

The Auk 117(4):1048–1051, 2000

Effects of Time of Sampling on Oocyst Detection and Effects of Age and Experimentally Elevated Testosterone on Prevalence of Coccidia in Male Dark-eyed Juncos

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The hormone testosterone (T) has been linked to enhanced expression of secondary sexual traits and acquisition of mates (Ligon et al. 1990, Alatalo et al. 1996, Raouf et al. 1997). However, high plasma levels of testosterone also have been suggested to suppress immune function (Folstad and Karter 1992, Zuk et al. 1995, Hillgarth and Wingfield 1997; but see Ros et al. 1997, Hasselquist et al. 1999). Thus, an obvious question is whether testosterone enhances reproductive success while simultaneously affecting susceptibility to disease.

In the Dark-eyed Junco (*Junco hyemalis*), experimental maintenance of plasma testosterone levels at their natural early season peak for the entire breeding season causes males to sing more frequently, and testosterone-treated males (T-males) in captivity are more attractive to females than are control males (C-males; Enstrom et al. 1997, Hill et al. 1999). However, T-males also have elevated plasma levels of the steroid hormone corticosterone (Ketterson et al. 1991, Klukowski et al. 1997) and show a greater response to handling stress than do C-males (Schoech et al. 1999), which can translate into suppressed immune function (Sapolsky 1992, Besedovsky and del Rey 1996, J. Casto pers. comm.). To investigate possible effects of elevated levels of plasma testosterone on the junco's susceptibility to disease, we asked whether free-living males treated with testosterone would be more likely than controls to be infected by coccidia (*Isospora* spp.), a protozoan gut parasite belonging to the *Eimeria* complex. We also asked whether yearling juncos were more likely than older adults to be infected and whether our ability to detect infection was affected by the time of day when fecal samples were collected.

Methods.—This research was conducted at the University of Virginia's Mountain Lake Biological Station (37°22'N, 80°32'W; see Chandler et al. [1994] for description of study area). We captured male juncos in mist nets and Potter traps during the early spring of 1995 and 1996 and classified them as second year (younger) and after second year (older) based on

plumage characteristics and iris coloration (Pyle et al. 1987, Mulvihill 1993). Males were randomly assigned a treatment and implanted with either control or testosterone-filled implants that consisted of two 10-mm lengths of silastic tubing placed subcutaneously above the thigh and under the wing (see Ketterson et al. 1992). T-implanted males maintained plasma testosterone levels that corresponded to the natural peak found in untreated males during territory establishment and pair formation (Chandler et al. 1997) and were higher than those of C-males (Ketterson and Nolan 1992).

Males captured in late summer (30 T-males, 23 C-males) were taken into the laboratory and placed in individual holding cages (22 × 28 × 30 cm) lined with fresh wax paper. Food and water were available *ad libitum*. In both years, samples produced between 1500 and 0500 (EST) were considered "night" samples, and those produced between 0500 and 1500 were considered "day" samples. Only night samples were used in analyses of the effects of testosterone and age on prevalence of infection, but samples collected at both times were used to determine whether time of day affects whether feces contained oocysts. In addition, we sampled feces produced by 14 individuals during both day and night in 1996. These males were brought into the laboratory after 1500 and held overnight for feces collection. Their cage linings were changed the following morning at 0500, and the birds were held until 1200.

Feces and residual food were separated by hand and the food discarded. Feces were placed in petri dishes containing approximately 7 mL of a 2.5% potassium dichromate solution for seven days, which is adequate time for oocyst sporulation to occur (D. W. Duszynski pers. comm.). After sporulation, samples were transferred from petri dishes to vials for storage. We followed the methods of Duszynski and Wilber (1997) for separating oocysts from fecal material and identifying them. Birds were classified as infected (positive) if we detected one or more oocysts when examining samples under a microscope. Samples were scanned only once if positive and twice if the first scan was negative; the latter samples were considered to be positive if one or more oocysts were detected.

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Coccidia typically are transmitted when a potential host ingests an infective propagule; the likely mode of transmission in juncos is via ground feeding. Although little is known about the coccidia that infect juncos, oocysts discharged by juncos belonged to the genus *Isospora* based on oocyst morphology (four sporozoites within each of two sporocysts). In addition, when we measured the length and width of the oocysts, the frequency distribution for each measurement was unimodal, which is consistent with infection by a single species.

Statistical analyses were performed using SYSTAT 5.0. We first performed pairwise *G*-tests to determine if detection of oocysts was dependent on the time that samples were collected. Because prevalence depended on when samples were collected (see Results), subsequent analyses were based on night samples only. We used a loglinear model to determine whether prevalence of coccidia was dependent on hormone treatment or age, as well as to determine if hormone treatment and age influenced prevalence independently.

Results.—Coccidial oocysts occurred more frequently in feces produced at night in both 1995 (night, 23 of 31; day, 3 of 15; $G = 12.57$, $df = 1$, $P < 0.001$) and 1996 (night, 19 of 22; day, 6 of 12; $G = 5.14$, $df = 1$, $P < 0.01$). For the 14 individuals sampled in both day and night, oocysts occurred more often in feces produced at night (day, 2 of 14; night, 11 of 14; $G = 12.64$, $df = 1$, $P < 0.001$). Therefore, subsequent analyses were based only upon samples produced at night.

