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Gut Microbiota Composition is Correlated to Host Hummingbird Protein Requirements

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ABSTRACT: The gut microbiome shapes and is shaped by a host animal's physiology. Avian taxa hold physiological characteristics unique from mammals and might inform novel pressures experienced by microbial communities. Further, the symbionts' relative abundance and their abilities to adapt to available resources are of critical importance to a holobiont's fitness in rapidly changing climates. Therefore, wild populations of hummingbirds *Selasphorus rufus* and *Calypte anna* were studied. The two systems differ in *S. rufus's* annual migrations from wintering grounds to their breeding grounds in the Pacific Northwest, whereas *C. anna* are resident in the latter region. Previous findings have indicated host microbiome composition varied with hummingbird fat score and the month during which fecal sampling occurred. Although fat is an important resource, especially for *S. rufus* in their migrations, protein requirements are critical because other annual activities, pressuring the organism to access nitrogen. Three of these activities are producing an egg, replacing molted feathers, and carrying parasites. The hypothesis for this study was that birds performing these activities will have microbiota that will make nitrogen available to them. Analysis of OTUs from 16S rDNA V3-V5 amplicon sequencing showed *Actinobacteria* are more abundant in these hummingbird species than in mammals, replicating our lab's previous findings. Notably, *S. rufus* adult females with evidence of recent or current egg production had significantly lower relative levels of *Actinobacteria* and significantly higher abundances of five of the other ten most abundant bacterial phyla than *S. rufus* males. The composition of major bacterial phyla in *C. anna* adults undergoing body, wing, and tail molt did not differ significantly from that of *C. anna* adults not undergoing molt. Tenericutes were significantly more abundant in *S. rufus* individuals with high numbers of *Mallophaga* eggs when compared with birds with few eggs. We described differences in nitrogen-limiting physiological processes and nutritional deficits in wild avian models to provide ways of understanding holobiont success in changing environments.

INTRODUCTION:

The microbiota, a community of microbes that live in a particular site, contributes to that location's combined metabolic capacity and influences community dynamics. In the gastrointestinal tracts of animals, the microbiota and their genes, collectively named the microbiome, have been shown to impact activities as diverse as immune and neurological system development and maintenance, small molecule signaling, and catabolism of a variety of macromolecules (1-4). Factors that affect the regulatory and metabolic capabilities of these microbes can alter the health, wellness, and success of host organisms; dysbiosis may occur when inappropriate or unbalanced interactions occur (1-3). Understanding and explaining the

relationship between the microbiome, host organisms, and the mechanisms by which they are interconnected, will inform models of community interactions in normal and dysbiotic states.

Catabolism and anabolism are performed in concert in these symbioses. Gastrointestinal microbiota rely on the host's maintenance of diet and intestinal linings for materials to catabolize; host animals use microbiota to extend their abilities of macromolecular digestion in the upper digestive tract, and the provision of vitamins and nutrient uptake in the lower tract (2, 5-7). This dependence suggests selection on the host to support microbiota adept in mobilizing the resources it needs in locations it can uptake them; selection on microbiota may proceed so microbiota and their genes are in proportions that might best support host fitness through supplementing host metabolism and physiology. Digestion and nutrient absorption present opportunities for microbiota to support host access to physiologically limited materials.

The microbiota must adapt to make available macronutrient resources that the host might access for its physiological activities at the time of those activities, or the community risks lowering its fitness and that of the host. Newborn humans *Homo sapiens* who drink breast milk carry *Bifidobacteria* and *Lactobacillus* strains which produce byproducts that induce local and immune responses. In turn, this action allows the gut to be more hospitable for these strains' proliferation, thereby promoting the digestion of oligosaccharides present in the breast milk (8). Additionally, the passerine sparrow *Zonotrichia capensis* have different communities of microbiota in their breeding and non-breeding seasons (9). Moreover, the microbiota of the adult female passerine European pied flycatcher *Ficedula hypoleuca* change while breeding and their nestlings mature (10). It is concluded that host life history events can influence host microbiota abundances. Investigating the categorical variables of the host's physiology and microbiota relative abundances jointly may clarify the selective pressures the microbiota experience.

Notably, the majority of studies into the microbiome have been conducted in mammalian model systems, particularly domesticated and murine ones (6, 11-13). These models, although important for understanding some physiological activities relevant to humans, do not capture the range of processes animals perform. Birds are highly divergent from mammals with different gastrointestinal structures (5), various complex tasks including flight, and distinct means of transfer of microbiota from adults to offspring (14). Studies of broiler chickens and other avian gastrointestinal tracts have revealed different communities of microbes exist at various microenvironments of the tract including the cecum, hindgut, and cloaca when compared to homologous regions in mammalian gastrointestinal tracts (2, 15-17). Further, these distinct communities within each gastrointestinal substructure had different physiological tolerances and metabolic capabilities, suggesting evolved symbiosis was driven in part by maintaining microbial communities that both had tolerance for particular gut environments and the ability to support the function of that substructure (2, 15, 18). Namely, cloacal communities were indicated to enable host macronutrient reabsorption (19). Investigations of birds and their microbiota are worthwhile to compare to mammalian symbioses and to elucidate the constraints of avian symbioses.

