Avian Antibody Responses to Fowlpox virus and a Mosquito Vector

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Fall 2009

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TO THE UNIVERSITY HONORS COLLEGE:

As thesis advisor for JÚLIA PÁSZTOR,

I have read this paper and find it satisfactory.

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Jeb Owen, Thesis Advisor

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Date

PRÉCIS

The relationships between vectors, pathogens, and hosts are critical topics in entomology and infectious disease biology. Many diseases are spread from animals to humans and within animal populations by ectoparasitic arthropods such as mosquitoes. Fowlpox virus poses an especially great threat to the poultry industry and native species alike. Vectors and many pathogens are known to possess immunosuppressive properties. Several studies have examined molecules present in the saliva of vectors, or surface coats of pathogens, and found that these molecules suppress immune function of the host through a delay of a response, or complete inhibition. However, little is known about the interactions of these effects within the host. Understanding the relationship between the vector, the host, and the pathogen may advance current preventative medicine and knowledge of pathogen transmission. Questions concerning vector-pathogen-host interaction dynamics were addressed in this study. This study hypothesized that blood-feeding by mosquitoes would suppress the immune response of the host to a virus infection, and that the reciprocal effect would occur in the presence of Fowlpox virus.

White-leghorn chickens (*Gallus gallus*), Fowlpox virus, and the mosquito species *Culex quinquefasciatus* were used as models to understand these relationships. In the study chickens were divided into three treatment groups, exposed to mosquitoes, Fowlpox virus, or both. The experiment took place over a 7-week period. Blood was collected weekly from every chicken in the last 5 weeks of the experiment. Serum was isolated from the blood samples and was used to determine antibody levels specific to mosquito-proteins and Fowlpox virus-proteins using a common immunological assay (ELISA). The assay was designed to measure antibody levels used to evaluate an immune response to the mosquito challenge and Fowlpox virus challenge.

The results from these assays were recorded and analyzed for statistical significance. Repeated-measures and a multiple analysis of variance (MANOVA) were used to interpret the data. The results did not support the original hypothesis of reciprocal immunosuppression. An enhanced immune response to mosquitoes was observed in the treatment group exposed to mosquitoes followed by exposure to Fowlpox virus. The immune response to Fowlpox virus appeared to be lower in the last weeks of the study in the treatment group exposed to both mosquitoes and Fowlpox virus, though the initial antibody response to the virus was unaffected.

This study improves our basic understanding of vector-pathogen-host interactions. The results of this study suggest that timing of parasite exposure plays a critical role in the subsequent interactions of the immune responses to the individual parasites. The study demonstrates an interaction between the vector and pathogen; however additional studies need to be conducted to investigate the mechanisms of these interactions.

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INTRODUCTION

Ectoparasitic arthropods (such as ticks, lice and mosquitoes) serve as vectors for numerous pathogens that infect humans, as well as domesticated and wild animal species. Mosquitoes are one of the most important vectors, able to transmit an array of serious pathogens that include protozoans, nematodes, bacteria, and viruses. For example, mosquitoes transmit the filarial worms *Wuchereria* and *Brugia*, to over 120 million people in over 80 countries, causing the crippling disease known as lymphatic filariasis (WHO Media Center; Mullen and Durden, 2002). Mosquitoes in the genus *Anopheles* transmit four species of the protozoan genus *Plasmodium*, which causes 1 million deaths out of the 350-500 million annual cases of human malaria worldwide (Centers for Disease Control and Prevention, Malaria). A critical example of an animal disease caused by a mosquito-transmitted pathogen occurs in the Hawaiian Islands where the avian pathogen *Haemoproteus*, a genus of protozoan carried by the *Culex* mosquito, has devastated an endemic bird species known as the Hawaiian Honeycreeper (subspecies *Drepanidinae* in family *Fringillidae*) (Atkinson et al., 2008) *Aedes* and *Culex* mosquitoes transmit West Nile Virus (WNV), a flaviveridae virus that naturally infects multiple avian species. When transmitted to mammals (e.g. humans and horses) the infection can cause severe disease (encephalitis). In 1999, WNV first appeared in the United States affecting many crow populations, domestic horses, and humans. Since then the virus has claimed the lives of over 1,200 people within the United States and has cost over $500 million in medical expenses (Centers for Disease Control and Prevention, WNV). These examples illustrate some of the widespread effects of pathogen transmission by mosquitoes among human and animal populations, in terms of health, economics and conservation. Though the effects of these diseases in the vertebrate hosts have received a great deal of research attention, it is still

poorly understood what attributes associated with the vector contribute to the transmission of the pathogens.

During feeding, many blood-feeding arthropods secrete immunosuppressive materials from their salivary glands that may exacerbate the infectivity and/or pathological affects of the invading pathogen. These proteins inhibit local immune responses in the host at the bite site and enhance blood uptake by the arthropod (Wikel, 1996). For example, Lerner et al. (1990) found that a single salivary protein called Maxadilan in sandflies (*Lutzomyia longipalpis*) acts as a potent vasodilator, causing localized erythema that is essential to the blood feeding success of the sandfly. Maxadilan increases the secretion of a cell-signaling molecule (cytokine interleukin-6) by local macrophages and T-cells, which results in an immune response that limits immigration of granulocytic cells that could impair sandfly feeding success. Importantly, Maxadilan also promotes the local recruitment of neutrophils, which are cells preferentially invaded by the intracellular parasite *Lesihmania* that is transmitted by the sandfly (Lerner et al. 1990; Martínez et al., 2005; Lerner et al., 2007). Thus, a salivary protein serves a vital function for the feeding success of the arthropod and plays a direct role in promoting infection from a vectored pathogen (Morris et al., 2001).

Wasserman et al. (2004) explored the inhibitory affects of *Aedes* mosquito saliva on the immune response of mice. Mice exposed to mosquitoes in this study showed significant decreases in circulating T-cell and B-cell concentrations, indicating an immunosuppressing action from the mosquito (Wasserman et al., 2004). A subsequent study by Schnieder et al. (2007) revealed that mice exposed to blood feeding by *Aedes* had significantly higher mortality rates from WNV infection when compared to mice not exposed to the mosquito. The study by Schnieder et al. (2007) provided evidence showing that the immunosuppressive properties of mosquito saliva could play an important role in the pathology of infection by WNV. However, the study focused on the mammalian host, though WNV is primarily an

avian pathogen. It remains unknown if a similar phenomenon may occur in birds infected with WNV and exposed to mosquitoes.

