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PATHOGENICITY OF *HAEMOPROTEUS DANILEWSKYI*, KRUSE, 1890, IN BLUE JAYS (*CYANOCITTA CRISTATA*)

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ABSTRACT: Although the impact of blood parasite infections on passerine birds is potentially great, little is known of their pathologic effects. We studied *Haemoproteus danilewskyi* in experimentally infected captive and naturally infected free-ranging blue jays (*Cyanocitta cristata*) to determine patterns of infection and examine the pathologic effects of the parasite on the host. Physiologic changes, such as elevated numbers of lymphocytes, heterophils, basophils, eosinophils, and monocytes and decreased packed cell volume in the peripheral blood were associated with the erythrocytic phase of experimental infections of captive juvenile jays. Sublethal pathologic changes associated with the pre-erythrocytic phase of infections were observed in the liver, lung, and spleen. Schizonts were observed in the pulmonary capillaries of a 1 yr old jay necropsied 31 days post-inoculation, but not in 20 juvenile jays necropsied 57 days post-inoculation. In free-ranging naturally infected jays plasma protein concentration increased with density of natural infections.

Key words: Blue jay, *Cyanocitta cristata*, *Haemoproteus*, hematozoa.

INTRODUCTION

The impact of blood parasites on the fitness of their avian hosts has been the focus of numerous studies since Hamilton and Zuk (1982) first suggested parasites influence plumage brightness and sexual selection. Over the past two decades, the cost of these infections on host fitness has been documented through both correlative (Allander, 1997) and experimental (Merino et al., 2000; Yorinks and Atkinson, 2000) studies that demonstrated infections can be costly to avian reproductive success. These studies were based on the assumption that hematozoa have a debilitating effect on their avian hosts and deter host energy from reproductive efforts and towards immune defenses.

Species of *Haemoproteus*, common arthropod-borne blood parasites, are frequently reported in surveys of wild birds. These infections were long considered to be nonpathogenic (Ashford, 1971; Bennett et al., 1988), although now they are known to negatively impact reproductive success of wild birds (Allander, 1997; Merino 2000). This misconception is in part because few species of *Haemoproteus* have been the focus of studies to assess their virulence (Atkinson, 1991), primarily because of the difficulties associated with experimentally infecting birds. Because schizogony does not occur in erythrocytes, infections cannot be experimentally transmitted by blood transfer and tissue transmission attempts rarely have been successful (O'Roke, 1930; Coatney, 1933; Lastra and Coatney, 1950; Bierer et al., 1959). Host specificity, limiting the number of feasible experimental models and few identified vectors also have restricted laboratory studies on the adverse effects of these parasites. In a landmark study, Atkinson et al. (1988) reported lesions associated with experimental infections of *Haemoproteus* in domestic turkey poults. They found acute hemorrhagic myositis, enlarged spleens, secondary bacterial and fungal infections in the intestines and lungs, reduced body weight, lower mean hemoglobin values, and lower plasma protein concentrations associated with infection. This work, like most studies (see Atkinson and Van Riper, 1991; Bennett et al., 1993) involved laboratory studies of a domestic avian species and, thus, do not elu-

cidate the impact of *Haemoproteus* spp. on wild birds.

Pathologic consequences of *Haemoproteus* infection have been reported in several species of wild birds (Atkinson and Forrester, 1987; Bosch et al., 1997; Ots and Horak, 1998). However, more studies are necessary to fully understand the mechanisms behind the evolutionary and ecological impact of *Haemoproteus* infections on natural bird populations. Here, we present results of a study to determine the pathologic effect of *H. danilewskyi* in experimentally infected captive and naturally infected free-ranging blue jays (*Cyanocitta cristata*).

MATERIALS AND METHODS

Because the greatest density (range=<1–150 infected cells/10,000) of natural *H. danilewskyi* infections occur in immunonaive hatching year (HY) birds (Garvin and Greiner, 2003a), we suspected that this age group would experience the greatest cost of infections and therefore make the most suitable model for experimental infections in the laboratory. We examined the adverse effects of initial infections of *Haemoproteus danilewskyi* in experimentally infected captive juvenile blue jays to better understand if infection contributes directly or indirectly to blue jay fitness. We combined these laboratory data with our observations of several blood parameters in relation to density of infection in naturally infected free-ranging blue jays.

