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A Comparison of the Bacterial Microflora Found on the Surface of American Kestrel and House Wren Eggs

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Abstract: It is generally accepted that avian eggs acquire a microflora during ovipositioning. The goal of this study was to identify and compare the eggshell microflora of two avian species, House Wrens and American Kestrels. Differences in the nesting habitats and incubation behavior of these species suggest that their eggshell microfloras also should differ. Culture-dependent techniques were combined with sequencing of the 16S rRNA gene to identify bacteria in samples taken from American Kestrel eggs during late incubation. These data were compared to previously collected data from House Wren eggs. In both studies, bacteria were isolated from three different phyla, Actinobacteria, Firmicutes and Proteobacteria. The eggshell microflora of Kestrels was dominated by bacteria in the Actinobacteria and Firmicutes phyla during late incubation, whereas the eggshell microflora of House Wrens was dominated by bacteria in the Gamma-Proteobacteria subphylum during pre- and late-incubation and bacteria within the Fimicutes phylum during early incubation. Actinobacteria genera on House Wren and American Kestrel eggshells differed, but the prominent genera in the Gamma-Proteobacteria and Firmicutes phyla, *Pseudomonas* and *Staphylococcus*, respectively, were similar between bird species. Thus, our results suggest that the microflora of avian eggshells have both variable (i.e., phyla) and conserved (i.e., specific genera) aspects of bacterial diversity.

Keywords: Actinobacteria, eggshell, firmicutes, microflora, proteobacteria, pseudomonas, staphylococcus.

INTRODUCTION

The surface of avian eggshells sampled in commercial hatcheries and in natural incubation harbors a community of bacteria and fungi referred to as a microflora [1-12]. This microflora is thought to have two important roles. One role is to protect the egg/embryo from infection by pathogenic bacteria [4-5]. Cook *et al.* (2005) showed that unincubated eggs were more likely to be infected than parentally incubated eggs because unincubated eggs harbored more pathogenic bacteria [4-5]. They suggested that parental incubation selects for beneficial bacteria and limits pathogenic bacterial growth and that parental incubation behaviors may be important in defining the microflora found on eggshells. The second role is to alter the egg microstructure to aid in embryonic development. Evidence for this was first shown by Board *et al.* (1979) when bacteria within the *Pseudomonas* genus were shown to degrade the cuticle of hens' eggs [13]. In addition, cuticle degradation of mandarin duck, *Aix galericulata*, eggs by *Bacillus licheniformis* has been linked to an increase in the conductance of water vapor in early incubation [14].

A better understanding of how bacteria facilitate these distinctly different functions requires identification of common bacteria that compose the microflora found on diverse avian eggshells. However, this endeavor is neither simple nor trivial given the variability in avian nesting environments, nest microclimates, and incubation behavior. For example, differences might be expected between birds breeding in grassland versus forested areas, tropical *versus* temperate climates, open-cup versus cavity nests, and based on nest materials used. Many, but not all, birds practice intermittent incubation, and the level of male participation in incubation and the length of the incubation period also differ. Authors of only a few studies have focused on identifying the bacteria that compose the microflora of avian eggshells. These studies were done on eggs of Pearly-eyed Thrashers [7], Western Bluebirds, Tree Swallows, and Violet Green Swallows [8], Pied Flycatchers [9], and House Wrens [12]. All avian species studied thus far have been characterized by female-only intermittent incubation behavior. The Pearly-Eyed Thrasher differs from the other species studied because it lives in a tropical climate.

More species must be examined to understand whether the diversity in the eggshell microflora is tightly constrained to specific bacterial strains and groups or is variable in composition. Identifying specific bacteria on eggs across a broad range of avian species would indicate these bacteria are integral to one or both of the proposed roles for the microflora. On the other hand, plasticity in bacterial population compositions might suggest that a species or group of species is endemic to the avian egg microflora as a function of species-specific behavioral and ecological characteristics. The goal of this study was to help distinguish between these possibilities by determining the composition of the eggshell microflora of a non-passerine bird, the American Kestrel (*Falco sparverius*).