Fowlpox virus (FPV) is an avian pathogen also transmitted by mosquitoes. Fowlpox virus is a member of the *Poxviridae* family that infects many bird species and is an important pathogen of the poultry industry. The virus causes two forms of disease. Inhalation of viral particles can form a false membrane of coagulated necrosis in the mouth, pharynx, larynx, and trachea. Transmission by mosquitoes causes skin lesions on the comb, wattles, and beak (Saif, 2008) Fowlpox virus poses an emerging threat to bird species in fragile ecosystems like the Galápagos archipelago (Thiel et al., 2005), where the recent introduction of both the virus and mosquito vectors are putting delicate avian species, such as the Darwin's Finch (*Geospiza fortis*), at risk of extinction (Kleindorfer and Dudaniec, 2006). Commercial vaccination for FPV is available and has effectively controlled the disease throughout the poultry industry. However, it appears that antigenically variant strains of the virus are surfacing and the current vaccine is ineffective against these strains (Wang et al., 2006). These evolved strains of FPV pose a new threat to the poultry industry, and due to the ability of the virus to cross over between species, FPV concurrently threatens native species (Akey et al., 1981).

The salivary gland of mosquitoes secretes proteins with immunosuppressive properties (Reisen et al., 2003). The viral coat of FPV also has similar immunosuppressive properties (Sedger and McFadden, 1996). Thus, the FPV-mosquito system provides an opportunity to explore the interactions between potential immunosuppressive properties of a blood-feeding arthropod and the immune response to a vectored pathogen. Improved knowledge of these interactions could directly benefit the efforts to predict, or prevent, the spread of arthropod-transmitted pathogens.

CENTRAL QUESTIONS

A bird is expected to develop immune responses to a blood-feeding ectoparasite (e.g. mosquito) and a viral infection. The combined effects of the ectoparasite and viral infection on the immune responses of the host remain poorly understood. This gap in the knowledge leads to two discrete questions: 1) Does blood-feeding by a mosquito affect the immune response of the host to a mosquito-transmitted virus? 2) Does the immunosuppressive effect of viral infection impair the immune response of the host to the ectoparasitic arthropod?

OBJECTIVE

The objective of this study was to characterize the antibody responses of the white leghorn chicken (*Gallus gallus*) to *Culex quinquefasciatus* mosquitoes (Cxq) and Fowlpox virus (FPV) challenges separately and in combination. An indirect-enzyme linked immunosorbent assay (ELISA) was used to determine the levels of antibody specific to Cxq and FPV proteins.

Based on the reported effects of mosquito feeding on host immunity and the immunosuppressive properties of FPV, I tested two hypotheses:

Hypothesis 1: Birds exposed to blood-feeding by *Culex* mosquitoes will have lower antibody responses to subsequent FPV infection.

Hypothesis 2: The production of antibodies against *Culex* proteins will decrease following infection with FPV.

METHODS

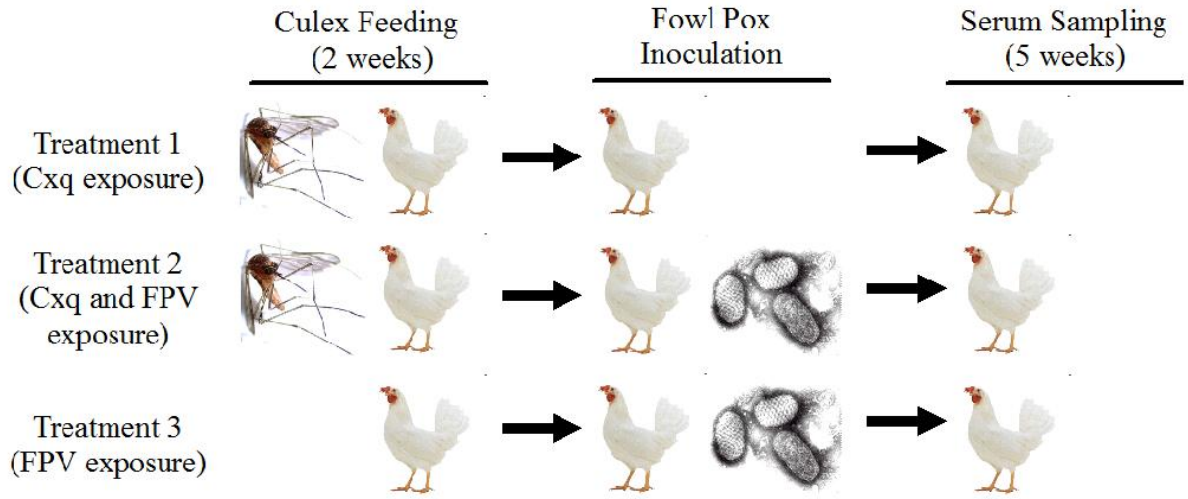
Antibody levels as an evaluation of antigen-host interaction.

The antibody levels of Cxq-specific antibodies and FPV-specific antibodies were used to determine the immune response of the birds to the Cxq and FPV challenges. Antibodies are a direct product of the adaptive immune response following the antigen recognition and effector cell activation phases of the humoral immune response (Janeway, 2005). An increase or decrease of antibody response correlates with changes in the activity of the adaptive immune system. Antibodies activate the complement system and effector cells, indirectly destroying invading foreign bodies (Janeway, 2005). A change in antibody response thereby also correlates with the ability of the host to destroy invading pathogens.

Description of animals and treatments.

Adult while leghorn female chickens (20 weeks old) were used to model the host immune response to Cxq and FPV challenges. A total of 18 hens were used for this study and were individually housed in separate wire cages (1.5' x 1.5') kept in the Animal Science Lab Building at Washington State University (Pullman, Washington). Three treatment groups, each consisting of 6 hens, were used to test the hypotheses of this study. The chickens were used as hosts for *Cx. quinquefasciatus* mosquitoes between the ages of 2-weeks and 8-weeks old. Prior to use in this experiment, the levels of Cxq-specific antibodies present in the birds were assayed (see below) to ensure that the range of Cxq-specific antibody levels were evenly distributed among the treatments. Following the initial assay, the hens were distributed among the treatment groups to represent equally the range of Cxq-specific antibodies already present in the host birds.