Experimental infections

Twenty nestling blue jays, ages 10–17 days post hatching, were collected from nests and housed in a vector proof room maintained between 24 and 28 C and natural photoperiod at Archbold Biological Station, Lake Placid, Florida, USA. A New Jersey Light trap (J. W. Hock, Co., Gainesville, Florida) was operated in the room at all times to detect the presence of biting flies. Nest mates were placed together in an artificial nest made of shredded newspaper and banded with unique combinations of colored leg bands. Thin blood smears were prepared daily from blood collected from the brachial vein of each bird for 1 wk after capture, then to reduce stress, birds were sampled only a single additional time 2 mo after capture to confirm absence of natural infection. Methods follow those described in Garvin and Greiner (2003a). At fledging, siblings were housed together in 1×1×1 m cages made of 1.3 cm hardware cloth. Each bird was assigned to either a treatment or control group using a random design stratified by age. Fresh oak and hickory branches, pine cones, and acorns were placed in each cage weekly to provide fledglings with cover, perches, and enrichment in an effort to reduce the stress of crowding and artificial conditions. When aggression associated with dominance behavior began to develop at 3 wk of age (prior to inoculation), each bird was housed in a separate 1×1×0.5 m cage.

During daylight hours, nestlings and fledglings less than 1 mo of age were fed a mixture of water and nonmedicated turkey starter (Manna Pro Corp., St. Louis Missouri, USA) every 30 min. After 1 mo of age, birds were fed approximately once an hr. By 5 wk of age, fledglings were able to feed themselves on moist turkey starter and received food and water ad libitum. After 3 mo of age, water, dry nonmedicated turkey starter, and high protein, low salt dog food (Nestlé Purina PetCare, St. Louis, Missouri, USA) was provided ad libitum.

At approximately 3–4 mo post-hatching, each of the 10 birds in the treatment group received an intraperitoneal inoculation of five infected *Culicoides edeni* (Diptera: Ceratopogonidae) known to support sporogonic development of *H. danilewskyi* (Garvin and Greiner, 2003b). Flies were infected 10 days earlier by feeding upon an infected blue jay with an density of approximately 40 erythrocytes infected with mature gametocytes per 10,000 erythrocytes. Inoculations were prepared by grinding flies in a Tenbrock glass tissue grinder with 1 ml RPMI tissue culture medium (Sigma Chemical Co., St. Louis, Missouri) in ice. Each inoculum contained approximately 3,000–4,000 sporozoites as determined by a hemocytometer. Each of the 10 control birds was inoculated with 1 ml RPMI tissue culture medium and fly body parts from uninfected male specimens of *Culicoides edeni.*

We measured five physiologic parameters for comparison between experimental groups: weight gain, packed cell volume (PCV), hemoglobin, plasma protein concentration, and white blood cell (WBC) count. These parameters were compared with density of infection. Prior to inoculation, and once each week for eight consecutive weeks post-inoculation, we measured these parameters for each bird. All samples and measurements were collected in the morning immediately following feeding. To determine PCV, total protein, and hemoglobin, we collected blood from the brachial vein of each bird into two heparinized capillary tubes. One tube was spun in a microhematocrit centrifuge for 5 min to determine PCV. Plasma from that tube was removed with a Hamilton

syringe and analyzed with a refractometer (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for protein concentration. Blood from the second tube was analyzed for hemoglobin concentration with a hand held hemoglobinometer (Leica #1010D, Fisher Scientific). Body mass was measured with a spring scale (Pesola®, Forestry Suppliers, Inc., Jackson, Missississi, USA) to the nearest 0.1 g. For determination of leukocyte count and density of infection, two thin blood smears were immediately prepared from one of the tubes, fixed and later stained with Wright-Giemsa (Fisher Scientific). Numbers of lymphocytes, monocytes, heterophils, eosinophils, basophils, and infected erythrocytes were determined by viewing smears under 100× oil immersion until approximately 100,000 erythrocytes were examined for each bird, then estimating the number of leukocytes and infected erythrocytes per 10,000 blood cells. Numbers of erythrocytes were determined by estimating the number per field of view, then reading the appropriate number of fields.

After we collected all measurements at 8 wk post-inoculation, birds were euthanized with an overdose of isoflurane and examined at necropsy to search for pre-erythrocytic schizonts and associated tissue damage in the pectoral muscle, heart, gizzard, kidney, liver, spleen, and lung. Organs were fixed in 10% buffered formalin, dehydrated in graded alcohols, cleared in xylene, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy.