Because the prevalence of coccidia did not differ significantly between years ($G = 1.20$, $df = 1$, $P = 0.27$), we combined data to assess the overall effect of hormone treatment, age, and the interaction of hormone and age on prevalence of coccidia in male juncos. Neither hormone treatment ($G = 0.00$, $df = 1$, $P = 0.97$) nor age ($G = 1.17$, $df = 1$, $P = 0.28$) influenced prevalence of coccidia (Fig. 1). In addition, the loglinear model revealed no interaction between hormone treatment and age ($G = 1.09$, $df = 1$, $P = 0.30$).

Discussion.—Laboratory experiments on House Sparrows (*Passer domesticus*) indicate that *Isospora* coccidia are shed on a circadian rhythm, with the highest numbers of oocysts being shed between 1600 and 0100 (Boughton 1988). Our field experiments on Dark-eyed Juncos strongly agree with these results. In random samples from both years, coccidial oocysts were more likely to be found in samples collected at night than during the day. Further, in 14 birds sampled during both day and night, we were more likely to detect oocysts in feces produced at night. Therefore, we recommend that future studies of coccidia focus on samples collected at night.

In juncos, treatment with testosterone results in an increase in plasma levels of testosterone (Ketterson and Nolan 1992) and corticosterone (Ketterson et al.

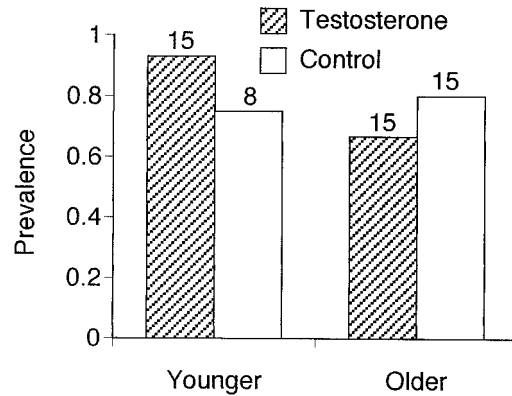


FIG. 1. Prevalence of coccidia by age in testosterone-treated and control male Dark-eyed Juncos at Mountain Lake Biological Station, Virginia. Data are prevalence estimates pooled between the 1995 and 1996 breeding seasons (sample sizes shown above bars).

1991, Klukowski et al. 1997). Although increased plasma testosterone and corticosterone are correlated with suppressed immune function in salmonid fish (Slater and Schreck 1993) and mammals (Hillgarth and Wingfield 1997), the effect of testosterone on avian immune function is not clear (Marsh 1992). Some studies suggest that testosterone is immunosuppressive in birds (Saino et al. 1995, Zuk et al. 1995), whereas others provide no support for such a relationship (Ros et al. 1997, Hasselquist et al. 1999). Treatment with T suppresses cell-mediated immunity and humoral immunity in juncos (J. Casto pers. comm.), but we found no evidence that experimentally elevated T produces a detectable effect on the prevalence of coccidia. Background prevalence of coccidia was high in our population, however, and our sample sizes were small. In addition, we had no measure of the intensity of infection. Therefore, we conclude that although we found no effect, another study of the effect of T on susceptibility to coccidia, especially one employing larger sample sizes and a measure of intensity of infection, might be warranted.

Laboratory studies of domestic chickens experimentally infected with coccidia (*Eimeria* spp.) revealed lower prevalence in adult birds, which was interpreted as indicating that older chickens had more time to build partial immunity to infection (Levine 1988). Similarly, younger male juncos were more likely to be infected than were older birds, but the effect of age was not significant (Fig. 1).

In summary, suppressed immune function has been suggested as a cost of elevated testosterone levels. Therefore, we tested whether male Dark-eyed Juncos with experimentally elevated testosterone would be more likely to harbor coccidia (*Isospora*

spp.), which are protozoan parasites that may cause the disease coccidiosis. The likelihood that an individual would shed coccidial oocysts depended on the time of day when feces were produced, supporting earlier laboratory studies by Boughton (1988). However, we found no evidence that treatment with testosterone or an individual's age affected the likelihood that it would harbor coccidia, and thus we found no evidence to support a tradeoff between reproduction and survival.

Acknowledgments.—We thank D. W. Duszynski for sharing his expertise during the early stages of this project and T. Kast, E. Kennedy, S. Lynn, M. Rosenshield, E. Snajdr, and C. Ziegenfus for assistance in the field. C. R. Chandler, D. W. Duszynski, J. L. Krstolic, S. L. Perkins, O. Pung, C. H. Slater, J. C. Wingfield, the GSU E&E discussion group, and four anonymous reviewers gave helpful comments on an earlier draft of this manuscript. We also thank our friends and colleagues at Mountain Lake Biological Station for stimulating conversations and constructive criticism and the Mountain Lake Hotel for allowing us to work on their property. This research was funded by NSF grants IBN-94 08061 and IBN 97-28384 to EDK and VN.