Domesticated model systems fail to capture wide-ranging ecological challenges presented to wild organisms due to their more stable environment, limiting knowledge of how microbial composition may change or be changed by the host. Environmental pressures for community adaptations are particularly important as climate change progresses. In addition to loss of access to abiotic factors such as fresh water in many environments, many organisms experience changes in the timing of life history events. Passerine breeding phenologies have shifted earlier in seasons in response to earlier minimum temperatures related to advancing climate change (20-22). Because a bird is assumed to be uncolonized by bacteria at hatching, environmental materials

and food available to it will affect its microbiome. If environmental materials and foods consumed differ between the advancing breeding dates and the evolved breeding dates, microbial community functional profiles, meaning the taxa and the activities the taxa perform, may shift in response to climate (22-25). To understand the pressures of animal-microbe symbiosis, resource mobilization within wild holobionts, or hosts and their symbionts, must be studied.

Therefore, a framework of holobiont studies may be used to expand current notions of how microbiota respond to host physiology: Host physiological activities bias certain microbial networks in the host gastrointestinal tract because those networks support those activities (5-7, 18). To test the theory's viability, we studied microbiota in feces of cohorts of wild *Selasphorus rufus* and *Calypte anna* undergoing contrasting life history events. These birds were selected for their common breeding grounds in the Pacific Northwest and similar life history events with the major exception of annual roundtrip *S. rufus* migration between Mexico and the Pacific Northwest while *C. anna* remain resident in the Pacific Northwest year-round (20, 26). They undergo physiological changes with high macronutrient demands under limited diets principally composed of nectar while sustaining high metabolic rates (26, 27).

Distinct physiological processes necessitate differential proportions of macronutrients. Fat score and fat metabolism have been demonstrated to be of particular interest in *S. rufus* due to the migration it undergoes (28). Protein metabolism, which dictates the majority of other life history event processes, has gone understudied. Large portions of bird bones, muscles, and feathers are composed of protein (18, 29, 30). As noted, however, these birds consume primarily nectar, which is composed of mono- and disaccharides (18). Although they may support the metabolic rate of these birds, these sugars do not supply necessary nitrogen for the maintenance of these birds' bodies (5, 18, 29). To gain nitrogen, nectarivores must consume arthropods (20,

29, 31). However, nitrogen is contained in proteins and polymers, such as chitin and bacterial cell walls, that animals are likely unable to digest (32). The catabolic activity to release this stored nitrogen is commonly attributed to bacterial species, and unidentified intestinal bacteria in *C. anna* degrade uric acid to ammonia, which is able to be absorbed by host tissues (18, 32). Due to this nitrogen limitation, activities that require building those three materials should place selective pressures on bird populations' abilities to access amino acids. It might be suggested that microbiota may support nitrogen liberation and absorption by host birds. Microbiome studies of the tropical passerine *Toxorhamphus poliopterus*, another primary nectarivore that consumes insects for nitrogen, showed that, within the phylum Tenericutes and the family Mycoplasmataceae, members of the genus *Ureaplasma* were found to be significantly more abundant in lower gastrointestinal structures than in other omnivorous and insectivorous birds and that the Actinobacteria *Conexibacter* was highly abundant (5). Bodawatta posited, on the basis of genetic work by Glass et al. 2000 and Pukall et al. 2010, that *Ureaplasma* and *Conexibacter* help the nectarivore conserve nitrogen (5, 18, 33, 34). Further work was completed in neotropical Apodiformes, the order to which hummingbirds belong, with different diets than *C. anna* and *S. rufus*; the study demonstrated approximately 80% of bacterial relative abundance can be attributed to Firmicutes and Proteobacteria in equal proportion (35). As a result of diet limiting nitrogen uptake in *C. anna* and *S. rufus*, the working hypothesis of this study is that Mycoplasmataceae and *Conexibacter* direct nitrogen recycling. Therefore, this study predicted that primary nectarivores experiencing processes with high nitrogen demands will host greater relative abundances of *Ureaplasma* and *Conexibacter* and related genera than primary nectarivores that are not under high nitrogen demands. Two nitrogen-demanding physiological processes critical to hummingbird life cycle and phenology are (A) egg production and (B)

feather molt. In this study, microbiota of egg producing female *S. rufus* and breeding male *S. rufus* have been examined (11, 36) while the microbiota of molting *C. anna* have been compared with the microbiota of non-molting *C. anna* (1, 37). Bird physiological demands do not exclusively dictate nutritional needs; parasite load may affect the proportion of macronutrients available to a host. Endo- and ectoparasites exact nutritional deficits, including protein deficits, on wild and domestic mice, and these hosts have different microbial diversity than those without endoparasites (38, 39). We aimed to learn if ectoparasite load is linked with microbiota composition in birds. Microbiota of *S. rufus* with few and those with many eggs from *Mallophaga*, or biting lice, have been compared, and hosts with many such tubular eggs were predicted to have distinct microbiota from those with few tubular eggs. This study's combined hypotheses were that birds producing an egg, molting, or carrying many *Mallophaga* eggs would have higher relative abundances of microbiota that support nitrogen recovery, such as Mycoplasmataceae and *Conexibacter*, than birds that were not producing an egg, not molting, or were hosting few *Mallophaga* eggs. Differences in microbiota were evaluated with sequencing of 16S rRNA V3-V5 gene regions liberated from *C. anna* and *S. rufus* feces.

