Avian immune responses to ectoparasites: a serological survey of ectoparasite-specific antibodies in birds in western Nebraska

Carol Fassbinder-Orth

Abstract

Birds serve as reservoirs for at least 10 arthropod borne viruses of wildlife and human concern (e.g. West Nile virus, Western Equine Encephalitis) and greater knowledge of the immune system dynamics of avian hosts and their disease vectors will have obvious economic benefits to agricultural, wildlife and human health interests. The more we begin to understand the host-vector-pathogen interactions that contribute to the emergence and transmission of arthropod borne diseases, we can better predict where outbreaks might occur and whose health and economic interests will be affected. Arthropod vectors (ectoparasites) exert strong direct selection pressures on their avian hosts by decreasing nestling survival, reducing future reproductive events and host lifespan. Levels of ectoparasites are known to vary widely across species with different life history strategies, and also across different life history stages of the same species. For example, colonial nesting birds (e.g. swallows and martins) have been shown to have enhanced levels of ectoparasites compared to non-colonial birds (e.g. sparrows) and nestlings are known to be highly susceptible to ectoparasites in multiple avian species. However, no studies have been performed that have investigated how immune responses to arthropod disease vectors variy across avian species with different life history characteristics (i.e. native vs. invasive), or the role that arthropod salivary proteins play in avian arbovirus disease ecology. The specific objectives of this proposal are aimed at quantifying the immune responses of free-living cliff swallows and house sparrows to the salivary proteins of an ecologically relevant ectoparasite, the swallow bug. Additionally, I will characterize the swallow bug-specific immune responses of nestling, juvenile and adult birds following experimental inoculation with swallow bug salivary proteins. To my knowledge, this will be the first study to document age-dependent immune responses of native and invasive avian hosts in response to ectoparasites, and will increase our knowledge of the immunomodulatory effects of vector salivary proteins on avian hosts.

I. Statement of Purpose

Arthropod vector-specific immune responses are found in many vertebrate populations (e.g. humans, livestock, wild birds and mammals), and salivary protein-specific antibody responses have been used as a marker for vertebrate exposure to ticks, mosquitoes, sand flies, nest flies, black flies and triatomines (Hostomska et al., 2008, Schwartz et al. 2009, Huber et al. 2010, Tsujimoto et al. 2010). Additionally, host immune responses to vectors that occur prior to and concurrent with pathogen exposure may influence the severity and etiology of vector-borne diseases (Donovan et al. 2007, Schneider et al. 2004). For this reason, it is important to understand not only the immune responses of hosts to arthropod-borne diseases, but the immune responses to the arthropod vectors as well. However, explicit comparative studies of the levels of arthropod-specific antibodies in multiple free-living species of birds exposed to the same ectoparasites have not been done. Additionally, to my knowledge, no studies have been performed that have explored antibody production and cytokine expression following experimental exposure of free-living bird species to arthropod salivary proteins.

This research will be the first to investigate the specific immunological mechanisms underlying the immune responses of free-living birds to an ectoparasite called the swallow bug. I will utilize a system in which two free-living avian species (one an endemic native and one a susceptible invasive) are the primary hosts of an arthropod borne virus (arbovirus) called Buggy Creek virus, which is transmitted by the swallow bug. I will not only be able to compare immunocompetence of these two species to this ecologically relevant antigen (swallow bug salivary proteins) but also be able to explore the development of immunocompetence with age by exposing the nestlings of these species to the antigen and quantifying their immune responses over time.

II. Significance of the Problem

This work has three major implications for our understanding of ecological immunology, the ontogeny of avian immune responses, and host-vector-pathogen evolution more broadly. (1) ***It addresses fundamental questions about differences in immunocompetence between invasive and native species, and how their life history strategies may have affected their susceptibility to specific diseases.*** The work proposed here is unique in that innate and adaptive immune responses will be measured in these species against an ecologically relevant pathogen, which will yield information about how invasive (house sparrow) and native (cliff swallow) species invest in their immune responses to relevant pathogens. (2) ***By categorizing the immune responses developing birds to a relevant pathogen, our understanding of the ontogeny of the avian immune system will be improved***. Furthermore, we will be able to identify the specific aspects of the immune response that change during nestling development, which may help to explain the differences in age-dependent disease susceptibility that is seen for several avian diseases. (3) ***This project also addresses key questions about host-vector interactions.*** By investigating the role that swallow bugs play in the stimulation of the avian innate and adaptive immune responses in native and invasive hosts we will expand our knowledge of the influence arthropod vectors may have in the etiology and evolution of vector-host interactions and vector borne diseases.