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Received 6 August 1999, accepted 26 April 2000.
Associate Editor: J. M. Wingfield

The Auk 117(4):1051–1056, 2000

Identification of the Extinct Hawaiian Eagle (*Haliaeetus*) by mtDNA Sequence Analysis

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Among the many bones of extinct birds discovered in the first extensive deposits studied in the Hawaiian Islands were a few from Molokai and Oahu that clearly belonged to a large sea-eagle of the genus *Haliaeetus*, as shown by their large size and the fusion of the phalanges of the inner toe (Olson 1982, Olson and James 1982). Even with the discovery of a nearly complete skeleton of this eagle on Maui, its specific identification could not be entirely resolved. Size and other characters eliminated most species of the genus, but no qualitative osteological characters could be discerned between the Hawaiian fossils and available skeletons of the Old World White-tailed Eagle (*H. albicilla*) and the New World Bald Eagle (*H. leucocephalus*; Olson and James 1991). Unable to make a morphological resolution, Olson and James (1991:62) were constrained to list this interesting specimen as "*Haliaeetus* sp., aff. *H. leucocephalus*/*H. albicilla*." To identify the Hawaiian eagle more satisfactorily, we

turned to "ancient" DNA methods to obtain mitochondrial DNA (mtDNA) sequences from its bones.

The bones used in ancient DNA analysis were obtained from a nearly complete skeleton found on 4 April 1988 in a good state of preservation in Puu Makua Cave, a lava tube at 1,463 m elevation on the south slope of Mt. Haleakala, Maui (Olson and James 1991). The eagle had died in a cave that served as a natural trap for flightless species such as moa-nalos, rails, and ibises. The only entrance to the cave is a skylight of approximately 5 m diameter above a vertical drop of approximately 22 m. The eagle may have flown into the cave to scavenge flightless birds that had fallen in beforehand. The eagle presumably became trapped because the entrance was too small for such a large bird to be able to fly back out.

Previous analyses showed that the skeleton contained 4.1% nitrogen and amino acids in proportions only slightly different from those of modern cow bone (T. Stafford, Jr. pers. comm.). An AMS radiocarbon date on the skeleton gave an age of 3,300 ± 60 years (two-sigma calibrated range 3,389 to 3,689 years; see James 1995). Because the nitrogen and amino acid content suggested that organic compounds

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were well preserved in the skeleton, and despite its antiquity, we chose this specimen for ancient DNA analysis.

We isolated DNA from two bones from the skeleton in two separate laboratories dedicated to ancient DNA analysis. DNA was amplified from the Hawaiian eagle bones for three different gene regions of mtDNA. We compared sequence from part of the cytochrome-*b* (*cyt b*) gene with *cyt b* sequences obtained from GenBank for seven of the eight species of *Haliaeetus* known worldwide (Seibold and Helbig 1996). We also obtained sequences of the ATP subunit 8 gene (ATPase8) and part of the 12S ribosomal RNA gene (12S rRNA) and compared them with sequences we obtained from individuals of *H. leucocephalus*, *H. albicilla*, and *H. leucogaster*.

Methods.—Two bones were selected for analysis from the nearly complete skeleton found in Puu Makua Cave (USNM 431238): a thoracic rib fragment and a pedal phalanx. We obtained two tissue samples of *H. albicilla* from the Swedish Museum of Natural History (nos. 501 and 502) and feathers of *H. albicilla* and *H. leucogaster* (White-bellied Fish-Eagle) from captive birds in the United States. DNA samples of *H. leucocephalus* were obtained from W. Piper.

All pre-PCR protocols were conducted in isolated ancient DNA laboratories located in buildings different from the ones containing the primary DNA laboratories. DNA isolations and PCRs for ATPase8 and 12S rRNA segments were conducted at the National Zoological Park (NZP) by A. C. Cooper, and DNA isolations and PCRs for *cyt b* and 12S rRNA were conducted at the University of Durham by R. Fleischer. Methods to reduce the probability of contamination followed Handt et al. (1994) and included the use of barrier pipet tips; sterile solutions; DNA destruction with UV illumination; liberal washing of equipment and bench surfaces with bleach solutions; and the use of extract, PCR, and "large DNA" controls. In addition, equipment and supplies were dedicated to the ancient DNA laboratories, and work was not conducted in an ancient DNA laboratory after working in the main laboratory on the same day.

Isolation protocols in the two laboratories were very similar. A small piece of each *Haliaeetus* bone was cut away and cleaned in sterile water. This was followed by a wash with rotation at room temperature in sterile 0.5 M EDTA, after which the EDTA was discarded. At Durham, the bone was wrapped in heavy aluminum foil (which had been decontaminated of DNA by UV illumination for 20 min) and crushed in a mortar and pestle (decontaminated by soaking in 50% bleach solution). At the NZP, a bleach-sterilized coffee grinder was used to pulverize the bone sample. The crushed bone was placed in 5 mL of sterile UV-treated lysis buffer (0.01 M Tris, pH 8.0; 0.002 M EDTA; 0.01 M NaCl; 10 mg/mL DTT; 1% SDS; and 1 mg/mL Proteinase K) and rotated overnight at 60°C. The sample was then extracted

twice with 5 mL of phenol and once with 5 mL of chloroform. The supernatant was placed in 1 mL of sterile water in a Centricon 30 microconcentrator and centrifuged until about 50 to 100 μ L covered the membrane. A second 1 mL of sterile water was added and the centrifugation repeated. The remaining sample was collected from the membrane by a short centrifugation and diluted to 200 μ L total volume. From 2 to 4 μ L of this were used in subsequent PCR experiments.