A presented macronutrient need in the host should be related to the microbiota present in the host; thus, we predict that (A) *S. rufus* breeding females producing an egg would have higher Mycoplasmataceae or *Conexibacter* proportions than *S. rufus* breeding females without an egg and *S. rufus* breeding males, (B) *C. anna* molting feathers both on their body and wings would have higher Mycoplasmataceae or *Conexibacter* proportions than *C. anna* not molting, and (C) *S. rufus* with high *Mallophaga* egg counts would have higher Mycoplasmataceae or *Conexibacter* abundances than *S. rufus* with low *Mallophaga* egg counts. Further, other taxa key for nitrogen recovery would also be specified.

METHODS:

The procedures for bird capture and sampling were approved by Swarthmore College under IACUC protocol #06-02-18 and were permitted by the Washington Department of Fish and Wildlife and US Geological Survey Bird Banding Laboratory Master Banding Permit #21487 to Sara Hiebert Burch.

Bird Capture: *C. anna* (Fig. 1A) and *S. rufus* (Fig. 1B) individuals were captured on San Juan and Shaw Islands, WA, USA between May and August in 2018 and 2019 and in October 2018 (Fig. 1C,D) using Hall traps (Fig. 1E). *S. rufus* occupy the islands following their spring migration north from wintering grounds between southern CA and LA, USA and southern Mexico (20, 26) from March until late August.

Physical Measurements, Cohort Categorization: Physical characteristics of captured birds were recorded, and birds were banded in the case repeated captures of the same individual occurred. Date of capture and sampling, sex, age, egg status (Fig. 2), molt statuses (body, wing, and tail) (Fig. 3A, 3B), and *Mallophaga* tubular egg number (Fig. 3C) data were used to generate comparison cohorts and to select bird samples for analyses of gut microbiomes.

Egg Status: S. rufus were divided by two metrics: first, the birds were sexed, and second, their cloacae were inspected to determine whether they were carrying or had recently carried an egg. Males were assigned a score of zero because they cannot carry eggs. Females were assigned a value of 2, which indicates no evidence of current or recent egg carrying (referred to hereafter as "*S. rufus* females without eggs"); a score ranging from 6 through 9 (referred to from now on as "*S. rufus* females with eggs or those that have recently laid one"); 6, for birds that had recently laid an egg, or 7 through 9, for birds with progressively larger eggs visible in the oviduct (Fig. 2).

These categories best distinguish between known nitrogen demands of the hosts; scores of zero and two suggest no further nitrogen limitation than is usual in matured adults, and birds with scores between six and nine were assumed to be under advanced nitrogen limitation (Fig. 2). Each cohort was compared to each of the others (Fig. 4).

Molt: C. anna were examined for pin feathers, which indicate feather replacement, on their bodies, wings, and tails (Fig. 3A,B) (40). Birds were categorized by which feathers were being replaced: no molt; body molt but no wing or tail molt; wing or tail molt but no body molt; and body, wing, and tail molt. To compare birds with the greatest assumed differential nitrogen needs, birds with no molt were compared with birds presenting body, wing, and tail molt.

Mallophaga Tubular Egg Count: Mallophaga tubular eggs were counted on *S. rufus* throats (Fig. 3C), *Mallophaga* nymphs and adults as well as bird mites were counted on bodies, and flower mites were counted around the birds' mandibles. Feather mites feed on bird feathers (41), bird mites feed on bird blood (42), and flower mites have not been found to harm the host bird (43). In establishing the comparison cohorts, only tubular egg counts were used. Egg laying and hatching proceed at consistent daily rates and are predicted by adult lice numbers, so tubular eggs counts are an apt proxy for total feather mite counts (44). As such, only tubular egg counts were used for accurate categorization. "Highly parasitized" birds with five or more tubular eggs were compared with lowly parasitized birds with one tubular egg (Fig. 5). Host birds without parasites were not studied because they may not have been exposed, or they may have resisted parasitization (38). Birds with few parasites may have been only newly colonized, but these birds engage in common antiparasitic behavior that chronically parasitized birds perform (45).

Feces Sampling: During physical measures, feces and urine were noninvasively collected into sterile, DNA-free 0.6 mL microcentrifuge tubes (Thomas Scientific, Swedesboro, NJ) held to the

cloaca. Control samples of microbiota were obtained by touching the tube cap to feathers, as happens in fecal sampling, or to the researcher's hand. This method of fecal sampling limits invasiveness, but it preferences bacteria in the hindgut (7, 46). Tubes were placed on ice immediately following sampling to stop bacterial growth in the feces, preserving microbiota within feces in their recently excreted state (47). Tubes were transferred to -20°C within seven hours and to -80°C within two to seven days (48). Samples were shipped to Swarthmore College on dry ice, where they were stored at -80°C until DNA was extracted and prepared for sequencing.

DNA Extraction, Purification: The QIAamp PowerFecal kit and August 2016 protocol (Qiagen, Hilden, Germany) were used for processing feces and controls (49). The protocol specified use of fecal samples of 0.1g to 0.25g. All of our samples were below this mass. Nucleic acid products were frozen at -20°C.

End-Point PCR: 1µL 5' universal eubacterial and archaeal [5'-GTGYCAGCMGCCGCGGTAA-3'] and 3' eubacterial [5'-CCGYCAATTYMTTTRAGTTT-3'] 16S rRNA gene primers specific for the V3-V5 regions, 1µL of extracted DNA, 25µL New England BioLabs Quick-Load Taq 2X Master Mix, and 25µL nuclease-free water were used in end-point PCR reactions prior to Next Generation Sequencing sample submission. Agarose gel electrophoresis bands suggested successful extraction and purification. A lack of bands suggested unsuccessful extraction of 16S rRNA genes, unsuccessful purification of uric acid that limits Taq polymerase, or a sample with low DNA concentrations (50). If no bands were seen, uric acid-filtering microcentrifuge tubes were used to capture nitrogenous waste, and end-point PCR was run on the purified sample (50).

