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ORIGINAL ARTICLE

Eggshell microbiota of a brood parasite reflects environment, not species

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Abstract

Microbiota affect many aspects of vertebrate biology and vertebrates provide diverse ecological niches for microorganisms. In avian systems, potential habitats for microorganisms include the feathers, skin, gastrointestinal tract, and eggshells. Eggshell microbiota may seed bird microbiota as the birds hatch and may be shaped by parental and environmental influences. Brood parasites are a natural system where parental and environmental influences are obligately decoupled. Here, we sequenced the V4 region of the 16S rRNA gene to characterize the eggshell microbiota of a brood parasite, the Brown-headed Cowbird (*Molothrus ater*), and its host, the Ovenbird (*Seiurus aurocapilla*), to explore the relationships between nest environment, host phylogeny, and the eggshell microbiota. Eggshell microbiota did not differ significantly between species. The observed variation was best explained by the nest in which an egg was laid and the collection site. Our study suggests that eggshell microbiota are influenced by the nest and local environment with significantly less influence by the species of the bird.

Keywords Microbiota · Avian microbiome · Brood parasite · 16S rRNA · Eggshells

Zusamemnfassung

Das Mikrobiom auf der Eischale eines Brutparasiten spiegelt die Umwelt, nicht die Art wider.

Das Mikrobiom beeinflusst viele Aspekte der Wirbeltier-Biologie, wobei Wirbeltiere den Mikroorganismen vielfältige ökologische Nischen bieten. Bei Vögeln sind die potenziellen Lebensräume für Mikroorganismen u.a. die Federn und Haut, der Verdauungstrakt und die Eischalen. Das Mikrobiom der Eischale kann die Mikrobiom der Vögel beim Schlüpfen prägen und durch Einflüsse von Eltern und Umwelt weiter geformt werden. Brutparasiten stellen ein natürliches System dar, das von den Einflüssen durch Eltern und Umwelt zwangsläufig abgekoppelt ist. In unserer Untersuchung haben wir die V4-Region des 16S rRNA-Gens sequenziert, um das Eischalen-Mikrobiom eines Brutparasiten, des Braunkopf-Kuhstärling (*Molothrus ater*) und seines Wirts, dem Pieperwaldsänger (*Seiurus aurocapilla*), zu bestimmen und die Beziehungen zwischen Nestumgebung, Wirts-Phylogenie und der Eischalen-Mikrobiom zu erforschen. Das Mikrobiom der Eischalen beider Arten unterschieden sich nicht signifikant voneinander. Die beobachteten Unterschiede lassen sich am besten durch das Nest, in das ein Ei gelegt wurde, und durch die Umgebung des Nests erklären. Unsere Untersuchung deutet darauf hin, dass das Mikrobiom einer Eischale durch das Nest und die lokale Umgebung beeinflusst wird und deutlich weniger von der Vogelart.

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Introduction

Animals house complex microbial communities, known as microbiota, and these microbes are important mediators of many aspects of host biology. Vertebrate microbiota influence innate immune system development and function (Thaiss et al. 2016), gastrointestinal function (Guinana and Cotter 2013), and whole organism development (Kohl et al. 2018; Sommer and Bäckhed 2013). Host-associated microbiota are also directly and indirectly involved in host health and disease (Kinross et al. 2011). The characterization of host-microbe interactions, therefore, is key to a complete understanding of vertebrate biology. Bird microbiota can reflect host ecology and evolution (Cao et al. 2020; Michel et al. 2018; Godoy-Vitorino et al. 2008) or host environment (Hird et al. 2014; Grond et al. 2019), and can impact host development (Kohl et al. 2018; Videvall et al. 2019). Much remains unknown, however, about the avian microbiota and its effects on birds.

The earliest events and contacts in a host's life can affect its microbiota (Bokulich et al. 2016; Wang et al. 2016), suggesting a need to understand microbial conditions during the nesting period in birds. To accommodate gas exchange, eggshells are porous, and eggs are, therefore, susceptible to invasion by microbes (Chen et al. 2019). The chicks of some species hatch without gut microbiota (Grond et al. 2017), although prenatal transfer of microbiota has been suggested for other species (Dietz et al. 2020), and the presence of microbes *in ovo* has been observed in lizards and birds (Trevelline et al. 2018).

