). Altricial birds undergo rapid growth in the nestling period, and food restriction encountered during that time has been shown to alter allocation patterns to growth, development, and maintenance metabolism. For example, house sparrows experiencing 25% food restriction throughout the nestling period reduced digestive organ and muscle masses and reduced body temperatures, yet maintained structural growth in line with well-fed control nestlings (Killpack and Karasov 2012a). This pattern of conserved skeletal growth at the expense of reductions in soft tissue growth has also been observed in several other species of growing birds (Lacombe et al. 1994, Negro et al. 1994, Burness et al. 2000, Brzek and Konarzewski 2001, Dahdul and Horn 2003, Moe et al. 2004).

Food-restricted nestlings may also face trade-offs in energy allocation to immune defense (Norris and Evans 2000). Innate and adaptive immune functions have been shown to develop slowly throughout the nestling period of altricial birds (Killpack et al., in press, Killpack and Karasov 2012b). Different arms of the immune system are proposed to have different costs associated with their development and use (Klasing and Leshchinsky 1999, Lee 2006). Constitutive innate immune functions, such as natural antibodies and complement, have purportedly low costs to develop because they are non-specific, and low costs to use because they do not increase with inflammatory challenge. Induced innate immune responses, though nonspecific, may be costly to use because they involve systemic inflammation and synthesis of acute phase proteins (such as haptoglobin) in the liver. Adaptive humoral (antibody-mediated)

and cell-mediated immune functions are purportedly costly to develop because they require generation of diverse receptor specificities and memory lymphocyte pools. Adaptive cell-mediated (but not humoral) immune function may also incur elevated costs to use because it involves systemic inflammatory response. Immune responses of animals to perturbations may depend on these relative costs.

Food restriction imposed during the nestling period is an important experimental manipulation to isolate key determinants of immune function in growing birds. Based on the hypotheses described above, if costs of immune function are high, then during food restriction we would expect there to be an effect on the given immune measure. If costs are low, then during restriction there would be no effect on the immune function. This hypothesis related to energetic costs proposes that direct trade-offs in energy allocation may occur between the immune system and other demanding tasks such as growth (Sheldon and Verhulst 1996, Hasselquist and Nilsson 2012). The costs of immune response may be of rather short duration, and it is useful to simultaneously measure multiple aspects of growth and maintenance during food restriction and also following the period of energy limitation. This allows us to examine whether some functions are sustained during restriction (reflecting perhaps relatively lower costs compared with others) and whether costs may be long lasting, respectively.

Relationships between growth and immune function in nestlings have been little studied until recently, and patterns vary with type of immune measure. The most commonly used immunity metric among studies in growing birds has been the skin-swelling response to phytohemagglutinin injection (PHA). PHA response involves both induced innate and adaptive cell-mediated immune components (Martin et al. 2006), making it difficult to evaluate costs of the different arms of the immune system. A positive correlation between PHA response and body

mass or body condition was observed in several species of young birds, indicating that increased investment in PHA response occurs in higher quality nestlings (Saino et al. 1997, Soler et al. 1999, Alonso-Alvarez and Tella 2001, Hoi-Leitner et al. 2001, Snoeijs et al. 2005, Martín-Vivaldi et al. 2006, Forsman et al. 2010). However, in some studies, this positive correlation was not observed when young birds were well-fed or were senior nestlings within a brood, indicating perhaps a threshold for the condition-dependence of PHA response (Alonso-Alvarez and Tella 2001, Snoeijs et al. 2005, Martín-Vivaldi et al. 2006).

In nestling sand martins, 60% food restriction for three days led to significantly slower body mass growth rates and PHA skin-swelling immune response compared with controls (Brzek and Konarzewski 2007). Among the food-restricted birds, those that showed the greatest body mass growth had the lowest immune responses to PHA, indicating a trade-off between growth and PHA response during restriction. In contrast, nestlings fed *ad libitum* showed a positive relationship between growth and PHA response (Brzek and Konarzewski 2007). This study demonstrates that cell-mediated and/or inflammatory immune function appears to be costly when resources are limited during the nestling period. This study also highlights the importance of imposing food restriction, and thus preventing birds from increasing food intake to meet increased energy demands, to evaluate immune costs.

Fewer studies have examined trade-offs in adaptive humoral and constitutive innate immune functions in growing altricial birds, though findings suggest relatively low costs of these functions. Adaptive humoral function, measured as antibody response to injected *Brucella abortus* antigen, did not significantly alter daily energy expenditure or feather growth in first-year green finches, but did result in increased fluctuating asymmetry in developing feathers (Amat et al. 2006). Constitutive innate natural antibody and complement activities were not

significantly related to body condition in house sparrow or tree swallow nestlings (Palacios et al. 2009, Killpack et al., in press). Natural antibody levels were not significantly correlated with growth rate among 17 species of free-living passerine nestlings (Arriero et al. 2013). No experimental food restriction was imposed in these studies, thus it is difficult to determine if lack of trade-offs reflects absence of nutritional stress or reduced costs of humoral and constitutive innate immune function compared with PHA.

The current study examined immune function during food restriction in growing altricial birds, *Passer domesticus*. We imposed 40% food restriction in early (5-7 days post-hatch) and late-stage (11-13 days post-hatch) nestlings in the laboratory and measured indices of induced and constitutive innate immune function and humoral adaptive immune function during restriction. To examine how trade-offs involving immune response compared with trade-offs in other life history traits, we compared the responses to restriction by immune components with responses to restriction in structural growth, organ growth and development, and body temperature. We also examined whether lasting differences in allocation were evident in birds refed after experiencing food restriction that occurred early in the nestling period. We predicted that induced innate immune response and adaptive humoral measures, but not constitutive innate function, would be reduced during food restriction, reflecting their high costs of use and development, respectively. We predicted that food restriction early rather than late in development would more likely lead to tradeoffs in allocation, and that more significant reductions would be observed in organ and muscle masses compared with immune and skeletal measures.

Methods

Study site and bird collection

House sparrow nest sites, both wooden nest boxes and natural nests, were located on the University of Wisconsin-Madison campus. In early May 2012, all potential nesting locations were checked weekly to note the onset of egg laying. From mid-May to late-June we visited the 29 known active nesting sites daily, between 1300 and 1500 hours CST to ensure accurate and consistent aging of nestlings. At each visit, we counted eggs and noted the number of nestlings in each nest (average brood size=3.5 nestlings). We recorded the date that a new nestling was found as 'day 0' of age for that nestling. Nestlings were marked with colored permanent marker and returned to the nest so that each bird could be distinguished from its nest mates on later dates. Nestlings were collected from the nest at 3 days (d) post-hatch and transported to our laboratory. Nestlings were placed in round tissue-lined plastic containers (445 cm^3) and housed in an environmental chamber under constant conditions of 15 hour light : 9 hour dark photoperiod (lights on 0600 h), 35°C chamber temperature, and 40–45% relative humidity.

Experimental design

A total of 60 nestlings were raised in the laboratory, assigned to one of five treatment groups differing in feeding regime and in age of sampling (n=12 per treatment group) (Fig 1). The two control treatment groups were fed age-specific meal sizes until 7d or 13d post-hatch, respectively. The two food-restricted treatment groups birds were fed age-specific meal sizes except for the final two days prior to euthanization at 7d or 13d post-hatch, when they were fed 40% less than age-specific meal sizes. The final "refed" treatment group was restricted early (5-

7d post-hatch) but was then returned to age-specific meals sizes and euthanized at 13d post-hatch. Nestlings from the same brood were randomly assigned to different treatment groups and there was no significant difference in body mass at 3d between groups ($F_{4,55}$=0.044, p=0.996).