Figure 1 Treatments administered over time



Each group was treated with a different combination of Cxq and FPV challenges (Fig. 1). Treatment group 1 was exposed to only blood-feeding by Cxq. Treatment group 2 was exposed to both blood-feeding Cxq and infection with FPV. Treatment group 3 was only exposed to infection with FPV.

Cxq mosquitoes used to feed on 12 of the 18 hens (Groups 1 and 2) were colonized and maintained in the Owen laboratory at Washington State University (Pullman, Washington). An estimated 2,400 mosquito pupae were placed in an enclosure with 12 hens. The enclosure consisted of two nested tents (15' x 20') covering the wire cages holding the chickens. The Cxq pupae were placed in the enclosure and were allowed to emerge. A small lamp with a 15W incandescent bulb was set on a timer to turn on for 2-hours at dusk and 2-hours at dawn each day. The bulb created a low-light level that mimicked the light conditions when *Cx. quinquefasciatus* feed. It was assumed that half the population was female and that all pupae enclosed, resulting in a 100:1 blood-feeding female mosquito to hen ratio. Blood-feeding on hens was observed over the 2 week period the mosquitoes and hens were kept together. In addition to observing blood-fed mosquitoes, egg rafts were deposited in water pans in the enclosure. The egg rafts indicated the mosquitoes were successfully obtaining the blood-meals required to reproduce. The chickens that were not exposed to the

mosquito feeding were housed in the same room, but were kept outside of the mosquito enclosure. No mosquitoes were observed outside the tent during the 2-week feeding period.

Following the mosquito exposure period, all birds in the enclosure were moved out into the main animal room. The remaining mosquitoes were collected and the enclosure was removed. The pox-exposed birds were then inoculated with FPV. Inoculation with commercially available attenuated FPV vaccine (Intervet™) was used as the FPV challenge. The vaccine was inoculated subcutaneously into the wing web of both the right and left wing of hens in treatment groups2 and 3. The hens were checked for pox lesions around the inoculation sites, 1-week after exposure to the virus. Observed pox lesions were considered evidence of infection. Following the FPV challenge (week 1), 0.5 ml of blood was collected weekly from the ulnar vein of each hen over a 5-week period. Collected blood was centrifuged to isolate serum and serum samples were stored in a freezer (-20 ºC) until they were assayed to measure levels of antibody specific to either Cxq or FPV proteins.

Design of the indirect-ELISA for measuring antibody levels specific to Cxq and FPV proteins.

An indirect-ELISA was used to determine antibody levels against both Cxq antigens and FPV antigens.

Diagram 1 Indirect-ELISA

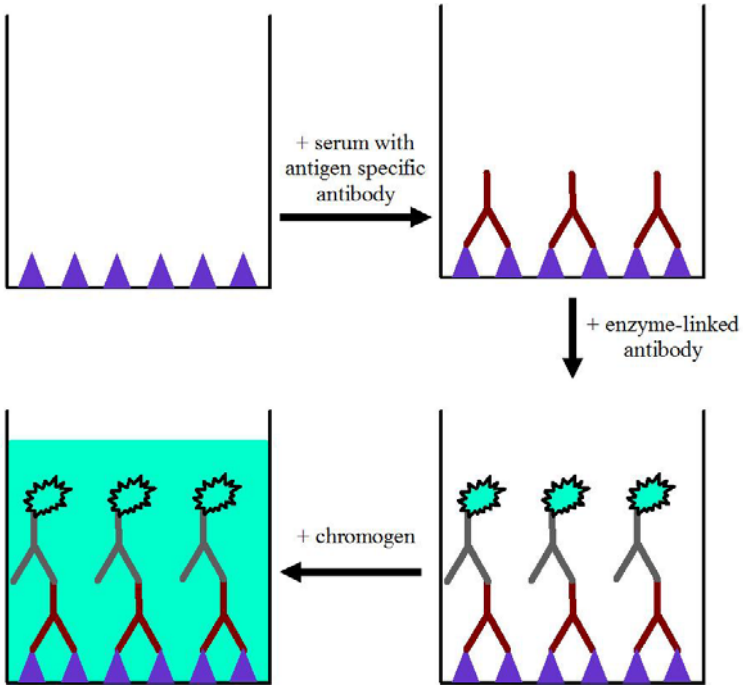


Diagram 1 illustrates how an indirect-ELISA functions. Antigens (purple triangles) are added to a well. Serum with antibody (crimson) is also added to the well. The antigen-specific antibodies (primary antibodies) bind to the anitgens. Then an enzyme-linked antibody (grey-cyan, secondary antibody) specific to the primary antibody is added to the well. The secondary antibodies will bind to the primary antibodies which are still bound to antigen. Finaly, a chromogen is added to the well. The chromogen will react with the enzyme linked to the secondary antibody causing a color-change reaction (white to cyan). The optical density of this reaction can be measured to evaluate the primary antibody level within the well.

The antigens used for this study were FPV-derived proteins and Cxq proteins. The FPV vaccine (live virus), which was used to inoculate hens with FPV, was used for the indirect-ELISA. Cxq proteins were obtained from adult, female *Cx. quinquefasciatus*. The anterior portion of individual mosquitoes (head and prothorax) was cut off and isolated from 500 mosquitoes in phosphate buffered saline (PBS). These anterior portions of the mosquitoes were expected to contain the salivary glands. The anterior portions were then

ground with a pestle in PBS and the material was centrifuged to pellet the debris. The supernatant (protein extract) was removed and stored (-20 ºC) until used in the ELISAs.