The nest material and uropygial gland both influence the eggshell microbiota of Eurasian Hoopoes (*Upupa epops;* Martínez-García et al. 2016). Similarly, in the Woodlark (*Lullula arborea*) and the Eurasian Skylark (*Alauda arvensis*), the eggshell microbiota is shaped by the microbial communities of the nest material, the maternal brood patch and maternal feathers, but not that of the maternal cloaca (van Veelen et al. 2018). After a chick hatches, its microbiota could be recruited from or supplemented by any number of environmental sources, including host diet, nest material, parental feathers, brood patch, beak, and/or eggshell fragments. Consequently, the eggshell microbiota may have an important influence on the chick's subsequent microbiota.

Avian brood parasites are birds that lay their eggs in the nests of other species. They provide a natural experimental system in which to separate the effects of phylogeny and environment on the microbiota, as the eggs of two species are raised under the same environmental conditions with the same parental care. The microbiota of brood parasites may be determined by both vertical (from the biological parent) and horizontal (from the environment, including the foster parent) transmission. The gut microbiota of the nestlings of the brood-parasitic Great Spotted Cuckoo (*Clamator glandarius*), for example, reflect both the microbiota of their biological mother and their Eurasian Magpie (*Pica pica*) foster family (Ruiz-Rodriguez et al. 2018). Alternatively, in adult Brown-headed Cowbirds (*Molothrus ater*) and a set of representative parasitized species, sampling locality was the strongest correlate to the gut microbiota (Hird et al. 2014). The eggshell microbiota of parasitic Common Cuckoos (*Cuculus canorus*) and their Great Reed Warbler (*Acrocephalus arundinaceus*) foster parents became more similar to each other over the incubation period (Geltsch et al. 2018).

Brown-headed Cowbirds are non-specific brood parasites that inhabit most of North America and parasitize over 250 bird species (Lowther 1993). Cowbirds remove or destroy one member of the foster brood and replace it with their own egg, but do not typically damage other members of the foster brood unless the foster parent rejects parasitic eggs (Hoover and Robinson 2007). We sampled the microbiota of the brood-parasitic cowbird and foster species eggs in the nests of Ovenbirds (*Seiurus aurocapilla*), a ground-nesting passerine in northeastern North America. Ovenbirds are exposed to high rates of cowbird parasitism (Porneluzi and Faaborg 2001), partly as a result of their ground-nesting behavior (Bisson and Stutchbury 2000). Ovenbird nests generally contain a clutch of about four eggs (Porneluzi et al. 2020). Here, we characterized the microbiota of both Ovenbird and cowbird eggshells and the Ovenbird nest cup at multiple sites using 16S rRNA gene sequencing. We then: (1) compared the eggshell microbiota of the two species, (2) compared the eggshell and nest cup microbiota at different sites and between different nests, and (3) quantified the relative contribution of the nest environment and the bird species to variation in eggshell microbiota.

Methods

Sample collection

We conducted our study at a set of forest fragment sites in northeastern Connecticut, USA, that were selected as part of a larger study of multispecies interactions (Bagchi et al. 2018). During May and June 2018, Ovenbird nests were located at four sites in Storrs, Connecticut, USA (41.807382° N, 72.235807° W), Gay City State Park (41.722237° N, 72.453683° W), and Meshomasic State Forest (41.686907° N, 72.50307° W; 41.675509° N, 72.481827° W). Nests were located during the construction, egg laying, and incubation periods by systematically walking back and forth within territories and watching for behavioral signs of nesting. Samples for this study were taken from 11 nests which contained 42 eggs (31 Ovenbird eggs

and 11 Brown-headed Cowbird eggs; Table 1). Swabs were taken from the exposed side of the eggshell exterior that was not touching the nest cup or other eggs in the nest. Sterile swabs were moistened with a sterile saline solution and were swiped back and forth across the full exposed side for three seconds, while simultaneously twisting the swab to sample the eggshells. For nest cup samples, moistened swabs were inserted into the nest cup material directly underneath the eggs and rotated while gently prodding the material for three seconds. Swabs were deposited in sterile Eppendorf tubes and stored at − 80 °C within 8 h of collection. Prior to freezing, samples were stored at ambient temperature. To determine the incubation status of each nest we checked them every three days to count the number of eggs and chicks and to see if the female was on the nest incubating. Because females were not necessarily present and incubating at the time of a nest check, we used temperature dataloggers to record the internal nest temperature of each nest every three minutes. Recorded temperatures from nest dataloggers above ambient for prolonged periods of time indicated incubation.