We attempted PCR amplifications with primers that amplify different-sized segments of the three mtDNA genes: 12S rRNA, ATPase8, and *cyt b*. We designed a set of primers (AncEag. 2: forward, 5'-AGGAATCTGCCTACTAACACA-3', 3' is base 15,026 of *Gallus gallus* sequence; Desjardins and Morais 1990; reverse, 5'-TTCGACATGTATGGGCTACG-3', 3' is base 15,090) from *Haliaeetus* eagle *cyt b* sequence (Seibold and Helbig 1996) such that they flanked a small (63 base pairs [bp]) region that contained four transitional substitutions between *H. albicilla* and *H. leucocephalus* and also differed by at least this much from the other five *Haliaeetus* species. The AncEag. 2 product size was 104 bp.

Primers *cyt b* 2-rc (5'-TGAGGACAAATATCC TTCTGAGG-3', 15,320 of *Gallus*, reverse complement of *cyt b* 2; Kocher et al. 1989) and *cyt b* ack (5'-CCTCCTCAGGCTCATTCTAC-3', 15,376 of *Gallus*) amplified a 98-bp segment of *cyt b* disjunct from AncEag. 2. The 12S rRNA segments were amplified and sequenced using primers 12SA, 12SH, 12SJ, and 12SB2 (Cooper 1994), producing 392 bp of sequence. The ATPase8 segment included 35 bp of tRNA-lysine and was amplified and sequenced with "Birds-R-Us" (5'-TGGTCCAAGAAGCTTAGGTTCA-3', 9,241 of *Gallus*) and t-lys (5'-CACCAGCACTAGCCTTTT AAG-3', 9,051 of *Gallus*).

Final PCR components in a 50- μ L reaction volume were 1X *taq* buffer (Perkin Elmer), 1.25 units of *Taq*-gold polymerase, 0.2 mM each dNTP, 2.0 mM MgCl₂, 3 mg/mL non-acetylated BSA, and 1 μ M of each primer. PCR was conducted for 45 cycles beginning with a "hot-start" for 10 min at 94°C and ending with a 6-min soak at 72°C. Conditions per cycle were 92°C denaturation for 40 s, 50°C annealing for 1 min, and 72°C extension for 1 min.

Successful amplification was determined by visualizing PCR products on a 2% agarose gel. Single-banded products were cleaned by centrifugation using a Qiagen kit following the manufacturer's instructions. About 100 ng of product were used for cycle sequencing of both strands using dye-labeled dideoxynucleotide terminators; resulting sequence reactions were run on an ABI 377 sequencer. We assessed chromatograms for accuracy and aligned sequences using Sequencher 3.0.

We compared sequences from the Hawaiian eagle bones directly with those of other *Haliaeetus* species. We also conducted a total-evidence phylogenetic

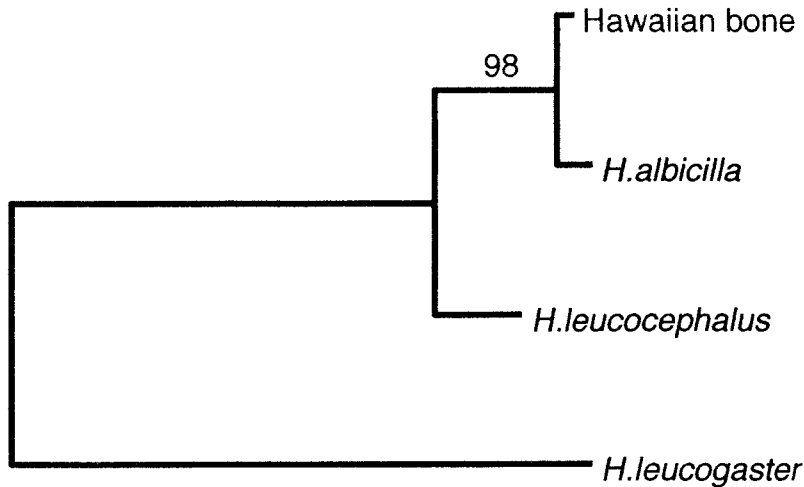


FIG. 1. Total-evidence parsimony phylogram showing relationships among mtDNA sequences from Hawaiian bone and from three species of *Haliaeetus* (see Table 1). Note that the outgroup sequences are chimeric for individuals: ATPase8 and 12S rRNA were obtained from individuals sequenced as part of this study, and *cyt-b* sequences are from different individuals described in Seibold and Helbig (1996). The 98% bootstrap value for the Hawaiian bone and *H. albicilla* clade is on the clade's node.

sents a postmortem artifact rather than a real substitution. Other than this single change, which does not modify our conclusions, no other differences are suggestive of artifacts owing to environmental modification.

The reconstructed phylogeny (Fig. 1) shows the close relationship of the Hawaiian bone sequences to those of *H. albicilla* and their more-distant relationship to *H. leucocephalus* and the outgroup taxon *H. leucogaster*. Bootstrap support for a Hawaiian bone/*H. albicilla* clade was very high (98%). Constraining the Hawaiian bone sequences to be sister to *H. leucocephalus* increased the tree length by five steps, but the Kishino-Hasegawa test was not quite significant in supporting the Hawaiian bone/*H. albicilla* clade over the Hawaiian bone/*H. leucocephalus* clade ($t = 1.9$, $P = 0.058$).