The ability of chickens to produce FPV- and Cxq-specific antibodies was verified prior to the start of the experiment. For FPV, two trial hens were inoculated subcutaneously with the FPV vaccine in wing web of both the left and right wing to stimulate an antibody response. Two weeks following inoculation with FPV, 0.5ml of blood was collected from the ulnar vein of each hen. The blood was then centrifuged and the serum was isolated. The serum was placed in a freezer until needed for optimizing the indirect-ELISA used for this study. Blood was also collected from hens that were never exposed to FPV and the serum was similarly isolated and stored. The serum from each set of birds (exposed and unexposed) was separately pooled and used in a checkerboard titration ELISA. This verified that birds exposed to FPV produced detectable FPV-specific antibodies, relative to the unexposed birds. In addition the titration was used to determine the optimal antigen and serum dilutions to minimize non-specific, background binding.

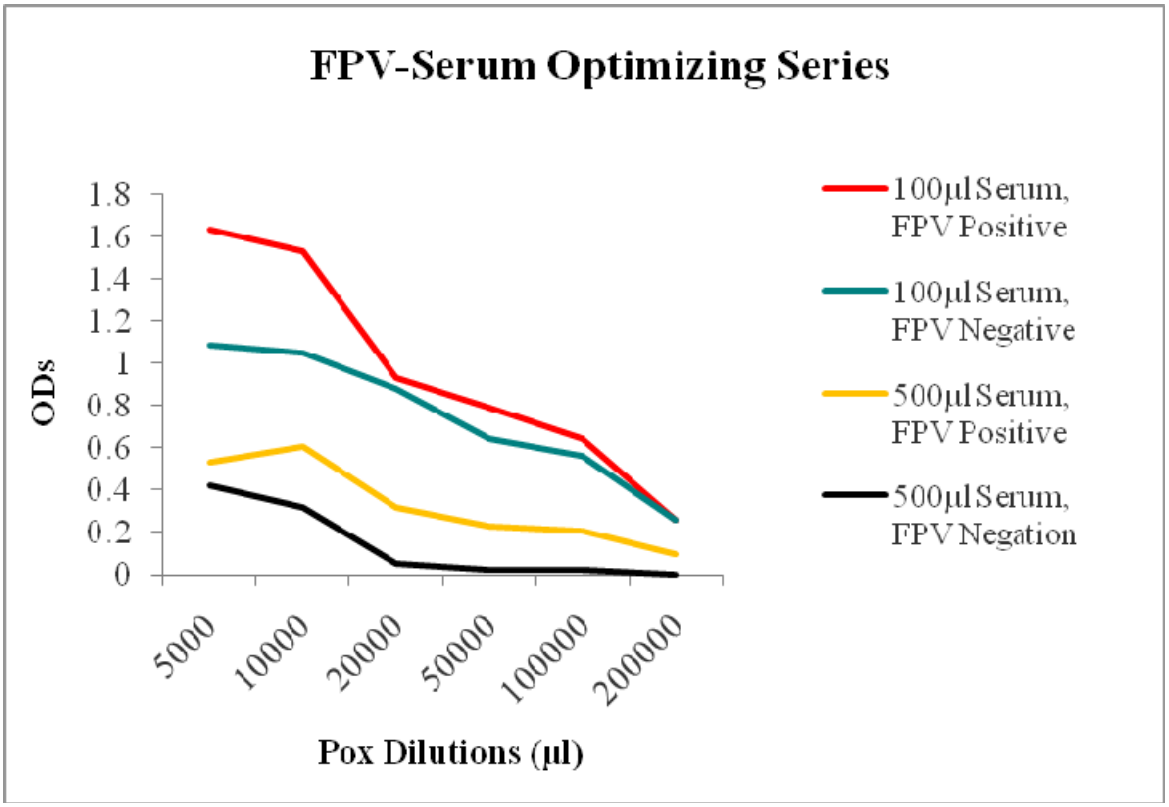
A similar procedure was performed to determine the optimal serum dilution and Cxq dilution concentration combinations. The Cxq protein extract (100 µl; 100 mg/mL) was injected subcutaneously into each of 4 hens. Blood (0.5 ml) was collected from the ulnar vein of each hen two-weeks after injection. The blood was then centrifuged and the serum was isolated. Serum was also isolated from 15 hens that were never exposed to Cxq feeding or antigen injection for use as a negative control. The serum samples were placed in a freezer until need for optimizing the indirect-ELISA used for this study.

The remaining serum collected from the FPV-infected trial hens and Cxq-injected trial hens was used as positive controls for the FPV-specific indirect-ELISAs and Cxq-specific indirect-ELISAs.

The dilutions used in the experiment were determined by controlled optimizing series.

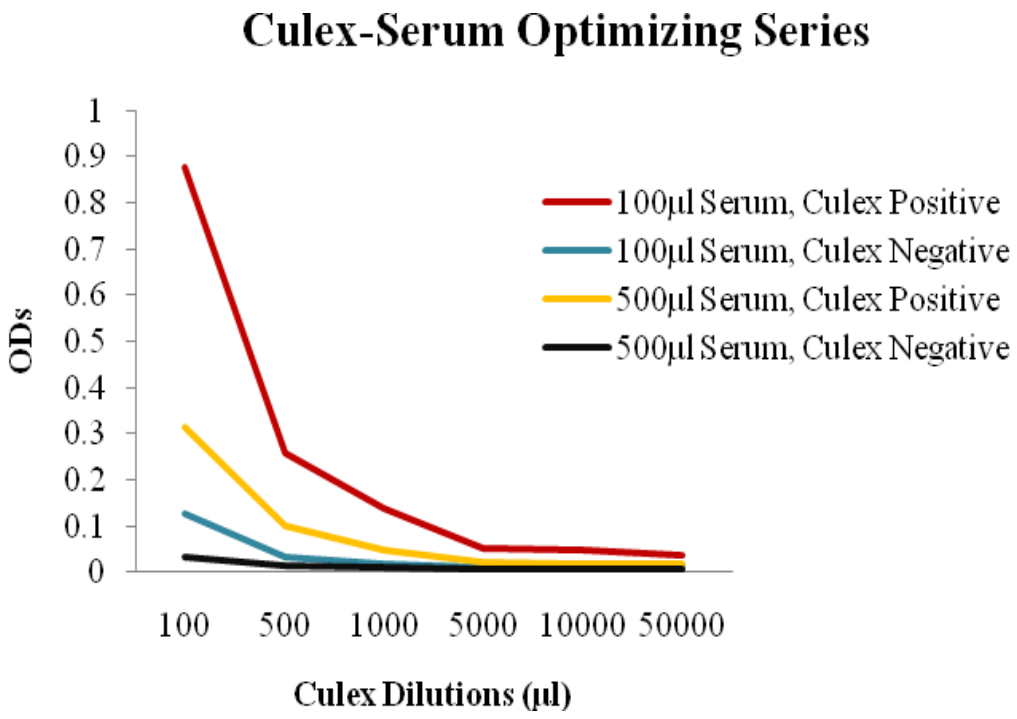
In each series, the optical density levels in relation to both serum dilution and antigen dilution was determined. Positive and negative controls were assayed side by side for comparison.

