INFLUENCE OF BODY CONDITION AND HOMO- VS. HETEROSUBTYPIC IMMUNITY ON INFLUENZA A VIRUS INFECTION IN MALLARD DUCKS: EXPERIMENTAL INFECTION DATA

By

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ABSTRACT

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Migrating waterfowl, particularly mallards (*Anas platyrhynchos*), are implicated in the global spread of influenza A viruses (IAVs). Experimental infection studies are used to assess a bird's ability to maintain IAV, but these studies have not represented the natural variation in physiological condition observed in migrating waterfowl and have only used captive-bred birds. We assessed how body condition affects susceptibility, viral shedding, and antibody production in wild-caught and captive-bred juvenile mallards challenged with IAV H5N9. We found wild mallards fed *ad libitum* were more susceptible to IAV infection, shed higher virus loads, and shed virus more frequently compared to birds in poor condition. No difference in viral load was detected between wild and captive-bred mallards. Antibody production did not vary according to condition. We conclude captive-bred mallards represent wild mallards during IAV infection when fed *ad libitum*, and IAV infection in mallards is largely influenced by body condition.

Waterfowl are frequently exposed to the same or different IAV subtypes during migration. We examined whether primary H5N9 infection in juvenile mallards provides long-term immunity towards reinoculation with the same or different IAV subtype. Our findings demonstrate mallards can mount long-term protective immunological responses against reinfection with the same H5N9 virus, but were susceptible to reinfection with a different H7N3 subtype 49 days after primary inoculation. Additional IAV immunity studies are needed using different virus subtypes and different challenge timings.

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CHAPTER 1

INFLUENCE OF BODY CONDITION ON INFLUENZA A VIRUS INFECTION IN MALLARD DUCKS

INTRODUCTION

Birds associated with aquatic environments including Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) serve as the natural reservoir for influenza A viruses (IAVs) (Webster et al. 1992). Among these birds, dabbling ducks within the *Anas* genus are recognized as the primary reservoir hosts (Olsen et al. 2006). Prevalence of IAV infection in dabbling ducks peaks during autumn when immunologically naïve juvenile waterfowl congregate before migrating south (Wallensten et al. 2007). During migration, many of these birds travel long distances and potentially spread low pathogenic avian influenza viruses (LPAIVs) among countries or continents. Migration is considered one of the most physiologically demanding activities in the animal world and animals vary in their ability to meet the associated energetic challenges. Despite peak IAV prevalence occurring during migration, studies have not fully evaluated how natural variation in waterfowl condition influences a bird's ability to serve as a reservoir host for LPAI.

An understanding of waterfowl host competence during the migratory period is needed to understand how LPAIV is maintained and transmitted. It has long been assumed that waterfowl are asymptomatic carriers of LPAI and may transmit the virus during migration (Webster et al. 1992). However, recent examination of migratory behavior in wild Bewick's swans (*Cygnus columbianus*) found that swans infected with LPAIV exhibited delayed migration, reduced feeding rates, and shorter flight distances compared to uninfected conspecifics (van Gils et al.

2007). Furthermore, Latorre-Margalef et al. (2009b) found that migrating mallard ducks (*Anas platyrhynchos*) infected with IAVs had significantly lower body mass than did uninfected birds. These studies concluded that LPAIV infection may incur larger physiological costs to migrating waterfowl than was previously thought. In response, Flint and Franson (2009) provide an alternative explanation, suggesting that birds in poorer condition exhibit reduced immune function and are more susceptible to IAV infection (i.e. "condition-dependent hypothesis") (Latorre-Margalef et al. 2009a). If their hypothesis is correct, host condition would predict susceptibility to infection, and concentration and duration of viral shedding.

Despite the suggested influence of host condition on IAV infection, laboratory experiments have used birds in normal physiological condition (Sturm-Ramirez et al. 2005, Brown et al. 2006, Keawcharoen et al. 2008, Fereidouni et al. 2009, Jourdain et al. 2010). These studies have not accurately represented the energetic and immunological condition of migrating ducks. For example, migrating waterfowl can experience declines in condition due to inclement weather (Robb et al. 2001) and decreased food availability (Moon et al. 2007). Furthermore, decreases in condition have been correlated with immunosuppression (Owen and Moore 2008, Buehler et al. 2009). Owen and Moore (2008) found that immune function in migrating thrushes (Family Turdidae) was positively related to body condition. Common eiders (*Somateria mollissima*) experienced reduced immune function during periods of mass loss caused by enhanced incubation effort (Hanssen et al. 2005). It remains unclear how natural fluctuations in body condition influence susceptibility and severity of IAV infection.

We evaluated the condition-dependent hypothesis through experimental inoculation of wild-caught juvenile mallards with LPAIV. Mallards were selected as the focal species because they are the most abundant migratory dabbling duck in North America and Eurasia, and have

accounted for more IAV recoveries than any other species of bird (Krauss et al. 2004, Olsen et al. 2006, Munster et al. 2007). Studies have shown that mallards in normal physiological condition often remain asymptomatic to IAV infection, but shed high concentrations of the virus (Sturm-Ramirez et al. 2005, Brown et al. 2006, Keawcharoen et al. 2008). We chose to use wild-caught mallards because previous experimental infection studies have used captive-bred mallards (Homme and Easterda.Bc 1970, Cooley et al. 1989, Sturm-Ramirez et al. 2005, Brown et al. 2006, Keawcharoen et al. 2008, Fereidouni et al. 2009, Jourdain et al. 2010), which may not be truly representative of wild mallard host competency. Accordingly, we included a group of captive-bred mallards for comparison and validation of past research.

In this study, we tested the effect of body condition on susceptibility to infection and viral shedding patterns in wild-caught juvenile mallards challenged with LPAIV. We hypothesized food restriction and subsequent reduced body condition will result in (1) increased susceptibility to infection, (2) increased concentration and duration of viral shedding, and (3) decreased antibody production. In addition, we compared susceptibility and viral shedding patterns in wild-caught vs. captive-bred juvenile mallards.

MATERIALS AND METHODS

Animals:

Wild mallards were trapped in September 2009 to coincide with increased abundance of staging waterfowl and seasonal peaks in avian influenza prevalence (Halvorson et al. 1985, Wallensten et al. 2007). Trapping sites (n = 5) were located in shallow marshes surrounded by cropland within the lower peninsula of Michigan. Birds were captured using portable swim-in traps (Mauser and Mensik 1992) and rocket nets at sites previously baited with whole kernel corn.

Mallards were aged in the field as juvenile or adult by examining wing plumage and cloacal characteristics (Carney 1992). All juvenile birds were immediately transported to a biosafety level 2 animal containment facility (see Housing, below), weighed to the nearest 1.0 g, and measured for length of flattened wing chord (nearest 1.0 mm), head (0.1 mm), and tarsometatarsus (0.1 mm). Upon capture, birds were tested for previous IAV exposure using the MultiS-Screen ELISA (see Serologic Assays, below). Seronegative birds were isolated from one another in separate cages to prevent any potential virus transmission within the facility. All birds were retested with the ELISA at 20 days post-capture to ensure no birds had been recently infected at time of capture. Previous research indicates 20 days is adequate time for seroconversion (Brown et al. 2006). Thirty seronegative wild mallards (20 males, 10 females) remained in the study, all other birds were released.

Once wild mallards were selected, 10 (8 males, 2 females) twelve week-old mallards were purchased from a closed-flock hatchery (Ridgeway Hatcheries Inc., Ohio, USA). Captive-bred birds were processed the same as wild mallards and were negative for previous IAV exposure.

Bird handling and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University (protocol 03-09-052-00).

Housing:

Mallards were kept in the Michigan State University Research Containment Facility. Birds were randomly assigned to three identical biosafety level 2 rooms and individually housed in 10.6 ft^3 stainless steel rabbit cages at 20°C. Cages were positioned to allow birds to view one another. Room lighting was adjusted weekly to match the natural photoperiod in Michigan at that time. Each bird received water and grit *ad libitum* daily. All birds were acclimated for 30 days before the start of the study. They were fed *ad libitum* a commercial maintenance food mash (21% crude protein, 2.7% crude fat, 4.75% crude fiber; true metabolizable energy = 2.82 kcal/g).

Body condition index:

Capture mass for wild juvenile mallards was adjusted by subtracting the estimated mass of remaining crop contents (1-25% = -28.2 g, 26-50% = -44.4 g, 51-75% = -68.9 g, 46-100% = -119.5 g), and 3.0 g were added for every hour a bird was held prior to recording its initial mass (Robb et al. 2001). Body condition was estimated for males and females separately using residuals from an ordinary least-squares (OLS) regression of adjusted mass vs. an index of body size (Devries et al. 2008). The body size index was developed by performing a principal component analysis (PC1) using wing chord, head and tarsus length (PROC PRINCOMP, SAS institute 2002). Condition scores were calculated individually by dividing a bird's residual from the OLS regression by its predicted mass. The condition index was assumed to represent normal pre-migration staging condition for juvenile mallards in Michigan. Body condition was not

estimated for the captive-bred birds. These closed-flock mallards exhibited significant structural differences (i.e. reduced wing size, large head and tarsus lengths), and sample size (n = 10 birds) was not large enough to produce a unique and reliable condition model.

Experimental design:

Wild mallards were randomly divided into three treatment groups (n = 10 birds/treatment). Sex and location of capture were stratified among the groups. After birds acclimated 30 days, diet treatments were initiated following protocols from a pilot study conducted in spring 2009 (Arsnoe unpublished data). Mean treatment conditions were manipulated through food availability to relative conditions decided *a priori* 1) poor treatment = -20% body mass, 2) lean treatment = -10% body mass, 3) *ad libitum* treatment = +0-5% body mass. Reduced conditions were selected to replicate natural (lean) and substantial (poor) decrease in body condition encountered by migrating waterfowl (Pawlina et al. 1993). Mallards were fed *ad libitum* to simulate optimal foraging conditions. Captive-bred mallards (n = 10) were also fed *ad libitum*.