Feeding protocol and daily measurements

Birds were hand-fed a synthetic starch-containing diet developed by E. Caviedes-Vidal (Lepczyk et al. 1998), which has been shown in previous studies to provide adequate nutrition and hydration for growing house sparrows (Lepczyk et al. 1998, Brzek et al. 2009, Killpack and Karasov 2012a). Age-specific and food-restricted meal sizes were based on previous studies (Lepczyk et al. 1998, Brzek et al. 2009, Killpack and Karasov 2012a) and preliminary hand-feeding trials in house sparrows. Beginning at 0630 h, nestlings were removed from the environmental chamber hourly and fed using a 0.50 ml or 1.0 ml syringe (depending on meal size), for a total of 15 meals per day. Food-filled syringe mass (±0.01 g) was recorded prior to feeding and weighed following feeding to calculate the exact meal mass delivered.

Body mass (±0.01 g) and body temperature (±0.01°C) using a thermocouple inserted approximately one centimeter into the bird's cloaca, were recorded daily before the first feeding at 0630 h.

Inflammatory challenge, blood samples and euthanization

Nestlings were injected with 50 µl of lipopolysaccharide (LPS) dissolved in phosphate buffered saline (PBS) at the concentration of 1 µg LPS per g body mass (Millet et al. 2007) between 1600 and 2000 hours CST on the day prior to euthanization to induce an acute phase response. Nestlings were euthanized with CO_2 at 15.2±0.1 hours post-injection and decapitated

for blood collection. Blood samples were centrifuged and the plasma was stored at -80 °C until three measures of immune function were performed: haptoglobin acute phase protein levels (an inducible innate response), hemolysis (a constitutive innate feature), and total circulating IgY levels (a constitutive adaptive immune feature). One side of the flight muscles of each bird was dissected immediately and stored in liquid nitrogen for analysis of citrate synthase enzyme activity (see below) and the remaining carcasses were frozen for later dissection and measurements.

Haptoglobin acute phase protein assay

We used an *in vitro* spectrophotometric assay to detect haptoglobin acute phase protein concentration, a measure of inducible innate immune function, in nestling plasma. Plasma samples used were visually inspected to ensure minimal presence of hemolysed blood prior to use in the assay. Haptoglobin was quantified (mg ml^{-1}) by modifying the 'manual method' instructions provided with a commercially available assay kit (#TP801; Tri-DD, LLC, Boonton Twp, NJ). Because of previous findings of relatively low haptoglobin concentrations in avian plasma samples (Buehler et al. 2009, Georgieva et al. 2010, Matson et al. 2012), we used 15µl of house sparrow plasma sample per well (twice the suggested protocol volume) and adjusted concentration calculations based on the standard curve accordingly (Matson et al. 2012).

Hemolysis assay

We used an *in vitro* spectrophotometric assay to detect complement-mediated lysis, a measure of constitutive innate immune function (Killpack et al., in press). Twenty microliters of plasma sample were diluted in 60µL of PBS in microcentrifuge tubes and 80µL of 2% rabbit red

blood cell solution were added to each tube. Negative control tubes were prepared using 20µl of heat-inactivated plasma (30 minutes at 56°C to inactivate complement) and positive control tubes were prepared using heat-inactivated plasma as well as 2% rabbit red blood cell solution vortexed with Triton-X 100 detergent (to lyse all red blood cells). Tubes were incubated in a 37°C water bath for 30 minutes, then centrifuged for 2 minutes at 2000 x g to pellet any unlysed red blood cells. Then, 40µl of supernatant from each tube were added in triplicate to a round-bottom 96-well plate, and absorbance (or optical density, OD) was read at 540nm using an automated microplate reader. Hemolysis measures are reported as (OD of sample)-(OD of negative control).

Total IgY Enzyme-linked immunosorbent assay (ELISA)

We used a previously described direct ELISA to determine total IgY in house sparrows (Killpack et al., in press). A flat-bottomed Immulon-4 96-well plate (Dynex Technologies) was coated in triplicate with 100 ul per well of house sparrow plasma samples (and positive and negative plate control samples), diluted to 1:100 in coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). The plate was incubated overnight at 37°C. The coating solution was removed, 200 ul of blocking buffer (PBS with 5% non-fat dry milk, 0.05% Tween) were added to each well, and incubated at room temperature for 30 minutes. The plate was washed four times with wash buffer (PBS with 0.05% Tween) using an automated plate washer. Fifty ul of the detecting horseradish peroxidase- conjugated goat anti-bird IgG (=IgY) (Bethyl Laboratories, Inc. #A140-110P) were added at 1:1000 in blocking buffer, incubated at 37˚ C for 1 hour, and washed. One hundred ul of ABTS substrate were added to each well, the plates were incubated for 5 minutes, and the reaction was stopped with 100 ul of 1% SDS. Optical densities

of the wells were read at 405 nm using an automated plate reader and average absorbance values of triplicate wells were recorded. Total IgY measure was reported as (OD of sample)-(OD of negative control).

Organ, muscle, and skeletal measurements

Carcasses were partially thawed and the flight muscles (pectoral + surpacoracoideus muscles), small intestine, liver, gizzard, and heart were dissected and wet masses were recorded (±0.10 mg). Tissues were dried to a constant mass in a 65°C drying oven and dry masses were recorded (±0.10 mg). Tarsus and skull (head+beak) lengths were recorded from the carcasses (±0.10 mm) using digital calipers.

Muscle Composition Analysis

We also used dissected flight muscles to examine the impact of food restriction on tissue maturation, indexed by decreasing tissue water content (Marsh and Wickler 1982, Choi et al. 1993). Tissue maturity is commonly indexed by lean tissue water content, because reductions in muscle water content occur with age and coincide with increasing lean content in birds (Ricklefs and Webb 1985).

Muscle samples were dried to constant mass in a 65 ˚C drying oven and dry masses were recorded and water mass (g wet tissue-g dry tissue) was calculated. Dried flight muscle samples were individually wrapped in pre-dried filter paper, and lipids were extracted from the samples using diethyl ether in a modified Soxhlet side-arm extractor. Lean dry mass after extraction and tissue water content (g water/g lean dry tissue) were calculated.

Citrate Synthase assay

We also examined the impact of food restriction on flight muscle citrate synthase (CS) activity, an index of muscle cellular aerobic capacity using a previously described assay (Liknes and Swanson 2011). Briefly, frozen muscle samples were minced, weighed to the nearest 0.1mg, homogenized 1:10 w:v in homogenizing buffer (2 mM EDTA, 100 mM potassium phosphate buffer, pH 7.3), and sonicated. Five microliters of homogenate (or citrate synthase standard (Sigma, C-3260) were added to a test tube with 100µl of 2.0 mM acetyl-CoA (sodium salt, Sigma A-2897), 100µl 1.0 mM 5.5'-dithiobis-(2-nitrobenzoic acid) (Sigma D-8130), and 695µl of assay buffer (100 mM triethanolamine-HCl, 2.5 mM EDTA and 10 mM potassium phosphate). Contents of each sample (and positive control) were pipetted in triplicate (225 µl per well) to a 96-well-plate. Initial absorbance for all samples was measured on a plate reader at 405nm. Then, 25 µl of a 5.0 mM oxaloacetic acid solution (Sigma O-4126) was added to each well, the plate was immediately placed back in the plate reader, and absorbance was measured every minute for five minutes. We used average values of triplicate wells for calculation of mean mass-specific activities (μmol min^{-1} g fresh mass $^{-1}$) and mean total activities (mass-specific activity x wet muscle mass; μmol min^{-1}).