PCR and MiSeq Amplicon Sequencing: Samples remained frozen in transit to Wright Labs at Juniata College in Huntingdon, PA 16652 on dry ice or freezer packs. Wright Labs utilized Illumina MiSeq to sequence the 16S rRNA gene V3-V5 amplicons of samples and controls using the Earth Microbiome protocol (51). The number of amplicons of a particular sequence produced in PCR were related to total amplicon count, producing relative abundances of those amplicons.

This sequencing technique is a cost effective means for identifying microbial taxa in environmental samples regardless of the ability to culture those taxa (52). The 16S rRNA gene contains stable sequences to which primers can anneal. These stable sequences are adjacent to regions that are variable to the taxonomic level of the genus. V4 and V5 bind tRNAs in the course of translation while the function of V3 is not well understood (53). Selection against mutations in V4 and V5 is high, thereby imparting low mutation rates in these regions. Sequences in these regions are similar in broad classes microbes; therefore, isolates can be grouped into high classifications of taxa. With lower selective pressures on V3 mutations, lower taxa may be identified to the genus level (53, 54). Sequencing variable regions provides a means for taxon identification to the genus level, but not the species level, present in a sample (55-57).

Operational Taxonomic Unit Table Construction: QIIME 2 organized sequence reads into Operational Taxonomic Units table (58). OTUs are the taxon defined by a given percent sequence similarity in the variable regions of the 16S rRNA gene; 97% similarity dictates relatedness to the genus level between sequences (54, 58). If the threshold for matching a sequence to a taxonomic level was not met, the sequence would then listed as unclassified at that level but would be classified under the taxonomic levels it had successfully matched; for example, if the class rank was not successfully matched to any known classes, the sequence would be identified at the kingdom and phylum it matched but would be listed as unclassified at

the class level. Sequences were grouped according to these parameters, and sequences were assigned to taxa from the domain to the genus (58). The amplicon relative abundances were then assigned to corresponding OTUs (58). QIIME also contains records of genera that have been identified in sequencing but not in cultures; it specifies in OUT classifications whether the sequence belongs to a taxon that has been cultured but without immediate reference (58).

Selecting OTUs: To assess differences in high taxa and classifications of organisms that may be broadly useful for host nitrogen mobilization, the OTUs for the ten most abundant phyla were compared; this choice guaranteed over 99% of microbial sequence matches were represented in comparisons in every cohort. However, keystone taxa and particular genetic and molecular roles are defined at lower taxa. Instances of sequence matches to genera across all samples were calculated in proportion to the total sample number. Median relative abundances for all sequences matched to a genus were calculated. To ensure cohorts of sequences were controlled, only the ten most abundant phyla, the four genera with nonzero median relative abundances, and *Mycoplasma moatsii* were compared within bird cohorts.

Data Analysis: Mann-Whitney tests and were run with Real Statistics Resource Pack Microsoft Excel add-on. Relative abundances of the ten most abundant phyla and six most abundant genera in cohorts were compared with Mann-Whitney tests. $p<0.05$ for comparisons of phyla and genera. Effect size: $r = Z / (\sqrt{(\text{sum of n})})$.

RESULTS:

Characterization of Microbial Relative Abundances: Sequences matching two archaeal, three fungal, and 823 bacterial genera were identified. Of these totals, 257 of the bacteria and one archaeon have yet to be cultured. 0.36% of sequences could not be matched to known phyla,

0.48% of sequences could not be matched to known classes, 1.33% of sequences could not be matched to known orders, 3.37% of sequences could not be matched to known families, 10.1% of sequences could not be matched to known genera, and 44.0% of sequences could not be matched to known species. The phyla Actinobacteria, Cyanobacteria, Firmicutes, and Proteobacteria were represented in nearly every sample sequencing result, and the phyla Tenericutes and Bacteroidetes were also represented in 62% and 83% of samples, respectively (Table 1). Actinobacteria matched 81% of the microbiota by sequencing reads across all cohorts; other phyla commonly occupied less than five percent of total microbial abundance (Table 1, Fig. 6). Four OTU at the genus level had nonzero median relative abundances across all fecal samples: one Actinobacterium genus *Corynebacterium*, one Actinobacterium genus *Rothia*, one unclassified Proteobacteria family Enterobacteriaceae, and one unclassified Proteobacteria family Mitochondria. Four others were identified in all fecal samples from one round of sample submission to Wright Labs but not represented in any sample in the other; these include one unclassified Actinobacterium family Corynebacteriaceae and three unclassified Cyanobacterium class Chloroplast. From here forward, these sequencing isolates are referred to as their lowest assigned known taxon. These genera were represented by sequences in 66 to 94% of samples, and they, with the next 24 most abundant genera, accounted for 90% of sequence matches in all uncategorized samples. Notably, *Corynebacterium* and Cyanobacteria occupied large ranges of abundances across samples, spreading as much as 0.9 out of 1 in one cohort of birds (Fig. 8A, 7B). Less abundant taxa at both the phylum and genus levels had higher instances of individual relative abundances beyond the fourth quartile. The Tenericutes were represented by four taxa, of which two species belonging to the family Mycoplasmataceae and the genus *Mycoplasma*, *M. moatsii* and an unclassified species. The unclassified species was present in only one sample and

therefore excluded from analysis. *Ureaplasma*, the genus hypothesized to be more abundant in nitrogen limited hummingbird cohorts, was not matched to sequence reads; *Conexibacter*, also hypothesized to support nitrogen recovery in nectarivores, was represented in only five samples and was also excluded.