III. Summary of Pertinent Literature

It is well known that host immune responses are costly physiological responses. The costs incurred upon the host may be in the form of immunopathologies, in which elements of the immune response may result in host damage, as in hyperactive inflammatory immune responses (i.e. Systemic Inflammatory Response Syndrome) or autoimmunity (Sadd and Schmid-Hempel 2009). The costs of immunity may also be evident during times of limited energetic and nutritional resources, and may result in trade-offs between investment in immunocompetence (ability of the host to prevent or limit infections) and life history decisions such as reproduction (Norris and Evans 2000, Zuk and Stoehr 2002, Bonneaud et al. 2003, Tschirren and Richner 2006, Martin et al. 2008). Immunocompetence and life history trade-offs have been shown to be in direct conflict for several avian species (Deerenberg et al. 1997, Ilmonen et al. 2000, Lochmiller and Deerenberg 2000, Hasselquist et al. 2001, Brommer 2004), with the innate immune system being considered the most costly to maintain and use and most likely to generate a cost to host fitness (Klasing 2004, Martin et al. 2008).

Differences in immune response strategies have been suggested as an explanation for some species' success, but few studies exist that have investigated this topic. One study compared the house sparrow with its less successful invasive congener, the Eurasian tree sparrow, and suggested that the innate, inflammatory immune response to phytohemagglutinin (PHA) and lipopolysaccharide (LPS) is less costly in metabolic, behavioral and reproductive measurements for the house sparrow than the tree sparrow (Lee et al. 2005), possibly due to a dampened systemic inflammatory response in the house sparrow. However, when the adaptive immune system (the pathogen-specific portion of the immune system that includes antibody and cell-mediated responses) responses of house sparrows and tree sparrows to keyhole limpet hemocyanin (KLH) were investigated, it was found that house sparrows mounted a stronger adaptive immune response to KLH than did tree sparrows (Lee et al. 2006). These two studies suggest that investment in some types of immune responses may be costlier to the host than others, and may be predictive of invasion success for some species. ***I am aware of no studies that compare the immune responses of an invasive avian species with a native species following pathogen infection or arthropod vector antigen.*** By documenting these responses, I will be able to determine if invasive species such as the house sparrow show differential immune responses to an ecologically-relevant disease or antigen compared to a native bird (as predicted by previous researchers; Klasing 2004, Lee et al. 2005, 2006, and Martin et al. 2008) and whether these immunological investments may be protective for the host.

Immunocompetence is known to vary with age in several mammalian species (Levy 2007), but comparable studies on the immunological ontogeny of free-living bird species are few, as the majority of studies on avian immunological ontogeny have focused on chickens and other domesticated birds with precocial development (Klasing and Leschinsky 1999, Palacios et al. 2009). The few studies that have been conducted present mixed results of both innate and adaptive immune system ontogeny in birds. Some have suggested that most nestlings are unable to mount a detectable adaptive immune response prior to 4 weeks of age (Apanius 1998, Moller et al. 2001), and that younger nestlings are highly dependent on trans-ovo passive immunity (e.g. maternal antibodies) and on their own innate immune system (Pihlaja et al. 2006). However, others report that some aspects of the adaptive immune response may reach adult levels in altricial nestlings sooner than predicted (Tella et al. 2002, Palacios et al. 2009, King et al. 2010). Although the specific explanatory mechanisms are lacking, nestlings have been shown to be more susceptible than adults to several pathogens, including flaviviruses and alphaviruses (Holden et al. 1973, Scott et al. 1988, Swayne et al. 2001, Nemeth and Bowen 2007, O'Brien et al. 2010). ***However, there are no studies***

published to date that have explored the development of the immune response of altricial birds to ectoparasites.