Statistical Analyses

Results are given as means ± s.e.m (*N* = number of individuals). All tests were carried out using R statistical package (R Development Core Team 2008). Parametric tests were used when data were normal or could be transformed to an approximately normal distribution. In all tests, the significance level was set at $P<0.05$ and $0.05<P<0.10$ was considered to indicate a trend.

To analyze treatment effects on daily morning body mass and body temperature within the early (7d) and late (13d) sampled groups, linear mixed models were fit using maximum likelihood criteria, with day, treatment, and their interaction as fixed effects and individual bird as a random effect (to account for repeated measures). Chi-square-based tests were used to determine the significance of the day by treatment interaction and post-hoc analyses were performed with simultaneous tests for general linear hypotheses, using defined contrasts of treatment differences on a given experiment day.

To compare the influences of age and food restriction on final body measures and immune parameters, we performed two-way ANOVAs of age, treatment, and their interaction. Post-hoc Tukey multiple comparisons of means were performed when interactions were significant. Non-significant interactions were removed to examine the significance of fixed effects alone.

To examine whether lasting allocation differences occur in birds refed after experiencing food restriction early in the nestling period, two-sample t-tests were performed to compare differences in final body measures and immune parameters between control and refed birds at 13d.

Results

Daily body measurements

Control and food-restricted 7d birds showed a significant day by treatment interaction in daily body mass (ChiSq=88, df=3, $p<0.001$), with significantly lower body mass in food-restricted birds relative to controls during restriction at 6d ($p=0.002$) and 7d ($p<0.001$) (Fig 2a).

There was a trend for a day by treatment interaction in daily body temperature (ChiSq=7.65, df=3, p=0.054), with significantly lower body temperature in food-restricted birds relative to controls during restriction at 6d (p=0.002), but no significant difference following LPS injection at 7d (p=0.340) (Fig 2b).

Control and food-restricted 13d birds showed a significant day by treatment interaction in daily body mass (ChiSq=19.7, df=9, p=0.020) (Fig 3a). There were no significant differences in body mass between groups, yet at 13d there was a trend for reduced body mass in food-restricted birds compared with controls (p=0.066). Control and refed 13d birds showed significant differences in body mass during the experimental period (day x treatment interaction: ChiSq=61.74, df=9, p<0.001). Refed birds had significantly reduced body mass during the food restriction treatment at 6d (p=0.037) and 7d (p<0.001). After return to age-specific meal sizes, refed birds showed persistently lower body mass relative to controls at 8d (p<0.001) and 9d (p=0.014), after which they did not differ significantly in body mass from controls (Fig 3a). There was no significant difference in body temperature between control and restricted 13d birds at any time throughout the experimental period (ChiSq=1.37, df=1, p=0.242) (Fig 3b). Repeated measures analysis of 13d refed and control body temperatures showed an overall pattern of reduced body temperature in the refed birds compared with 13d controls throughout the experimental period (ChiSq=4.72, df=1, p=0.030) (Fig 3b).

Immune measures during restriction and after re-feeding

Plasma haptoglobin concentration, our index of induced innate immune response to LPS injection, significantly increased with age from 7d to 13d ($F_{1,45}$=6.7, p=0.013). There was a trend for lower haptoglobin concentration in food restricted birds compared with controls ($F_{1,45}$=3.9,

p=0.054) (Fig 4a). There was a significant age by treatment interaction in lysis activity, our index of constitutive innate immune function ($F_{1,44}$=6.3, p=0.016) (Fig 4b). Among control birds, there was a significant increase in lysis activity from 7d to 13d (p=0.020). However, food restricted birds at 7d did not significantly differ from food restricted birds at 13d (p=0.955). There were no significant treatment differences in lysis activity at 7d (p=0.192) or 13d (p=0.430). Total circulating IgY, our index of adaptive immune function did not significantly differ with age ($F_{1,43}$=2.72, p=0.106) or feeding treatment ($F_{1,43}$=0.28, p=0.602) (Fig 4c). There were no significant differences in lysis activity (p=0.532), haptoglobin concentration (p=0.248), or total IgY levels (p=0.849) between re-fed and control birds at 13d.

Structural measures during restriction and after re-feeding

Tarsus length significantly increased with age ($F_{1,44}$=221, p<0.001), and there was an overall trend for reduced tarsus length in food restricted birds compared with controls ($F_{1,44}$=3.1, p=0.084) (Fig 5a). There was a significant interaction between age and treatment in skull length ($F_{1,44}$=4.6, p=0.038) (Fig 5b). Skull was significantly shorter in 7d compared with 13d birds, regardless of treatment (p<0.001). Skull length was significantly shorter in 7d food restricted birds compared with same-aged controls (p=0.008). However, there was no significant reduction in skull length if food restriction occurred later in the nestling period, as 13d food restricted birds did not have significantly different skull length compared with 13d controls (p=0.983). There were no significant differences in tarsus length (p=0.169) or skull length (p=0.357), despite reductions observed at the time of restriction in 7d food-restricted birds.

Organ measures during restriction and after re-feeding

Dry intestine mass significantly increased with age ($F_{1,44}$=121, p<0.001), and was significantly lower in food-restricted birds compared with controls regardless of the timing of food restriction during the nestling period ($F_{1,44}$=50.4, p<0.001) (Fig 6a). Dry liver mass (Fig 6b) and dry heart mass (Fig 6c) showed similar patterns with age (liver: $F_{1,44}$=40.9, p<0.001; heart: $F_{1,44}$=162, p<0.001) and with food restriction (liver: $F_{1,44}$=14.8, p<0.001; heart: $F_{1,44}$=14.3, p<0.001). Dry gizzard mass was significantly decreased with food restriction ($F_{1,44}$=14.0, p<0.001) though it did not significantly increase with age from 7d to 13d ($F_{1,44}$=2.5, p=0.123) (Fig 6d). There were no significant differences in dry intestine (p=0.252), liver (p=0.114), heart (p=0.972), or gizzard (p=0.436) mass between refed and controls at 13d, despite reductions observed in food-restricted birds at 7d.

Muscle measures during restriction and after re-feeding

Dry flight muscle mass significantly increased with age ($F_{1,45}$=745, p<0.001), and was significantly lower in food-restricted birds compared with controls regardless of the timing of food restriction during the nestling period ($F_{1,45}$=7.07, p=0.012) (data not shown). Tissue composition analysis of the flight muscle revealed that lean dry mass of the flight muscle also significantly increased with age ($F_{1,44}$=505, p<0.001), and was overall significantly lower in food-restricted birds compared with controls ($F_{1,44}$=7.07, p=0.008) (Fig 7a). Lean tissue water content, an index of tissue immaturity, was significantly higher in 7d compared with 13d birds ($F_{1,42}$=106, p<0.001) and there was a trend for increased water content in food restricted compared with control birds ($F_{1,42}$=3.39, p=0.073) (Fig 7b). Flight muscle citrate synthase (CS) activity was also measured to index tissue maturity. Mass-specific CS activity significantly

increased with age ($F_{1,42}$=152, p<0.001) but was not significantly altered with food restriction treatment ($F_{1,42}$=1.25, p=0.269) (Fig 7c). There was a significant age by treatment interaction in mean total activities ($F_{1,41}$=25.89, p<0.001). Mean total CS activity significantly increased with age (p<0.001). There was significant reduction in mean total CS activity in 7d food-restricted birds compared with same aged controls (p<0.001), but no significant treatment effect at 13d (p=0.943) (Fig 7d).