Body condition was assessed every five days by weighing birds to the nearest 1.0 g. In addition, we monitored each bird's body reserves by scoring their keel protuberance and breast muscle development on a 0-3 point scale (Gregory and Robins 1998). When treatment groups reached their desired condition levels, all birds were oropharyngeally inoculated with 1.5 mL of 10^6 PFU/mL LPAIV H5N9. Following inoculation birds were maintained on their treatment diets to keep them at desired conditions. Cloacal and oral swabs were collected the first 3 days post inoculation (dpi) and every 2 days thereafter until 28 dpi. Swabs from individual birds were pooled together in 1.5 mL of BHI media with antimicrobial drugs (100X Anti-Anti, 1.0 mL/100 mL BHI media), and transported on dry ice to a -80 °C freezer. Blood serum was collected from

the brachial vein on 14, 21, and 28 dpi for serologic testing. At 28 dpi 20 mallards were euthanized using CO2 asphyxiation, followed by cervical dislocation. The remaining 20 mallards were retained for the study described in Chapter 2 (see below).

Virus:

The LPAI virus used was A/Northern pintail/California/44221-761/2006 (H5N9), obtained from USGS National Wildlife Health Center, Wisconsin, USA. This strain of IAV was selected as it has been well characterized and serves as a model waterfowl-derived IAV in our laboratory. Virus was propagated by inoculating the allantoic cavity of 9-11 day old embryonated chicken eggs with 200 μL (1:10 dilution in DMEM media) (Woolcock 2008). Allantoic fluid was harvested after 4 days, centrifuged and stored in 2 mL aliquots at -80 °C. Stock virus was titrated using MDCK plaque assay as described by Tobita et al. (1975).

Matrix gene RRT-PCR:

Swab samples were thawed at 37 °C and thoroughly homogenized by vortexing. RNA extractions were performed using the QIAamp viral RNA mini kit (QIAGEN, QIAGEN Sciences, Maryland, USA) using 140 μl of sample material, according to the manufacturers' instructions (Jonassen and Handeland 2007). Real-time RT-PCR assays were performed using protocols targeting the matrix (M) gene (Spackman et al. 2002). Samples were processed at the Michigan State University Genomics Laboratory on an ABI Prism 7900 Sequence Detection System machine using the TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA, USA). We detected the matrix gene of LPAIV H5N9 at 100nM and 500nM final concentration, respectively. Two microliters of the final RNA prep were used as template in a 10

µl final reaction volume. Cycle threshold (ct) values were standardized by setting the baseline to a threshold of 0.028 for all runs. All ct-values <40 were considered LPAI virus positive.

Virus titration:

Swab samples were prepared and processed (as described above) with known concentrations of control viral RNA. We created RNA controls by serially diluting our stock virus of known concentration (10^1 - 10^7 PFU/mL) in BHI media. RNA controls were used to generate a standard curve, and sample titers were determined by entering the observed ct-value into the standard curve equation (Lee and Suarez 2004). Swab samples from all birds were tested through the first 7 dpi. When a bird tested negative for viral RNA on two consecutive sampling events (i.e. minimum 4 days after last positive sample) it was considered no longer shedding virus.

IDEXX FlockChek ELISA:*

Serum was tested using a commercially available IAV antibody ELISA kit (FlockChek* AI MultiS-Screen, IDEXX Laboratories Inc., Maine, USA). According to the manufacturers' instructions, samples with a signal-to-noise ≤ 50% were considered positive. Comparison of the FlockChek* ELISA with the more recent NP-ELISA revealed both tests are equally reliable in detecting IAV antibodies, and more specific and sensitive than traditional HI and AGP tests (Wu et al. 2007).

Hemagglutination inhibition (HI) assay:

To quantify post inoculation serum antibodies, HI assays were performed using standard protocols (Swayne et al. 1998), using chicken erythrocytes and four HA units of stock virus used

for inoculation. Serum samples were treated with 10% erythrocytes solution to remove nonspecific inhibitors and agglutinins. Samples were processed in duplicate using a 0.5% suspension of chicken erythrocytes. Antibody titers were expressed as the reciprocal of the highest serum dilution yielding complete inhibition of hemagglutination. Samples with HI titers ≥ 8 were considered positive.

Data analysis:

The body size index, estimated by PC1, accounted for 60% and 56% of the variance associated with structural measurements (wing chord, head length, and tarsus length) for females and males, respectively. Diet treatments were monitored and adjusted using the mean condition of all birds within a group. Treatment condition scores were compared at the time of capture and when desired condition levels were met using one-way analysis of variance (ANOVA). Post-hoc analyses were carried out using two sample t-tests assuming equal variances.

Elevated viral shedding occurred through 5 dpi for most birds. Therefore, analysis of shedding concentration was performed during the first 5 dpi using repeated-measures ANOVA. In the analysis, samples that did not exhibit detectable virus were assigned a value of zero, and birds that did not shed virus during the course of the study were included in the comparisons. Viral titers were transformed using log (base 10).

Shedding duration was calculated for each bird as the last day post inoculation where viral RNA was detected. In addition, shedding frequency was calculated for each mallard as the number of positive samples detected from the 4 sampling days between 1-5 dpi. Mallards that did not shed detectable virus were excluded from both analyses. Shedding duration and frequency data were not normally distributed (Shapiro-Wilk, $p < 0.05$). Therefore, overall group

comparisons were done using a nonparametric Kruskal-Wallis ANOVA on ranks, and post-hoc analyses were conducted using Mann-Whitney rank sum test.

Serologic response was compared among treatment groups on 14, 21, and 28 dpi. For analysis, negative samples (HI titer < 8) were assigned a value of half the minimum detectable titer as described by Stephenson et al. (2009). All HI titers were transformed using log (base 2). Analyses of antibody production were conducted using repeated-measures ANOVA. All repeated-measures post-hoc analyses were done using paired t-tests. The alpha level was set at 0.05 for all analyses, and derived p values correspond with two-tailed tests. Analyses were performed using PASW 18.0 (PASW, 2010).

RESULTS

A total of 81 (43 males; 38 females) juvenile mallards were captured during September 2009. Influenza A virus antibodies were detected in 51% of birds (17 males; 24 females) using ELISA. Of the 40 seronegative mallards available for the study, 10 were released because they had problems acclimating to captivity. Mean condition scores for the three wild treatment groups were similar at the time of capture (one-way ANOVA; $F_{2,27} = 1.12$, $p = 0.34$) (Figure 1.1). All (wild and captive-bred) birds (n = 40) adjusted well to captivity and were eating and drinking normally at the end of the acclimation period. Food manipulation significantly separated wild treatment group conditions by day 60 ($F_{2,27} = 18.0$, $p = < 0.001$) (Figure 1.1). Mean condition score for mallards in the poor treatment (-21%) was significantly reduced compared to birds in the lean treatment (-13%) (two-sample t-test; $t = 2.13$, $p = 0.046$) and *ad libitum* treatment (2%) ($t = 5.79$, $p = < 0.001$). Condition score for lean birds was lower than for mallards in the *ad libitum* treatment ($t = 3.77$, $p = 0.001$). Wild mallards in the *ad libitum* treatment surpassed their predicted mass during the first five days of diet manipulation, and their mean condition remained elevated (1.0 - 2.4%) for the remainder of the study. Nine of 10 captive-bred mallards gained mass during diet manipulation (Table 1.1).

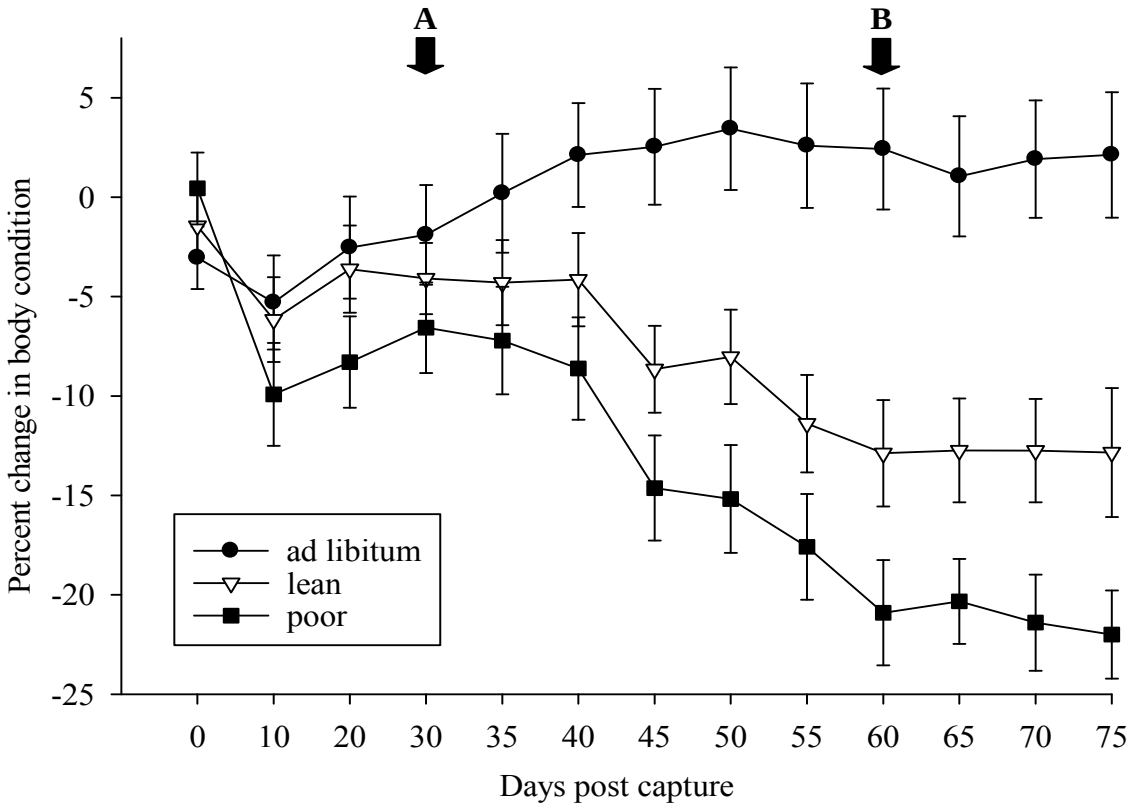

Figure 1.1. Body condition for wild mallard treatment groups throughout the study. Data points represent mean condition (± 1 standard error), A = start of diet manipulation, B = LPAIV H5N9 inoculation.

Table 1.1. Body mass (g) for 10 captive-bred mallards (C1-C10) fed *ad libitum* from time of purchase through LPAIV H5N9 challenge.