We used a repeated measures analysis of variance (ANOVA; SPSS Inc., Chicago, Illinois, USA) to test for differences in weight, PCV, hemoglobin, plasma protein, and leucocyte counts between experimentally infected and uninfected jays. Week 0 was used as a simple contrast for all mean values at all other weeks. When treatment*week interactions were significant at $P=0.15$, we proceeded to conduct independent sample t-tests to compare infected with control birds at each week post-inoculation. However, by chance alone, we expected one in 20 differences to be significant at a P value of 0.05. We controlled for this problem with a Bonferonni correction (Scheiner, 1993) which lowered the critical P value to 0.005.

We also examined tissue from one adult jay that had been experimentally infected as described above for determination of vector species (Garvin and Greiner, 2003b) and euthanized on day 31 post-inoculation. Histologic sections prepared from the pectoral muscle, heart, gizzard, kidney, liver, lung, and spleen of this bird were examined for pre-erythrocytic schizogony and associated tissue damage.

Natural infections

To determine the effect of naturally acquired infection on PCV and plasma protein and hemoglobin concentration, we captured free-ranging adult and juvenile jays in nylon mist nets at Archbold Biological Station, (Archbold; 27°10′N, 81°21′W) Highlands County, Florida (Garvin and Greiner, 2003a) in 1994–95. We then collected 0.66 ml blood via the jugular vein with a 1.0 cc syringe rinsed with 50 mM ethylenediaminetetraacetec acid and equipped with a 27 G 12 mm needle. We filled two heparinized microhematocrit tubes with blood to determine PCV and plasma protein and hemoglobin concentration as described above. The remaining 0.5 ml of blood was collected for ongoing studies on encephalitis virus, immunoglobulins, hormones, and DNA analysis of parentage. We used a Spearman's rank correlation coefficient to examine correlations between density of infection and PCV, hemoglobin, and plasma protein. A representative infected blood smear was deposited in the US National Parasite Collection (Beltsville, Maryland, USA; Accession number 091708).

RESULTS

Experimental infections

Infections were first detected in the peripheral circulation on day 14 post-inoculation in nine of the challenged birds. Infection in the remaining bird was detected 21 days post-inoculation. Density of infected birds dropped from week 2 post-inoculation ($\bar{x}=204.7$, 74–300 infected cells/10,000) to week 8 post-inoculation ($\bar{x}=3.9$, 0.5–13 infected cells/10,000, Fig. 1). Parasitemia dropped most rapidly between 2 and 4 wk post-inoculation. Numerous cells were infected with two and occasionally three gametocytes and all infections remained patent for at least 57 days. No infections were detected in the control birds during the patent period.

Tissue stages of *Haemoproteus* were not detected in the liver, spleen, kidney, gizzard, heart, or pectoral muscle of any of the infected juvenile birds at necropsy on day 58 post-inoculation. Schizonts were found in the pulmonary capillaries of one adult bird that was experimentally infected (Garvin and Greiner, 2003b, Fig. 2).

Several lesions were observed micro-

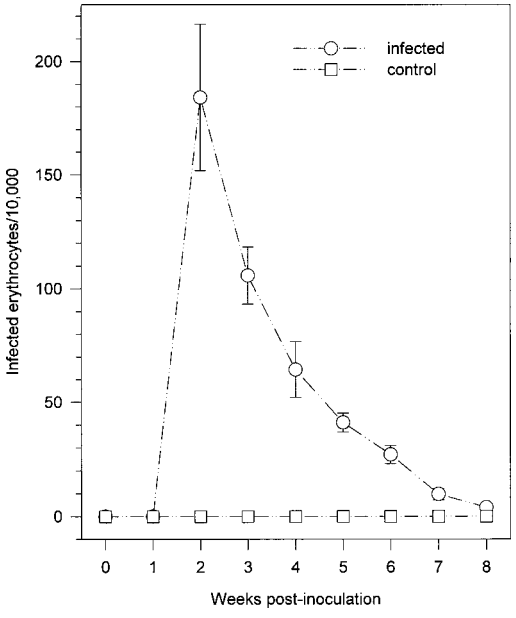

FIGURE 1. Density of infection of *Haemoproteus danilewskyi* in experimentally infected (n=10) and control (n=10) blue jays. Bars indicate standard error.