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American Kestrel eggs were sampled near the end of incubation, and bacteria were identified using culture-dependent techniques. The bacterial community of Kestrel eggs was compared with community data previously published from House Wren eggs [12]. The incubation behavior and nesting environments of these two avian species differ strongly. American Kestrels, like House Wrens, begin incubating their eggs before clutch completion. However, the timing of incubation initiation is highly variable among female American Kestrels [15]. The incubation period for American Kestrels is approximately 30 days [16] compared to 14 days for House Wrens [12]. Unlike House Wrens, American Kestrel males participate in incubation for short periods of time during incubation [17]. Moreover, the American Kestrel females are typically too small to cover all their eggs during incubation [15]. Male incubation coupled with the female's inability to cover the clutch completely might contribute to higher variability in Kestrel egg surface temperatures over the course of incubation relative to House Wrens. We think the data presented in this comparative study will contribute to the current understanding of the avian eggshell microflora and provides insight into the potential for variability in bacterial composition.

MATERIALS AND METHODOLOGY

Study Species and Field Site

The American Kestrel is the smallest of the falcons and can be found throughout the Western hemisphere [18]. They prefer open areas and are considered secondary cavity nesters, but will readily occupy nest boxes [18]. Data for this study were collected during the 2011 breeding season from a network of 40 nest boxes in southwestern New York (Chautauqua County) and northwestern Pennsylvania (Warren County). The nest boxes have been maintained for more than twenty years and are used exclusively by American Kestrels. All nest boxes were constructed of wood, measure approximately 41 cm x 25 cm x 25 cm, and were mounted on ten-foot-high telephone poles.

The House Wren data was collected during the 2010 breeding season from a network of 50 standard-size bluebird boxes that have been exclusively used by House Wrens since 2002. These nest boxes are also in southwestern New York (Chautauqua County) and northwestern Pennsylvania (Erie County) [12]. Next box locations for the two species were in the same general area, but American Kestrels and House Wrens prefer very different nest habitats, so study sites did not overlap completely and environmental conditions may have differed between sites or study years.

Bacterial Sampling

American Kestrel eggs were swabbed on May 30th during the 2011 breeding season. Thirteen nest boxes were sampled and 1-2 eggs of each clutch (avg. 4-5 eggs) were swabbed. All nests sampled were within one week of hatching. Repeated samples were not taken from American Kestrel nests because of concerns that the disturbance associated with sampling would result in abandonment or force fledging. Samples were obtained as previously described for House Wren samples [12]. Briefly, aseptic techniques were used to the furthest extent possible in a field setting. Eggs were removed with a gloved hand, and a sterile plastic stencil (7 x 10-mm rectangle) was placed over the egg to limit microflora samples to a standardized area. Samples were taken with a sterile swab slightly wet with sterile nutrient broth. Each swab was placed in nutrient broth in an individually labeled microfuge container and stored at 4°C until processed with culture-based techniques.

Bacterial Identification Methods

All American Kestrel samples were processed the same as previously described for House Wrens [12]. Briefly, the tubes containing the swabs and nutrient broth were vortexed for 30 seconds. After vortexing, the broth was immediately sampled and used for serial dilutions and plating. Nutrient agar plates were incubated at 30°C and 37°C (most of the growth was isolated on plates incubated at 30°C) for 48 hrs. After incubation, colonies on plates containing 30-300 total colony forming units (CFUs) were counted, and CFUs were characterized based on morphology. Unique colonies were streaked for isolation.