DNA extraction and sequencing

DNA was extracted from swabs using a QIAGEN PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with an added 10-min heat step at 65 °C prior to bead beating to assist in denaturing the DNA. The extractions were sent to the Microbial Analyses, Resources, and Service facility at the University of Connecticut, for library preparation and sequencing on the Illumina MiSeq platform. The Quant-iT PicoGreen kit was used to quantify DNA concentrations. The V4 region of the 16S rRNA gene was amplified using 30 ng of extracted DNA as a template and using the 515F and 806R primers with Illumina adapters and dual index barcodes (Caporaso and Lauber 2011; Kozich et al. 2013). PCR conditions consisted of 95˚C for 3.5 min, 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 90 s at 72 °C, followed by final extension at 72 °C for 10 min. PCR products were normalized based on the concentration of DNA from 250 to 400 bp and pooled. Pooled PCR products were cleaned using the Mag-Bind RxnPure Plus (Omega Biotek) according to the manufacturer's protocol, and the cleaned pool was sequenced on the MiSeq using a v2 2 × 250 base pair kit (Illumina, Inc, San Diego, CA). Two blank extraction controls, one positive PCR control and one negative PCR control were also sequenced.

Sequence quality control and analyses

Data were processed and analyzed using R 3.6.1 (R Development Core Team 2018). The bioinformatics pipeline DADA2 version 1.16.0 (Callahan et al. 2016) was used for sequence quality control, to merge forward and reverse reads, and to remove chimeric sequences. Forward and reverse reads were truncated to 240 and 200 base pairs, respectively, before merging. Paired reads that did not align at 100% of bases were discarded. All other DADA2 default quality control parameters were used. We used Amplicon Sequence Variants (ASVs) as the operational taxonomic units, i.e., all unique sequences that passed the quality thresholds and were not singletons were considered individual taxonomic units ($N = 10{,}262$ ASVs). Taxonomy was assigned to each ASV using DADA2's native naive Bayesian classifier (Wang et al. 2007) based on the SILVA reference database version 132 (Quast et al. 2012). After assigning taxonomy, we removed all non-target sequences (mitochondria, chloroplasts and

Table 1 Number and origin of samples collected and extracted from each site and nest

Site	Nest ID number	Incubation status	Origin and number of samples		
			Ovenbird eggs	Cowbird eggs	Nest cup swabs
Meshomasic medium	53	Yes	1	1	1
	92	No	0	1	1
	94	No	2	2	1
	167	Yes	3	1	0
Storrs	109	No	2	1	1
	153	No	2	2	1
	171	Yes	2	1	1
Meshomasic large	87	Yes	1	0	1
	184	Yes	4	0	1
Gay City	65[a]	N/A	0	0	1
	130	No	3	0	1
Totals			20	9	10

[a]Nest 65 was depredated

any sequence not assigned to Bacteria) leaving behind 7075 ASVs. The presence and abundance of potential contaminants was assessed using the decontam package version 1.8.0 in R (Davis et al. 2018) and employing the "prevalence" method, which compares sequences in the negative extraction and PCR controls to those in the samples. Eleven ASVs were identified as likely contaminants and were removed.

For phylogenetic analyses, we conducted a multiple-alignment using the DECIPHER package version 2.12.0 in R (Wright 2016), and built a phylogenetic tree of the microbial ASVs by constructing a neighbor-joining tree based on a generalized time-reversible (GTR) model with a gamma distribution using the phangorn package version 2.4.0 (Schliep 2011).

Data and variables

We examined the following variables: (1) collection site, (2) nest, (3) sample type (eggshell vs. nest cup), (4) species, (5) incubation status (whether or not a parent had begun incubating the eggs), and (6) parasite presence (whether or not a cowbird egg was present in the nest). We used a subset of the data excluding nest cup samples for certain statistical analyses.

Statistical analysis

For all diversity analyses, samples were rarefied to an even depth as determined by the size of the smallest sample ($N = 4378$ sequences). We used total observed ASVs and Shannon (Shannon and Weaver 1949) and Simpson (Simpson 1949) diversity indices to assess alpha diversity using the *vegan* R package version 2.5.6 (Oksanen et al. 2018). We used analysis of variance (ANOVA) to compare Shannon and Simpson indices among sites, nests, incubation status, and sample type, and ran a Tukey Honest Significance test (TukeyHSD) for pairwise comparisons.

Bray–Curtis dissimilarity (Bray and Curtis 1957) and weighted and unweighted UniFrac (Lozupone and Knight 2005) distance matrices were constructed for each data set and visualized with Non-metric multi-dimensional scaling (NMDS) plots. To assess the relative contributions of each variable to the variation in microbial communities, we ran a permutational multivariate analysis of variance (PerMANOVA) using the *adonis2* function in vegan. Three PerMANOVA models were generated considering the following variables: site, nest, incubation status, sample type (replaced with species for models where only eggshell samples were considered), and parasite presence (only in a model considering Ovenbird eggs exclusively). We ran a differential abundance analysis (DESeq2 version 1.24.0; Love et al. 2014) to identify specific differences between the Ovenbird and cowbird eggshell microbiota at the genus level. Finally, we ran a Mantel test (Mantel 1967) to assess whether differences in beta diversity between samples from different nests and sites could be explained strictly by the geographic distance between sampling locality.