scopically in the liver, lung, and spleen of seven of the 10 infected juveniles, but not in control birds. Periportal and random individual cell necrosis was observed in liver sections of one of the infected birds. Hyperplasia of white pulp arteriolar endothelium, possibly subsequent to necrosis of lymphocytes, was found in spleen of three of the infected birds and pyknotic nuclei and random necrosis of lymphocytes were found in spleen of two other infected birds. Spleen of another infected bird had an increased number of macrophages, plasma cells, and Mott's cells, as well as scattered necrotic lymphocytes. In the lung of two infected birds, lymphocytic infiltrates and epithelial hyperplasia were observed around the tertiary bronchi. Sections of kidney, gizzard, heart, and pectoral muscle appeared normal in both control and infected birds. No gross lesions were observed in any of the infected or control birds. Furthermore, no deaths occurred and no behavioral abnormalities were observed among the 10 infected fledgling jays used in this experiment.

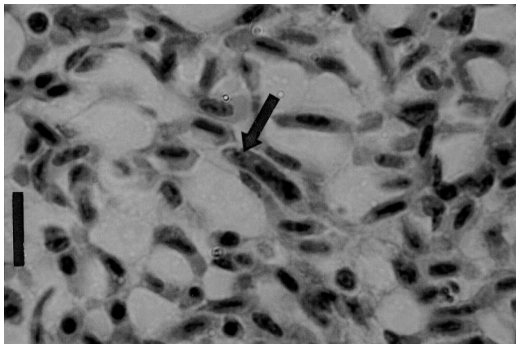

FIGURE 2. *Haemoproteus danilewskyi* schizont (arrow) in the lung of an experimentally infected blue jay necropsied at 31 days post-inoculation. Stained with hematoxylin and eosin. Bar=10μm.

A significant effect of infection on several blood parameters was detected during the 8 wk experiment. We found significant elevation in number of lymphocytes, heterophils, basophils, eosinophils, and monocytes in infected over control groups. Once established, the difference between infected and control groups were maintained for lymphocytes, heterophils, basophils, and monocytes. Despite considerable fluctuation in eosinophils between treatment and control, overall the treatment and control groups varied significantly. We found no significant overall difference in plasma protein concentration, hemoglobin concentration, PCV, or weight between infected and uninfected birds. However, because the interaction between treatment and week was considerable (P=0.110), we compared mean PCV at each week and found a significant difference between infected and control groups at week 5 post-inoculation (P=0.002).

Natural infections

Plasma protein, PCV, and hemoglobin were measured from free-ranging birds captured 1994–1995. Because these parameters did not vary with age of bird (Table 1), all ages were combined to test for correlations between each parameter and density of infection. We found a positive relationship between plasma protein concentration and density of infection (Fig. 5).

TABLE 1. Analysis of variance of age on plasma protein, packed cell volume (PCV), and hemoglobin for free-ranging blue-jays, 1994–95.

	Sum of squares	DF	Mean square	*F*	*P*
Plasma protein	61.16	1	61.10	2.25	0.136
PCV	2.99	1	2.99	0.94	0.334
Hemoglobin	0.18	1	0.18	0.29	0.590

However, neither PCV nor hemoglobin concentration was associated with density of infection.

DISCUSSION

We evaluated the impact of experimental *H. danilewskyi* infections on juvenile blue jays under controlled laboratory conditions and in free-ranging naturally infected juvenile and adult blue jays. We found increased leukocyte counts and decreased PCV in experimental infections in captive birds. In addition, increased plasma protein concentrations positively correlated with density of natural infections in free-ranging jays. Although plasma protein concentration in natural populations of birds may vary with season, we were unable to evaluate this due to sample size limitations. Admittedly, caution must be used when drawing conclusions about natural populations from laboratory studies. However, because, experimentally infected birds in this study reached peak densities similar to the highest density infections observed in free-ranging blue jays, we be-