Bird ID	**Acclimation period**				**Diet manipulation**							**H5N9 challenge**			
	0*	***10***	***20***	***30***	***35***	***40***	***45***	***50***	***55***	***60***	***%*** [1]	***65***	***70***	***75***	***%*** [2]
C1	1139	1249	1264	1219	1216	1186	1221	1211	1204	1193	*4.7*	1212	1235	1185	*-0.7*
C2	935	1042	1096	1129	1125	1157	1170	1175	1167	1148	*22.8*	1156	1144	1137	*-1.0*
C3	872	853	893	927	929	938	933	961	947	938	*7.6*	935	903	916	*-2.3*
C4	1367	1383	1357	1329	1314	1282	1276	1245	1219	1217	*-11.0*	1226	1219	1270	*4.4*
C5	1055	1283	1347	1316	1331	1345	1333	1356	1333	1331	*26.2*	1347	1355	1358	*2.0*
C6	796	1042	1133	1171	1196	1186	1190	1248	1263	1254	*57.5*	1290	1277	1304	*4.0*
C7	702	849	932	946	1001	994	1028	1043	983	1016	*44.7*	1006	978	1020	*0.4*
C8	948	1107	1176	1192	1212	1222	1225	1227	1194	1208	*27.4*	1236	1213	1226	*1.5*
C9	1043	1126	1183	1225	1166	1188	1206	1223	1212	1205	*15.5*	1223	1197	1185	*-1.7*
C10	857	950	972	998	994	994	999	1008	993	989	*15.4*	990	986	1004	*1.5*

***** The numbers indicate days after captive-bred mallards were purchased.

1 Change in mass for each bird at the end of diet manipulation (day 60), expressed as percent change from purchase mass (day 0).

2 Change in mass for each bird 15 days post challenge (day 75), expressed as percent change from inoculation mass (day 60).

Susceptibility to LPAIV (H5N9) infection:

A total of 36 out of 40 (90%) study birds shed detectable virus (≥ 1.0 $\log_{10}$ PFU/mL). All four birds without detectable viral shedding were wild mallards in the poor condition treatment. Body condition scores for these birds at the time of LPAIV challenge were -18%, -21%, -24% and -37%. Thirty-five of 36 (97%) infected birds shed virus by the second day post inoculation. One captive-bred mallard delayed viral shedding until five days post inoculation. After LPAIV challenge, no birds exhibited clinical signs of disease.

Concentration of viral shedding:

The mean virus titer ($\log_{10}$ PFU/mL) for all treatment groups peaked at 2 dpi (wild *ad libitum* = 3.8; lean = 2.20; poor = 1.33; captive-bred *ad libitum* = 4.03) and the bulk of shedding continued through 5 dpi (Figure 1.2). Virus excretion in wild mallards during the first 5 dpi among treatment groups varied significantly (repeated-measures ANOVA; $F_{2, 27} = 7.24$, $p = 0.003$). In general, higher levels of viral shedding were observed in treatment groups with higher relative condition scores (i.e. greater food availability) (Table 1.2, Figure 1.2). Wild mallards in the poor treatment shed less virus than birds fed *ad libitum* (paired t-test; $t = 2.39$, $p = 0.001$) but not significantly less than lean mallards ($t = 1.31$, $p = 0.065$). We were unable to detect a difference in shedding concentration between ducks in the *ad libitum* and lean treatments ($t = 1.34$, $p = 0.097$). Wild and captive-bred birds fed *ad libitum* shed similar virus concentrations ($F_{1, 18} = 0.22$, $p = 0.64$).

Concentration of viral shedding was highly variable within treatment groups during the first five days of infection. Daily mean titers and standard errors are presented for groups in Table 1.2. Inspection of individual shedding patterns found a total of 14 mallards that excreted virus at

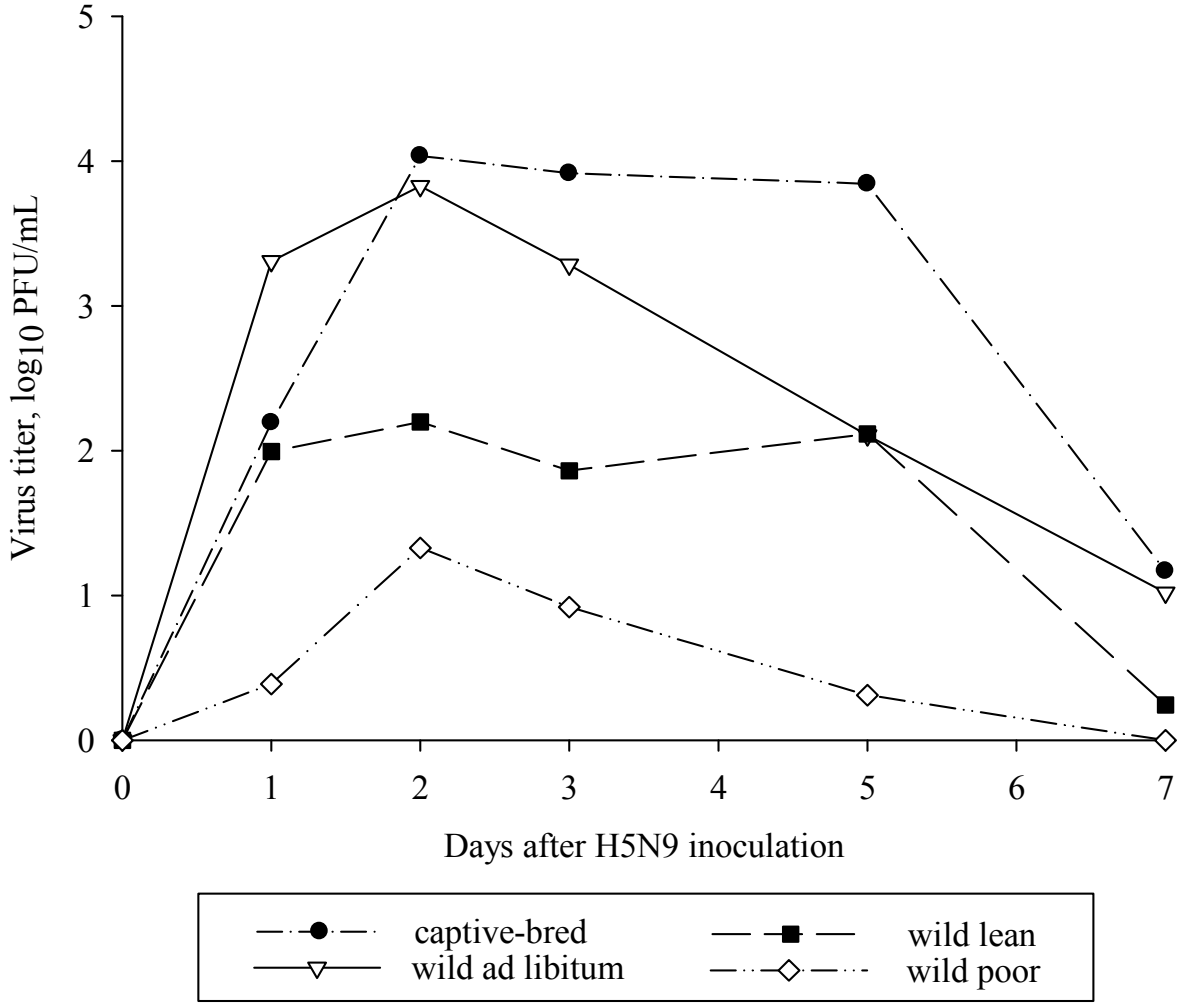

Figure 1.2. Virus shedding concentration results for captive-bred and wild mallard treatment groups. Data points represent mean titer (log_{10} PFU/mL) for each day sampled.

Table 1.2. Daily mean (± 1 standard error) virus titers (log_{10} PFU/mL) for mallards inoculated with LPAIV H5N9. Mean titer includes birds without detectable viral RNA, whereas minimum and maximum titers are reported for the number of birds *(n)* with detectable viral RNA on the given day.

Treatment group	**Days post inoculation**				
	1	**2**	**3**	**5**	**7**
Captive-bred *ad libitum*	2.2 ± 0.54	4.0 ± 0.73	3.9 ± 0.87	3.8 ± 0.59	1.2 ± 0.41
min-max (n)	*1.6-4.6 (7)*	*2.8-5.8 (8)*	*4.5-6.7 (7)*	*1.1-5.8 (9)*	*1.7-3.2 (5)*
Wild *ad libitum*	3.3 ± 0.56	3.8 ± 0.52	3.3 ± 0.66	2.1 ± 0.72	1.0 ± 0.45
min-max (n)	*1.3-5.4 (9)*	*1.7-6.2 (10)*	*1.9-5.7 (8)*	*3.4-4.9 (5)*	*1.8-3.3 (4)*
Wild lean	2.0 ± 0.42	2.2 ± 0.50	1.9 ± 0.59	2.1 ± 0.72	0.2 ± 0.24
min-max (n)	*0.9-4.3 (8)*	*1.8-5.7 (8)*	*1.1-6.1 (7)*	*1.2-6.6 (6)*	*2.4 (1)*
Wild poor	0.4 ± 0.26	1.3 ± 0.69	0.9 ± 0.57	0.3 ± 0.31	-
min-max (n)	*1.6-2.3 (2)*	*2.0-6.8 (4)*	*1.8-5.6 (3)*	*3.1 (1)*	-

high titer ($\geq$ 5.0 $\log_{10}$ PFU/mL). The majority of these birds were wild and captive-bred mallards fed *ad libitum* (wild, n = 5; captive-bred, n = 7), whereas only two birds came from reduced condition treatments (lean, n = 1; poor, n = 1) (Figure 1.3). However, peak viral titers in the above mentioned poor bird (6.8 $\log_{10}$ PFU/mL) and lean bird (6.6 $\log_{10}$ PFU/mL) were the highest observed across all wild mallards (Table 1.2).

Duration of viral shedding:

Duration of infection among all study birds was large (1-20 dpi). Among wild mallards, mean duration (days) of shedding was largest in groups with higher condition scores (wild *ad libitum* = 6.4; lean = 5.4; poor = 3.3) (Figure 1.4). Intermittent shedding beyond 5 dpi was more common in wild mallards fed *ad libitum* (n = 5), whereas only two birds in reduced condition treatments (lean, n = 1; poor, n = 1) shed virus past 5 dpi. Despite these relationships, we were unable to detect a significant difference in shedding duration among wild mallards (Kruskal-Wallis ANOVA; H = 2.86, 2 d.f., p = 0.24). In addition, mean duration of shedding in captive-bred birds (7.8 days) was similar to wild birds fed *ad libitum* (H = 0.52, 1 d.f., p = 0.47).