Results

Of 53 swabs collected, 39 samples yielded sufficient DNA for sequencing (10 nest cup samples, 20 Ovenbird eggs, and 9 cowbird eggs, Table 1). Sequencing yielded a total of 1,262,465 reads. Eleven ASVs were identified as likely contaminants because they were present in blank extraction controls and were removed from our dataset using decontam. After contaminant removal, samples ranged from 4378 to 85,113 reads, with a mean of 32,371 reads per sample. For diversity analyses, all samples were rarefied 5 times to 4378 reads.

Alpha diversity

Shannon diversity differed significantly among sampling sites, but not between nests, sample origins, or incubation status (Fig. 1, Table 2). The Simpson diversity index did not significantly differ for any variable, nor did total observed richness. The TukeyHSD pairwise comparison we conducted for the Shannon diversity site comparison revealed no significant differences between site pairs. The test revealed a marginal difference between the Meshomasic large and medium forest fragments ($p = 0.056$).

Beta diversity

We detected a significant difference between eggshell and nest cup samples (PerMANOVA, $F = 2.99$, $df = 2$ and 38, $p < 0.001$; Table 3), although in Bray–Curtis NMDS plots there was overlap between the nest cup and the eggshells of both species (Fig. 2), while eggshell and nest cup samples collected from the same nest tended to cluster together. We found no differences between the eggshell microbiota of Ovenbirds and cowbirds ($F = 1.00$, $df = 1$ and 28, $p = 0.41$). Among eggshell samples, nest explained 30% ($F = 1.94$, $df = 6$ and 28, $p < 0.001$) and collection site explained 21% ($F = 2.66$, $df = 3$ and 28, $p < 0.001$) of all observed variance in microbial communities. Incubation status did not contribute to the variation in microbiota composition. The Ovenbird eggshell microbiota was unaffected by the presence or absence of a cowbird egg in that same nest ($n = 20$ eggs).

We detected a weak positive linear relationship between geographic distance between sites and Bray–Curtis dissimilarity (all samples; Mantel: $r = 0.10$, $p = 0.03$; only eggshell samples; Mantel: $r = 0.12$, $p = 0.04$). In this context, a null result would indicate that there is no relationship between geographic separation and Bray–Curtis dissimilarity.

