Leukocyte Profiles of House Wrens (*Troglodytes aedon*) in Northwestern Ohio: The Relationship Between Parental Health and Reproductive Success

Research Thesis

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Abstract

Evolution occurs when species adapt to changes in their environment. Pathogen prevalence in the environment may affect the health of an individual, and in turn, affect that individual's reproductive success. This study uses leukocyte profiles to quantify immune function in House Wrens (*Troglodytes aedon*), and to determine whether this aspect of the immune system affects individual mass, laying date, clutch size, and offspring size. Blood was drawn from 95 adult House Wrens and physical morphology was recorded. The nestlings' physical morphology was measured at ten days after hatching and averaged per brood. A principal components analysis was used to summarize four differentiated types of white blood cells with two principal component scores. The PC1 score indicated more heterophils and fewer lymphocytes, but was not related to any measures of reproductive success. A high female PC2 score, indicating more eosinophils and basophils, correlated with a smaller nestling tarsus. Eosinophilia and basophilia often result from an allergic immune response or parasitism. For females, clutch size decreased with a higher white blood cell (WBC) count. Male hematology did not relate to clutch size or nestling size. Neither PC1 nor PC2 correlated with laying date. These findings suggest that immune function positively impacts the number and size of offspring. This is the first study to describe the leukocyte profiles of wild House Wrens. Because it may be that only healthy birds can reproduce, future work should examine the hematology of non-breeding House Wrens to see if abnormal blood counts can account for their lack of reproduction.

Introduction

Natural selection favors individuals that can successfully survive and reproduce despite pathogen prevalence, predation and parasite pressure. Although much research has focused on predator-prey relationships historically, there has been increasing interest in how an individual's health and immunity affects its ability to reproduce successfully. Good health of the individual can be described as being free from disease and parasites, having an adequate nutritional intake, a normally functioning immune system, and a safe environment for living and breeding. The correlation between immune function and reproductive success can help us to better understand how natural selection may function to enhance survival for both parents and offspring.

The overall health of adult birds plays a significant role in the ability to reproduce successfully. A stronger immune system positively correlates with health and survival which in turn, promotes reproductive success due to the yearly survival of adults (Horuk et al. 1999; Råberg and Stjernman 2003; Møller and Saino 2004). For example, Råberg and Stjernman (2003) found that higher antibody titers in Blue Tits (*Parus caeruleus*) positively correlated with survival in the following years. Immunity is essential for protection against naturally-occurring pathogens and parasites in the environment (Zuk and Stoehr 2002).

Leukocyte profiles and peripheral slide examination are useful diagnostic laboratory tests that can aid in determining health status. It includes a breakdown of the cellular components within the blood (white blood cells or WBCs, red blood cells, and platelets). Peripheral slide examinations aid in the detection and presence of parasitemia as well as allow

for quantifying and differentiating the types of WBCs in the immune system. The number and types of WBCs are useful in determining the health and/or immunological stress of the individual (Krams et al. 2013). Different types of WBCs can be activated as part of the immune response to different health threats (Krams et al. 2013). Heterophils are a type of WBC that is part of the innate immune system in birds. They respond as a non-specific, first line-of-defense against foreign antigens (Davis et al. 2004). Lymphocyte WBCs belong to the acquired part of the immune system and are specialized leukocytes that recognize foreign antigens and form highly specific antibodies that later protect against the specific antigen that caused the initial immune response (Roitt et al. 1993; Davis et al. 2008; Campbell and Ellis 2013). They also aid in the removal of viruses. Eosinophils and basophils are primarily involved with allergic responses but also play a role in parasitic infections (Roitt et al. 1993; Campbell and Ellis 2013; Beutler 2004). The heterophil/lymphocyte ratio, or H/L ratio, refers to the number (and distribution) of heterophils per lymphocytes in the WBC differential. The two inversely correlate with each other, meaning when one goes up the other goes down and vice versa. The H/L ratio can be used in the diagnosis of certain diseases as well as stress due to their redistribution upon their activation in the immune system (Briscoe et al. 2010; Davis et al. 2008; Maxwell 1993; Müller et al. 2011). Heterophils and lymphocytes are the two most numerous WBCs within the immune system and are inversely correlated to one another (Roitt et al. 1993; Müller et al. 2011). The total protein measures the amount of albumin and globulins in the blood. High levels are commonly associated with infection and inflammation while low levels can indicate anemia, malabsorption, and abnormal kidney function (Roitt et al. 1993; Woerpel and Rosskopf 1984). The packed cell volume (PCV thereafter), is also known as blood hematocrit. It is a