Discussion.—We obtained mtDNA sequences from two subfossil Hawaiian eagle bones that are nearly identical to ones from *Haliaeetus albicilla*. Our analyses were conducted in two separate laboratories using independently purchased supplies, solutions, and synthesized primers. No outgroup samples of *Haliaeetus* were present in the laboratories used at Durham University (where the *cyt-b* and some 12SrRNA sequences were obtained). In the NZP laboratory (where the ATPase8 and 12SrRNA sequences were obtained), all outgroups were analyzed after DNA isolation from the subfossil. Thus, it is extremely unlikely that these sequences are the result of contamination by external sources of DNA.

It is remarkable that DNA and amino acids are well preserved in this skeleton that has lain in a cave in a

subtropical climate for perhaps 3,500 years, especially when considering that the eagle skeleton was exposed to dripping water in the cave and was moist when collected. Factors that may have favored preservation of organic compounds include the relatively constant, cool temperatures of this high-elevation cave, and the fact that the eagle corpse was always protected from UV radiation, being preserved in darkness.

Our results show that DNA sequences of the Hawaiian *Haliaeetus* are more similar to sequences of the White-tailed Eagle than they are to those of the Bald Eagle. They do not support a hypothesis that the Hawaiian eagle skeleton is conspecific with the Bald Eagle. The protein-coding sequence divergence (i.e. *cyt b* and ATPase8, combined) between the two (3.5%) is higher than might be expected for a within-species level divergence in birds and more typical of divergence between congeneric species (Klicka and Zink 1997, Avise and Walker 1998). On the other hand, the divergence between the White-tailed Eagle and Hawaiian bone protein-coding sequences (1.0%) is much lower and is within the typical range of within-species variation. Our results do not allow us to reject the hypothesis that the Hawaiian skeleton is that of a White-tailed Eagle, or its very close relative. Therefore, we suggest that the Hawaiian eagle is conspecific with and represents a disjunct Hawaiian population of the White-tailed Eagle.

The similarity of the sampled sequence from the Hawaiian eagle to that of the White-tailed Eagle also suggests that this species colonized the archipelago at a relatively late date, although one that apparently

preceded the arrival of humans (James 1995). The relative recency of colonization is also supported by the absence of eagle fossils in the sediments of a Pleistocene lake at Ulupau Head, Oahu (James 1987) and the lack of skeletal differences between the Hawaiian eagle and *H. albicilla* (Olson and James 1991). The Hawaiian eagle and the Black-crowned Night-Heron (*Nycticorax nycticorax*) are the only lineages of Hawaiian birds that successfully colonized the archipelago without the aid of humans, but that have not differentiated at least to the level of subspecies. This, and other evidence (e.g. Olson and James 1991, Fleischer and McIntosh 2000) suggest that colonization of the Hawaiian Islands is an ongoing process and that "equilibrium" diversities predicted by theories of island biogeography have not yet been reached.

Although the eagle appears to have been the only indigenous predator capable of depreeding the large flightless waterfowl of the islands (see Olson and James 1991), genetic and other data indicate that these waterfowl existed in the islands well before the arrival of *H. albicilla* (Paxinos 1998, Sorenson et al. 1999).

Our study demonstrates the utility of analyzing fossil or ancient DNA for identifying species when other approaches will not work. The identification of the Hawaiian eagle adds another Palearctic element to the sources of colonization of the Hawaiian avifauna. Most avian lineages in Hawaii appear to have colonized from the New World, particularly North America, and very few from the Palearctic (Mayr 1943, Fleischer and McIntosh 2000). Other lineages of Palearctic origin include the Laysan Rail (*Porzana palmeri*; Olson 1973a,b; Fleischer and McIntosh 2000) and possibly the *Acrocephalus* warblers and the drepanidines (Groth 1994, Fleischer et al. 1998). Historical records of eagles in the Hawaiian chain include a Golden Eagle (*Aquila chrysaetos*) resident on Kauai for 17 years (Berger 1981, Pyle 1997) that may have originated in either North America or Asia, and the Asian Steller's Sea-Eagle (*H. pelagicus*) that was recorded from the Northwestern Hawaiian Islands of Kure and Midway (Pyle 1997). *Haliaeetus albicilla* is a latitudinal migrant in Eurasia, especially in the eastern part of its range (del Hoyo et al. 1994), and sometimes is a vagrant and thus could be expected to have reached the islands.

Acknowledgments.—We thank R. Michael Seaverns and Pauline Fiene-Seaverns for guiding us to Puu Makua Cave and helping to collect the eagle skeleton. C. Pickett (National Zoological Park), P. Erickson (Swedish Museum of Natural History), and W. Piper generously contributed samples of outgroup *Haliaeetus*, and R. Hoelzel and C. Shaw provided lab facilities in Durham. We also thank Thomas Stafford, Jr., for helping to collect the skeleton and for providing results of his CHN, amino acid, and radiocarbon analyses. We thank R. Chandler, R. Prum, and an

anonymous reviewer for comments on the manuscript, including the request for phylogenetic analysis. The research was supported by the National Geographic Society; Friends of the National Zoo; and the Wetmore, Walcott, and Scholarly Studies Funds of the Smithsonian Institution.

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Received 27 May 1999, accepted 27 April 2000.