Ectoparasites exert strong direct selection pressures on their avian hosts by decreasing nestling survival, reducing future reproductive events and host lifespan (Hamilton and Zuk, 1982, Møller et al 1993, Fitze et al. 2004). Levels of ectoparasites are known to vary widely across species with different life history strategies, and also across different life history stages of the same species (Hamstra and Badyaev 2009). For example, colonial nesting birds (e.g. swallows and martins) have been shown to have enhanced levels of ectoparasites compared to non-colonial birds (Poulin 1991, Davis and Brown, 1999), and nestlings are known to be highly susceptible to ectoparasites in multiple avian species (Richner et al. 1993, Møller et al. 2009).

The influence of arthropod vectors on arthropod-borne disease etiology has been elucidated in several disease systems in mammals, with particular focus on the immunomodulatory effects of arthropod salivary proteins. For instance, *Borrelia burgdorferi*, the causative agent of Lyme disease, utilizes the salivary protein Salp15 from the tick vector, *Ixodes scapularis,* to enhance Lyme disease infection in mammals (Ramamoorthi et al., 2005). ***However, no studies have been performed that have investigated how immune responses to arthropod disease vectors vary across avian species with different life history characteristics, or the role that arthropod salivary proteins may play in avian arbovirus infections.***

IV. Research Questions

Invasive avian species (e.g. house sparrow) have been shown to significantly differ in their immune responses to innocuous and infectious challenges (Holden et al. 1973, Lee et al. 2005, 2006, O'Brien et al. 2010), which may reflect differential investment in immune defenses compared to other life history decisions (Sadd and Schmid-Hempel 2009). For example, house sparrows are more susceptible than their native endemic counterpart (cliff swallows) to an alphavirus called Buggy Creek virus (BCRV), with nestlings being the primary amplifiers of the virus (Huyvaert et al., 2008, O'Brien et al. 2008, O'Brien et al. 2010). Additionally, arbovirus infections in mammals have been shown to be modulated by salivary proteins of their arthropod vector, but equivalent studies in birds are lacking. The specific objectives of this proposal are aimed at quantifying the immune responses to swallow bug salivary proteins that exist in natural populations of cliff swallows and house sparrows, and characterizing the swallow bug-specific immune responses of birds following experimental inoculation with swallow bug salivary proteins. My hypotheses are as follows:

(1) **I predict that free-living adult house sparrows and cliff swallows that have been exposed to swallow-bug infested nests will have detectable levels of swallow-bug specific antibodies.** To test this, I will first do a serological study of 50 free-living adult cliff swallows and 50 house sparrows at Cedar Point Biological Station in western Nebraska to test for swallow bug–specific antibodies.

(2) I predict that captive birds will produce measurable immune responses to swallow bug salivary proteins and that the level of the immune response will be correlated to life history strategies and evolutionary history with the ectoparasite. Predicted incidence and strength of swallow bug-specific immune responses: cliff swallow> house sparrow. To test this I will experimentally inoculate nestling house sparrows and cliff swallows with swallow bug salivary gland extract and test for the development of swallow-bug-specific immune responses

V. Design and Methods

Serological survey. The levels of swallow-bug specific antibodies present in wild populations of cliff swallows and house sparrows will be measured. Adult house sparrows (n=50) and cliff swallows

(n=50) will be captured in mist nets near the Cedar Point Biological Station, where over 175 cliff swallow colonies with accompanying house sparrow nests can be found (Brown et al., 2008). Blood will be collected via jugular venipuncture. Sera will then be separated and stored at -80 °C until needed for enzyme immunoassay processing.