The number of positive samples through 5 dpi differed among wild mallard groups (H = 6.20, 2 d.f., p = 0.045) (Figure 1.4). Mean number of positive samples in poor mallards (1.7) was significantly less than for *ad libitum* birds (3.2) (Mann-Whitney rank sum; U = 8.0, p = 0.016), but not less than for lean mallards (2.9) (U = 13.5, p = 0.071). No differences were found between wild *ad libitum* and lean treatments (U = 45.0, p = 0.71), or between wild and captive-bred birds fed *ad libitum* (H = 0.007, 1 d.f., p = 0.93).

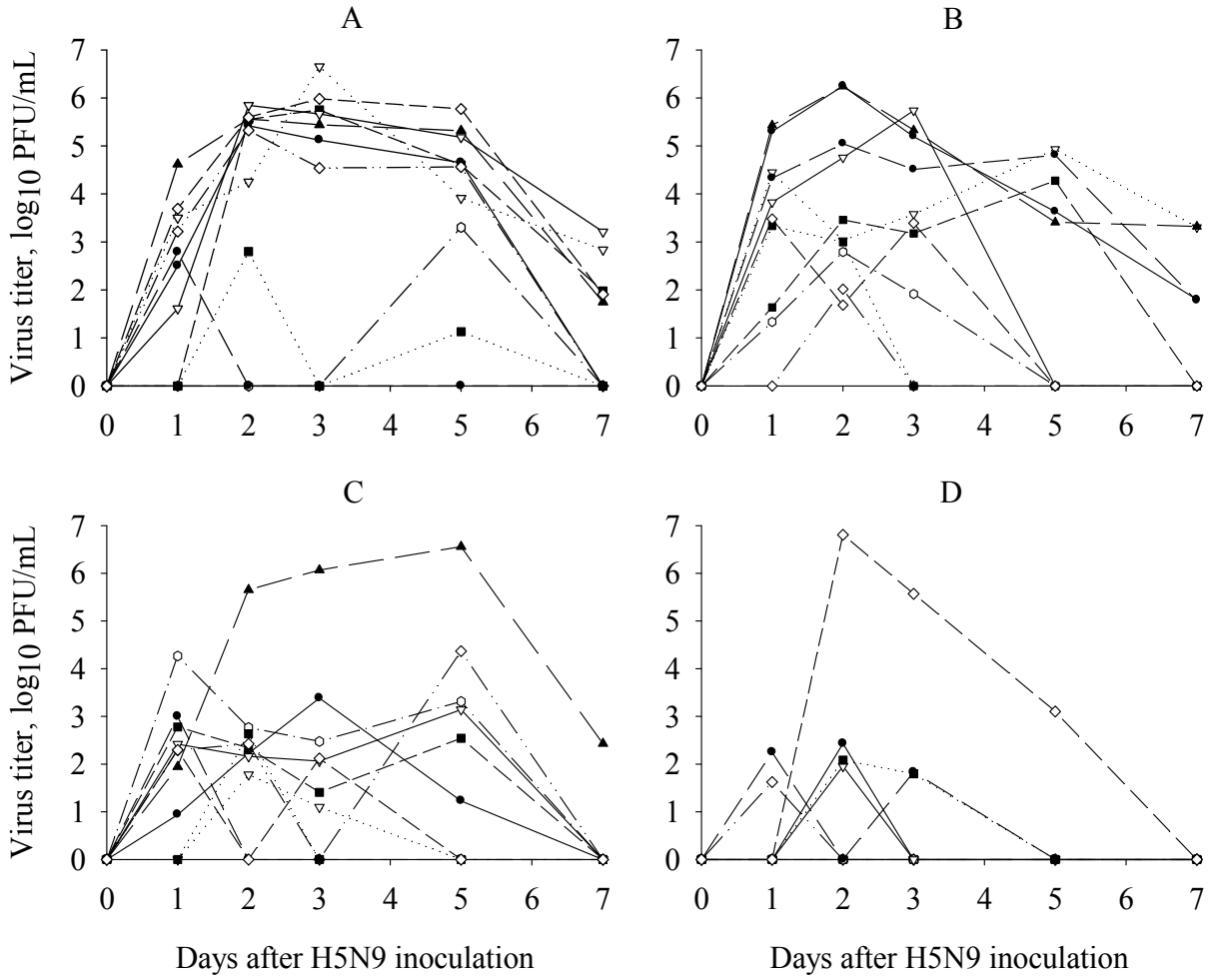

Figure 1.3. Virus shedding concentration (log_{10} PFU/mL) results for all mallards with detectable viral RNA using matrix gene RTT-PCR. A: captive-bred (n = 10), B: wild ad libitum (n = 10), C: wild lean (n = 10), D: wild poor (n = 6).

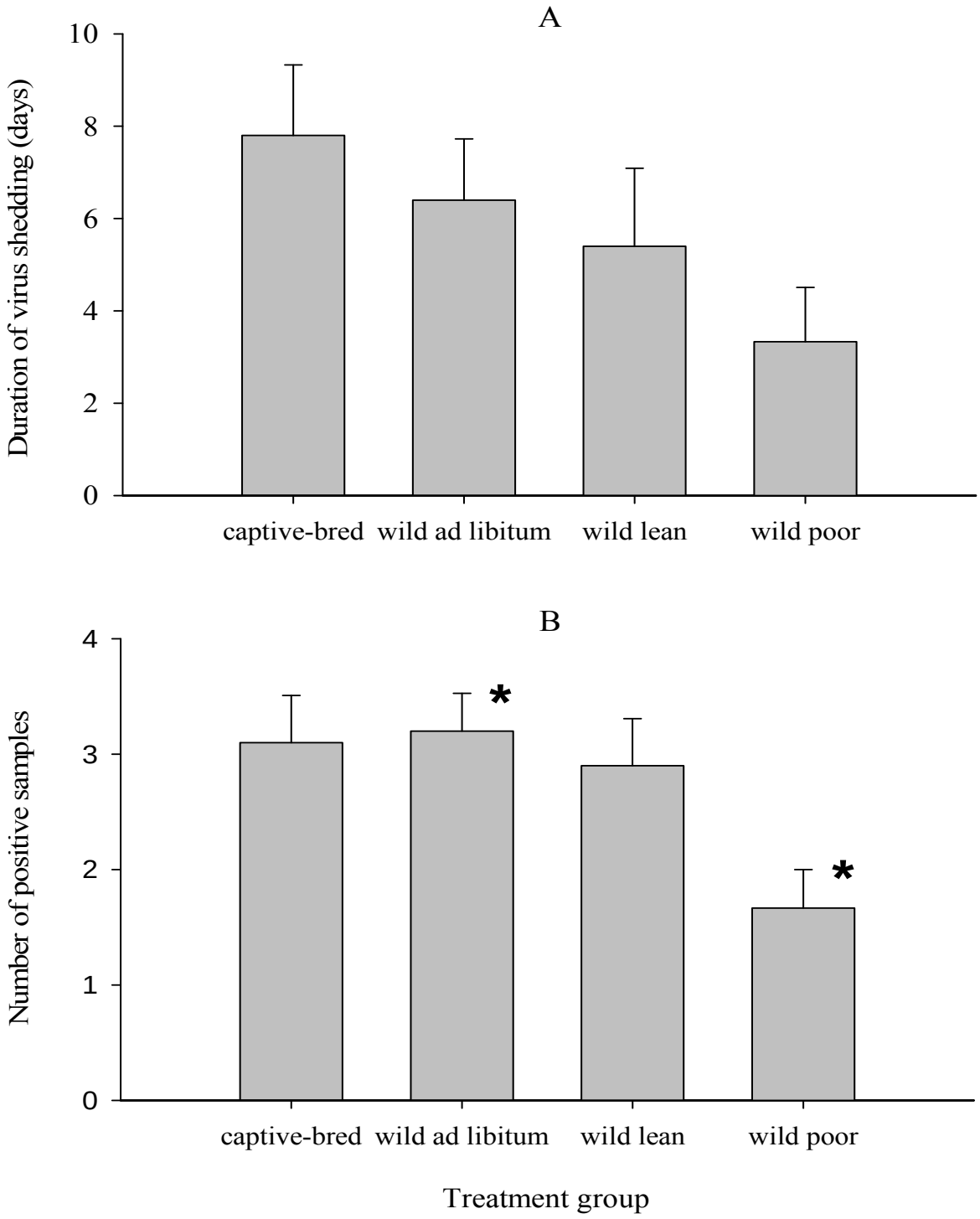

Figure 1.4. Duration of viral shedding (A) and number of positive samples (B) for captive-bred and wild mallard treatment groups. Bars represent means ± 1 standard error, asterisks identify groups significantly different from one another (Mann-Whitney rank sum, alpha = 0.05). The number of positive samples was calculated from the 4 sampling days between 1-5 dpi.

Serologic response:

After LPAIV H5N9 challenge, 33 out of 40 (82.5%) study birds tested seropositive using ELISA, whereas 32 out of 40 (80%) had detectable levels of H5-specific antibodies according to HI tests. Among wild mallards, mean HI titers peaked at 14 dpi for *ad libitum* and lean treatments (3.4, 3.2 $\log_2$, respectively), while HI titers in poor mallards were highest at 21 dpi (3.3 $\log_2$) (Table 1.3). Overall antibody production at 14, 21, and 28 dpi was similar across wild mallard treatments (repeated-measures ANOVA; $F_{2, 23} = 0.29$, $p = 0.75$). Mean HI titer for captive-bred birds (4.6 $\log_2$) peaked at 14 dpi, and did not differ from wild mallards fed ad libitum ($F_{1, 17} = 2.47$, $p = 0.13$).

Table 1.3. Serological status of mallards before and after LPAIV H5N9 challenge using ELISA and HI tests. Mean (± 1 standard error) test scores include all birds in each treatment.