Associate Editor: R. O. Prum

The Auk 117(4):1056–1060, 2000

Are Nest Predation and Brood Parasitism Correlated in Yellow Warblers? A Test of the Cowbird Predation Hypothesis

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Brown-headed Cowbirds (*Molothrus ater*) are generalist brood parasites that reduce the productivity of many of their passerine hosts through egg removal and competition between cowbird and host nestlings (Lowther 1993, Payne 1998, Lorenzana and Sealy 1999). Recently, it has been suggested that cowbirds exert a previously unappreciated influence on the demography of their hosts through predation (Arcese et al. 1992, 1996; Smith and Arcese 1994; Arcese and Smith 1999). The cowbird predation hypothesis (Arcese et al. 1992) was suggested to explain the frequently observed link between nest predation and interspecific brood parasitism in many passerines (e.g. Wolf 1987, Payne and Payne 1998). Specifically, the hypothesized link is a direct result of cowbird

predation of host nests that are discovered too late in the host's nesting cycle for successful parasitism. This creates future opportunities for parasitism by causing hosts to renest (Arcese et al. 1996). Alternatively, a link between predation and parasitism may result if cowbird parasitism facilitates predation, or if both factors are correlated with a third variable such as nest vulnerability (Arcese and Smith 1999). Determining the independent effects of brood parasitism and nest predation on host populations is difficult because of this potential functional dependence (Pease and Grzybowski 1995, Arcese and Smith 1999).

In a resident population of Song Sparrows (*Melospiza melodia*) on Mandarte Island, British Columbia, the frequency of nest failure was positively related to the frequency of cowbird parasitism (Arcese et al. 1996). In 5 of 19 years when cowbirds were rare, absent, or removed on the island, nest failure was lower

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than when one or more cowbirds were present on a consistent basis. To explain these trends, Arcese et al. (1992) proposed the cowbird predation hypothesis, which they subsequently tested directly (Arcese et al. 1996). A critical prediction of the hypothesis was supported when Arcese et al. (1996) found that parasitized nests failed less often than unparasitized nests when female cowbirds maintained exclusive territories, because cowbirds should regularly depredate unparasitized nests but not nests they have already parasitized.

To assess whether results from this island population were unique, J. N. M. Smith and M. J. Taitt (unpubl. data) and Rogers et al. (1997) analyzed comparable data from Song Sparrows on Westham Island, near mainland British Columbia. In both studies, lower cowbird numbers were associated with higher host nesting success, as predicted. However, Rogers et al. (1997) found no difference in the frequency of nest failure in parasitized and unparasitized nests, contrary to the key prediction of the hypothesis. Published analyses of potential correlates of cowbird predation behavior are limited to these populations of Song Sparrows. Arcese et al. (1996) cited several anecdotal accounts of cowbirds destroying passerine nests and also called for additional independent tests of the cowbird predation hypothesis (see also Arcese and Smith 1999).

We compared the frequency of failure of parasitized versus unparasitized nests in a population of Yellow Warblers (*Dendroica petechia*) in the Delta Marsh region of southern Manitoba, using data collected from 1974 to 1976. The cowbird predation hypothesis predicts that unparasitized nests should fail more often than parasitized nests when cowbird egg-laying ranges do not overlap. In contrast, parasitized nests may fail more often than unparasitized nests if cowbird egg-laying ranges overlap, or if cowbirds facilitate rather than cause predation. We also examined the evidence in support of the prediction that unparasitized hosts whose nests are depredated should have a high probability of their replacement nests being parasitized. We further tested the general hypothesis that nest predation is linked to brood parasitism, either directly or through facilitation, by examining the relationship between frequency of nest predation and parasitism in seven Yellow Warbler populations across North America (cf. Arcese and Smith 1999).

Yellow Warblers are a common host of the cowbird at Delta Marsh (19.1% of 2,163 nests parasitized; Neudorf and Sealy 1994) and elsewhere. They typically accept cowbird eggs laid after the midpoint of the laying stage but respond to eggs laid earlier either by burial of the cowbird egg (and any host eggs) under a new nest, or by desertion followed by re-nesting at a different site (Clark and Robertson 1981, Sealy 1995).

Methods.—During 1974 to 1976, Yellow Warblers at

Delta Marsh suffered high cowbird parasitism and high predation. Further details of the study site, species, and methods used to find and follow nests are given in Goossen (1978) and Sealy (1995). We examined data for 331 Yellow Warbler nesting attempts. Parasitism frequency (proportion of all nests that received one or more cowbird eggs) was 26%. We calculated the frequency of failure of parasitized and unparasitized nests in the subset of nests surviving at least to the second day of incubation to eliminate the bias of unequal exposure to parasitism. Nest-failure frequency is defined as the proportion of all nests that fledged no young. A parasitized nest in which the cowbird egg (and Yellow Warbler eggs, if present) was buried and a new clutch was laid was considered a single parasitized nesting attempt. Where possible, we identified cause of nest failure for each unsuccessful nesting attempt. For example, weather or predation could be implicated confidently in many cases. Only nests that clearly failed as a result of loss of most or all of the eggs or nestlings were considered to have been depredated.