To isolate DNA from a representative unique colony, a pure colony of bacteria was placed in 50 µL of water and cells were lysed with a freeze-thaw method. Samples were placed in methanol/dry ice bath for two minutes, and then in a heat block (99°C-105°C) for 2 minutes. This cycle was repeated twice to ensure lysis of cellular membranes and release of the DNA. Lysed cells were centrifuged for five minutes at 13,000 rpm. The supernatant containing DNA was removed and retained. The 16S ribosomal gene was amplified from the extracted DNA with the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') [19]. After polymerase chain reaction (PCR), DNA amplification was verified using electrophoresis, and the PCR product was sent for sequencing at the Genomics Core Facility at Penn State University Park. Forward and reverse sequences were used to construct a consensus sequence with Geneious software (Biomatters Ltd). Consensus sequences were run through NCBI-BLAST/EzTaxon to determine the genus and species identifications of bacterial isolates.

Presentation of Data

The authors understand that the two avian species were sampled in two different years and that it is possible that any differences observed in the microflora composition could be due to these confounding factors. Differences in location were unavoidable because of difference in nesting habitat between species. To address whether yearly temperature differences could have had an effect, the average daily temperatures for the months of May, June, and July for Erie County and Chautauqua County were downloaded from the National Oceanic and Atmospheric Administration. A Student's *t*-test (Microsoft Excel) was used to compare temperatures at both sampling locations for the two years. No differences were found between years at the same locations or between locations in the same year (Erie County 2012 *vs.* Erie County 2013: $p = 0.792$, Chautauqua County 2012 *vs.* Chautauqua County 2013: $p = 0.810$, Erie County 2012 *vs.* Chautauqua County 2012: $p = 0.344$, Erie County 2013 *vs.*

Fig. (3). The distribution of bacterial populations found within the Gamma-Proteobacteria subphylum reveals Pseudomonas as a prominent genus on eggshells of both the American Kestrel and House Wren (*R*-value = -0.0044 and *p*-value = 0.513).

Fig. (4). The bacterial profiles of the eggshells microflora within the Firmicutes phylum are similar between the American Kestrel and House Wren and is dominated by the Staphylococcus genus (*R*-value = -0.0561 and *p*-value = 0.670).

given the patterns described in the previous paragraph, we think it is appropriate to open a discussion about the possibility that these differences arise, at least in part, from differences among species in breeding habitat, foraging behavior, and nest structure. For instance, House Wrens, Pied Flycatchers, and Pearly Eyed Thrashers nest and forage preferentially in areas of dense shrub and deciduous woodland. American Kestrels, Western Bluebirds, and Tree Swallows prefer open grassland areas for nesting and foraging. House Wrens build complex, densely packed cavity nests with tightly woven egg cups at the center of a large volume of twigs [Voss, personal observation]. The nest material almost fills the entire cavity. The nest-building behaviors and habitat preferences of the Pearly-eyed Thrasher are very similar to those of House Wrens [23]. Pied Flycatchers build a substantial base of dried deciduous plant material, which supports a woven cup to hold eggs [24]. Nests of all three species are characterized by a large volume of plant material commonly found in woodland habitats. In contrast, Western Bluebirds and Tree Swallows build loosely constructed nests in boxes [Voss, personal observation]. The nest material is predominantly grass, with a few feathers lining a loosely formed (not woven) cup. The volume of nest material used is far less than that observed in the nests of the woodland species described above, and eggs are often in close contact with the wooden base of the nest box or cavity. American Kestrels are on the extreme end of this spectrum because they do not build nests at all. Eggs are laid in a small quantity of wood shavings or sawdust that lacks a formed cup [25]. Eggs are always in contact with the floor of the nest cavity [25]. Thus, nest structure and bacterial composition on the eggs appear to be correlated. We suggest that elaborate nests with a large volume of densely packed nest material may create a microclimate that selects for a fundamentally different microbial community than is found in smaller, more loosely constructed nests. Such differences may account for the observed variation in Actinobacteria genera. A larger volume of nest material densely packed into a cavity may increase the likelihood for microbial transfer to eggs, whereas loose, minimal structure might limit microbial diversity and reduce bacterial transfer to eggs. The same nest characteristics were used to explain trends in the bacterial profiles of the cloacal microflora of various avian species [26, 27].