Figure 2 Comparing optimal levels of FPV dilution and serum dilution.



As expected, the more dilute the FPV-antigen solutions, the smaller the values became. At a FPV-buffer dilution of 1:10000 and serum dilution of 1:100, the difference between positive and negative values was large enough to observe FPV-specific antibody levels.

Figure 3 Comparing optimal levels of Cxq dilution and serum dilution.



Similar to FPV, Cxq-specific antibody levels dropped as both serum and antigen dilutions increased. At *Culex*-buffer dilution of 1:1000 and serum dilution of 1:100, the difference between positive and negative values was large enough to observe various Cxq-specific antibody levels.

Figure 4 Indirect-ELISA plate map

	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	C1000/ P10000	Column 10	Column 11	Column 12
S100	G1 C2B10 Trial1	G1 C2B10 Trial2	G1 C2B10 Trial3	G2 C2B12 Trial1	G2 C2B12 Trial2	G2 C2B12 Trial3	G3 C2B19 Trial1	G3 C2B19 Trial2	G3 C2B19 Trial3			
S100	G1 C2B3 Trial1	G1 C2B3 Trial2	G1 C2B3 Trial3	G2 C2B8 Trial1	G2 C2B8 Trial2	G2 C2B8 Trial3	G3 C2B15 Trial1	G3 C2B15 Trial2	G3 C2B15 Trial3			
S100	G1 C2B2 Trial1	G1 C2B2 Trial2	G1 C2B2 Trial3	G2 C2B1 Trial1	G2 C2B1 Trial2	G2 C2B1 Trial3	G3 C2B5 Trial1	G3 C2B5 Trial2	G3 C2B5 Trial3			
S100	G1 C1B13 Trial1	G1 C1B13 Trial2	G1 C1B13 Trial3	G2 C1B12 Trial1	G2 C1B12 Trial2	G2 C1B12 Trial3	G3 C1B14 Trial1	G3 C1B14 Trial2	G3 C1B14 Trial3			
S100	G1 C1B22 Trial1	G1 C1B22 Trial2	G1 C1B22 Trial3	G2 C1B8 Trial1	G2 C1B8 Trial2	G2 C1B8 Trial3	G3 C1B5 Trial1	G3 C1B5 Trial2	G3 C1B5 Trial3			
S100	G1 C1B9 Trial1	G1 C1B9 Trial2	G1 C1B9 Trial3	G2 C1B7 Trial1	G2 C1B7 Trial2	G2 C1B7 Trial3	G3 C1B3 Trial1	G3 C1B3 Trial2	G3 C1B3 Trial3			
Row G												
S100	NSB Trial1	NSB Trial2	NSB Trial3	NC Trial1	NC Trial2	NC Trial3	PC Trial1	PC Trial2	PC Trial3	Blank	Blank	Blank

C1000/P10000 refers to the antigen dilution concentration where in Cxq-specific indirect-ELISAs 1 µl of extracted Cxq-protein was used with 1000 µl of buffer, and in FPV-specific indirect-ELISAs 1 µl of FPV vaccine was used with 10000 µl of buffer.

G1 C2B10 refers to individual hens where G is the treatment group, C is the colony of chickens the hen was raised in, and B is the bird number in the given colony.

NSB refers to cells with only Cxq or FPV antigens used to measure non-specific binding. NC and PC refer to negative controls and positive controls, respectively. Blank cells were only inoculated with buffer to measure plate the binding that occurred without antigen.

The ELISAs used 96-well flat bottom, no lid, non-sterile Costar® polystyrene plates. Based on the optimizations of the assays (described above), serum samples were diluted 1:100. The Cxq protein extract was diluted 1:1000 and the FPV was diluted 1:10,000. All serum samples were run in triplicate and each ELISA plate also contained a known positive control (for antigen of interest) and controls for non-specific binding (binding of detection antibody with antigen).

The following procedure was used for the indirect-ELISAs. A dilution of the antigen (Cxq-protein extract or FPV vaccine) and coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) was made in concentrations of 1000 µl buffer per 1 µl Culex antigen and 10,000 µl buffer per 1 µl FPV antigen. 100 µl of this dilution was placed in wells according to the plate set-up to coat the plastic with the antigen. The plate was then incubated over night. After

incubation, the plate was washed using the Bio-Rad ImmunoWash (Model 1575) with de-ionized water and a washing solution (50 mM Tris buffer with 0.14 M NaCl, 0.05% Tween 20, and pH 8.0). Following washing of the plate, 200 µl of blocking buffer (50 mM Tris buffer with 0.14 M NaCl, 1% BSA, and pH 8.0) was placed in all previously coated wells to block all possible open areas on the plastic wells not already coated with the antigen. This was incubated for an hour on a Fisher Scientific orbital shaker (Model 2314F5) followed by a wash. Dilution of serum and conjugate buffer (50 mM Tris buffer with 0.14 NaCl, 1% BSA, 0.05% Tween 20, and pH 8.0) were made in concentrations of 100µ buffer per 1 µl chicken serum. Serum from the three treatment groups and serum from positive and negative controls were used. In each well, 100 µl of serum dilution was added, followed by a 1-hour incubation on the orbital shaker. A dilution was made for the detector enzyme-linked HRP Conjugate, goat-derived chicken-antibody-specific antibody (A30-104P; Bethyl Laboratories) and conjugate buffer; the dilution consisted of 1 µl of HRP Conjugate and 20,000 µl of buffer. The plate was then washed and 100 µl of HRP Conjugate and conjugate buffer dilution was added to all the previously coated wells. The plate was incubated on the orbital for 1 hour followed by a wash. A substrate of 5 ml TMP peroxidise substrate with 5 ml peroxidise substrate solution was mixed and 100 µl of the solution was added to each well in the plate. The color reaction between the enzyme and chromogen was allowed to incubate for seven minutes, after which 100 µl of 2M H_2SO_4 was promptly added to stop the color reaction. The Bio-Rad iMark™ Microplate Reader spectrophotometer was then used to measure the optical density of each well at a wave length of 450 nm. The optical density was then interpreted as antibody levels.