measurement of the percentage (and size) of red blood cells compared to the total fluid portion of the blood in the circulatory system. There is a lot of variation among PCV values due to sex, age, reproductive status, elevation, migratory status, and habitat so it must be used in conjunction with other laboratory values to determine health status (Fair et al. 2007). A low PCV can indicate anemia (low blood volume) and/or non-specific inflammation, and a high PCV can indicate a high total blood volume, dehydration and also non-specific inflammation (Fair et al. 2007; Velguth et al. 2010). Variations of the PCV can also be related to changes in hormone levels as well as mating behaviors (Fair et al. 2007). By examining multiple hematological findings simultaneously, we can get a more complete picture of health of the individual (Davis et al. 2008; Maxwell 1993; Müller et al. 2011).

The House Wren (*Troglodytes aedon*) is a small songbird that is found all over the Americas. It prefers to live in open woodlands, city parks, and residential areas (Johnson 1998). They readily occupy artificial nest boxes, allowing for easy access to nests and large samples sizes. The wrens are small (11-13 cm long, 10-12 g) and their markings mainly consist of a uniform brown color (Johnson 1998). What the House Wren lacks in color, it makes up for in song. The small birds sing a vast repertoire of melodies (Johnson 1998). Despite its abundance across its range, little research has examined its blood parasite prevalence nor its cellular blood components in *vivo*.

The objective of this study is to utilize peripheral blood smears and hematological findings to determine whether individual health affects individual size, nestling size and number, and reproductive success. In total, 120 nest boxes were monitored to obtain laying

date and clutch size. Offspring mass and size were measured when the nestlings were 10 days old. Adults were captured and weighed during routine nestling feedings. We obtained blood samples from the adult House Wrens by performing a venipuncture on the brachial wing vein. Hematological tests of the blood samples were used to quantify the leukocyte profiles and PCV, as well as determine presence of blood parasites. This study established the leukocyte profiles for a common bird species that has been previously unknown. It was our prediction that the bird's health, as indicated by leukocyte profiles, positively correlated with the adult wren's mass, laying date (earlier laying), and the mass of their nestlings.

Methods

Field Methods:

The study was conducted from April to August 2014 near Lima, Ohio, USA. We monitored forty nest boxes at each of three locations: a wooded habitat in the northwest corner of the OSU- Lima campus, 40.7363927 ° N, -84.0266254 ° W; a park habitat, 40.736104 ° N, -84.029907 ° W; and a golf course, 40.75551 ° N, -84026012 ° W. Nest boxes were checked every three to four days until the nest cup appeared. Then, we checked the boxes daily to determine the exact laying date and clutch size. Following clutch completion, nests continued to be checked every three to four days until 12 days after clutch completion (Johnson 1998). After that time, they were checked daily to determine exact hatch date (≥50% of eggs hatch). At day 10 after hatching, the nestlings were weighed (±0.1g) and measured (tarsus ±0.1mm).

Hematological Methods:

When the nestlings were between 5 and 10 days old, the adult House Wrens were caught using a sliding door to trap them inside the nest box when they entered to feed the nestlings. This time period was selected because they are unlikely to abandon the nest during the nestling feeding stage. We recorded the mass, tarsus, and wing length of the birds and performed a venipuncture using a standard lancet and heparinized hematocrit tubes. Approximately 50 μl of blood was drawn out of the basilic (wing vein) for analyses (Owen 2011). We made two peripheral blood smears for the WBC count and WBC differential and used the remaining blood for the PCV and total protein.

Each peripheral smear was examined for the presence of blood parasites. All smears were stained with Romanowsky stain (Marshall et al. 1975) and an estimated WBC count was performed by counting 10 fields under high power and taking the average number per field, +/- 10%, and multiplying by 200 to get the estimated WBC count. A standard 100 WBC differential (determining the type of WBC for 100 WBCs total) was calculated for each smear. The cell counts and parasite examination were then verified by a second skilled avian hematologist for verification. The PCV was measured using a standard hematocrit centrifuge. They were spun for 5 minutes at 2500 rpm. The total protein was measured by a refractometer. All hematological analyses were conducted by Avian and Exotic Animal Clinical Pathology Lab in Wilmington, Ohio.