Our data suggests plasticity in the overall composition of eggshell microfloras, but it also suggests conservation of diversity within specific genera. *Pseudomonas* was the most prominent Gamma-Proteobacteria genus on eggs of both species. Gamma-Proteobacteria genera are ubiquitous, and this broad distribution may be the primary factor contributing to their presence on eggshells. The question is whether this persistence is potentiated by the bacteria or the birds. For instance, *Pseudomonas* produces antibacterial proteins, pyocins, which may provide them with a competitive advantage over other bacterial species on eggshells [28, 29]. It is also possible that inherent avian antimicrobial mechanisms may not be effective against all bacteria introduced to the eggshell. For example, uropygial gland secretions have been shown to be ineffective against *Pseudomonas* [30]. Inherent antimicrobial properties of nest materials (e.g., feathers and aromatic plant material) also might be responsible for the bias if they selectively maintain specific bacterial genera [31-33]. Another possibility is that bacterial populations are periodically replenished by repeated parental transmission to the eggs. Whether maintenance of specific bacterial populations is beneficial to the development of particular avian species is not known. For example, Pseudomonas produces a protease that can degrade the cuticle of eggshells [13]. Cuticle degradation could be advantageous to developing avian embryos if it enhances gas exchange across the egg shell late in incubation. On the other hand, the associated eggshell degradation may also allow trans-shell migration of bacteria and subsequent hatching failure. Thus, variations in the dominance of this genus might indicate varying levels of alterations to the egg microstructure. *Staphylocccus* was prominent in both American Kestrels and House Wrens and was one of the most common genera found on Western Blue Bird, Tree Swallow, and Violet Green Swallow eggs [8]. Several investigators have identified *Staphylococcus* as a member of the bacterial microflora of the cloaca in various avian species [34-36]. In addition, *Staphylococcus aureus* and *Staphylococcus epidermidis* have been implicated as potential pathogens, but these species were not identified in this study. The potential pathogenicity of the *Staphylococcus* species isolated in this study requires further study.

In conclusion, our comparison of the eggshell microfloras of American Kestrel eggs in late incubation and House Wren eggs throughout incubation suggests plasticity in diversity at the level of bacterial phyla and some conservation of bacterial diversity at the genera level within the microflora of avian eggshells. Confounding factors may have contributed to the differences observed between avian species, but we think some plasticity may be the result of differences in habitat, diet, or nest construction. Similarities in the structure of the microflora may be a consequence of narrow temperature and moisture regimes in nests or of specific avian or bacterial processes that affect bacterial composition. Additional studies are necessary to substantiate this hypothesis. Many questions still surround the eggshell microflora, including how bacterial populations are initially acquired and whether their maintenance is guided by the bacterial physiology or avian antimicrobial practices/parental behavior. This study broadens our knowledge of the microbial communities on avian eggshells. It has also raised some interesting questions regarding how specific bacteria may affect the egg and embryonic development.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Appendix. Characterization of all bacterial phylotypes identified from the surface of House Wren eggs throughout incubation and American Kestrel eggs in late incubation.