Egg Status: *S. rufus* males (n=19) and females without an egg (n=20) had significantly higher representation than in males in five of the most abundant phyla: the Cyanobacteria ($p=0.021$, $r=0.37$), Acidobacteria ($p=0.013$, $r=0.4$), Planctomycetes ($p=0.017$, $r=0.38$), Verrucomicrobia ($p=0.0042$, $r=0.46$), and other unclassified taxa ($p<0.0083$, $r=0.42$) were significantly greater in abundance in females with scores of two than in males (Fig. 7B, C; Table 2). These results are similar to those comparisons of females with an egg or had recently laid one (n=22) with males. These females had feces that were significantly less abundant in Actinobacteria ($p=0.0089$, $r=0.41$; Fig. 7A) and significantly more abundant in Cyanobacteria ($p=0.030$, $r=0.34$), Tenericutes ($p=0.0074$, $r=0.42$), Acidobacteria ($p=0.017$, $r=0.37$), Planctomycetes ($p=0.013$, $r=0.39$), and other unclassified taxa ($p=0.0031$, $r=0.46$) than feces from the breeding males were (Fig. 7B, C; Table 2). However, the two female cohorts demonstrated did not differ in relative abundances of any tested phyla ($p>0.1$, $r<0.25$ for all; Fig. 7, Table 2).

At the genus level, the abundances of Mitochondria ($p=0.0057$, $r=0.44$) and *M. moatsii* ($p=0.0054$, $r=0.44$) in *S. rufus* females without eggs were significantly more abundant than in *S. rufus* breeding males (Fig. 8B). The abundance of *Corynebacterium* in *S. rufus* females without eggs was significantly higher in abundance than those abundances in males ($p=0.018$, $r=0.38$; Fig. 8A). The relative abundances of Mitochondria in the *S. rufus* females with eggs were significantly higher than the abundances in the males ($p=0.015$, $r=0.39$; Fig. 8B). The remaining genera were not significantly different within comparisons of any two of the three cohorts.

Molting: The most abundant phyla were not found to differ significantly in abundance between molting (n=21) and nonmolting *C. anna* (n=21, $p>0.05$, $r<0.30$ in all) (Fig. 9). The abundance of *M. moatsii* in molting *C. anna* did not differ from the abundance of that strain in nonmolting *C. anna* ($p>0.5$, $r<0.1$; Fig. 10B). The prominently abundant genera did not significantly differ between molting and nonmolting *C. anna* ($p>0.1$, $r<0.25$ in all; Fig. 10A,B).

Parasitization: Tenericutes were significantly more abundant in *S. rufus* with few *Mallophaga* tubular eggs (n=22) than in *S. rufus* with many tubular eggs (n=21, $p=0.037$, $r=0.32$) (Fig. 11A). The remaining phyla were not significantly different from one another in abundance ($p>0.1$, $r<0.3$ in all) (Fig. 11). Within Tenericutes, *M. moatsii* abundances did not significantly differ in abundance in *S. rufus* with one *Mallophaga* tubular egg in comparisons with abundances in *S. rufus* with many tubular eggs ($p>0.3$, $r<0.2$; Fig. 12B). *Corynebacterium* ($p>0.1$, $r<0.2$), *Rothia* ($p>0.5$, $r=0.1$), unclassified Enterobacteriaceae ($p>0.05$, $r=0.27$), and unclassified Mitochondrion ($p>0.1$, $r=0.24$) relative abundances did not differ between differently parasitized *S. rufus*.

DISCUSSION:

We investigated the relative abundances of microbiota by 16S rRNA gene sequencing and taxon assignment in the fecal samples of *S. rufus* and *C. anna* hummingbirds in relation to host life history events that are expected to limit nitrogen resources. We found that strains hypothesized to be important to nitrogen mobilization to be lacking and that the majority of sequences were dominated by *Corynebacteria* and other Actinobacteria.

Firstly, *Conexibacter* and *Ureaplasma* where genera hypothesized to be more abundant in egg producing *S. rufus*, molting *C. anna*, and *S. rufus* with many *Mallophaga* tubular eggs. However, because they were found in few to no samples, our data were unable to provide

sufficient support for our hypotheses. One member of the Mycoplasmataceae family, which was also proposed to be more abundant in nitrogen-limited birds, *M. moatsii*, was well represented in samples and was significantly more abundant in female *S. rufus* without eggs than in male *S. rufus*. Strains of this species isolated from primates and rats have demonstrated arginine metabolism but not urea metabolism *in vitro* (59, 60). Therefore, *M. moatsii* may be considered a candidate for amino acid absorption in the hind gut of *S. rufus* but not a candidate for nitrogen recycling through uric acid mobilization.