Treatment group	**ELISA** [1]				**H5N9 HI** [2]			
	B.I. [3]	**14** *	**21**	**28**	**B.I.**	**14**	**21**	**28**
Captive-bred *ad libitum* Seropositive *(n)*	0.93 ± 0.03 *(0)*	0.36 ± 0.07 *(7)*	0.42 ± 0.09 *(6)*	0.42 ± 0.09 *(6)*	< 3 *(0)*	4.6 ± 0.70 *(7)*	4.1 ± 0.55 *(7)*	3.9 ± 0.53 *(7)*
Wild *ad libitum* Seropositive *(n)*	0.87 ± 0.05 *(0)*	0.30 ± 0.06 *(9)*	0.36 ± 0.07 *(6)*	0.37 ± 0.07 *(6)*	< 3 *(0)*	3.4 ± 0.43 *(7)*	3.2 ± 0.29 *(8)*	2.8 ± 0.20 *(7)*
Wild lean Seropositive *(n)*	0.86 ± 0.04 *(0)*	0.21 ± 0.03 *(10)*	0.33 ± 0.08 *(8)*	0.36 ± 0.09 *(8)*	< 3 *(0)*	3.2 ± 0.25 *(8)*	3.0 ± 0.26 *(8)*	2.8 ± 0.25 *(5)*
Wild poor Seropositive *(n)*	0.84 ± 0.03 *(0)*	0.36 ± 0.10 *(7)*	0.47 ± 0.09 *(6)*	0.47 ± 0.08 *(6)*	< 3 *(0)*	3.1 ± 0.38 *(6)*	3.3 ± 0.58 *(5)*	3.1 ± 0.31 *(6)*

***** The numbers indicate days after LPAIV H5N9 inoculation.

1 The ELISA scores represent the signal to noise (S/N) ratio where values ≤ 0.50 are considered seropositive.

2 The hemagglutination inhibition values represent the mean titer (log_2) of sera samples.

3 Before LPAIV H5N9 inoculation.

DISCUSSION

Our study demonstrates body condition significantly influences susceptibility to infection and viral shedding patterns in wild-caught juvenile mallards challenged with LPAIV H5N9. However, our findings were opposite of our original predictions based on the condition-dependent hypothesis in which birds in poor condition would experience reduced immune function and increased susceptibility to infection (Flint and Franson 2009). Here we find birds in good condition were more susceptible to LPAIV infection, and shed significantly higher virus titers for a longer duration compared to birds in poor condition. Four mallards in the poor condition treatment were resistant to infection, whereas all remaining mallards shed detectable virus. A clear trend was observed among wild birds showing a positive relationship between body condition and host competence.

Until recently, it has been assumed LPAIV infections remain asymptomatic in wild waterfowl with little or no effects on life-history parameters. Then van Gils et al. (2007) determined LPAIV infection decreased migratory performance in Bewick's swans, and Latorre-Margalef et al. (2009b) found mallards infected with IAV were leaner than uninfected conspecifics. The latter study has generated ongoing debate on whether IAV infection influences body condition of migrating waterfowl, or vice versa (Flint and Franson 2009, Latorre-Margalef et al. 2009a). Both sides acknowledge the possibility that birds may become immunosuppressed during migration due to reduced energy stores, and therefore suggest further studies are needed to conclusively discriminate between these two hypotheses. To our knowledge, our research represents the first experimental study that has evaluated the condition-dependent hypothesis using LPAIV and waterfowl as an experimental model. We have provided evidence that body

condition influences IAV infection in wild juvenile mallards, however, the mechanisms responsible for our findings remain unclear and contrary to those suggested by the condition-dependent hypothesis (Flint and Franson 2009).

Current research examining host nutrition and susceptibility to infectious disease provides overwhelming support for our original hypotheses. In general, these studies found malnutrition increases susceptibility and severity of infection with most microbial agents (Scrimshaw et al. 1968, Louria 2007). In the case of IAV infection, deficiencies in vitamins A and C, selenium, and protein have increased susceptibility and burden of disease (Stephensen et al. 1993, Beck 2001, Li et al. 2006, Louria 2007, Ritz et al. 2008). These findings were attributed to reduced immune function caused by limitations in one or several essential nutrients, vitamins, and/or dietary protein (Pollett et al. 1979, Ritz et al. 2008). If we assume mallards in poor condition were malnourished, as indicated by significant decrease in breast condition score (Figure 1.5), then our findings contradict the well established trend described above. Therefore, we propose the relationship between body condition and LPAIV infection in waterfowl is more complex than previously thought.

Review of previous research, however, would suggest three additional factors that may influence the observed relationship: (1) duration of food restriction, (2) depletion of subcutaneous fat reserves, and (3) changes in intestinal composition due to reduced food intake. Past studies have shown certain conditions of malnutrition increase hosts resistance to viral infection (Sprunt 1942, Flanigan and Sprunt 1956). Sprunt and Flanigan (1956) found mice and chickens fed reduced protein diets exhibited a cyclic pattern of susceptibility relative to those fed high protein diets. Protein restriction increased susceptibility for the first two weeks, then decreased susceptibility from three to six weeks. Beyond seven weeks, mice and chickens

became more susceptible to viral infection. Animals were less susceptible to infection once they depleted subcutaneous fat reserves, and initiated catabolism of available protein reserves. Diets high in protein have been correlated with increased resistance to viral infection (Pollett et al. 1979). Our findings support those of Sprunt and Flanigan (1956), as mallards in poor condition were less susceptible to infection after four weeks of food restriction. Moreover, birds in the poor condition treatment had significantly reduced keel scores when challenged with LPAIV (Figure 1.5); thereby suggesting these mallards transitioned to catabolism of protein reserves.

Changes in intestinal composition from reduced food availability may be responsible for decreased susceptibility and viral titers among mallards in reduced condition. Food deprivation has been shown to reduce the relative amount of mucin glycoprotein in the intestinal tract. Smirnov et al. (2004) examined intestinal mucin in chickens fasted for 72 hours, and found acute food deprivation decreased mucin thickness throughout the intestinal tract. In rats deprived 50% of their daily intake for five weeks, the concentration of intestinal mucin was significantly reduced compared to control animals fed normally (Sherman et al. 1985). In waterfowl, LPAI viruses preferentially bind to sialic acid (SA) receptors which occupy terminal positions on mucin glycoproteins within the intestinal tract (Webster et al. 1978, Kida et al. 1980). Therefore, it seems plausible that decreased abundance of mucin may reduce SA expression and inhibit viral attachment and propagation. Further investigations concerning reduced food availability, intestinal mucin, and SA expression in waterfowl are needed to evaluate this hypothesized interaction.

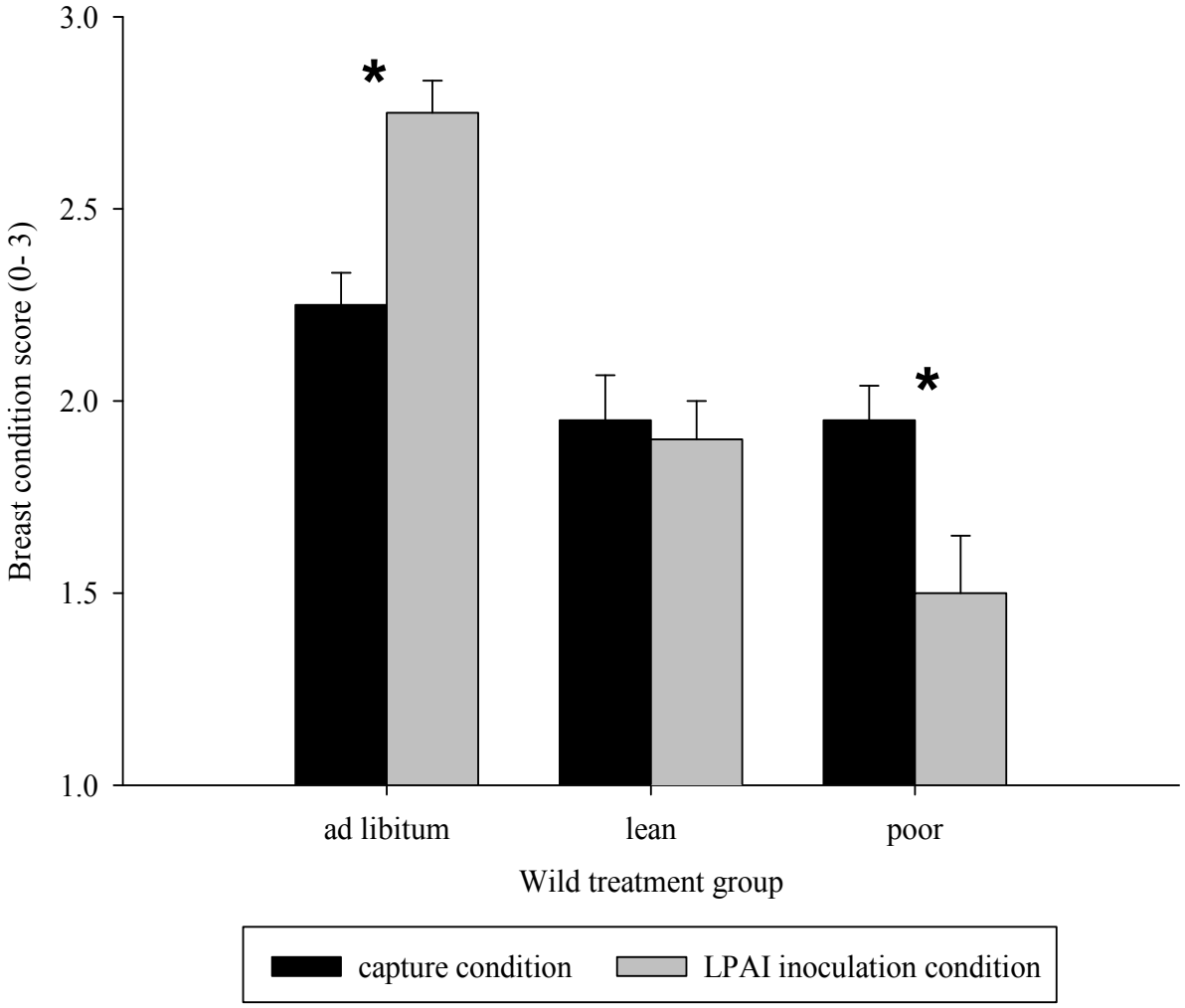

Figure 1.5. Breast condition score for wild mallard treatment groups at capture vs. LPAIV H5N9 challenge. Bars represent mean condition score ± 1 standard error (n = 10), asterisks represent significant within group differences (paired t-test, alpha = 0.05).