Experimental inoculation. To investigate the immune response of birds to swallow bug salivary proteins, an experimental inoculation of swallow bug salivary proteins will be performed with nestling cliff swallows and houses sparrows. Nests at fumigated cliff swallow colonies (see Brown and Brown 1996) where bugs have been removed will be monitored closely for cliff swallow and house sparrow nesting. All nests will be monitored daily, bugs removed if found, and nestlings will be taken from nests in the field immediately upon hatching. Nestlings will be hand-reared in the laboratory using methods developed by Oesterle (2008), and Blem (1975). When nestlings are 5 days old, blood will be collected from all birds via jugular venipuncture, and processed via quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) to screen for BCRV and to assess immune gene expression. Sera will also be separated from the blood and will be screened for swallow bug- specific antibodies using Enzyme Immunoassay. Only birds negative for BCRV and swallow bug antibodies will be included in the study. Ten birds of each species will be inoculated subcutaneously with swallow bug salivary gland extract (SGE) and three birds of each species will serve as controls, receiving subcutaneous saline injections only. Blood and tissue samples will be collected according to the schedule shown in Figure 1.

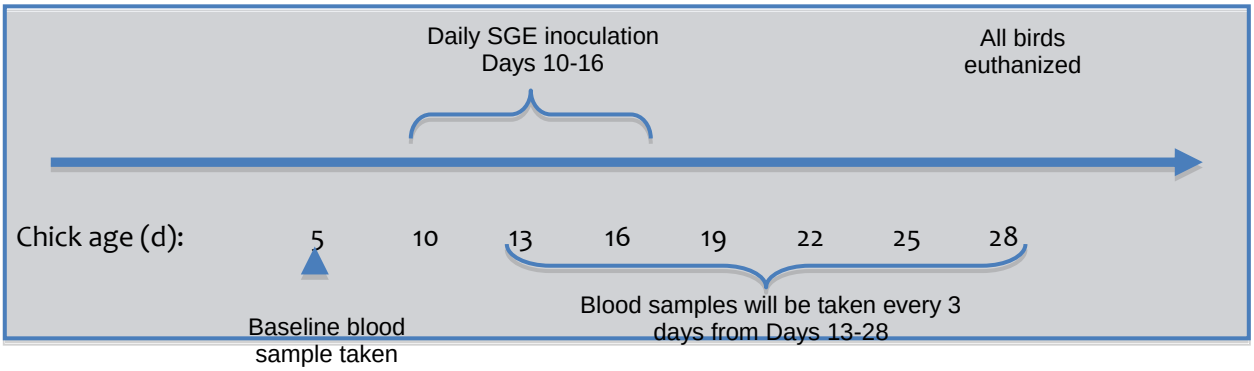

Figure 1. Experimental Schedule for Swallow Bug Salivary Gland Extract Inoculation Experiment

Each SGE extract inoculation will contain the equivalent of 2 pairs of salivary glands, a level of inoculation that has been shown to induce immune responses in mammals (Schneider et al 2004). The repeated inoculation used in this experiment will mimic the repeated swallow bug exposure that occurs in natural nest settings. The initial inoculation at age 10 days was based on previous research on the ontogeny of the adaptive immune response in altricial birds. The limited studies performed in birds suggest that the adaptive immune response begins to develop around 7-12 days of age (Koppenheffer et al. 1981, Mast and Goddeeris 1999, Gaunson et al. 2006, King et al. 2010). Additionally, cliff swallows cannot be held in captivity past approximately 28 days of age (when fledging normally occurs) due to their being aerial insectivores (Oesterle 2008). This timeframe allows for a 2 ½ week period to measure the adaptive immune responses of all three species to SGE. All blood samples will be assayed by Enzyme Immunoassay (described below) for the presence of SGE-specific antibodies.

Swallow bug salivary gland extract (SGE) preparation. Bugs will be collected from cliff swallow nests according to Moore et al. (2007). Then, salivary glands will be dissected and homogenized according to Schwarz et al. (2009).

Enzyme-linked immunoassay. An Enzyme-linked immunoassay (EIA) will be developed to detect the presence of antibodies specific for swallow bug salivary components. Avian species-specific antibodies will be purchased from a commercial source. Then, an EIA will be standardized according to Huber et al. (2010), with modifications. Briefly, 96 well plates will be coated with SGE diluted in coating buffer and allowed to coat overnight at 4 °C. Then, the plate will be washed and diluted cliff swallow and house sparrow sera will be added to each well and allowed to incubate at 40 °C for 1 hour. During the initial standardization phase of this assay, sera already collected from cliff swallows and house sparrows with known swallow bug contact will be used. Approximately 250 sera samples are currently available for assay, and will be provided by Charles Brown (University of Tulsa) and colleagues. Next, the wells will be washed, and anti-passerine IgY detecting antibodies (conjugated to horseradish peroxidase) will be added to each well and allowed to incubate for 1 hour. Appropriate substrate for the chromatophore will be added, and sample absorbances will be measured on a 96 well plate reader.