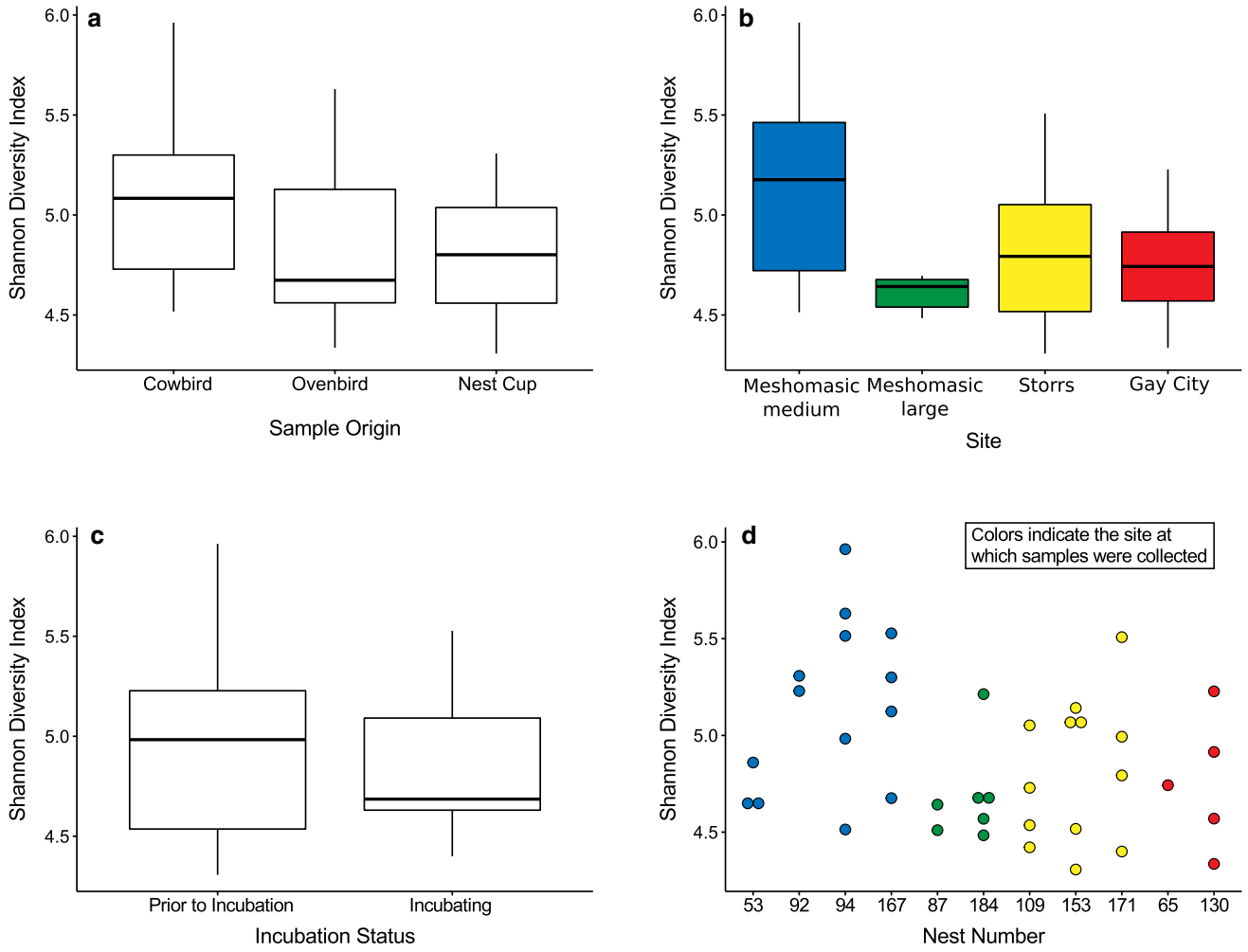

Fig. 1 Shannon diversity index by **a** sample origin with points overlaid to represent variable sample counts for each condition, **b** sampling site, **c** incubation status, and **d** nest number with box plots excluded given the small number of eggs in each nest

Table 2 Analysis of variance test results for amplicon sequence variant diversity

	Shannon index		Simpson index		Observed richness	
Variable (*df*)	*F* =	*p* =	*F* =	*p* =	*F* =	*p* =
Site (3, 35)	**3.195**	**0.035**	2.481	0.077	1.934	0.142
Sample origin (2, 36)	1.284	0.289	1.208	0.311	2.735	0.078
Nest (10, 28)	1.551	0.174	0.907	0.540	1.560	0.171
Incubation (2, 36)	0.522	0.475	0.300	0.587	2.455	0.100

Rows include a one-way ANOVA for each variable and each index

Results significant at $p < 0.050$ are bolded

Community composition

In both eggshell and nest samples Proteobacteria was the most abundant phylum, comprising 63.8 ± 0.9% (mean ± SE) and 65.8 ± 1.3% of the eggshell and nest cup microbiota, respectively (Fig. 3a). Nest cup samples averaged more Bacteroidetes (13.3 ± 1.5% of the overall community) than eggshell samples (8.8 ± 0.9%). Actinobacteria were less abundant in nest cup samples at 10.0 ± 0.5% compared to 13.4 ± 0.8% of the eggshell community.

The abundance of certain groups of bacteria also differed among sites. For instance, eggshell samples from the medium-sized Meshomasic State Forest site on average

Table 3 Permutational multivariate analysis of variance test results for contribution and significance of individual variables to each dissimilarity or distance matrix

	Variable	Bray–Curtis		UniFrac		Weighted UniFrac	
		R^2	p	R^2	p	R^2	p
Eggshell and nest cup samples	**Site**	**0.163**	**< 0.001**	**0.133**	**< 0.001**	**0.188**	**< 0.001**
	Nest	**0.247**	**< 0.001**	**0.212**	**0.002**	**0.249**	**0.006**
	Sample Origin	**0.111**	**< 0.001**	**0.079**	**< 0.001**	**0.111**	**0.003**
Only eggshell samples	**Site**	**0.207**	**< 0.001**	**0.176**	**< 0.001**	**0.256**	**< 0.001**
	Nest	**0.302**	**< 0.001**	**0.258**	**< 0.001**	0.282	0.012
	Species	0.026	0.409	0.028	0.641	0.018	0.586