Statistical Analysis:

A principal component analysis was used to obtain two independent measures of the variation in blood characteristics. PC scores were compared to the adult's mass, clutch size, laying date, and average nestling mass and tarsus. All statistical analyses were conducted in JMP (version 9.0.0, SAS Institute, Cary, NC). Sample sizes vary because it was not possible to measure every characteristic for every bird. Means are presented with their standard errors.

Results:

Of the 120 nest boxes that were monitored, blood was collected from 95 adult House Wrens. Only PCV% varied between males and females (Males: 68.1 ± 2.4, Females: 74.7 ± 1.7, $F = 5.14$, $N = 40$, $P = 0.03$). All other hematological measurements did not differ between the sexes ($F<2.09$, $P>0.15$), and showed little variability (Table 1). The principal components analysis combined heterophils with lymphocytes for PC1 and used eosinophils and basophils for PC2 (Table 2).

The average adult mass was 10.6 ± 0.1g ($N = 94$) and the average adult tarsus was 17.4 ± 0.1mm ($N = 95$). Mass did not differ by sex ($t = -1.48$, $N = 94$, $P = 0.14$), but tarsus was longer for males than females (males: 17.61 ± 0.08mm; females: 17.23 ± 0.06mm; $t = 3.90$, $N = 95$, $P = 0.0002$). Adult mass and tarsus length was not affected by hematological findings (Table 3).

The average laying date was 2 June ± 2.1 days (median = 25 May; mode = 20 May). The average clutch size was 6.3 ± 0.1 eggs and decreased with laying date (Figure 1, Table 3). Laying date was not affected by hematological findings of either males or females (Table 3). After

controlling for laying date, females with higher WBC counts had smaller clutches (Figure 2, Table 3). Clutch size did not vary with hematological variables in males (Table 3).

Average nestling mass was 9.5 ± 0.1g and average nestling tarsus length was 16.7 ± 0.1mm. Nestling mass was not affected by hematological findings of either males or females (Table 3). Females with high PC2 values, indicating higher basophils and eosinophils and fewer heterophils, had nestlings with shorter tarsi (Figure 3, Table 3). Male hematology did not affect nestling size (Table 3).

There were two male birds with significantly higher WBC counts. The average adult WBC count was 8.3 ± 0.31 (10-3/ μl). One male had a WBC count of 21 with a differential of 58 heterophils, 41 lymphocytes, and one basophil (indicating a high H/L ratio). Its mass was 10.1g and tarsus was 17.5mm. This indicates that he was an overall healthy bird with an average mass and tarsus but was suffering from an unspecified bacterial infection and/or stress. The other adult male had a WBC count of 22 and differential of 16 heterophils, 77 lymphocytes, 4 basophils and 3 eosinophils (indicating a low H/L ratio). His mass was 10.6g and tarsus was 18.0mm. This indicates that he too, was an otherwise healthy bird but was suffering from an unspecified viral infection or secondary immune response (indicated by the low H/L ratio). Both males actively fed nestlings signifying that their immune function was not inhibiting their parental behavior.

One female bird was infected with microfilaria (an early stage of a parasitic nematode which was not further classified). Her WBC count was 6.0 and differential values were: 39 heterophils, 46 lymphocytes, 3 basophils, and 2 eosinophils. Her mass was 10.8g and tarsus was

16.7mm. Her WBC count was slightly lower than the standard and her eosinophils and basophils were within normal range. The PCV value was 72%. Microfilaria are an early, post-egg stage of the parasitic life cycle and can potentially cause anemia due to the destruction of red blood cells.

Discussion:

Our hypothesis was supported in that a positive correlation was found between immune function and clutch size as well as size of offspring, but only in females. The males that were found to have a high WBC count were actively participating in normal nestling feeding and were seemingly unaffected by their immunological condition. This indicates that their immune system was working appropriately and their WBC count and differential would most likely return to normal levels following the infection or removal of the stressor. The remaining birds seemed to have adequate immune function for reproductive success.