In contrast to immune, skeletal, and organ measures, there were significant reductions in flight muscle measures in refed birds compared with controls at 13d. Lean flight muscle mass (p=0.009) was reduced in refed birds, indicating that reductions observed in 7d food restricted birds were not recovered with refeeding (Fig 7a). Refed birds showed a trend for increased muscle water content compared with controls (p=0.069), indicating persistent reductions in flight muscle maturity that were observed in 7d restricted birds (Fig 7b). Mass-specific CS activity did not significantly differ between control and refed birds at 13d (p=0.543), though mean total CS activity was significantly lower in 13d refed birds compared with 13d controls (p=0.036) because of persistent reductions in flight muscle mass (Fig 7c,d).

Discussion

Altricial nestlings grow rapidly post-hatch, and patterns of allocation may differ depending on whether food restriction occurs early, when growth demands are high, or late, when body mass has reached asymptotic size prior to fledging. Our food restriction treatment was severe enough to initiate allocation trade-offs among nestlings, and there were variations depending on the age of restriction. More significant reductions in both body mass and body temperature were observed when food restriction was imposed during the period of rapid growth

(5d-7d) compared with the time after adult body size was reached (11d-13d). In birds restricted early and then refed until 13d, final body mass did not significantly differ from 13d controls, indicating that birds have capacity to recover from slowed early growth. This pattern of slowed growth with early restriction, and recovery upon re-feeding, mirrors scenarios observed in free-living birds. Alpine swifts (fledging age 50-76d) experiencing food restriction early in the nestling period (15-21d) showed slowed body mass growth that was quickly recovered upon re-feeding. Later-stage nestlings (21-27d) showed no significant reduction in body mass growth in response to food restriction (Bize et al. 2006). We were interested in examining how the timing of food restriction in house sparrows nestlings impacted levels of immune function, trade-offs with body growth and metabolism, and lasting allocation differences after re-feeding.

Because of their purportedly different costs, we assessed the effects of food restriction on three different types immune responses in house sparrow nestlings. We observed a trend for reduced haptoglobin concentration with food restriction in both age groups (treatment $p=0.054$), but no significant impact of feeding treatment on circulating adaptive IgY levels or constitutive innate lysis activity in food restricted birds. Significant reductions in liver mass, where acute phase proteins are synthesized, may have contributed to reductions in induced haptoglobin response to LPS injection. Our findings support the notion that the induced acute phase response is relatively more costly compared with other arms of the immune system (Iseri and Klasing 2013). We saw no lasting impact of early food restriction on induced haptoglobin response. Birds re-fed following restriction from 5d-7d and sampled at 13d showed no significant differences from 13d controls. This is similar to findings in young chickens, that, despite reductions in pro-inflammatory components during restriction, measures were generally not significantly lower than controls after 20 days of re-feeding (Jang et al. 2009). Therefore, investment in

inflammatory acute phase responses appears to be related to immediate costs of use, and thus its magnitude is flexible to immediate environmental resource availability.

The observed lack of difference in circulating IgY levels between control and restricted birds reinforces the idea about low costs of development of the humoral arm of immune function. Total circulating IgY levels reflect stimulation of IgY-secreting B-cells by constitutive environmental antigens. Levels in the current study were similar to those in free-living house sparrow nestlings of similar age (Killpack et al. in press), indicating a similar magnitude of constitutive B-cell stimulation in wild-collected nestlings in the laboratory compared with the wild. Though food restriction only lasted for two days during the nestling period, our finding of unaltered circulating IgY levels is in agreement with studies of longer duration food restriction in young birds. Young chickens showed no change in circulating IgY levels following a 7-day food restriction of 30% of food intake (Jang et al. 2009) and nestling starlings showed no decrease in circulating IgY levels when raised in enlarged broods where competition likely limits food (Bourgeon et al. 2011). Thus, it appears that constitutive development of B-cells and circulating IgY levels incur low energetic costs during the nestling period.

No significant difference in lysis activity was observed with food restriction treatment. However we did observe a pattern of slightly higher lysis activity with food restriction in 7d birds compared with 13d birds in comparison with their same-aged controls. Food-restricted 7d birds also demonstrated increased body temperature following LPS injection, perhaps indicating a fever response to LPS. Enhanced immune activity and fever in response to antigen stimulation leads to the production of reactive oxygen species, which may cause immune-pathological damage to host tissues if they are not effectively neutralized by antioxidants (Costantini and Møller 2009). Increased reactive oxygen intermediates or reduced antioxidant capacity have been

observed in response to food restriction zebra finches (Blount et al. 2003), chickens (Hangalapura et al. 2005), and starlings (Bourgeon et al. 2011). Elevated lysis activity with food restriction in 7d compared with 13d house sparrows may reflect relatively increased oxidative stress in early-stage nestlings responding to LPS injection. This finding supports the hypothesis that oxidative stress could be an important 'currency' for trade-offs between immune function and other life history traits (Hasselquist and Nilsson 2012) Future quantitative studies of fever, antioxidant capacity, and oxidative status in immune-challenged nestlings are necessary to determine differences with age and food restriction treatment.

To compare the relative costs of immune function to those of growth and maintenance metabolism, we also examined organs, structural growth, and muscle maturity during restriction. We found significant reductions in organ masses with food restriction, regardless of its timing during the nestling period. Several studies in growing birds have demonstrated the flexibility of digestive organs and muscle masses to feeding (Lacombe et al. 1994, Negro et al. 1994, Burness et al. 2000, Brzek and Konarzewski 2001, Dahdul and Horn 2003, Moe et al. 2004, Killpack and Karasov 2012a), with down-regulation in size possibly reflecting the high metabolic costs of maintaining those tissues. There were no significant differences in organ masses in 13d refed birds compared with 13d controls, indicating that reductions in organ mass are recovered upon return to adequate feeding conditions.

In contrast to previous studies, we did observe slight reductions in skeletal growth in food-restricted nestlings, particularly in 7d food restricted birds. This indicates that food restriction had a more significant impact on structural growth early in the nestling period, during the time of elevated growth demands in house sparrows (5d-7d). There were no persistent reductions in skeletal measures in birds that were refed following early restriction, indicating that

slowed growth during restriction was recovered upon re-feeding and may not have lasting consequences for post-fledging fitness.

Reductions in flight muscle mass and muscle maturity, indexed by total water content and mean total CS activity, were observed in response to food restriction. In contrast to organ and skeletal measures, these reductions in muscle size and maturity persisted even following return to control feeding. Additionally, 13d refed birds had significant reductions in body temperature throughout the experimental period following restriction compared with controls. Thus, early restriction may have permanently altered metabolism and/or thermoregulatory ability in these birds and could have significant fitness consequences post-fledging.

Conclusions

By using controlled food restriction and simultaneous measurement of multiple immune functions, we found support for the hypothesis that induced innate responses are significantly more costly than constitutive innate or humoral responses. However, the impact of food restriction treatment on nestling immune response was relatively small when compared with the significant reductions observed in flight muscle and organ masses. If the energetic costs hypothesis is true, then these allocation patterns suggest that growth and maintenance of muscle and organ tissues may be relatively more expensive than development and use of immune functions measured (Sheldon and Verhulst 1996, Hasselquist and Nilsson 2012). Alternatively, immune function and body growth and maintenance may both incur costs, but perhaps altered immune function is irreversible if reduced during food restriction, and thus immune function is conserved. But, this does not appear to be the case because significantly lower haptoglobin levels during food restriction in the current study were recovered with refeeding. With the exception of

muscle measures that likely relate to level of maturity, reductions observed with early food restriction were recovered upon refeeding later in the nestling period. This study demonstrates that nestling growth and immune response patterns are flexible to energy limitation resulting from common environmental perturbations during the period of rapid growth in the nest. Possibly lasting impacts of reduced flight muscle maturity found in the current study should be further examined.