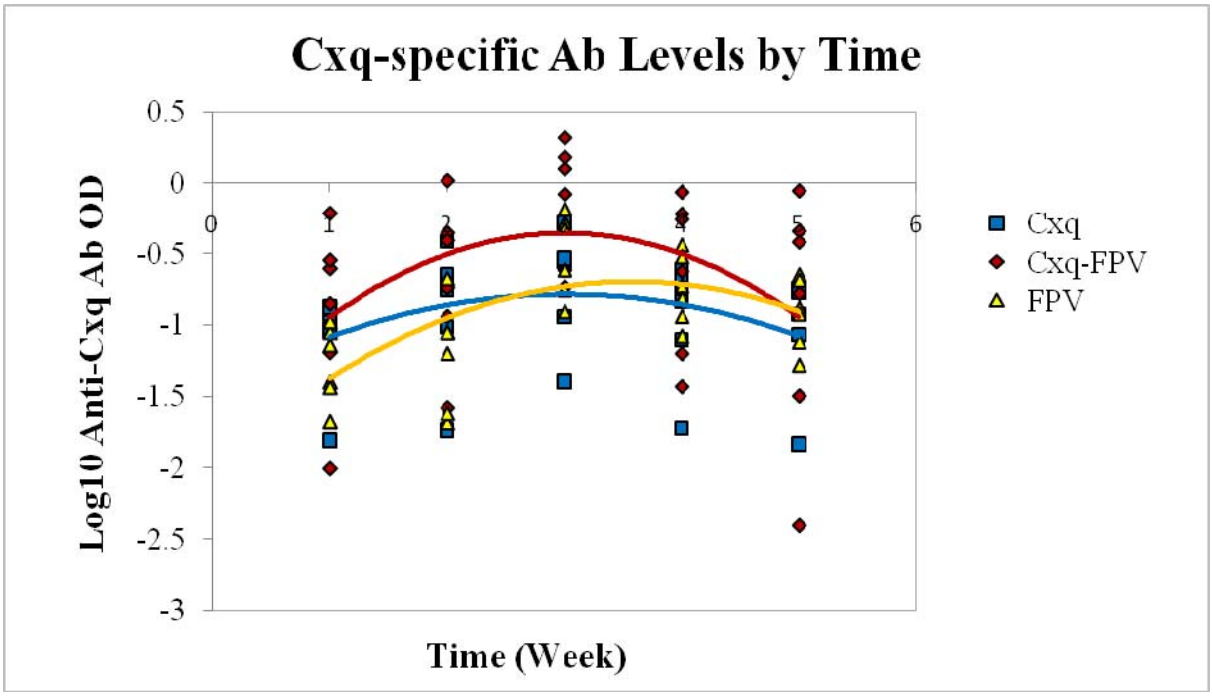
Analysis and statistics used to interpret data.

Each treatment group was assayed for levels of Cxq- and FPV-specific antibody levels over the 4-week period following the termination of the mosquito-exposure period and the inoculation with FPV (week 1). A repeated-measure, multiple analysis of variance (MANOVA) was used to compare treatment groups (SYSTAT 15). The OD values were log-transformed to meet the assumption of normality. The transformed values were used as response variables with "treatment" and "time" as fixed-factors and "week 1" as a covariate. Initially, all three treatment groups were analyzed together, and a post-hoc test was used to compare antigen-exposed groups with the antigen-unexposed (control) group. Subsequently, the MANOVA was run with only the antigen-exposed groups and the two groups were compared using a post-hoc test of the treatment effect by time between the two groups.

RESULTS

Prior to initiation of the experiment, all hens were determined to have detectable levels of Cxq-specific antibodies (OD range 0.04 - 0.12) that were higher than negative control values (OD Mean ± SE = 0.02 ± 0.002). After birds were sorted, the three treatment groups reflected identical distributions of Cxq-specific antibody levels at the start of the experiment (OD Mean ± SE = 0.08 ± 0.008). In the first two weeks following mosquito feeding, Cxq-specific antibody levels increased in the groups of mosquito-exposed hens (Fig. 5). The Cxq-specific antibody levels were higher in the exposed hens, compared to the unexposed (FPV-only) hens (MANOVA F-ratio = 4.157, df = 8. 20, p = 0.005).There was a significant interaction of treatment and time, as Cxq-specific antibody levels increased up to week 3 and subsequently decreased (MANOVA F-ratio = 17.07, df = 3, p = 0.001).In week 3, the Culex-Pox exposed birds had higher Cxq-specific antibody levels than birds exposed to mosquitoes only (MANOVA F-ratio = 8.843, df = 1, p = 0.016).