The unclassified mitochondrial genus was significantly more abundant in each female *S. rufus* cohort relative to males. This result may be due to differences in fecal excretion of epithelial cell, which would be checked by 18S rRNA gene sequencing; if relative abundances of 18S rRNA genes were proportional to the Mitochondria's relative abundances, it would be likely that 16S rRNA gene sequencing captured genes from animal or fungal mitochondria (61, 62). Peptide nucleic acid polymers might be used in conjunction with these sequencing techniques to distinguish symbiotic mitochondria from animal cells and highly related bacterial species (62).

Two genera, *Rothia* and the unclassified Enterobacteriaceae, did not differ significantly in any comparison of abundances in different bird cohorts. However, species within both of these taxa have been suggested to influence nitrogen mobilization. *Rothia mucilaginosa* from oral sampling of humans hold homologs for nitrite transporters and nitrite reductase, and *Rothia* in cystic fibrosis patients carry nitrate reductase and nitrogenase homologs while ammonia builds in the microbiota and sputum *in vitro* (63, 64). Moreover, Enterobacteriaceae species have been found to synthesize amino acids (65). These genetic and *in vitro* metabolic tests suggest these taxa may help support nitrogen mobilization. Because these taxa have not proliferated in nitrogen-limited settings, however, gene expression must be assessed to determine if they

differentially produce essential proteins for nitrogen metabolism under different conditions; if they do not, they may be outcompeted by other microbes adept at nitrogen metabolism to fill this role.

Corynebacteria have been understudied in their potential role for mobilizing nitrogen in symbiotic settings. Conserved genetic motifs in the genus suggest wide ranging abilities for nitrogen uptake and use. Strains of this genus have been utilized extensively in industrial microbiology due to their ability to produce several amino acids, and these were artificially selected from an environmental isolate *C. glutamicum* (66-68). Another environmental isolate, *C. efficiens*, maintains a suite of enzymes for amino acid catabolism and synthesis, ammonium uptake, urea transport proteins, and ureases (67). These genetic units for amino acid synthesis and ammonium uptake are largely maintained in pathogenic species that associate with animals, including *C. diphtheriae*, although the urea transport systems and ureases are less conserved (67, 69). It is therefore likely that these motifs would be largely conserved in symbiotic models as well; because both have evolved with animal interactions, the symbionts matched to sequences in this study may reflect the genetic profile of pathogenic *Corynebacteria*, and would therefore may play critical roles in a host access to amino acid macronutrients (69-72). However, due to the extensive presence of these uptake mechanisms in free-living *Corynebacteria* and ability of hummingbird gut microbes to catabolize uric acid, whole genome sequencing of isolated *Corynebacteria* from this study would be required to define their genetic profile and functional capacity within the hummingbird gut (18, 67, 69, 72). Further, due to their majority abundance in hummingbird microbiota and therefore high gene copy numbers, this clade may dominate most aspects of nitrogen metabolism or those aspects of metabolism for which they have functional proteins. To discern the sufficiency of the *Corynebacteria* to support nitrogen metabolism,

individual strains must be identified, and genome, transcriptome, and *in vitro* metabolic work would be required.

Phyla were also noted to differ in some cohorts of birds. First, the Tenericutes were significantly more abundant in *S. rufus* females with eggs than in *S. rufus* males. The *S. rufus* with few *Mallophaga* tubular eggs were carried significantly more Tenericutes than highly parasitized birds (39, 45). These two finding are not consistent within the framework of nitrogen limitation because it would be expected highly parasitized birds would hold more Tenericutes by way of Mycoplasmaceae (33, 35, 60). As a result, the interaction of ectoparasites with the gut microbiome may be considered. Unlike endoparasites that may occupy the same location as or interact with gut microbiota, ectoparasites interact at a distance (39). Its effect, therefore, on the microbiota may be in systematic changes in bird physiology and behavior (45). Newly parasitized birds, which would be expected to carry fewer eggs, might be hypothesized to experience short-term nitrogen demands due to the onset of colonization or that of new antiparasitic actions that may result in increases in Tenericutes. Once these needs are accommodated, other selective pressures upon the microbiota may result in a return to lower Tenericutes abundances. Alternatively, because the *M. moatsii* were not significantly different in the parasitized birds, nitrogen metabolism may not be a sufficient explanation for change; the birds with one *Mallophaga* tubular egg may be under another stressor that is mediated by other metabolic or community activities among Tenericutes or Tenericutes and other taxa (5, 60). Comparisons between chronically parasitized, newly parasitized, and unparasitized *S. rufus* would be required to clarify these ideas.

In the *S. rufus* cohorts with birds of different sexes and egg statuses, many more phyla differed significantly between groups than in any other life history event under consideration.

Importantly, the female cohorts did not differ in any phyla abundance, but more than half do between each female cohort and the male cohort. These results do not fully represent the hypothesis of this study. In considering the lack of differences between females with different apparent egg statuses, it may be assumed that the female birds without eggs are nitrogen-limited in ways other than egg production. For example, swelling and prolapse in the cloaca following egg laying are impermanent, and a bird that is raising hatchlings might be nitrogen-limited without evidence of recent egg laying in the cloaca (18, 27, 31). Without monitoring female cloaca and nests for eggs and hatchings repeatedly, this proposed nitrogen-limiting mechanism cannot be resolved. Regarding phyla that were notably different in abundance, Cyanobacteria in both *S. rufus* female cohorts were significantly more abundant than in males. This phylum is largely composed of photosynthetic organisms, but a discrete clade of isolates in mammals have been suggested to support fiber digestion (73). Although the diet of these birds is not composed of fiber, if both female cohorts manage nitrogen through arthropod consumption, chitin, another complex polymer, would be important for nitrogen mobilization from the polymer and from materials held behind the arthropod exoskeletons (18, 31). Without whole genome information from this taxon, this assumption is not apt and prompts future DNA sequencing. Lastly, the female cohorts had significantly more diverse microbiota generally, resulting in the significant lowering of relative abundances of Actinobacteria. With regards to raising young, these increases in abundance of less prominent taxa may be hypothesized to aid in seeding hatchlings' gastrointestinal tracts with a biased community to promote their development by means of regurgitation (1, 11, 12). Again, fecal samples are biased toward hindgut and cloacal microbiota, but assuming these changes in abundance are represented, perhaps proportionally, through the rest of the gut, this hypothesis might be tested with gizzard sampling (2, 4, 17, 74). Hypotheses