This cellular analysis of adult House Wrens quantifies leukocyte profiles for the species in this region. Results indicate that adult House Wrens have a low H/L ratio, indicating more lymphocytes than heterophils during active breeding. The H/L ratio is ever changing and fluctuates based on habitat, stress, nutritional intake, time of day, fat stores, parasite load, and hatch date, with a high H/L ratio correlating with the worse conditions (Müller et al. 2011). Other studies have noticed the H/L fluctuation among the different developmental stages nestlings and adults. Jakubas et al. (2015) noted numerous H/L ratio differences among Little Auks (*Alle alle*), across life stages from nestlings to fledglings to adults. Similarly, Parejo and

Silva (2009) found that H/L ratio differed among age and sex of Eurasian Kestrels, noting that the proportion of each could signify the different branches of nestling's developing immune system. They further found that breeding adults are more susceptible to stress which in turn, raises their H/L ratio (Parejo and Silva 2009, Palacios et al. 2009). Elevated H/L ratios are also seen in clinically abnormal birds in which the immune response is triggered by a stressor so it must be used in conjunction with other clinical data to determine overall fitness of the individual (Davis et al. 2004). Further work should seek to determine if House Wren's H/L ratio fluctuates with age, in response to stress, during breeding vs. non-breeding periods, during migration vs. breeding or wintering residence as well.

Both ectoparasites (outside the body) and endoparasites (inside the body) are common in birds. Many endoparasites are able to be detected in the blood stream of infected hosts using microscopic methods for visual analysis (as we used) and by PCR based detection methods. Both methods are equally accurate in detecting parasites (Valkiūnas et al. 2008). In many studies, parasite load is compared to hematological values aimed at distinguishing health status of the infected individuals. Parasitism can potentially cause both acute and chronic anemia in their infected hosts (Harrison and Harrison 1986). The use of PCV values aid in diagnosing anemias so many researchers utilize this method in conjunction with other clinical tests to determine whether or not anemia is present. Results are conflicted in the avian community however, because other studies have found that birds are frequently unaffected by parasitic infections and are able to maintain normal PCV values when parasites are present (Dawson and Bortolotti 1997; Ots et al. 1998; Ots and Hõrak 1998). The same phenomenon has been found in ectoparasite infections involving nestling House Wrens (Johnson and Albrecht

1993). This study also reports an individual infected with microfilaria and having a normal WBC count and leukocytic profile.

One drawback to our study is that many samples clotted upon collection. Of the 95 House Wrens drawn for analyses, PCV values were only reported in 40 specimens due to the presence of blood clots within the tube. There was also a high incidence of hemolysis (the destruction of red blood cells in *vitro*) which could in turn, falsely elevate PCV values. Normal PCV values in caged birds are 35-55% (Campbell and Ellis 2013). PCV ranges among wild birds have been shown to vary significantly among avian species (Fair et al. 2007; Balasch et al. 1976; Lavin et al. 1992; Smith and Bush 1978; Cooper 1975; Gee 1981; Rehder et al. 1982). PCV values also vary naturally depending on sex, age, reproductive status, sexual selection, hormone changes, and habitat (Fair et al. 2007). Our elevated PCV value of 71% could reflect hemolysis that occurred during the specimen collection. We cannot conclude however, that adult House Wrens do not naturally have a high PCV value so further research is needed to determine an adequate PCV range for the species. Dawson and Bortolotti (1997) also suggest that certain parasitic infections can falsely elevate PCV values in some species. For this reason, it is suggested that PCV values not be used as an indicator of health alone but as an aid in determining health in conjunction with other clinical and laboratory data.

In establishing reference ranges for adult House Wrens in this region, this study aids research in providing leukocyte profiles for future comparisons among other age groups, breeding vs non-breeding, and migrating House Wrens in other geographical regions. Future research should examine the hematological findings as it relates to survival in conjunction with

breeding status. If wrens have a lower WBC count, it could potentially correlate with the notion that their immune system is too weak for active reproduction and may interfere with the adult's long term survival rate. It could also establish valuable insight as to when wrens may not breed.

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Table 1. Hematological means and standard errors obtained from blood samples and smears from House Wrens near Lima, OH.