Immune gene expression. RNA in swallow bugs and bird blood and tissues will be extracted according to standard procedures and levels will be quantified by qRT-PCR. Viral RNA will be quantified using established BCRV protocols for viral RNA (Moore et al. 2007, Huyvaert et al. 2008, O'Brien et al. 2008). Quantification of messenger RNA (mRNA) expression of immune genes will be performed using standard techniques according to Barber et al. (2010). To perform the qRT-PCR on the immune genes in both bird species, sequences and primers that have already been established in my lab will be used and assays will be run on a real time PCR machine available in the Biology Department. The genes included in this immune gene expression experiment include pattern recognition receptors TLR2, TLR3, TLR4 and TLR7, which recognize conserved sequences in micro and macro parasites and also cytokines (protein messengers that coordinate immune responses). The cytokines will include interferons (IFN-α and IFN-γ) and interleukins IL-1β and and IL-10. For all qRT-PCR assays performed, beta-actin will be used as an endogenous control to normalize gene expression levels.

Project Schedule

Now-April, 2011: Finalize gene expression optimization and start Enzyme Immunoassay development
May 1-21: Collect sera samples from birds at Cedar Point Biological Station (predicted time required for collection: 2-3 weeks, based on previous experience with collaborator Charles Brown)
May 21-June 1: Collect swallow bugs and house sparrow and cliff swallow nestlings and bring them back to Creighton University.
June 1- June 30: Perform experimental inoculation of nestling birds (prior IACUC approval will be obtained)
July 1-August 31: Perform enzyme immunoassays and gene expression assays
September 1-December 1: Data Analyses, start manuscript preparation for publication

Data Analyses

1.The results of the Enzyme immunoassays, which will yield the levels of antibodies in free living and captive birds will be standardized using a standard curve and will be analyzed by either ANOVA or Repeated Measures Statistical Analyses.

2. The levels of immune gene expression will be normalized according to endogenous control genes to normalize expression. Then, results of this expression will be analyzed by ANOVA and necessary pairwise comparison tests (e.g. Tukey's).

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Appendices

A1. Biographical sketch

Carol Fassbinder-Orth received her BS from Iowa State University in Genetics in 2003 and her PhD in Zoology from the University of Wisconsin-Madison in 2008. Dr. Fassbinder-Orth has been a faculty member in the Biology Department at Creighton University since 2008. Dr. Fassbinder-Orth teaches courses in animal physiology and zoonotic disease ecology, and her research focuses on comparative avian physiology and disease ecology. She is a member and councilor of the Nebraska Physiological Society and a member of the Society for Integrative and Comparative Biology. Currently Dr. Fassbinder-Orth's lab is investigating two aspects of arbovirus disease ecology in wild bird species. The first deals with identifying the variation in the longevity of the immune response of wild birds to selected arboviruses. The second aspect of her current research focuses on determining the innate immune response mechanisms that are involved in the avian immune response to arboviruses. Dr. Fassbinder-Orth regularly engages undergraduates in her research and currently mentors 6 undergraduate students in her laboratory.

A2. Budget

Expense Description	**Estimated Expense**
3 Weeks Room and Board and Cedar Point Biological Station @ $280/week	**$840**
Detecting Antibody for Enzyme Immunoassay	**$120**
Animal care in Creighton's Animal Resource Facility (26 animals x $0.90/animal/day)	**$655**
Blood collection supplies, Reagents for Enzyme immunoassay and qRT-PCR experiments	**$1500**
Total Project Budget	**$3,115**

Total requested from the Faculty Research Fellowship Program:
$1000 ($500 from the allocated direct cost allowance and $500 from stipend)

The remaining $2,115 will come from Dr. Fassbinder-Orth's Faculty start-up fund