We did not detect a significant difference in susceptibility to infection and viral shedding patterns between wild-caught and captive-bred mallards fed *ad libitum*. Therefore, we support the use of captive-bred birds in LPAIV experimental models where birds are fed *ad libitum*. We are unable to conclude whether captive-bred birds exhibit similar host competency during periods of food restriction. Mallards bred and raised in captivity do not encounter prolonged food shortages, and may respond differently to food deprivation. Additional research is needed to validate the use of captive-bred mallards in future LPAIV experimental models incorporating food restriction.

Our serological findings indicate reduced body condition does not affect mallard antibody production in response to LPAIV H5N9 challenge. These findings do not support our initial prediction in which resource limited birds experience decreased antibody production (Hangalapura et al. 2005, Hanssen et al. 2005). It is well understood that maintaining and using the immune system is energetically costly (Klasing et al. 2004). During periods of limited food access, Buehler et al. (2009) found migrating shorebirds suppress more costly acute-phase immune responses (phagocytosis, fever, inflammation) in order to maintain a baseline level of immune function. Thus, mallards fed restricted diets may have down regulated some aspects of immune function and retained the ability to produce specific antibodies. Alternatively, mallards in reduced condition may have adequate resources to enable production of a low-level humoral response typical of LPAIV infections (Kida et al. 1980, Jourdain et al. 2010).

If the observed influence of body condition on LPAIV infection is at play in nature, prolonged periods of reduced food availability may inhibit virus transmission. Reduced transmission of LPAI viruses may decrease the development of highly pathogenic avian influenza virus (HPAIV). In the past two decades, HPAIV outbreaks have occurred frequently in

domestic poultry after introduction of H5 and H7 LPAI viruses (Perdue and Swayne 2005). These outbreaks resulted in high morbidity, mortality, and severe economic losses (Olsen et al. 2006). Understanding the ecology of LPAIV in waterfowl hosts remains a critical task.

A pattern between host condition and IAV infection has been observed in recent studies (Hanson et al. 2008, Latorre-Margalef et al. 2009b), and our study provides novel insight towards explaining these relationships. Hanson et al. (2008) found migrating ruddy turnstones (*Arenaria interpres*) with elevated stopover masses exhibited higher IAV prevalence rates. The researchers attributed their findings to on-site IAV amplification, as lighter birds were presumed to have recently arrived at the stopover site. However, according to our findings, such differences could be attributed to decreased IAV susceptibility among birds in poor condition. On the other hand, Latorre-Margalef et al. (2009b) observed a significant decrease in body mass in mallards infected with IAV compared to uninfected ducks (mean difference of 20 g). Although infected birds were lighter, we point out a 20 g reduction in mass would not indicate a mallard was in poor condition. Mean body mass loss among mallards in our poor treatment exceeded 200 g. Therefore, the relationship observed by Latorre-Margalef et al. (2009b) does not refute our findings.

In summary, we have taken a large step in showing how host body condition may play a significant role in the epidemiology of LPAIV infection in mallards, and presumable other waterfowl species. We provide evidence that (1) mallards in poor condition are less susceptible to infection compared to birds in good condition, (2) concentration and duration of viral shedding are positively correlated with host condition, and (3) body condition does not affect mallard specific antibody response after LPAIV challenge. The study also suggests that captive-bred mallards may replace wild mallards in future experimental models where birds are fed *ad*

libitum. The precise mechanisms of decreased host competence among mallards in reduced condition remains unknown. If susceptibility follows a cyclic pattern, as indicated by Sprunt and Flanigan (1956), then birds would first encounter a period of enhanced virus transmission in response to food deprivation. Additional field and laboratory studies under varying durations of food restriction are encouraged to clarify this proposed relationship. Furthermore, these studies should incorporate more comprehensive analyses of immune function and host nutritional reserves. Such data would help explain how body condition influences waterfowl host competency during LPAIV infection, and improve future LPAIV transmission models.

CHAPTER 2

ROLE OF HOMO- AND HETEROSUBTYPIC IMMUNITY ON REINFECTION OF MALLARD DUCKS WITH INFLUENZA A VIRUS

INTRODUCTION

In Europe and North America, the prevalence of influenza A virus (IAV) isolation in waterfowl peaks during autumn migration when juvenile ducks congregate before migrating south (Hinshaw et al. 1985, Olsen et al. 2006). Low pathogenic avian influenza virus (LPAIV) prevalence can reach 60% in these birds, and generally includes a large diversity of virus subtypes (Krauss et al. 2004, Wallensten et al. 2007). As a result, migrating waterfowl are frequently exposed to the same (homosubtypic) or different (heterosubtypic) IAV subtypes (Sharp et al. 1997). Recent studies suggest recurring IAV exposures induce transient immunity, thereby providing an explanation for the low IAV infection prevalence (< 1%) among wintering waterfowl (Latorre-Margalef et al. 2009b, Jourdain et al. 2010). To better understand transmission and seasonal maintenance of IAV by migrating waterfowl, we must improve our understanding of naturally occurring homo- and heterosubtypic immunity.

Previous experimental studies have demonstrated prior LPAIV infection protects against homosubtypic (Kida et al. 1980) and heterosubtypic (Jourdain et al. 2010) LPAIV reinfections. Kida et al. (1980) found that after primary LPAIV H7N2 inoculation, ducks resisted homosubtypic reinfection for 28 days, but were susceptible beyond 46 days post inoculation (dpi). Jourdain et al. (2010) demonstrated LPAIV H5N7 infection in juvenile mallards (*Anas platyrhynchos*) reduced viral RNA excretion during homosubtypic reinfection, and protected some birds against heterosubtypic LPAIV H5N2 reinfection. These studies demonstrate

waterfowl can mount immunological responses against recurring LPAIV exposures; however, more studies are needed to clarify homo- vs. heterosubtypic interactions and examine duration between viral challenges.

Understanding the role of homo- and heterosubtypic immunity is essential for several reasons. Immunity induced by prior IAV infection has been shown to reduce susceptibility and viral shedding in waterfowl during subsequent infections (Kida et al. 1980, Fereidouni et al. 2009, Jourdain et al. 2010). As a result, maintenance and spread of LPAIVs may be greatly reduced if herd immunity develops in the population (Latorre-Margalef et al. 2009b, Jourdain et al. 2010). However, previous LPAIV infection may enhance transmission of highly pathogenic avian influenza virus (HPAIV) by waterfowl. Recent findings indicate LPAIV infection reduces disease burden during HPAI reinfection, thereby allowing birds to migrate while shedding intermediate concentrations of HPAIVs (Fereidouni et al. 2009). In addition, acquired immunity may reduce the occurrence of simultaneous IAV infections, which play a role in producing novel virus subtypes through genetic reassortment (Hinshaw et al. 1980, Sharp et al. 1997).

In this study, we tested whether LPAIV H5N9 infection in juvenile mallards 1) provides long-term homosubtypic immunity to the same LPAIV strain and 2) provides long-term heterosubtypic immunity to a different LPAIV H7N3 strain. Long-term immunity was defined as seven weeks between primary and secondary LPAIV inoculation. We hypothesized mallards would be resistant to reinfection with the homosubtypic H5N9 strain, but susceptible to heterosubtypic H7N3 reinfection.

MATERIALS AND METHODS

Animals:

Wild-caught juvenile mallards (n = 20) were retained from the previous study described in Chapter 1 (hereafter "condition study"). During the condition study, birds were placed on diet treatments to manipulate their body condition before inoculation with LPAIV H5N9. To minimize variation in starting condition for the present study, birds with similar body condition scores were selected. In addition, only birds with detectable levels of H5-specific antibodies were included in this experiment. Mallards were housed at the Michigan State University Research Containment Facility using protocols described in Chapter 1 (see Housing).

Experimental design:

Following completion of the condition study (starting at 28 dpi), all birds were maintained with *ad libitum* access to water and food. Mallards were randomly divided into two treatment groups (homosubtypic, n = 10; heterosubtypic, n = 10), each housed in a separate biosafety level 2 room. One day prior to experimental reinoculation (at 48 dpi), cloacal and oral swabs were collected from all mallards to confirm absence of viral shedding using RRT-PCR as described in Chapter 1 (see Matrix Gene RRT-PCR). In addition, blood serum was collected to test for IAV antibodies using ELISA (Chapter 1, see IDEXX FlockChek* ELISA).

At 49 dpi the homosubtypic treatment group was oropharyngeally reinoculated with the same LPAIV H5N9 virus (A/Northern pintail/California/44221-761/2006) using 1.5 mL of 10^6 PFU/mL. The heterosubtypic treatment group was oropharyngeally reinoculated with a different LPAIV H7N3 virus (A/Northern pintail/Nevada/44252-242/2006) using 1.5 mL of 10^6 PFU/mL.

Both LPAIV isolates were obtained from USGS National Wildlife Health Center, Wisconsin, USA. Virus was propagated and titrated as described in Chapter 1 (see Virus). Following reinoculation, cloacal and oral swabs were collected daily for the first 3 days and then on days 5, 7, 9, 12, and 14 post reinoculation (dpr). Mallards in the homosubtypic treatment were always sampled before heterosubtypic birds to prevent spillover of the H7N3 LPAIV subtype. Swabs samples were pooled together for individual birds and tested for the presence of viral RNA. Blood serum was collected on 7 and 14 dpr for serologic testing. Swab and sera samples were collected and stored using protocols described in Chapter 1. All mallards were euthanized at 14 dpr using CO2 asphyxiation, followed by cervical dislocation.

RESULTS

One day before LPAIV reinoculation (48 dpi), swab samples from all mallards (n = 20) tested negative for viral RNA using RRT-PCR, indicating birds were not shedding IAV immediately prior to experimental reinoculation. Serology testing using ELISA found 14 of 20 (70%) mallards remained seropositive 48 days after primary LPAIV H5N9 infection (Table 2.1). The distribution of seropositive birds was equal among the treatment groups (homosubtypic, n = 7; heterosubtypic, n = 7).