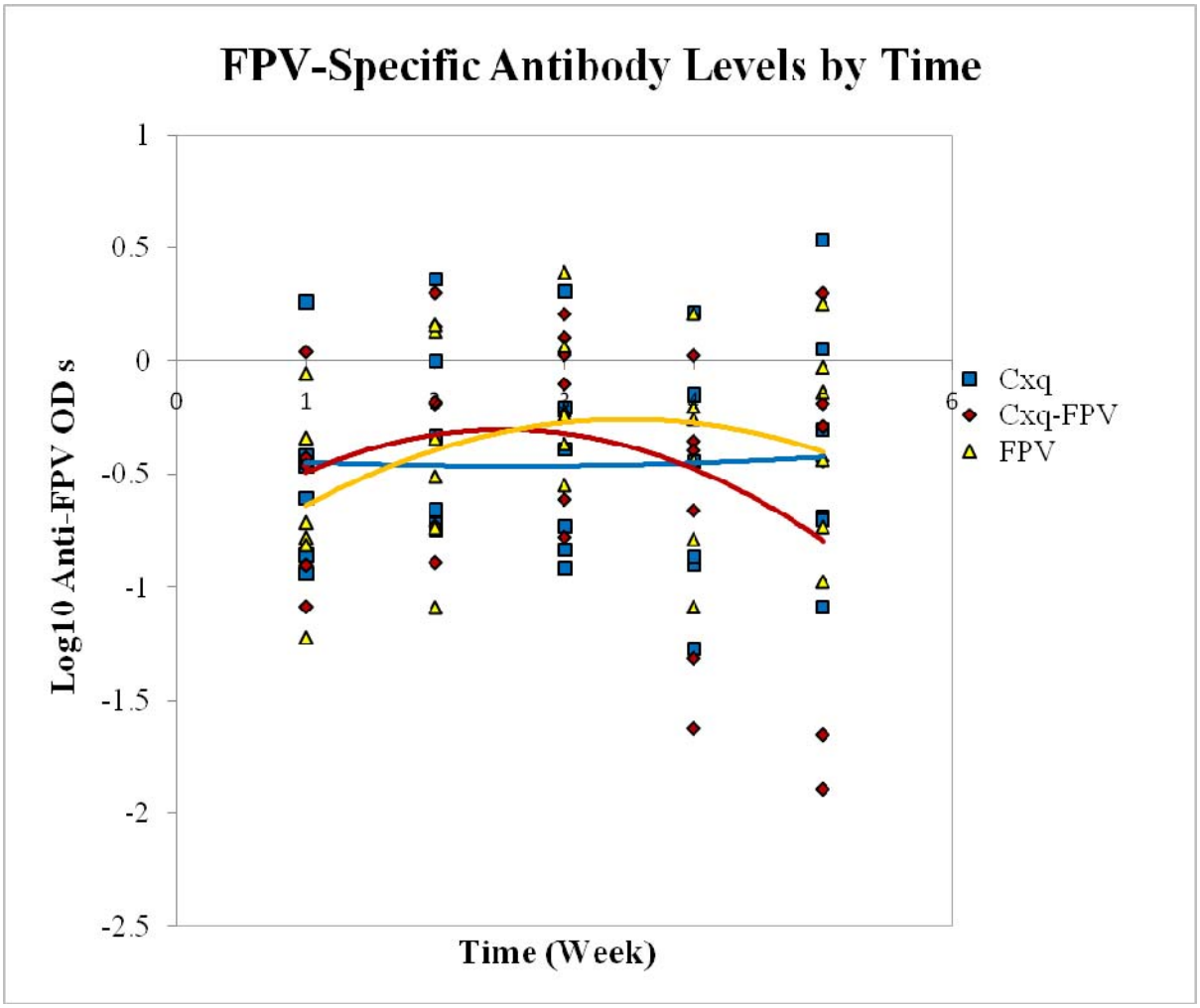
Figure 5 Log-transformed scatter plot of Cxq-specific antibody responses.



Cxq-protein-binding antibodies increased in birds exposed to mosquito feedings (Cxq-only, blue, and Cxq-FPV, red, treatments) in comparison to those that did not (FPV-only, yellow, treatment), indicating an adaptive humoral immune response.

The time and treatment interaction was significant from week 3 to week 4 (Fig. 6.) During this period, FPV-only hens maintained higher FPV-specific antibody levels than controls, whereas Culex-FPV hens had antibody levels that decreased to the level of controls (MANOVA F-ratios = 7.84, df = 2, p = 0.005). From week 3 to week 4, the antibody levels of Culex-FPV birds decreased more than the antibody levels of FPV-only birds (MANOVA F-ratio = 15.21, df = 1, p = 0.004). The mean antibody levels did not differ among the two FPV-exposed groups within any week.

Figure 6. Log-transformed scatter plot of FPV-specific antibody responses.



Fowlpox virus-binding antibodies increased in FPV infected birds in week 3 (FPV-only, yellow, and Cxq-FPV, blue, treatments), indicating an adaptive humoral immune response.

DISCUSSION

Suppression of the host immune system is hypothetically an advantageous effect for both the mosquito and invading FPV, because the invasive effects of both blood-feeding vector and pathogen are inhibited by the host immune response. Though host immunosuppression has been independently demonstrated for mosquitoes and FPV (Wikel, 1996; Morris et al., 2001; Wasserman et al., 2004; Schnieder et al. , 2007), it is unknown how these effects may interact within hosts exposed to both challenges. As proposed, a hypothetical outcome of the combined exposures to mosquitoes and FPV is the reciprocal suppression of the immune response against each challenge – the vector and the pathogen. This central hypothesis was not supported by the results of this experiment. Chickens with recent mosquito exposure produced higher Cxq-specific antibody responses following infection with FPV (Fig. 5), instead of producing lower antibody responses that would result from systemic immunosuppression by the pox virus. Chickens with FPV exposure produced similar levels of FPV-specific antibodies (week 3) in both mosquito-exposed and unexposed groups (Fig. 6). This suggested that the FPV-specific antibody response was not delayed due to any immunosuppressive effect of the prior mosquito exposure. As discussed below, the timing of the parasite exposures may play an important role in the activation and effects of the antibody response.

The FPV-specific antibody levels for both FPV-exposed groups were similarly highest in week 3 and indicated an unaltered peak of the antibody response (Janeway, 2005). However, the FPV-specific antibody levels of group 2 (*Culex* and FPV) were significantly lower than levels of group 3 (FPV only) in week 4. This rapid decrease of the antibody level following the peak response (week 3) suggests antigen (virus) may have been removed more effectively in mosquito-exposed birds, resulting in lowered antigenic stimulation and lowered

antibody production. A similar phenomenon was reported by Fonseca et al. (2007). Those authors observed that mice exposed to *Anopheles* mosquitoes produced a higher T-cell response (CD4+) to subsequent infection with rodent malaria (*Plasmodium chabaudi chaubaudi*), in comparison to mice not exposed to mosquito blood-feeding. Furthermore, they found that parasitemia was lower in the mice previously exposed to the mosquitoes. They proposed that the enhanced T-cell response resulting from mosquito exposure was producing a protective effect against the *Plasmodium* infection. An adaptive immune response against mosquitoes was activated in birds exposed to mosquitoes, as confirmed by an increase of Cx-specific antibody levels. The adaptive immune response follows 94 hours after an innate immune response is activated in the host; the innate immune response continues to be stimulated even after the adaptive immunity is up-regulated (Janeway, 2005). Potentially, the activated adaptive or innate immune responses of birds exposed to mosquitoes could have made the FPV infection less intense by decreasing the amount of virions present in the serum. A decrease in virion levels would also decrease the amount of FPV-specific antibody produced. Lack of antigens to present would result in less antibody-on-antigen binding.