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Figure Legends

Figure 1. Experimental design. Hand-feeding in the laboratory began at 3d post-hatch. Solid lines represent days when birds were fed age-specific meal sizes that support normal growth, and dashed lines represent days when birds were fed 40% less than age-specific meal sizes. Lipopolysaccharide injection occurred 15.2±0.1 hours prior to euthanization at 7d or 13d.

Figure 2. Daily body mass (A) and body temperature (B) in 7d birds. Data are means ± s.e.m. (error bars are smaller than the circles in some cases) for control (filled triangles) and food-restricted (unfilled circles) birds (N 12 per group). Dashed lines represent the 48h of food restriction. Significant differences between groups are described in the text.

Figure 3. Daily body mass (A) and body temperature (B) of 13d birds. Data are means ± s.e.m. (error bars are smaller than the circles in some cases) for control (filled triangles), food-restricted (unfilled circles), and refed (grey squares) birds (N 12 per group). Dashed lines represent the 48h of food restriction. Significant differences among groups are described in the text.

Figure 4. Immune measures for control (unfilled bars), food-restricted (filled bars), and refed (grey bars) birds. Data are means ± SEM (n=12 for each treatment group). Bars that share the same letter are not significantly different as determined by Tukey's *post hoc* tests following two-way ANOVA. Periods denote a trend for treatment effect in haptoglobin response (p=0.054). There were no significant differences between 13d-Refed and 13d-Ctrl groups in any immune measure.

Figure 5. Structural measures for control (unfilled bars), food-restricted (filled bars), and refed (grey bars) birds. Data are means ± SEM (n=12 for each treatment group). Bars that share the same letter are not significantly different as determined by Tukey's *post hoc* tests following two-way ANOVA. There were no significant differences between 13d-Refed and 13d-Ctrl groups in either structural measure.

Figure 6. Dry organ masses for control (unfilled bars), food-restricted (filled bars), and refed (grey bars) birds. Data are means ± SEM (n=12 for each treatment group). Bars that share the same letter are not significantly different as determined by Tukey's *post hoc* tests following two-way ANOVA. There were no significant differences between 13d-Refed and 13d-Ctrl groups in any organ measure.

Figure 7. Flight muscle measures for control (unfilled bars), food-restricted (filled bars), and refed (grey bars) birds. Data are means ± SEM (n=12 for each treatment group). Bars that share the same letter are not significantly different as determined by Tukey's *post hoc* tests following two-way ANOVA. An asterisk above 13d-Refed bar indicates a significant difference from 13d-Ctrl.

Figure 1.

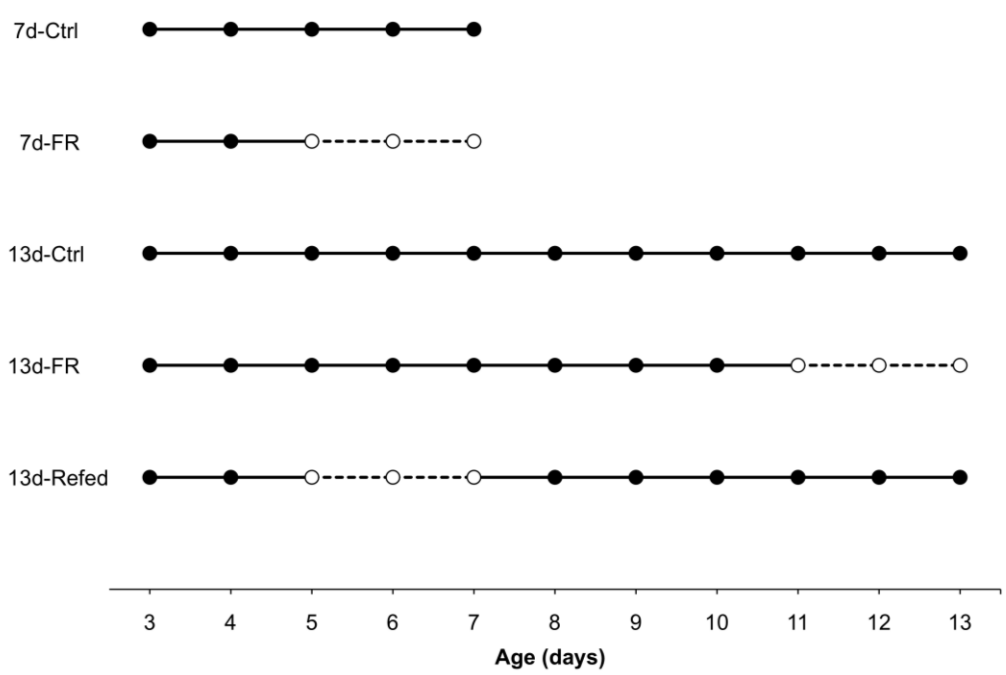

7d-Ctrl
7d-FR
13d-Ctrl
13d-FR
13d-Refed
3
4
5
6
7
8
9
10
11
12
13
Age (days)

Figure 2.

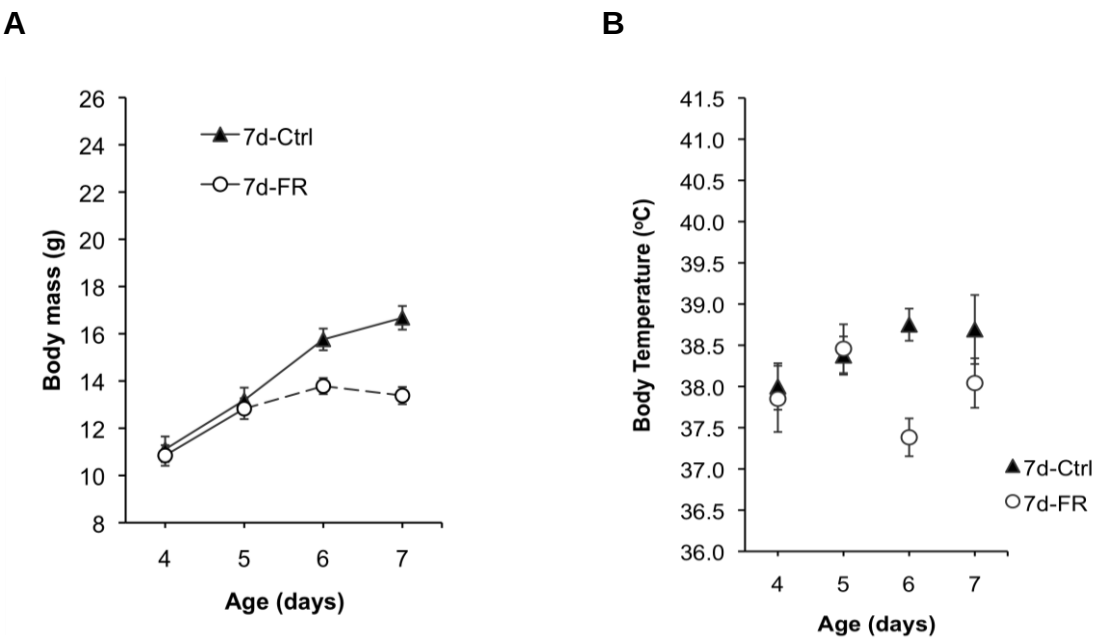
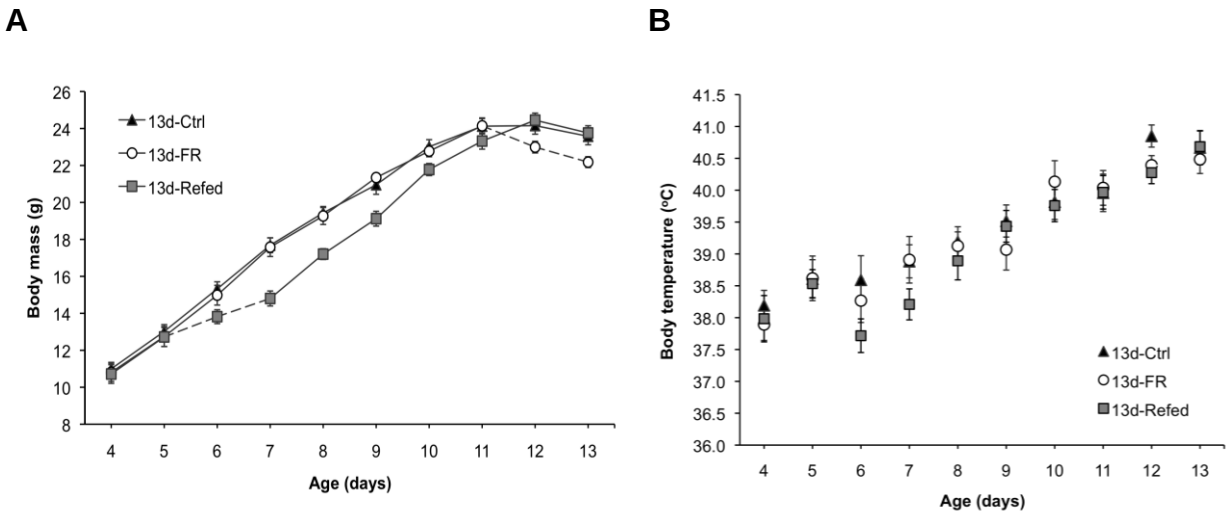