Results significant at $p < 0.010$ are bolded

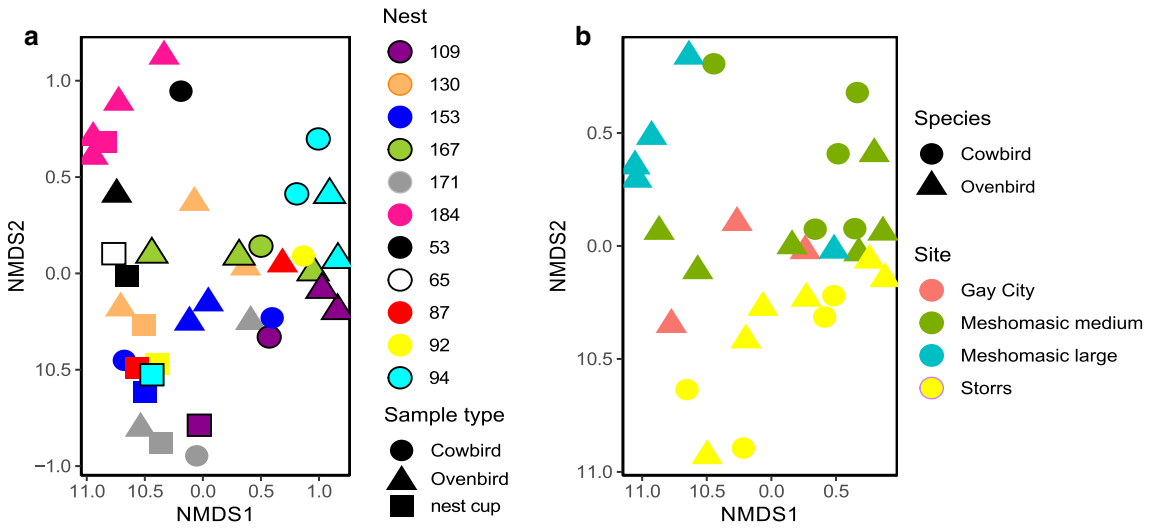

Fig. 2 Bray–Curtis NMDS plots for **a** all samples, colored by nest and **b** eggshell samples, colored by site. Shapes represent sample origin

contained more Planctomycetes (3.6 ± 0.9% of ASVs) than other locations (1.0 ± 0.2%), while those from the Storrs site contained fewer Acidobacteria (2.7 ± 0.9%) than other locations (6.7 ± 0.8%). Eggshell samples from Gay City State Park and the Storrs site contained more than twice as many Bacteroidetes as those from the two Meshomasic sites (13.1 ± 1.2% vs 5.2 ± 0.4%; Fig. 3b, c).

Seven differentially abundant taxa between cowbird and Ovenbird samples were identified by deSEQ. All seven taxa were derived from a single cowbird sample; when that sample was removed from analysis, no taxa displayed significantly differential abundances.

Discussion

In our study, we characterized the eggshell microbiota of Brown-headed Cowbirds, a brood parasite, and Ovenbirds, whose nests they parasitize. We also tested whether the species that laid an egg, or the environment in which the egg was laid, had the greatest influence on the egg's microbiota. Our results provide new insights into the microbiota of the nest environment prior to chick microbiota colonization and suggest that local environment has the most prominent influence on the eggshell microbiota.

We found that eggs in the same nest had more similar microbiota than eggs from different nests, but found no evidence that eggs from different species had different microbiota. Our differential abundance analysis supports this result, as we found no evidence for differences between cowbird and Ovenbird eggs in the abundance of individual microbe taxa, consistent with our main result that the nest and local environment had the greatest influence on an egg's microbiota. The very weak correlation of the Mantel test compared to the strong explanatory power of site-to-site and nest-to-nest differences indicates that spatial separation alone does not sufficiently account for differences across sites and nests. Given that local environmental factors are not assorted in any orderly manner across our study area, the Mantel test result leaves room for variables which do not correlate directly with geographic distance to explain some observed differences between sites.