as to the physiological benefit to the host itself, other than its fitness gains from having healthy offspring, may also be implicated in nitrogen metabolism, but the presence of chitinases or peptidases must be tested with particular strains of microbiota (71, 72, 75, 76).

Lastly, molting and nonmolting *C. anna* did not differ significantly in the relative abundance of any taxon examined. This result does not reflect the hypothesis that nitrogen mobilizing taxa would be more abundant in molting birds. Similarly to the lack of difference in the two female *S. rufus* cohorts, the birds not undergoing molt are likely experiencing nitrogen demands unaccounted for in the statistical controlling of cohort. Alternatively, molt is a fairly regular physiological process (37). There may be cues for the microbiota, just as there are cues for the host organism to molt, to change their gene expression in ways that result in the support of molting without the need for changes in the abundance of the microbiota.

V3-V5 Sequencing was utilized in the course of this study. It has been shown in recent studies that use of these variable regions do not delineate between low level taxa most effectively relative to other variable regions of the ribosome (53). As a result, some of the taxa identified, although they are likely accurate, capture more clades or strains of microbes then might be captured in other sequencing techniques (53). In order that more granularity and understanding of microbiota present in animal guts might be gained, V2-V3 sequencing in future efforts should be adopted. This will also shed light on the metabolic capacities of the community due to the greater abundance of genome sequences availability for scrutiny (53).

To assess whether changes in gene expression are reasonable means by which microbiota alter metabolic output and thereby resources to the host, transcriptomics and *in vitro* metabolic work should be explored. Particularly to discern the activities and capabilities of *Corynebacteria*

in nitrogen mobilization and recycling, arginine and urea metabolic tests might be applied under anaerobic conditions to simulate macronutrient catabolism in the gut (18, 67).

These results confirm previous analyses of the microbiota in these two species but contrast with previous findings in other non-migratory, tropical hummingbirds (35). These findings also suggest distinct core nitrogen mobilizing from other nectarivore species as proposed by others, particularly *Corynebacteria* (67). This study furthers our understanding that life history events have some influence over microbial communities that host animals carry with them. The physiology related to catabolism and anabolism by the microbiota have been suggested to influence development and other crucial processes; future work in isolating gene, protein, and small molecule products from this community will inform how well the microbiota influences the life history events host animals experience.

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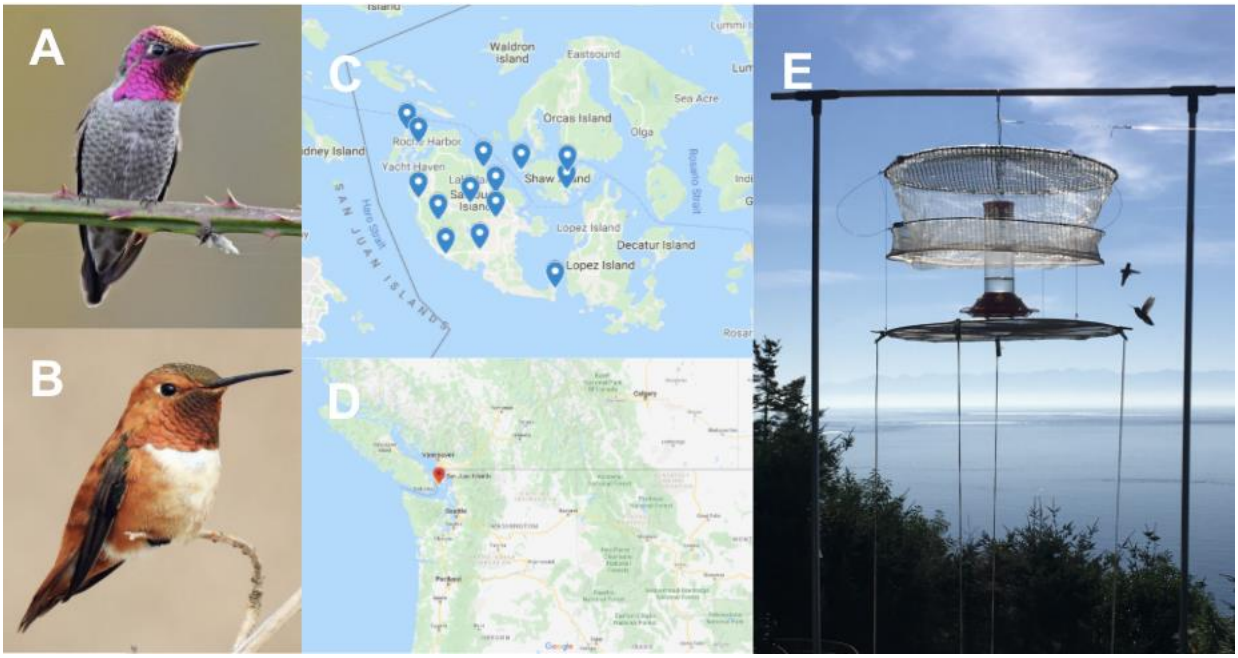