Figure 3.



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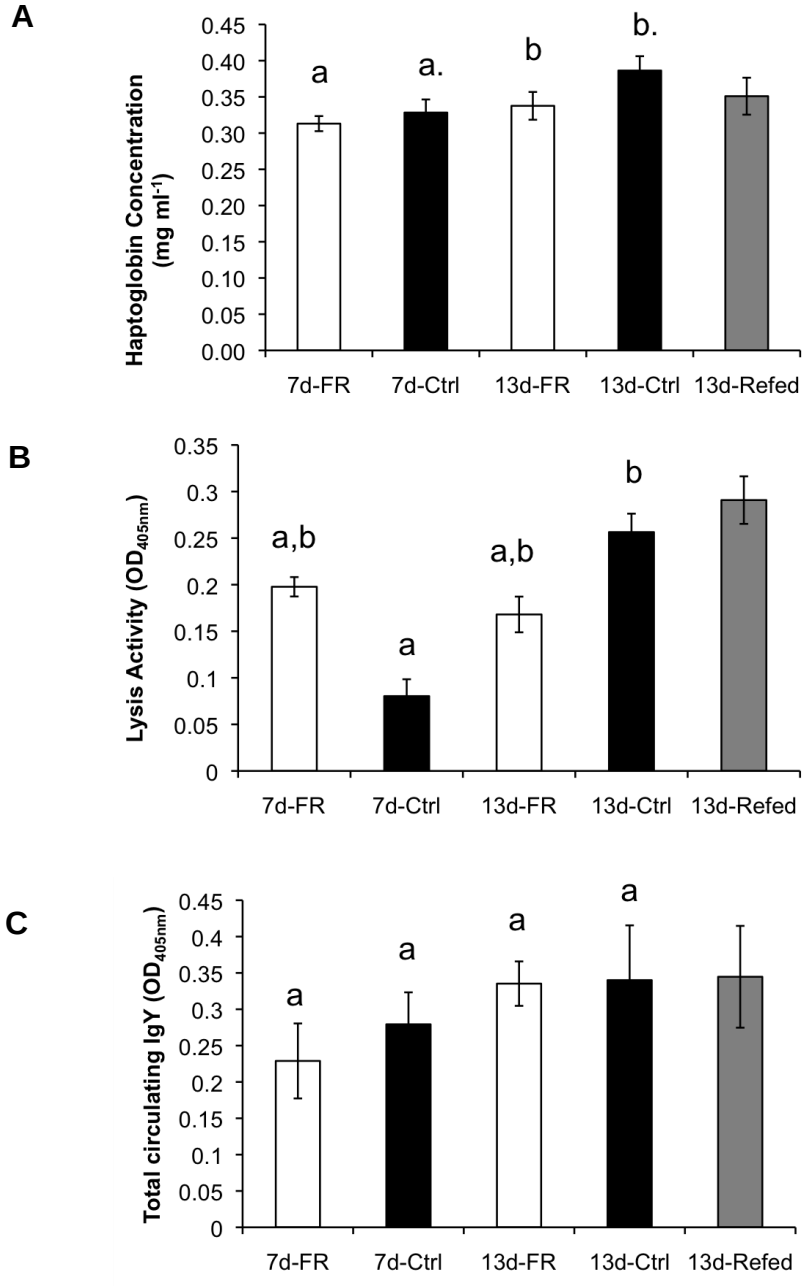

A
Haptoglobin Concentration (mg ml-1)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a.
b
b.
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
B
Lysis Activity (OD405nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a,b
a
a,b
b
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
C
Total circulating IgY (OD405nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
a
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed

Figure 5.

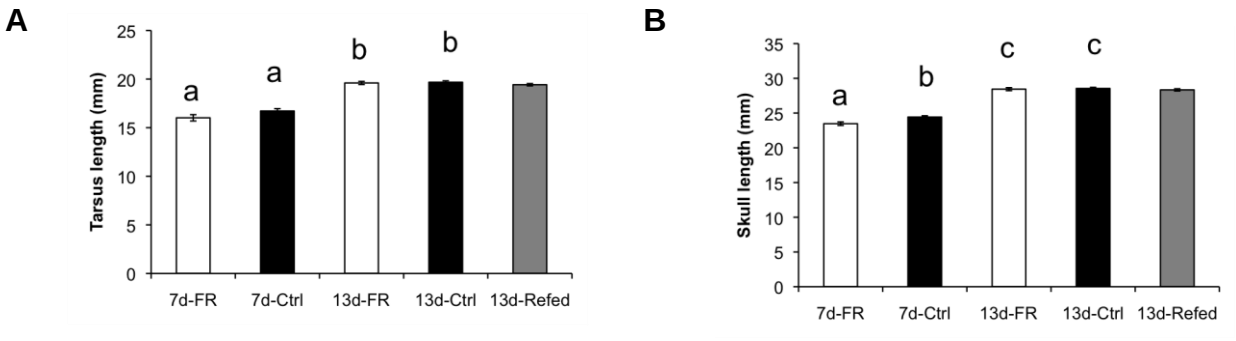

A
Tarsus length (mm)
25
20
15
10
5
0
a
a
b
b
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
B
Skull length (mm)
35
30
25
20
15
10
5
0
a
b
c
c
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed

Figure 6.