	Mean ± SE	N
WBC 10-3/µl	8.3 ± 0.31	95
PCV%	71.6 ± 1.54	40
Total Protein	8.79 ± 0.32	36
Heterophils	33.0 ± 1.03	95
Lymphocytes	54.6 ± 1.31	95
Eosinophils	5.83 ± 0.72	95
Basophils	6.79 ± 0.48	95

Table 2. Results of a principal component analysis that included the relative abundances of the four types of white blood cells obtained from House Wrens near Lima, OH.

Blood Characteristic	PC1	PC2
Heterophils	0.58	-0.54
Lymphocytes	-0.72	0.01
Eosinophils	0.27	0.54
Basophils	0.28	0.64
% Explained	46.7%	30.0%
Eigenvalues	1.87	1.20

Table 3. ANOVA results comparing how morphology and reproductive success vary with hematological analyses and differentiated by sex of the adult House Wren. Significant correlations highlighted in bold.

Response Variable	WBC	PC1	PC2	Laying Date	Overall Model
Females					
Mass	F=0.83, P=0.37	F=0.31, P=0.58	F=0.02, P=0.88		$F_{3,50}$=0.38, P=0.77
Tarsus	F=0.08, P=0.78	F=2.17, P=0.15	F=1.02, P=0.32		$F_{3,51}$ =1.15, P=0.34
Laying Date	F=0.22, P=0.64	F=2.55, P=0.12	F=0.003, P=0.95		$F_{3,51}$=0.88, P=0.46
Clutch Size	**F=9.80, P=0.003**	F=0.77, P=0.38	F=0.21, P=0.64	**F=9.17, P=0.004**	$F_{4,49}$ =5.69, P=0.008
Nestling Mass	F=2.71, P=0.11	F=0.07, P=0.79	F=0.63, P=0.43		$F_{3,46}$ =1.26, P=0.30
Nestling Tarsus	F=1.41, P=0.24	F=1.77, P=0.19	**F=4.12, P=0.05**		$F_{3,46}$ =2.51, P=0.07
Males					
Mass	F=0.39, P=0.54	F=0.02, P=0.89	F=2.53, P=0.12		$F_{3,36}$ =0.90, P=0.45
Tarsus	F=0.25, P=0.62	F=2.92, P=0.10	F=0.18, P=0.67		$F_{3,36}$ =1.02, P=0.39
Laying Date	F=2.32, P=0.14	F=1.39, P=0.25	F=3.52, P=0.07		$F_{3,36}$ =1.86, P=0.15
Clutch Size	F=0.02, P=0.88	F=0.10, P=0.76	F=0.40, P=0.53	**F=19.89, P=<0.0001**	$F_{4,34}$ =5.22, P=0.002
Nestling Mass	F=0.01, P=0.91	F=1.78, P=0.19	F=1.94, P=0.17		$F_{3,31}$ =1.003, P=0.40
Nestling Tarsus	F=0.02, P=0.90	F=0.33, P=0.57	F=0.23, P=0.64		$F_{3,31}$ =0.26, P=0.85

Figure 1. Clutch Size declines with later laying in House Wrens near Lima, OH.