Homosubtypic LPAIV (H5N9) reinoculation:

One of 10 mallards in the homosubtypic treatment shed detectable viral RNA ($\geq$ 1.0 $\log_{10}$ PFU/mL). The positive mallard tested seropositive by ELISA one day prior to reinoculation. Viral shedding peaked at 2 dpr (5.6 $\log_{10}$ PFU/mL), and viral RNA was detected in all pooled swabs samples through 9 dpr (Figure 2.1). All birds tested positive for IAV antibodies at 7 and 14 dpr (Table 2.1).

Heterosubtypic LPAIV (H7N3) reinoculation:

All mallards (n = 10) in the heterosubtypic treatment shed detectable viral RNA on the first date post reinoculation. Mean virus titer peaked at 2 dpr (5.9 $\log_{10}$ PFU/mL) and the majority of viral shedding occurred through 5 dpr (Figure 2.1). All birds excreted virus concentrations that exceeded 5.0 $\log_{10}$ PFU/mL, and three birds exhibited viral titers greater than 7.0 $\log_{10}$ PFU/mL. The duration of infection among mallards ranged from 5-14 dpr, with mean duration being 9.2

days. Only one mallard tested positive for viral RNA at 14 dpr. Influenza A virus antibodies were detected in all mallards at 7 and 14 dpr using ELISA (Table 2.1).

Table 2.1. Serological status of mallards before and after homosubtypic LPAIV H5N9 and heterosubtypic LPAIV H7N3 reinoculation using ELISA. Mean (± 1 standard error) test scores include all birds in each treatment.

Treatment group	**ELISA** [1]		
	B.I. [2]	**7 ***	**14**
Homosubtypic (LPAIV H5N9) Seropositive *(n)*	0.42 ± 0.07 *(7)*	0.11 ± 0.02 *(10)*	0.17 ± 0.04 *(10)*
Heterosubtypic (LPAIV H7N3) Seropositive *(n)*	0.39 ± 0.07 *(7)*	0.10 ± 0.01 *(10)*	0.15 ± 0.02 *(10)*

***** The numbers indicate days post reinoculation.

1 ELISA scores represent signal to noise ratio (S/N), values ≤ 0.50 are considered seropositive.

2 One day before reinoculation (48 days after primary LPAIV H5N9 challenge).

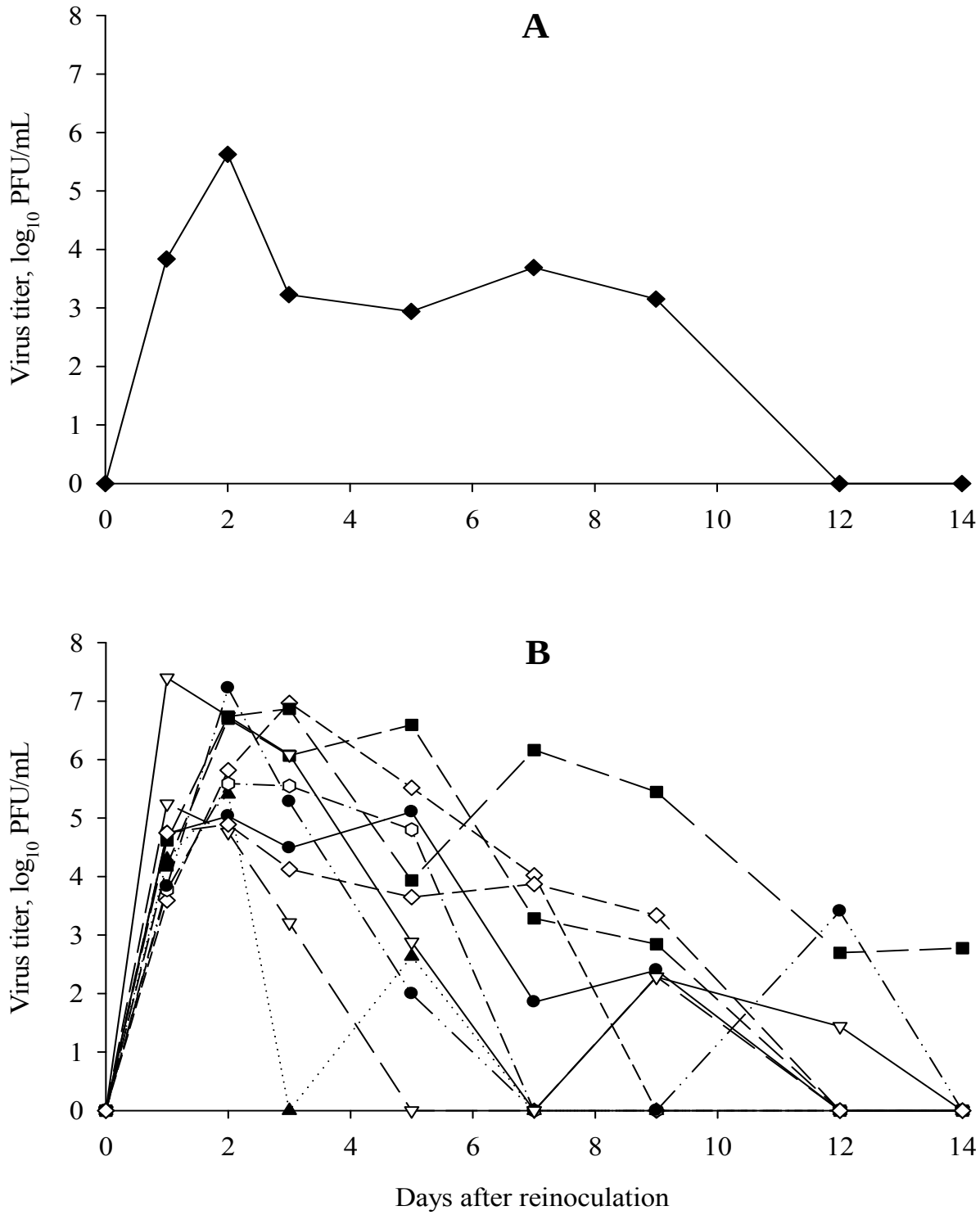

Figure 2.1. Virus shedding concentration (log_{10} PFU/mL) for all mallards with detectable viral RNA after reinoculation with (A) homosubtypic LPAIV H5N9 (n = 1), and (B) heterosubtypic LPAIV H7N3 (n=10). Birds were challenged 7 weeks after primary LPAIV H5N9 inoculation.

DISCUSSION

During autumn migration, juvenile mallards congregate with waterfowl from other areas and are frequently exposed to homo- and heterosubtypic IAVs (Sharp et al. 1997, Krauss et al. 2004, Wallensten et al. 2007). Our study was designed to test long-term (i.e. 7 weeks after primary infection) protective immunity throughout the migratory season. We provide evidence that juvenile mallards can mount an immune response protective against reinfection with the same LPAI H5N9 virus. Alternatively, we demonstrate mallards were susceptible to infection with a heterosubtypic LPAI H7N3 virus.

Previous studies successively inoculating juvenile mallards with LPAIV strains have found birds resistant to homosubtypic reinfection for up to 28 days (Kida et al. 1980, Jourdain et al. 2010). Our findings support these observations, as 9 of 10 mallards resisted reinfection with the same LPAIV. We did observe one mallard whose positive serological status did not protect it from homosubtypic reinfection. This observation is in agreement with Kida et al. (1980), who found Pekin ducks became susceptible to homosubtypic reinfection after 46 days. However, our findings suggest a greater proportion of wild juvenile mallards resist homosubtypic reinfection beyond 49 days.

Jourdain et al. (2010) found juvenile mallards are able to mount short-term (i.e. 21 days after primary infection) immunological responses protective against heterosubtypic LPAIV reinfections. Our study detected no strong evidence of heterosubtypic immunity, as all mallards in our heterosubtypic treatment shed detectable viral RNA. We did not have a control group of birds (not previously exposed to H5N9) to test the response to LPAIV H7N3 alone. However, the high concentration of virus shedding (≥ 5.0 $\log_{10}$ PFU/mL) observed in all mallards

reinfected with heterosubtypic LPAIV H7N3 suggests little or no cross-reactive immunity. We suggest immunological responses protective against heterosubtypic LPAIV infections may be short-term (less than 7 weeks).

Current research suggests the transmission dynamics of LPAIVs by migrating waterfowl are significantly influenced by homo- and heterosubtypic immunity (Kida et al. 1980, Sharp et al. 1997, Jourdain et al. 2010). Our findings contribute to available information in describing the interaction of more long-term immune responses. In summary, we provide evidence that (1) juvenile mallards can mount long-term protective immunological responses against reinfection with the same LPAI H5N9 virus, and (2) are susceptible to heterosubtypic LPAIV H7N3 reinfection 49 days after primary inoculation. Future studies should incorporate new virus subtypes, different challenge timings, and analysis of immune function to improve our understanding of homo- and heterosubtypic immunity.

REFERENCES

REFERENCES

Beck, M. A. 2001. Antioxidants and viral infections: host immune response and viral pathogenicity. J Am Coll Nutr **20**:384S-388S; discussion 396S-397S.

Brown, J. D., D. E. Stallknecht, J. R. Beck, D. L. Suarez, and D. E. Swayne. 2006. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. Emerging Infectious Diseases **12**:1663-1670.

Buehler, D. M., F. Encinas-Viso, M. Petit, F. Vezina, B. I. Tieleman, and T. Piersma. 2009. Limited Access to Food and Physiological Trade-Offs in a Long-Distance Migrant Shorebird. II. Constitutive Immune Function and the Acute-Phase Response. Physiological and Biochemical Zoology **82**:561-571.

Carney, S. M. (1992) U. S. Department of the Interior, U.S. Fish and Wildlife Service, Washington, D.C. 144pp.

Cooley, A. J., H. Vancampen, M. S. Philpott, B. C. Easterday, and V. S. Hinshaw. 1989. Pathological Lesions in the Lungs of Ducks Infected with Influenza-a Viruses. Veterinary Pathology **26**:1-5.

Devries, J. H., R. W. Brook, D. W. Howerter, and M. G. Anderson. 2008. Effects of spring body condition and age on reproduction in Mallards (Anas platyrhynchos). Auk **125**:618-628.

Fereidouni, S. R., E. Starick, M. Beer, H. Wilking, D. Kalthoff, C. Grund, R. Hauslaigner, A. Breithaupt, E. Lange, and T. C. Harder. 2009. Highly Pathogenic Avian Influenza Virus Infection of Mallards with Homo- and Heterosubtypic Immunity Induced by Low Pathogenic Avian Influenza Viruses. Plos One **4**:-.