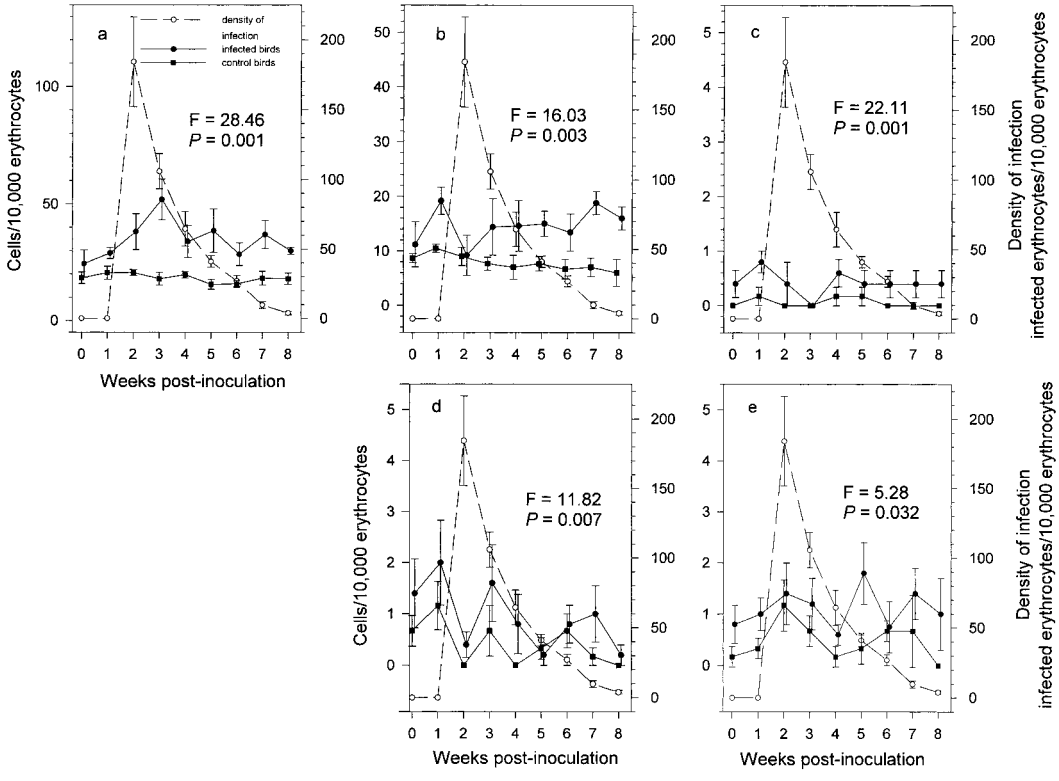

FIGURE 3. Mean number of a) lymphocytes, b) heterophils, c) basophils, d) eosinophils, and e) monocytes in experimentally infected (n=5) and control (n=6) blue jays in relation to parasitemia of *Haemoproteus danilewskyi* in infected jays. Bars indicate standard error.