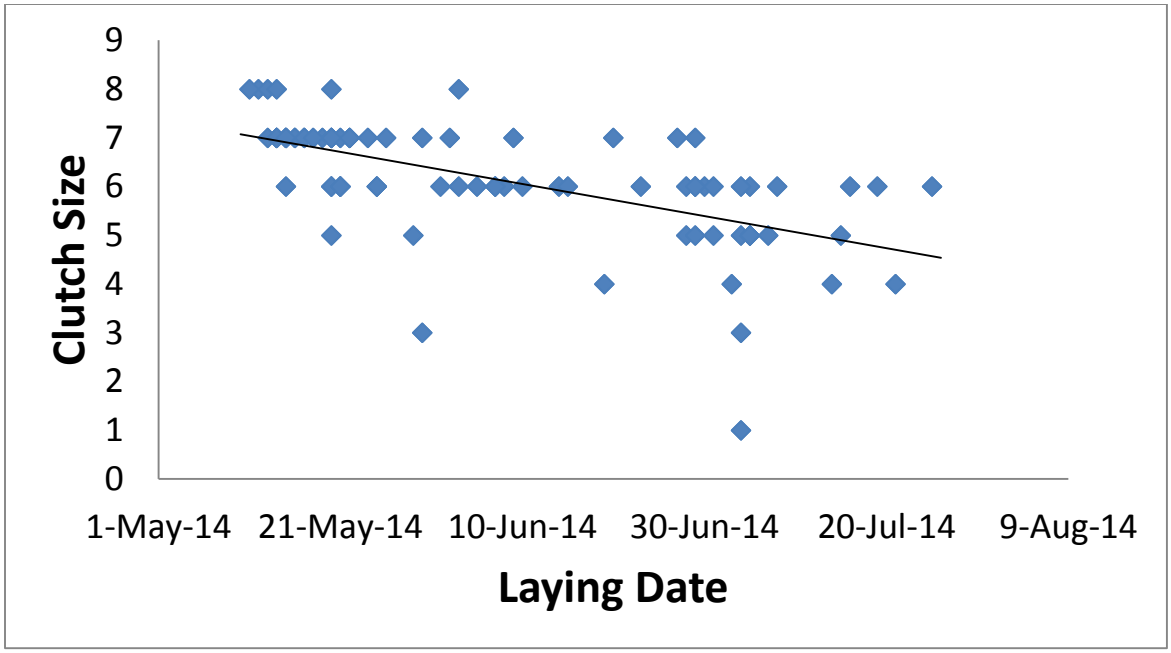

Figure 2. After controlling for laying date, female House Wrens with higher WBC counts had smaller clutches near Lima, OH.

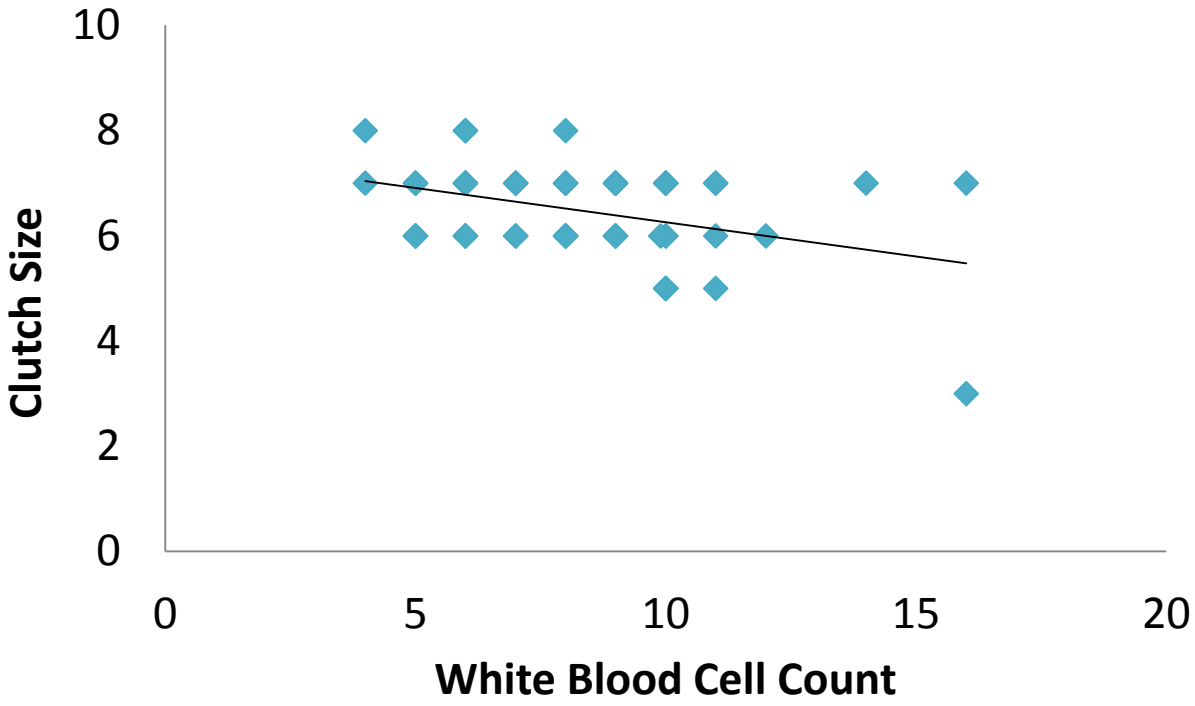

Figure 3. Female House Wrens with higher basophils and eosinophils and fewer heterophils had smaller nestlings near Lima, OH.