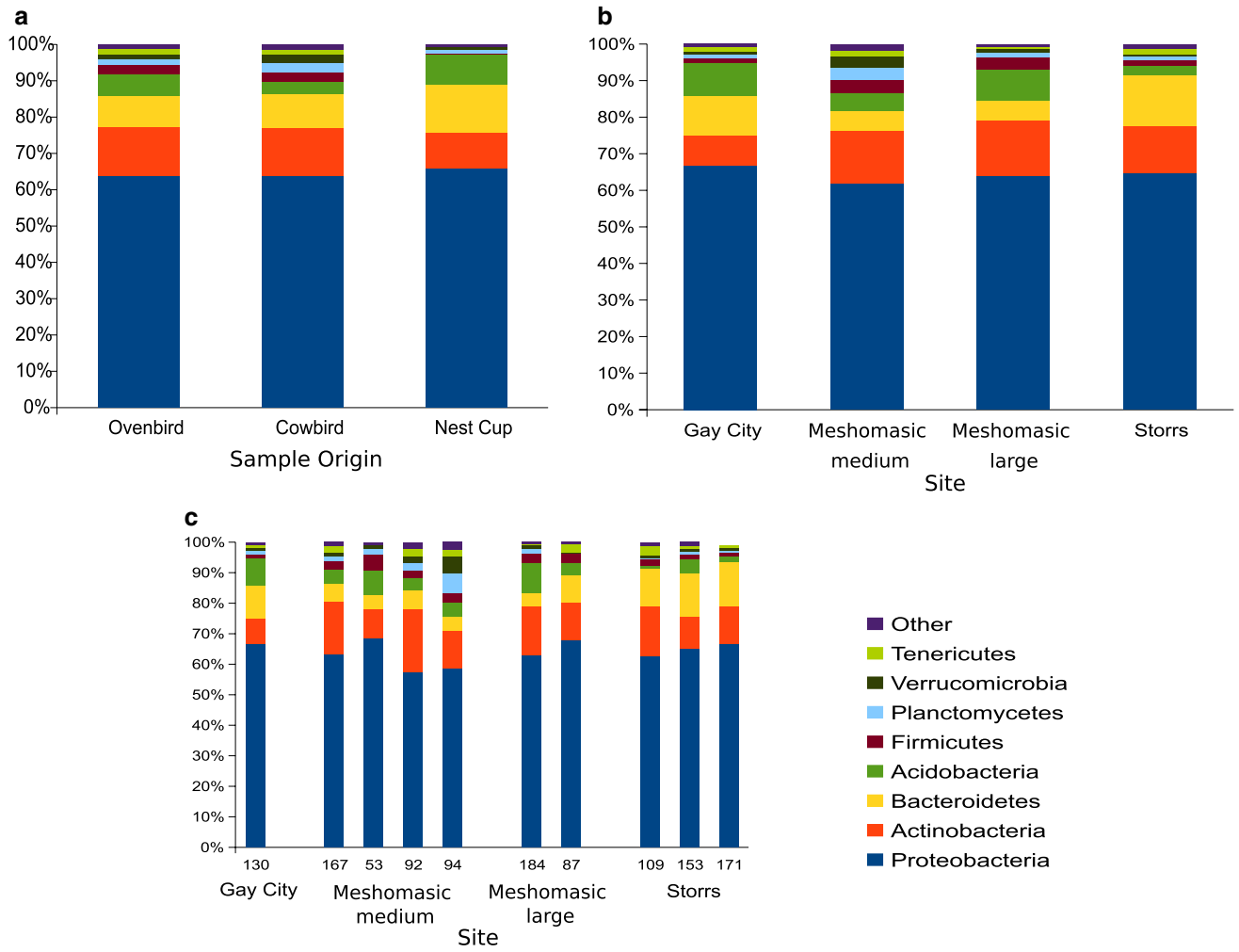

Fig. 3 Stacked bar plot of community composition by **a** sample origin, **b** site, and **c** nest, grouped by site

Our results are consistent with previous studies on eggshell microbiota in wild birds (Martínez-García et al. 2016; van Veelen et al. 2018), and with a study that showed geographic location was a more significant correlate to Brown-headed Cowbird gut microbiota than bird taxonomy (Hird et al. 2014). Similarly, the microbiota of chicks have been shown to be influenced by their local environment (Grond et al. 2017; Teyssier et al. 2018; Knutie 2020), suggesting host-filtering later in life. Consideration of our study serves as a reminder that environmental and parental contribution are often confounded. Without examination of foster and biological parent microbiota, we cannot rule out the incubating female Ovenbird or the visiting cowbird out as contributors to the microbiota of the nest, as they are most likely it is only visitors (Porneluzi et al. 2020). Future studies separating parental influence in an experimental setting could elucidate potential differences in microbiota associated with individual parents. Indeed, significant variation between individual adult birds was suggested as a factor driving nest-to-nest differences similar to our observations (van Veelen et al. 2017). The incubating parent themselves may contribute to differences observed between nests in our study.