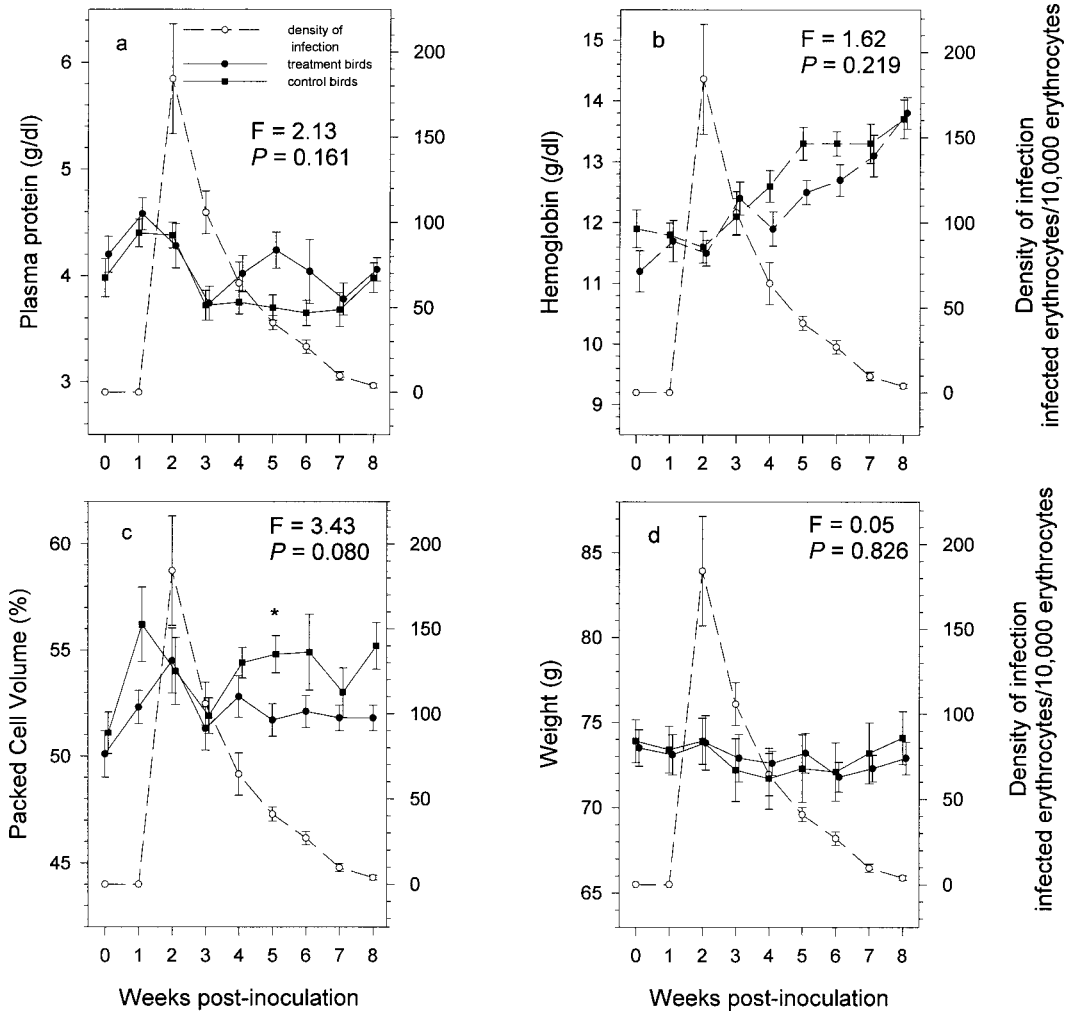

FIGURE 4. Mean a) plasma protein concentration b) hemoglobin concentration c) packed cell volume (PCV) and d) weight for infected ($n=10$) and control ($n=10$) blue jays in relation to mean density of *Haemoproteus danilewskyi* in infected jays. Bars indicate standard error. For PCV (Figure 4c) the P value of the interaction term between treatment and week was 0.110, therefore independent samples t-tests were used to search for differences between infected and control jays at each week. After Bonferonni correction, a $P<0.005$ was considered significant. An * indicates week when infected jays differed significantly from control jays.

lieve our findings are appropriate for extrapolations from the laboratory to nature and, therefore, valuable in assessing the ecological and evolutionary impact of *Haemoproteus* infections on wild birds.

Our observations of increased numbers of circulating lymphocytes in experimentally infected jays indicate allocation of resources towards a cell mediated immune response. These results are similar to Ots and Horak (1998) who found a similar response in natural infections of juvenile great tits, but different from work by Dufva and Allander (1995) who found no such response. The relationship between infection and number of other leukocytes in peripheral circulation is less clear. Observations of increased numbers of eosinophils, basophils, and heterophils in experimentally infected birds may reflect the inflammatory response to lesions observed in the liver, lung, and spleen of experimentally

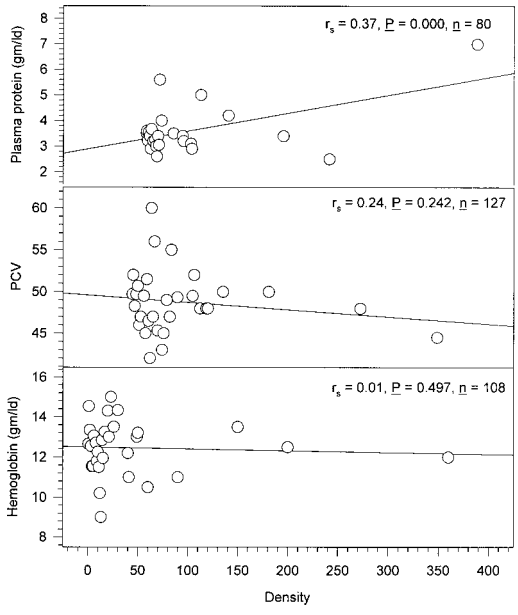

FIGURE 5. Mean packed cell volume (PCV), plasma protein concentration, and hemoglobin concentration in relation to density of *Haemoproteus danilewskyi* infection (infected erythrocytes/10,000 erythrocytes) in naturally infected free-ranging blue jays.