A study investigating the effectiveness of the innate immune response needs to be further investigated. Innate immunity would need to be activated followed by a challenge that would activate adaptive immunity. This type of experiment would demonstrate whether prior activation of the innate immunity affects the antibody levels produced in response to a second challenge.

The high Cxq-specific antibody response in FPV-exposed birds suggests the immune response against Cxq-proteins was enhanced in the presence of FPV. This is interesting, because FPV challenges occurred after birds were removed from mosquito exposure. In

other words, the antibody response to mosquitoes was boosted in the absence of mosquitoes. A possible explanation for this effect is that the immune response to FPV infection stimulated the pre-existing antibody response to *Culex*.

Cytokines are substances secreted by cells of the immune system as signalling molecules between cells. Resting memory B cells are activated by such cytokines and produce antibodies specific to a previously encountered antigen. Cytokines interleukin-1, interleukin-4, interleukin-6, interleukin-12 and tumor necrosis factor-α contribute to activation of B-cell proliferation and differentiation for antibody production (Janeway, 2005). Inoculation of FPV resulted in an antibody response (Fig. 6), indicating release of cytokines. These same cytokines used to induce an antibody response against FPV may be re-activating Cxq-specific memory B-cells.

The effects of salivary compounds on subsequent responses to a pathogen were seen in the study by Fonseca et al. (2007). Prior exposure to mosquitoes not infected with *Plasmodium chabaudi chaubaudi* resulted in low levels of spleen cells and interleukin-4 once exposed to *Plasmodium*-infected mosquitoes. The opposite may be occurring in this study. FPV may have increased cytokine activity leading to an increase in Cxq-specific antibody in birds with prior mosquito exposure. There are no published reports showing a positive reverse-effect of a pathogen on a pre-existing antibody response to an ectoparasite. However, there is evidence in the literature that a pathogen can influence the existing adaptive immune response to a parasite. For example, Ganley-Leal et al. (2006) showed that individuals exposed to the parasitic helminth *Schistosoma mansoni* developed acquired resistance to the parasite that was mediated by the antibody IgE and eosinophils (defensive cells). Individuals in the population that were infected with human immunodeficiency virus (HIV-1) appeared to lack this IgE-eosinophil response to *S. mansoni*, suggesting their adaptive immunity to the helminth was impaired by the virus. The reports of Fonseca et al. (2007) and Ganley-Leal et

al. (2006) indicate that immunological responses to different parasitic challenges can have diverse outcomes.

The effects of FPV on Cxq-specific antibody response need to be further understood. The results of this study give rise to new questions in regards to the affects of FPV on mosquito-specific antibody levels in the host. A study could be designed to explore the activation of Cxq-specific memory B-cells by cytokines. In such a study exposure to mosquitoes would be followed by activation of the Cxq-specific memory B-cells formed at the time of the initial mosquito exposure. Inoculation with a commercial cytokine mixture could activate memory B-cells, if present. The study would also be complemented with an experiment designed to investigate the possible indirect activation of Cxq-specific memory B-cells by FPV. Such an experiment would consist of 3 treatment groups – one where the host species was exposed to only Cxq, another exposed to only FPV, and the final group where the host species was exposed to Cxq followed by an FPV challenge. Cxq-specific antibody levels should be measured throughout the entire duration of both the cytokine and FPV effect on Cxq-specific immune response experiment; this should be done to confirm a primary and secondary immune response to Cxq. Presence of Cqx-specific antibodies produced by Cqx-specific memory B-cells should also be confirmed using simple a western blot technique; this should be done to verify that FPV-specific antibodies are binding to only FPV-proteins and Cxq-specific antibodies are binding to only Cxq-proteins. Theoretically, the group exposed to Cxq and FPV should appear positive for MSG-protein binding and FPV-protein binding in the final stages of the experiment because of Cxq-specific memory B-cells activation.

CONCLUSION

Due to the nature of this study, where FPV-proteins and Cxq-proteins did not co-occur, it is still unclear if the mosquito enhances the pathogenicity of an arthropod transmitted pathogen, such as FPV, in a bird host. There is evidence in the literature supporting that mosquitoes have the ability to suppress the immune response of the vertebrate host at the feeding site, which may create a "window of opportunity" for an invading pathogen to spread within the host without initial recognition by the immune system of the host. However, this study suggests the possibility of an enhanced immune response to FPV due to prior mosquito exposure. Moreover, the data strongly indicate a positive effect of FPV on a pre-exisiting response to *Culex*. The actual impact of these immune responses on the fitness of the respective parasites remains to be determined.

Further studies investigating the direct interactions of ectoparasitic arthropod immunosuppressive properties, the local adaptive and innate immune response of the host, and affects of the invading pathogens need to be conducted. Host immune suppression by mosquitoes may play a role in the transmission dynamics of FPV, if lower antibody responses result in persistence of viral activity. Alternatively, prior mosquito exposure may decrease the infectivity of FPV, which would influence the prevalence and pathogenicity of FPV in populations of birds. This work provides the basis for future studies concerning ectoparasitic arthropod suppression of the immune system of the host.

ACKNOWLEDGEMENTS

I would like to the Washington State University College of Agricultural, Human, and Natural Resource Sciences for providing the funding for this project. I also thank Wade H. Petersen in the Washington State University Department of Entomology for his assistance in various laboratory procedures. I give particular thanks and appreciation to Dr. Jeb P. Owen in

the Washington State University Department of Entomology for his patience, guidance and support throughout this project.

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