Phylum	Family	Genus and species	House Wrens	American Kestrels
Actinobacteria	Cellulomonadaceae	*Cellulomonas parahominis*	Y	N
Actinobacteria	Microbacteriaceae	*Curtobacterium flaccumfaciens*	N	Y
Actinobacteria	Microbacteriaceae	*Microbacterium hatanois*	N	Y
Actinobacteria	Microbacteriaceae	*Microbacterium testaceum*	N	Y
Actinobacteria	Microbacteriaceae	*Plantibacter auratus*	Y	N
Actinobacteria	Micrococcineae	*Arthrobacter gandavensis*	N	Y
Actinobacteria	Micrococcineae	*Arthrobacter rhombi*	N	Y
Actinobacteria	Nocardiaceae	*Rhodococcus erthropolis*	Y	N
Actinobacteria	Nocardiaceae	*Rhodococcus qingshengii*	N	Y
Actinobacteria	Sanguibacteriaceae	*Sanguibacter keddieii*	Y	N
Firmicutes	Bacillaeceae	*Bacillus circulans*	Y	N
Firmicutes	Bacillaeceae	*Bacillus fusiformis*	Y	N
Firmicutes	Dermabacteriaceae	*Brachybacterium alimentarum*	N	Y
Firmicutes	Leuconostocaceae	*Leuconostoc mesenteroides*	N	Y
Firmicutes	Paenabacillaceae	*Paenibacillus amylolyticus*	N	Y
Firmicutes	Paenabacillaceae	*Paenibacillus borealis*	Y	N
Firmicutes	Paenabacillaceae	*Paenibacillus odorifer*	Y	N
Firmicutes	Paenibacillaceae	*Brevibacillus invocatus*	Y	N
Firmicutes	Planococcaceae	*Filibacter limicola*	N	Y
Firmicutes	Planococcaceae	*Sporosarcina aquimarina*	N	Y
Firmicutes	Staphylococcaceae	*Staphylococcus epidermidis*	Y	N
Firmicutes	Staphylococcaceae	*Staphylococcus equorum*	Y	Y
Firmicutes	Staphylococcaceae	*Staphylococcus hominis*	N	Y
Firmicutes	Staphylococcaceae	*Staphylococcus lentus*	N	Y
Firmicutes	Staphylococcaceae	*Staphylococcus succinus*	Y	N
β-Proteobacteria	Burkholderiaceae	*Burkholderia glathei*	Y	N
β-Proteobacteria	Burkholderiaceae	*Burkholderia phytofirmans*	Y	N
β-Proteobacteria	Burkholderiaceae	*Burkholderia sordidicola*	Y	N
β-Proteobacteria	Oxalobacteriaceae	*Massilia species*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Enterobacter amingenus*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Enterobacter cowanii*	N	Y
ɣ-Proteobacteria	Enterobacteriaceae	*Enterococcus durans*	N	Y
ɣ-Proteobacteria	Enterobacteriaceae	*Enterococcus faecalis*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Enterococcus moraviensis*	N	Y
ɣ-Proteobacteria	Enterobacteriaceae	*Erwinia billingiae*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Pantoea agglomerans*	Y	N

(Appendix) contd….

Phylum	Family	Genus and species	House Wrens	American Kestrels
ɣ-Proteobacteria	Enterobacteriaceae	*Pantoea vagans*	N	Y
ɣ-Proteobacteria	Enterobacteriaceae	*Pectobacterium carotovorium*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Rahnella aquatilis*	Y	N
ɣ-Proteobacteria	Enterobacteriaceae	*Salmonella enterica*	N	Y
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas aeruginosa*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas alcliigenes*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas brenneri*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas fluorescens*	Y	Y
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas frederiksbergensis*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas gingeri*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas graminis*	Y	Y
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas jessenii*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas koreensis*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas libanensis*	N	Y
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas lurida*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas migulae*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas poae*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas putida*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas rhizospaceae*	Y	N
ɣ-Proteobacteria	Pseudomoadaceae	*Pseudomonas syringae*	Y	N
ɣ-Proteobacteria	Xanthomonadaceae	*Luteibactor rhizovincina*	Y	N
ɣ-Proteobacteria	Xanthomonadaceae	*Rhodanobacter lindaniclasticus*	Y	N
ɣ-Proteobacteria	Xanthomonadaceae	*Stenotrophomonas maltophilia*	Y	N
ɣ-Proteobacteria	Xanthomonadaceae	*Stenotrophomonas rhizophilia*	Y	N

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