Nesting stage at which failure occurred was assigned on the basis of laying date of the first egg for nests found during building or laying or by back-dating from the first day of incubation (i.e. the day after the last warbler egg is laid) or from the date of hatching for nests found later. For nests that were partially depredated over several days, the stage at the first day of egg or nestling loss was considered the stage of predation. Common nest predators at Delta Marsh include red squirrels (*Tamiasciurus hudsonicus*), Common Grackles (*Quiscalus quiscula*), Yellow-headed Blackbirds (*Xanthocephalus xanthocephalus*), Red-winged Blackbirds (*Agelaius phoeniceus*), Baltimore Orioles (*Icterus galbula*), and Gray Catbirds (*Dumetella carolinensis*; see Sealy 1994).

We used corrected χ^2 tests to compare the frequency of failure of parasitized and unparasitized nests, combining data for all years. We also performed an additional test confined to nests for which failure could be confidently attributed to predation. We examined the relationship between parasitism and predation in seven populations of Yellow Warblers from Ontario (S. Yezerinac unpubl. data), Michigan (Batts 1958, DellaSala 1985), Colorado (C. Ortega and J. Ortega unpubl. data, Howe and Knopf 2000), Montana (J. Tewksbury unpubl. data) and Manitoba (this study). We used Spearman rank correlation, weighted by sample size, to determine whether the percentage of nests depredated was related to the percentage of nests parasitized.

Results and Discussion.—The frequencies of failure of parasitized and unparasitized nests were opposite to predictions of the cowbird predation hypothesis: parasitized nests failed more often (52% of 64 nests) than unparasitized nests (37% of 214 nests; $\chi^2 = 3.81$, $P = 0.05$). To assess whether predation and parasitism are linked, we compared the frequency of failure

of parasitized and unparasitized nests for failures that we could confidently attribute to predation. Again, the trend was opposite to predictions, although the difference was not statistically significant (40% of 52 parasitized nests vs. 30% of 192 unparasitized nests depredated; $\chi^2 = 1.69$, $P = 0.19$). These results did not support the cowbird predation hypothesis.

In contrast to Arcese et al.'s (1996) results, unparasitized nests were less likely to fail than were parasitized nests. This result was not unexpected because Yellow Warblers frequently respond to parasitism by nest desertion (Clark and Robertson 1981, Burgham and Picman 1989, Sealy 1995). However, because we considered only nests that failed after the window for parasitism (after the second day of incubation), any nests deserted in response to parasitism within this window were not included. Similarly, Payne and Payne (1998) found that parasitized Indigo Bunting (*Passerina cyanea*) nests failed more often than unparasitized nests. This result may occur if cowbirds facilitate predation by their activities near host nests (Arcese and Smith 1999). However, because the frequency of failure was not significantly higher in parasitized nests, we have no evidence to support the cowbird facilitation hypothesis.

Identification of nest predators is crucial to understanding the association between nest failure and brood parasitism. Field signs after nest predation only occasionally are useful in identifying nest predators (Arcese and Smith 1999, Thompson et al. 1999); hence, we have no clear record of whether cowbirds or some other predator were responsible for nesting failures. However, many nest predators exist in the study area, and of several observed predation events at Yellow Warbler nests, none involved cowbirds (Sealy 1994). In a similar habitat, Thompson et al. (1999) documented only one case out of 25 predation events recorded on video of nest predation by a cowbird. We conclude that cowbirds probably were not responsible for many of the predation events at the Yellow Warbler nests in our sample.

The critical prediction of the cowbird predation hypothesis is that parasitized nests should fail less often than unparasitized nests, but this prediction would hold only when individual female cowbirds have distinct egg-laying ranges, because a female should destroy unparasitized nests but not nests she has parasitized (Arcese et al. 1996). When female cowbirds defend exclusive intraspecific nesting territories, any parasitized nest a female encounters is likely to contain her own egg. On the other hand, if cowbird egg-laying ranges overlap, different females would be likely to discover the same nests if some nests are especially conspicuous to cowbirds in general, and subsequent females may depredate a parasitized nest. Therefore, parasitized nests should fail more often than unparasitized nests (Arcese et al. 1996).

One could argue that the trend we documented resulted from overlap of egg-laying ranges among female cowbirds. However, recent DNA work has shown that egg-laying ranges of female cowbirds do not overlap at Delta Marsh (Alderson et al. 1999), and we have no reason to believe that this was not the case 25 years ago. Indeed, egg-laying ranges likely overlapped less often then, because Yellow Warbler nesting density was about twice what it is at present, but cowbird densities were similar (S. Sealy unpubl. data). If host nests are abundant, female cowbirds should not need to search wide areas for host nests. The low frequency of multiple parasitism in the Yellow Warbler data set (8 of 85 parasitized nests received more than one cowbird egg) also supports the claim that egg-laying ranges overlapped little.

Yellow Warblers frequently respond to parasitism by deserting (burying) the cowbird egg. In the present sample, burial occurred at 39% of parasitized nests. A nest at which burial has occurred and a new clutch is laid may suffer increased exposure to predation compared with a nest that proceeds directly to incubation. This difference would bias our results against the prediction of higher frequency of failure of unparasitized nests. When we excluded nests at which burial had occurred, our results were unchanged. In addition, the frequency of nesting failure did not differ between parasitized nests in which cowbird eggs were buried versus accepted ($\chi^2 = 3.02$, $P = 0.08$), and the trend was opposite to expectation because "burial" nests failed less often (36% of 25 nests) than "acceptance" nests (62% of 39 nests).