Flanigan, C. C., and D. H. Sprunt. 1956. The effect of malnutrition on the susceptibility of the host to viral infection. J Exp Med **104**:687-706.

Flint, P. L., and J. C. Franson. 2009. Does influenza A affect body condition of wild mallard ducks, or vice versa? Proceedings of the Royal Society B-Biological Sciences **276**:2345-2346.

Gregory, N. G., and J. K. Robins. 1998. A body condition scoring system for layer hens. New Zealand Journal of Agricultural Research **41**:555-559.

Halvorson, D. A., C. J. Kelleher, and D. A. Senne. 1985. Epizootiology of Avian Influenza - Effect of Season on Incidence in Sentinel Ducks and Domestic Turkeys in Minnesota. Applied and Environmental Microbiology **49**:914-919.

Hangalapura, B. N., M. G. B. Nieuwland, G. D. Reilingh, J. Buyse, H. Van Den Brand, B. Kemp, and H. K. Parmentier. 2005. Severe feed restriction enhances innate immunity but suppresses cellular immunity in chicken lines divergently selected for antibody responses. Poultry Science **84**:1520-1529.

Hanson, B. A., M. P. Luttrell, V. H. Goekjian, L. Niles, D. E. Swayne, D. A. Senne, and D. E. Stallknecht. 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? Journal of Wildlife Diseases **44**:351-361.

Hanssen, S. A., D. Hasselquist, I. Folstad, and K. E. Erikstad. 2005. Cost of reproduction in a long-lived bird: incubation effort reduces immune function and future reproduction. Proceedings of the Royal Society B-Biological Sciences **272**:1039-1046.

Hinshaw, V. S., W. J. Bean, R. G. Webster, and G. Sriram. 1980. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102**:412-419.

Hinshaw, V. S., J. M. Wood, R. G. Webster, R. Deibel, and B. Turner. 1985. Circulation of Influenza-Viruses and Paramyxoviruses in Waterfowl Originating from 2 Different Areas of North-America. Bulletin of the World Health Organization **63**:711-719.

Homme, P. J., and Easterda.Bc. 1970. Avian Influenza Virus Infections .4. Response of Pheasants, Ducks, and Geese to Influenza a/Turkey/Wisconsin/1966 Virus. Avian Diseases **14**:285-&.

Jonassen, C. M., and K. Handeland. 2007. Avian influenza virus screening in wild waterfowl in Norway, 2005. Avian Diseases **51**:425-428.

Jourdain, E., G. Gunnarsson, J. Wahlgren, N. Latorre-Margalef, C. Brojer, S. Sahlin, L. Svensson, J. Waldenstrom, A. Lundkvist, and B. Olsen. 2010. Influenza Virus in a Natural Host, the Mallard: Experimental Infection Data. Plos One **5**:-.

Keawcharoen, J., D. van Riel, G. van Amerongen, T. Bestebroer, W. E. Beyer, R. van Lavieren, A. D. M. E. Osterhaus, R. A. M. Fouchier, and T. Kuiken. 2008. Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5NI). Emerging Infectious Diseases **14**:600-607.

Kida, H., R. Yanagawa, and Y. Matsuoka. 1980. Duck Influenza Lacking Evidence of Disease Signs and Immune-Response. Infection and Immunity **30**:547-553.

Klasing, K. C., B. D. Humphrey, A. J. Mireles, and E. A. Koutsos. 2004. What are the costs of immunity? Poultry Science **83**:444-444.

Krauss, S., D. Walker, S. P. Pryor, L. Niles, C. H. Li, V. S. Hinshaw, and R. G. Webster. 2004. Influenza A viruses of migrating wild aquatic birds in North America. Vector-Borne and Zoonotic Diseases **4**:177-189.

Latorre-Margalef, N., G. Gunnarsson, V. J. Munster, R. A. M. Fouchier, A. D. M. E. Osterhaus, J. Elmberg, B. Olsen, A. Wallensten, T. Fransson, L. Brudin, and J. Waldenstrom. 2009a. Does influenza A affect body condition of wild mallard ducks, or vice versa? A reply to Flint and Franson. Proceedings of the Royal Society B-Biological Sciences **276**:2347-2349.

Latorre-Margalef, N., G. Gunnarsson, V. J. Munster, R. A. M. Fouchier, A. D. M. E. Osterhaus, J. Elmberg, B. Olsen, A. Wallensten, P. D. Haemig, T. Fransson, L. Brudin, and J. Waldenstrom. 2009b. Effects of influenza A virus infection on migrating mallard ducks. Proceedings of the Royal Society B-Biological Sciences **276**:1029-1036.

Lee, C. W., and D. L. Suarez. 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. Journal of Virological Methods **119**:151-158.

Li, W., N. Maeda, and M. A. Beck. 2006. Vitamin C deficiency increases the lung pathology of influenza virus-infected gulo-/- mice. J Nutr **136**:2611-2616.

Louria, D. B. 2007. Undernutrition can affect the invading microorganism. Clin Infect Dis **45**:470-474.

Mauser, D. M., and J. G. Mensik. 1992. A Portable Trap for Ducks. Wildlife Society Bulletin **20**:299-302.

Moon, J. A., D. A. Haukos, and L. M. Smith. 2007. Declining body condition of northern pintails wintering in the Playa Lakes Region. Journal of Wildlife Management **71**:218-221.

Munster, V. J., C. Baas, P. Lexmond, J. Waldenstrom, A. Wallensten, T. Fransson, G. F. Rimmelzwaan, W. E. P. Beyer, M. Schutten, B. Olsen, A. D. M. E. Osterhaus, and R. A. M. Fouchier. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. Plos Pathogens **3**:630-638.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier. 2006. Global patterns of influenza a virus in wild birds. Science **312**:384-388.

Owen, J. C., and F. R. Moore. 2008. Relationship between energetic condition and indicators of immune function in thrushes during spring migration. Canadian Journal of Zoology-Revue Canadienne De Zoologie **86**:638-647.

Pawlina, I. M., D. A. Boag, and F. E. Robinson. 1993. Population-Structure and Changes in Body-Mass and Composition of Mallards (Anas-Platyrhynchos) Wintering in Edmonton, Alberta. Canadian Journal of Zoology-Revue Canadienne De Zoologie **71**:2275-2281.

Perdue, M. L., and D. E. Swayne. 2005. Public health risk from avian influenza viruses. Avian Diseases **49**:317-327.

Pollett, M., J. S. Mackenzie, and K. J. Turner. 1979. The effect of protein-deprivation on the susceptibility to influenza virus infection: a murine model system. Aust J Exp Biol Med Sci **57**:151-160.

Ritz, B. W., I. Aktan, S. Nogusa, and E. M. Gardner. 2008. Energy restriction impairs natural killer cell function and increases the severity of influenza infection in young adult male C57BL/6 mice. J Nutr **138**:2269-2275.

Robb, J. R., G. M. Tori, and R. W. Kroll. 2001. Condition indices of live-trapped American black ducks and mallards. Journal of Wildlife Management **65**:755-764.

Scrimshaw, N. S., C. E. Taylor, and J. E. Gordon. 1968. Interactions of nutrition and infection. Monogr Ser World Health Organ **57**:3-329.

Sharp, G. B., Y. Kawaoka, D. J. Jones, W. J. Bean, S. P. Pryor, V. Hinshaw, and R. G. Webster. 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. Journal of Virology **71**:6128-6135.

Sherman, P., J. Forstner, N. Roomi, I. Khatri, and G. Forstner. 1985. Mucin depletion in the intestine of malnourished rats. Am J Physiol **248**:G418-423.

Smirnov, A., D. Sklan, and Z. Uni. 2004. Mucin dynamics in the chick small intestine are altered by starvation. J Nutr **134**:736-742.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology **40**:3256-3260.

Sprunt, D. H. 1942. The Effect of Undernourishment on the Susceptibility of the Rabbit to Infection with Vaccinia. J Exp Med **75**:297-304.

Stephensen, C. B., S. R. Blount, T. R. Schoeb, and J. Y. Park. 1993. Vitamin A deficiency impairs some aspects of the host response to influenza A virus infection in BALB/c mice. J Nutr **123**:823-833.

Stephenson, I., A. Heath, D. Major, R. W. Newman, K. Hoschler, W. Junzi, J. M. Katz, J. P. Weir, M. C. Zambon, and J. M. Wood. 2009. Reproducibility of Serologic Assays for Influenza Virus A (H5N1). Emerging Infectious Diseases **15**:1250-1259.

Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. P. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. M. Peiris, and R. G. Webster. 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of Virology **79**:11269-11279.

Swayne, D., D. Senne, and C. Beard. 1998. *Avian influenza* **4,** 150-155.

Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama. 1975. Plaque Assay and Primary Isolation of Influenza a Viruses in an Established Line of Canine Kidney-Cells (Mdck) in Presence of Trypsin. Medical Microbiology and Immunology **162**:9-14.

van Gils, J. A., V. J. Munster, R. Radersma, D. Liefhebber, R. A. M. Fouchier, and M. Klaassen. 2007. Hampered Foraging and Migratory Performance in Swans Infected with Low-Pathogenic Avian Influenza A Virus. Plos One **2**:-.

Wallensten, A., V. J. Munster, N. Latorre-Margalef, M. Brytting, J. Elmberg, R. A. M. Fouchier, T. Fransson, P. D. Haemig, M. Karlsson, A. Lundkvist, A. D. M. E. Osterhaus, M. Stervander, J. Waldenstrom, and B. Olsen. 2007. Surveillance of influenza A virus in migratory waterfowl in northern Europe. Emerging Infectious Diseases **13**:404-411.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and Ecology of Influenza-a Viruses. Microbiological Reviews **56**:152-179.

Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti. 1978. Intestinal Influenza - Replication and Characterization of Influenza-Viruses in Ducks. Virology **84**:268-278.

Woolcock, P. R. 2008. Avian influenza virus isolation and propagation in chicken eggs. Methods Mol Biol **436**:35-46.

Wu, R., S. Hu, Y. Xiao, Z. Li, D. Shi, and D. Bi. 2007. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Veterinary Research Communications **31**:631-641.