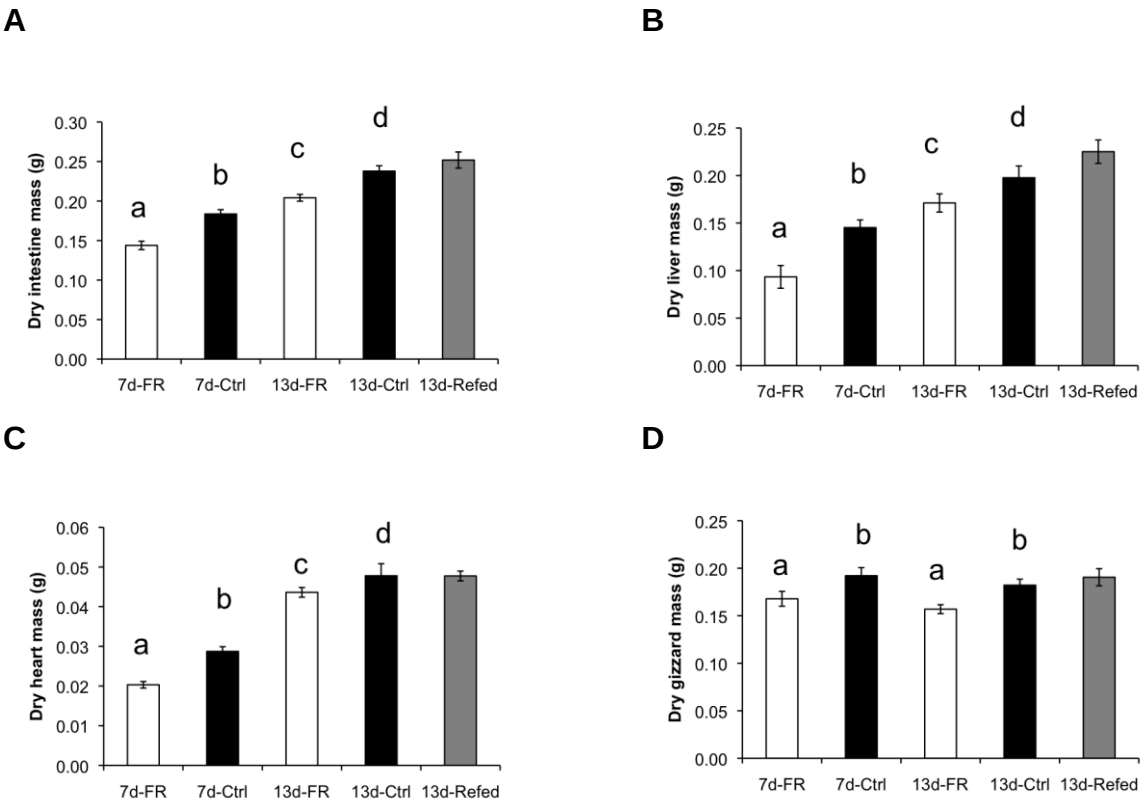
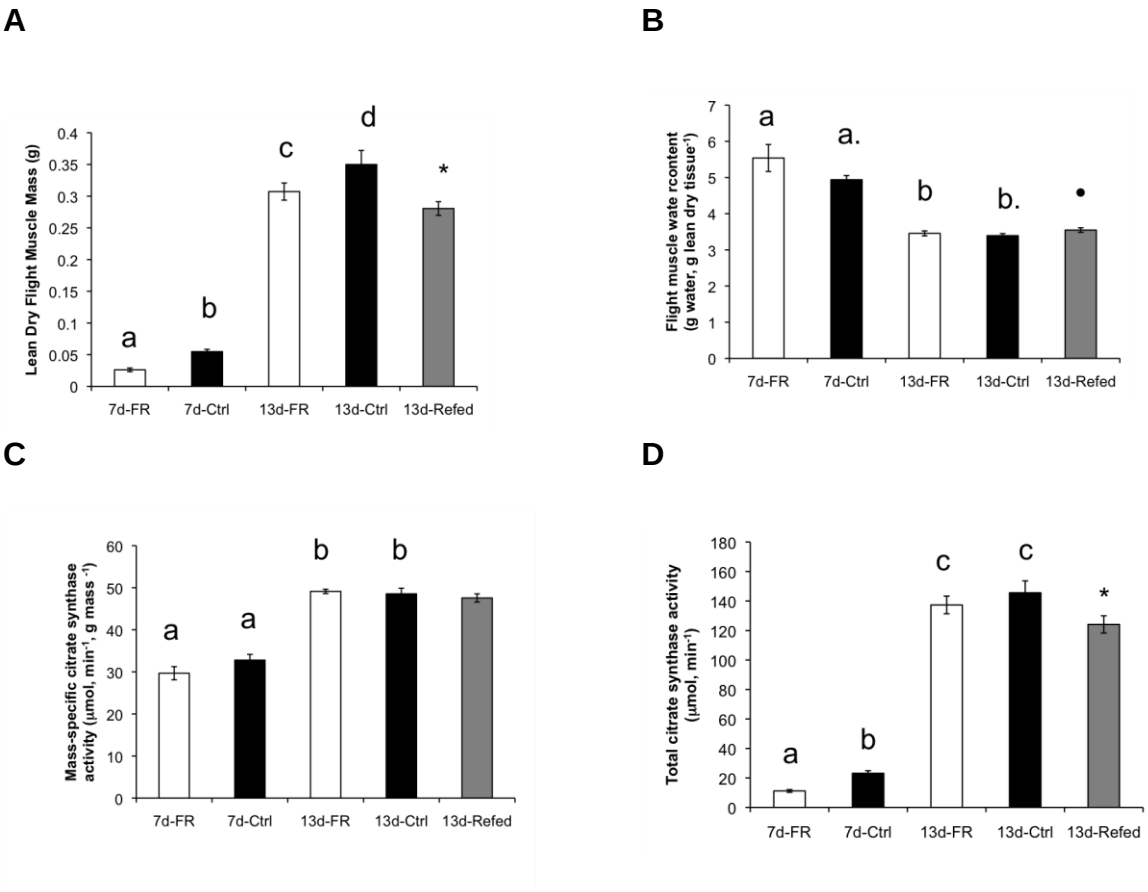

A
Dry intestine mass (g)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
d
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
B
Dry liver mass (g)
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
d
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
C
Dry heart mass (g)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a
b
c
d
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
D
Dry gizzard mass (g)
0.25
0.20
0.15
0.10
0.05
0.00
a
b
a
b
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed

Figure 7.


A
Lean Dry Flight Muscle Mass (g)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
c
d
*
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
B
Flight muscle wate rcontent (g water, g lean dry tissue-1)
7
6
5
4
3
2
1
0
a
a.
b
b.
•
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
C
Mass-specific citrate synthase activity (µmol, min-1, g mass -1)
60
50
40
30
20
10
0
a
a
b
b
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed
D
Total citrate synthase activity (µmol, min-1)
180
160
140
120
100
80
60
40
20
0
a
b
c
c
*
7d-FR
7d-Ctrl
13d-FR
13d-Ctrl
13d-Refed

CONCLUSION AND FUTURE DIRECTIONS

The main findings of this thesis were (1) innate and adaptive immune functions I studied developed slowly during the nestling period of altricial passerines, and levels in fledglings were significantly lower than in adults and (2) immune functions in nestlings I studied were not substantially or irreversibly impacted by food restriction during the nestling period. These findings can be applied to make comparisons in timing of immune development among altricial and precocial bird species, as well as mammals. In order to draw conclusions about the relative timing of maturation across species, it would be useful to determine a developmental analog to fledging in altricial birds in precocial and mammalian models. Additionally, tools could be further developed to measure and quantify immune responses in altricial birds. Designing an ELISA assay that utilizes a dilution series or standard curve of antibody concentrations would allow for further quantification of adaptive antibody responses from optical density measures.

The studies in this thesis also serve as a framework for future work in the fields of developmental and comparative immunology, ecological immunology, and physiological ecology. Future studies of immune function in altricial nestlings should consider the following topics related to ontogeny of immune function and influences on the strength and variation in immune response: (1) Maternal antibody transfer and impact on nestling endogenous immune response, (2) Immune response to live infectious agents and trade-offs with growth and development, and (3) Development of gut immune function in altricial birds.