Predation of a parasitized nest in which the cowbird egg is buried is predicted from the cowbird predation hypothesis only if cowbird egg-laying ranges overlap. Otherwise, a female probably remembers the nests she has parasitized (Sherry et al. 1993), and predation of these nests would be a form of the "Mafia" behavior described in parasitic cuckoos (Zahavi 1979, Soler et al. 1995). If Mafia-like behavior occurs, failures due to predation of parasitized Yellow Warbler nests should occur more often when egg rejection (i.e. burial) has taken place than when it has not. This was not the case in our study.

Another prediction of the cowbird predation hypothesis is that unparasitized hosts whose nests are depredated should have a high probability of their replacement nest being parasitized. We could not test this prediction because the identities of Yellow Warblers that renested were not known. However, in five cases where a first nest was depredated (two during laying, three during incubation), the same nests were reused for the new nesting attempts, presumably by the same adults. None of these replacement clutches was parasitized.

We present two explanations for our failure to support predictions of the cowbird predation hypothesis. First, host density is high at Delta Marsh, and opportunities for parasitism should not be limiting. In

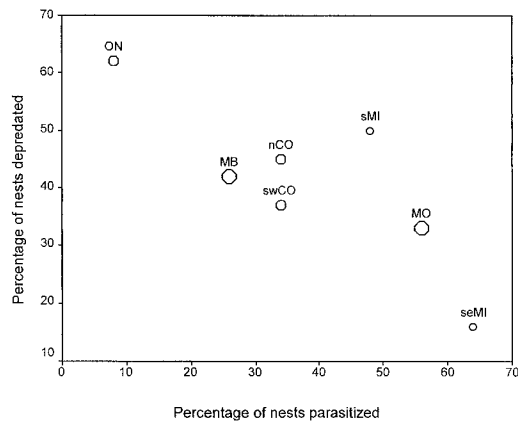


FIG. 1. Percent of nests depredated versus percent parasitized in seven populations of Yellow Warblers, weighted by the number of nests studied. Size of open circles indicates sample sizes for each population: Ontario (ON, $n = 74$; S. Yezerinac unpubl. data); Manitoba (MB, $n = 331$; this study); northern Colorado (nCO, $n = 73$; Howe and Knopf 2000); southwestern Colorado (swCO, $n = 93$; C. Ortega and J. Ortega unpubl. data); southern Michigan (sMI, $n = 20$; Batts 1958); Montana (MO, $n = 358$; J. Tewksbury unpubl. data); southeastern Michigan (seMI, $n = 25$; DellaSala 1985).

contrast, this may not be the case on Mandarte Island, where few alternate host species occurred. However, Smith and Taitt (unpubl. data) documented further support for the cowbird predation hypothesis in Song Sparrows on Westham Island, British Columbia, where alternate hosts were present. Despite this range of opportunity, alternate hosts are rarely used on Westham Island (J. N. M. Smith pers. comm.). Second, the timing of breeding of alternate host species is less synchronous at Delta Marsh than on Mandarte Island, and the presence of hosts late in the breeding season means that opportunities for parasitism are greater there. On Mandarte Island, cowbirds sometimes continue to lay until the end of the Song Sparrow laying season (Smith and Arcese 1994), but after this date, they are rarely seen on the island. At Delta Marsh, however, several other frequently parasitized hosts continue to breed later in the season, including Song Sparrows that lay second clutches. Both of the explanations noted above presuppose that nest predation confers a cost for cowbirds. If a cowbird incurs no cost by depredating a host nest, nests discovered too late for parasitism should always be destroyed regardless of the number of opportunities for parasitism.

Surprisingly, we found that across seven populations of Yellow Warblers, predation and parasitism were significantly negatively correlated ($r_s = -0.85$, $P = 0.008$; Fig. 1), in contrast to what has typically

been documented (e.g. Johnson and Temple 1990, Donovan et al 1997, Arcese and Smith 1999). We suggest that the frequently observed link between predation and parasitism cannot be explained by the cowbird predation hypothesis or the cowbird facilitation hypothesis, but instead represents a coincidental preference by nest predators and brood parasites for particular habitat features (Donovan et al. 1997, Tewksbury et al 1998). The only way to separate the independent influence of cowbirds as predators is to compare predation frequencies in populations where cowbirds are naturally or experimentally variable in abundance (Arcese and Smith 1999). In Song Sparrows, this test suggests that changes in the frequency of parasitism are directly linked to changes in the frequency of nest predation (Arcese and Smith 1999). Further experiments on other locally abundant hosts, such as Yellow Warblers, are needed.

Acknowledgments.—We thank J. P. Goossen for collecting the original data and the staff of the University of Manitoba Field Station at Delta Marsh for logistical support. The comments of P. Arcese, A. L. A. Middleton, S. I. Rothstein, J. N. M. Smith, J. J. Tewksbury, and an anonymous reviewer greatly improved the manuscript. We also thank C. P. Ortega, J. N. M. Smith, J. J. Tewksbury, and S. Yezerinac for kindly supplying unpublished data. Funds were provided by a research grant to SGS from the Natural Sciences and Engineering Research Council of Canada.

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Received 27 September 1999, accepted 28 April 2000.

Associate Editor: S. I. Rothstein