infected birds. Each of these white blood cell types when appropriately stimulated responds chemotactically, either directly or indirectly, to infectious agents. All are involved in the inflammatory process and tissue necrosis in a variety of ways and eosinophil numbers increase in response to parasitic infection (Slauson and Cooper, 1990). It should be noted, however that white blood cell numbers could be an artifact of the per red blood cell basis of quantification. Interestingly, the expected corresponding increase in plasma protein level was not observed in experimental infections, but did occur with increased density of natural infection in free-ranging jays, perhaps a result of increased synthesis of immunoglobulins or dehydration. The lack of a similar increase in experimental infections was possibly because of good body condition due to ad libitum food and absence of other stressful environmental factors, such as predation. Atkinson et al. (1988) reported increased plasma protein concentrations in domestic turkey poults experimentally infected with *Haemoprotoeus* and suggested either dehydration or increased synthesis of immunoglobulins as possible causes. Such immunoglobulin increases also have been reported in association with infections of *Plasmodium* and *Leucocytozoon* (Congdon et al., 1969; Morii, 1972).

Although no lesions were associated with exoerythrocytic schizonts observed in the pulmonary capillaries 31 days post-inoculation, lesions were observed 57 days post-inoculation in lung and spleen of experimentally infected jays that previously had high density infections. This may indicate that tissue damage occurs only after schizonts have matured and ruptured.

In the most thorough study of both blood and tissue phases of a *Haemoproteus* species in birds, Atkinson et al. (1988) reported the greatest pathogenic effects occur with the tissue phase. They found experimental infections in domestic turkey poults affected growth and weight gain and resulted in severe myositis. They also found hemorrhagic inflammatory infiltrates in association with schizonts in skeletal and cardiac muscle. Similar effects also were found in wild turkeys (Atkinson and Forrester, 1987). Pneumonia-like signs have been reported in association with the schizont stage of intense infections of *Haemoproteus columbae* in rock doves (*Columba livia*) by Garnham (1966). In naturally infected northern bobwhites with high parasitemia, Cardona et al. (2002) reported large megaloschizonts in skeletal muscle, especially in the thighs and back, which may have contributed to observed muscle dysfunction. Earle et al. (1993) also saw such signs in captive mourning doves and suggested that all species of *Haemoproteus* are probably capable of forming schizonts in a variety of tissue and that the number of different tissues containing schizonts depends on the density of infection.

Decreased PCV at week 5 post-inoculation also is notable and may reflect the removal of parasitized erythrocytes from

the circulation at a greater rate than the differentiation and release of erythrocytes into circulation from bone marrow (Lucas and Jamroz, 1961). However, this decrease might have been expected earlier given that the number of gametocytes in the peripheral circulation peaked shortly after they were first detected, between 14 and 21 days post-inoculation. Furthermore, a corresponding effect of infection on hemoglobin concentration was not observed, indicating that the removal of infected erythrocytes by the liver and spleen was balanced by the production of erythrocytes. This was suggested by Atkinson et al. (1988), who found no evidence of anemia associated with infection. Similar results are reported by Dufva (1996) and Ots and Horak (1998) in natural populations of breeding great tits. In contrast, O'Roke (1930) found severe anemia associated with high densities of erythrocytic stages of infection in naturally infected California quail. Similarly, Cardona et al. (2002) found severe anemia that resulted in lethargy and in some cases death in captive northern bobwhites. Manwell and Loeffler (1961) found that erythrocytes infected with *H. columbae* consumed 100 times more glucose than unparasitized cells, thus considerably lowering host glucose concentrations in pigeons. Other clinical signs reported in pigeons include weakness, anemia, and anorexia (Acton and Knowles, 1914; Coatney, 1933)

We were not able to determine if infected jays have lower survivorship than uninfected jays, however, repeatedly sampled free-ranging jays survived periods of elevated parasitemias. Although sublethal effects of *Haemoproteus* spp. may be manifested in ways too subtle to measure, species of two closely related genera, *Plasmodium* and *Leucocytozoon,* caused acute infections and mortality in nonimmune juvenile birds (Herman et al., 1975; Gabaldon and Ulloa, 1980). The sublethal pathologic conditions observed in this study are likely to be of greater consequence if experienced during periods of extreme environmental conditions, such as low food availability (Korpimaki et al., 1993), poor weather (Bennett et al., 1976) or stress associated with breeding.

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