1. Maternal antibody transfer and impact on nestling endogenous immune response

The lack of mature functional antibody response during the early weeks post-hatch may be partly compensated by maternal provision of IgY via egg yolk, which is absorbed into nestling circulation during development. Detection of specific maternal antibodies has shown varied persistence in nestling circulation during the early weeks post-hatch (Darbyshire and Peters 1985, Lozano and Ydenberg 2002, Hahn et al. 2006, Nemeth and Bowen 2007, Baitchman et al. 2007, Nemeth et al. 2008, Grindstaff 2009, King et al. 2010) and the half-life of maternal antibodies in the circulation of altricial nestlings appears to be shorter than in precocial birds (Figure 1). The mechanism of clearance of maternal antibodies in the plasma of altricial nestlings is understudied. It is possible that maternal antibodies are cleared more rapidly from circulation if they form complexes with foreign or self antigen. Examining decay of specific maternal antibodies in circulation in naïve nestlings compared those injected with the antigen specific to the maternal antibodies would shed light on this question.

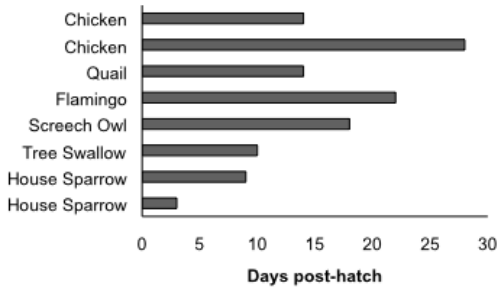


Figure 1. Persistence of maternal antibodies in the circulation of birds**.** References in text.

Estimates of the persistence of maternally derived antibodies in the circulation overlap with the time when altricial birds generate circulating B cells capable of humoral immune response. Contrasting hypotheses exist regarding the influence of maternal antibodies on nestling humoral immune development. Maternal Igs may have a 'priming effect' on nestling immune development by functioning as internal antigens and stimulating proliferation of those Ig-specific B cells (Hasselquist and Nilsson 2009). Maternal antibodies had protective effects against live

virus or bacterial infection in precocial turkeys and chickens even after being catabolized from circulation (Fadly and Nazerian 1989, Nemeth and Bowen 2007), and have been shown to increase specific antibody responses in mature offspring that had received specific maternal antibodies as neonates (Gasparini et al. 2006, Grindstaff et al. 2006, Reid et al. 2006).

Alternatively, maternal antibodies have been shown to exert a short-term 'blocking effect' when in circulation. Maternal antibody-antigen complexes may be rapidly cleared by phagocytosis, preventing activation of B- and T-cells and triggering of nestling adaptive antibody response (Hasselquist and Nilsson 2009). Significantly lower nestling antibody responses have been observed when vaccine-specific maternal antibodies were present in the circulation of young birds at the time of vaccination (Spencer and Robertson 1972, Nemeth and Bowen 2007, Staszewski et al. 2007, Elazab et al. 2009, 2010, Gasparini et al. 2009, Staszewski and Siitari 2010). However, the 'blocking' effect observed in the noted studies did not persist in birds vaccinated after maternal antibodies were catabolized from circulation.

Future studies should examine the persistence of specific maternal antibodies in the circulation of altricial nestlings and investigate whether maternal antibodies interact with the development of the humoral immune system, through a blocking or priming effect. Those studies will shed light on when nestlings are most susceptible to infection and whether lasting benefits are garnered even after maternal antibodies have been catabolized from circulation.

2. Immune response to live infectious agents and trade-offs with growth and development

Studies in this thesis demonstrated that nestlings are immunologically immature at the time of fledging, when they leave the nest environment and experience an increased antigenic environment. It is of interest to examine how exposure to infectious agents and parasites changes

from the nest to the post-fledging environment. It is also if interest to examine whether nascent immune functions in nestlings and fledglings translate to relatively increased morbidity and mortality in these juvenile birds. Nestling birds are rarely sampled in the field, though a recent study showed that 4% of house sparrow nestlings in a study system were infected with West Nile Virus (WNV), and that in August alone, when WNV activity is high, over 12% of nestlings sampled for positive for the virus (O'Brien et al. 2010). Future studies of the infection rates and host potential of nestlings for zoonotic diseases are warranted.

Limited trade-offs between growth and immune function were observed during and after resource limitation in our studies. Lack of reduction in immune functions with food restriction may reflect that these functions are relatively energetically inexpensive compared with growth. Alternatively, maintenance of immune levels may reflect the relative importance of conserving immune function for a growing animal. Incorporating more frequent measures of body mass and body temperature, as well as metabolic rate, following injection with inflammatory antigens would allow for finer-scale analysis of physiological costs of the actual immune response in nestlings.

It is of interest to evaluate whether patterns of immune maintenance with food limitation hold when nestlings are responding to live infectious agents and parasites. A recent study showed that house sparrow nestlings infected with Buggy Creek alphavirus had reduced tarsus, culmen, and wing length, indicating that physiological changes associated with infection may lead to trade-offs with growth (Hiatt et al. 2013). Immunopathology as well as behavioral changes (such as anorexia) associated with inflammatory responses to parasites and other infectious agents may increase the potential for trade-offs in growth and development (Schulenburg et al. 2009). Because immunopathology associated with inflammatory immune

responses can be costly, stronger immune responses may not necessarily translate to increased fitness and survival (Sears et al. 2011). Finally, experiments including infectious agents may allow for more sophisticated evaluation of host defense strategies by measuring resistance (minimization of parasite burden) versus tolerance (minimization of damage caused by a parasite burden) (Råberg et al. 2009).

3. Development of gut immune function in altricial birds

While studies in this thesis focused on development of systemic immune components, the gut is estimated to comprise more immune cells than any other tissue in the body (Davison et al. 2008), and should be a focus of future ontogeny studies. Lymphoid aggregates, Peyer's Patches, and cecal tonsils sample antigens from the gut flora, and the bursa of Fabricius also samples cloacal antigen (Davison et al. 2008). The development of gut-associated lymphoid tissue (GALT) in altricial nestlings is largely understudied. In chickens, innate immune components, indexed by expression of pro-inflammatory cytokines, antibacterial β-defensins, toll-like receptors, and inducible nitric oxide synthase, are present in GALT during the first week post-hatch (Bar-Shira and Friedman 2006, Miyazaki et al. 2007). Maturation of adaptive immune function in GALT has been shown to occur in 2 stages (Bar-Shira et al. 2003, Miyazaki et al. 2007). The first stage occurs during the first week post-hatch, when increases in $CD3^+$ cells (likely T cells or Natural Killer cells) and B cells are observed in the intestine. During the second week post-hatch, increasing levels of IL2 and interferon-gamma cytokines are observed, indicating a time lapse between establishment of immune cell populations and their expression of functional cytokines. No analogous studies, to our knowledge, have been performed in altricial nestlings.

Studies in chickens have also examined how GALT development is impacted by food restriction and food composition early post-hatch. Feed-withholding for 72h immediately post-hatch led to significant reductions in systemic and intestinal antibody responses and significant delay in colonization of the hindgut by T and B cells. Full recovery upon re-feeding did not occur until 2 weeks of age (Bar Shira et al. 2005). Feeding a high fat diet early post-hatch was shown to delay maturation of GALT in all sections of the gut. (Miyazaki et al. 2007). Given that altricial nestlings commonly experience food restriction in the early post-hatch period, it is of interest to examine how altered feeding may impact gut immune development.

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