Individual nest cup microbiota tend to bear more resemblance to the eggs within them than they do to eggs in other nests (Fig. 2a), supporting the notion that the nest itself may influence the microbial load of an eggshell (Walls et al. 2012). Because the eggshell microbiota and cup microbiota were statistically distinguishable, we stop short of suggesting that the eggshell microbiota is simply a reflection of the nest microbiota. Other variables such as local vegetation, moisture levels, predominant parental food sources, parental behavior, and the parental microbiota itself could influence eggshell microbiota.

While we found no evidence that incubation influences the eggshell microbiota, it can reduce the microbial load both on and inside the eggshell (Cook et al. 2005) and can protect against invasion by pathogenic bacteria (Brandl et al.

Table 4 Nest site descriptions for ten Ovenbird nests in eastern CT, USA, at which eggshell and nest microbial communities were sampled in May 2018

Site name	Nest ID number	Soil	Nearby features	Dominant understory vegetation
Gay City State Park	130	Dry	Rocky outcropping	Unknown saplings
Meshomasic State Forest, medium-sized forest fragment	167	Wet	Nearby swamp	Cub moss, unknown saplings
	53	Dry	Nearby stream	Minimal vegetation
	92	Dry	Nearby swamp	Grass
	94	Wet	Near hiking trail	Ferns
Meshomasic State Forest, large forest fragment	184	Dry	Near top of hill	Thick vegetation, unknown saplings
	87	Dry	On steep incline	Ferns
Storrs	109	Dry	Near hiking trail	Ferns
	153	Dry	Near hiking trail	Grass
	171	Dry	Multiple vegetation types	Grass, ferns, barberry

All observations are qualitative and were recorded at the time of sample collection

2014). Our study sampled a small number of nests at different points in the egg laying and incubating periods, making it difficult to draw a strong conclusion regarding this effect. As any effect of incubation may be short-lived, sampling eggs at variable lengths of time after the last incubation may not allow detection of such effects; sampling multiple times over the entire incubation period would be a valuable additional study.

A variety of parental behaviors could potentially influence the eggshell microbiota. Resemblance between parental beak and eggshell microbiota (Soler et al. 2016) could help to explain how the environment beyond the nest cup exerts an effect on the eggshell microbiota. Egg-turning behavior and transit in and out of the nest by the egg laying and incubating female Ovenbird could traffic microbes from outside the nest and soil below the nest cup to the egg surface. The effect of local biotic and abiotic variables on nest and eggshell microbiota is poorly understood. Although our sample sizes are too small for formal analysis, qualitative observations of the nesting sites (Table 4) suggest that soil moisture and proximity to water could explain some of the site-to-site differences in microbiota. All of the nests in the medium-sized Meshomasic forest fragment were located on moist soil or in close proximity to water, and all nests at the other sites were located on dry or rocky soil without nearby water sources. This difference in moisture may explain the over-representation of Planctomycetes at the former site, as this group of bacteria are commonly found in bogs and wetlands (Bodelier and Dedysh 2013).

Our study characterizes the microbial environment of Ovenbird nests and the parasitic eggs they contain, and supports the hypothesis that eggshell microbiota are influenced more by the nest environment than by the species of the egg's inhabitant. The biggest limitation of this study is sample size, which could strongly affect our results. Due to the nature of field work, we were limited in available nests but believe some conclusions can be drawn from this dataset. This limitation and others lend themselves to future work; time series data throughout the entire nesting period could reveal the impact of events like parasite egg introduction and incubation, while larger sample sizes collected from more diverse nesting sites could solidify the connection between local environment and microbiota.

Looking beyond the eggshell, research on chick microbiota could help to elucidate a connection between the eggshell microbiota and chick microbiota recruitment and assembly. The microbiota could be transferred directly from eggshell to chick, or the chick microbiota could be recruited from the environment in a similar manner to the eggshell microbiota. In the first case, the environment of the eggshell might provide an early selective environment to prepare a first microbial inoculate to the hatchling; in the latter, the eggshell would serve as a single-habitat model for chick microbiota recruitment.

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Author contributions BB: analyzed data and wrote the manuscript. EP: performed experiments and analyzed data. EG: conceived idea, designed methods, and performed experiments. KG: oversaw data analysis and contributed to the manuscript. SAK: contributed materials/resources/funding, trained EP on DNA extractions. SMH: contributed materials/resources/funding, oversaw analyses, edited manuscript.

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Availability of data All data will be uploaded to the NCBI SRA.

Availability of code Code will be made available by the authors by request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethics statement All applicable institutional guidelines for the care and use of animals were followed (IACUC #A17-007).

Consent to participate Not applicable.

Consent for publication Not applicable.

Legality All experiments were conducted according to local and national laws and regulations.

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