Figure 1. Model systems and capture conditions for birds of interest. A. Adult male *Calypte anna*. **B.** Adult male *Selaphorus rufus*. **C.** Bird capture locations on San Juan and Shaw Islands. **D.** Location of San Juan and Shaw Islands in the Pacific Northwest of North America. **E.** Feeder and net apparatus for hummingbird capture; a hummingbird is seen in the bottom right of the panel. Images of birds were provided by The Cornell Lab's All About Bird's website. Mapping and Hall trap photos by Sara Hiebert Burch.

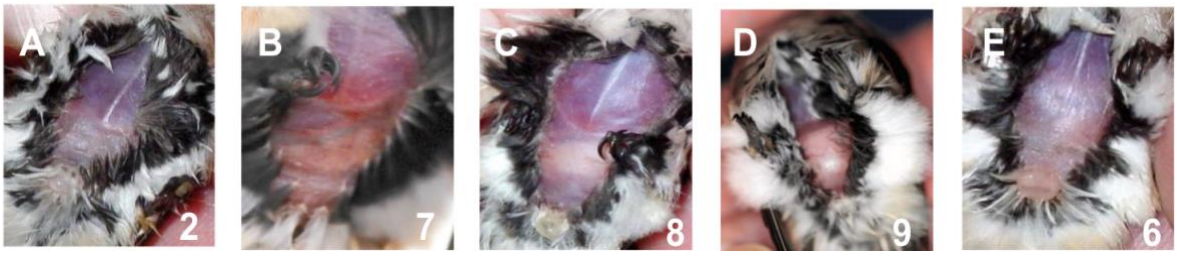


Figure 2. Egg status score. Female hummingbird cloacae at stages in bird egg development. **A.** Score of 2 indicates no egg visible, concave abdomen posterior to the breastbone. Scores of 7 (**B**), 8 (**C**), and 9 (**D**) demonstrate progressively more swelling and therefore eggs in later stages of development. **E.** Score of 6 indicates a normally prolapsed vent, which provides evidence of a recent egg laying. All photos by Sue Elwell, Princeton, BC.

Figure 3. Feather characteristics and ornaments. A. Pin feathers on the dorsal side of a hummingbird head. **B.** Pin feather of wing feather 7 of captured adult female *S. rufus* **C.** Pin feathers with basal sheaths along the body of a hummingbird. **D.** *Mallophaga* eggs glued to the base of hummingbird feathers. Photos in panels A, C, and D by Sue Elwell, Princeton, BC. Photo in panel B is provided by Hilton Pond Center's website.

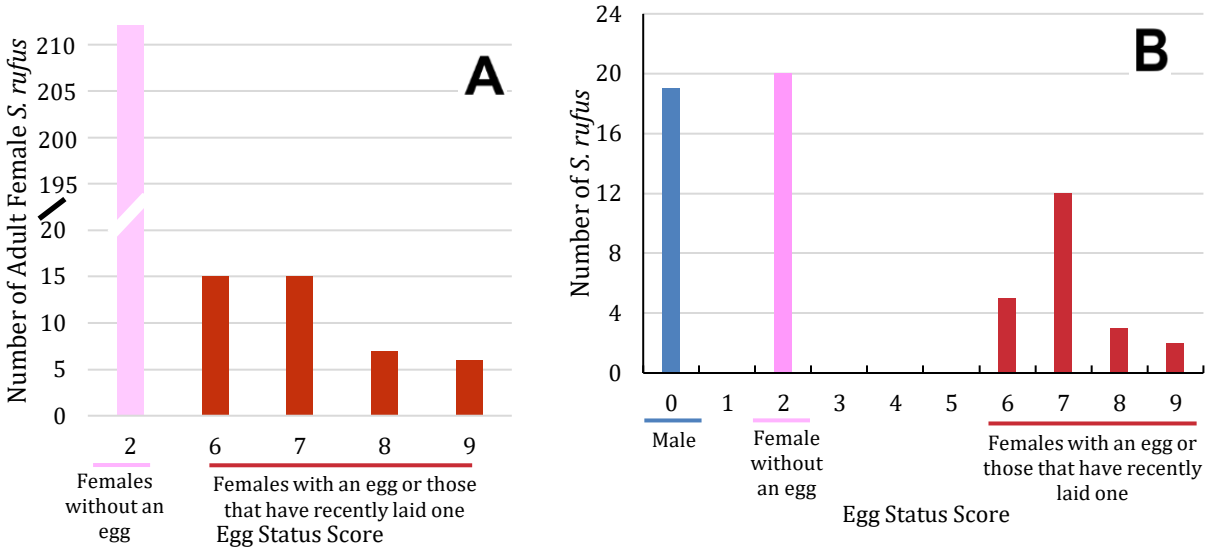


Figure 4. Sampling distribution of *S. rufus* egg status scores. A. Total numbers of adult female *S. rufus* sampled in the May 2018 to August 2019 by egg status score recorded. **B.** Numbers of *S. rufus* used for Wilcoxon rank sum analysis by egg status score.

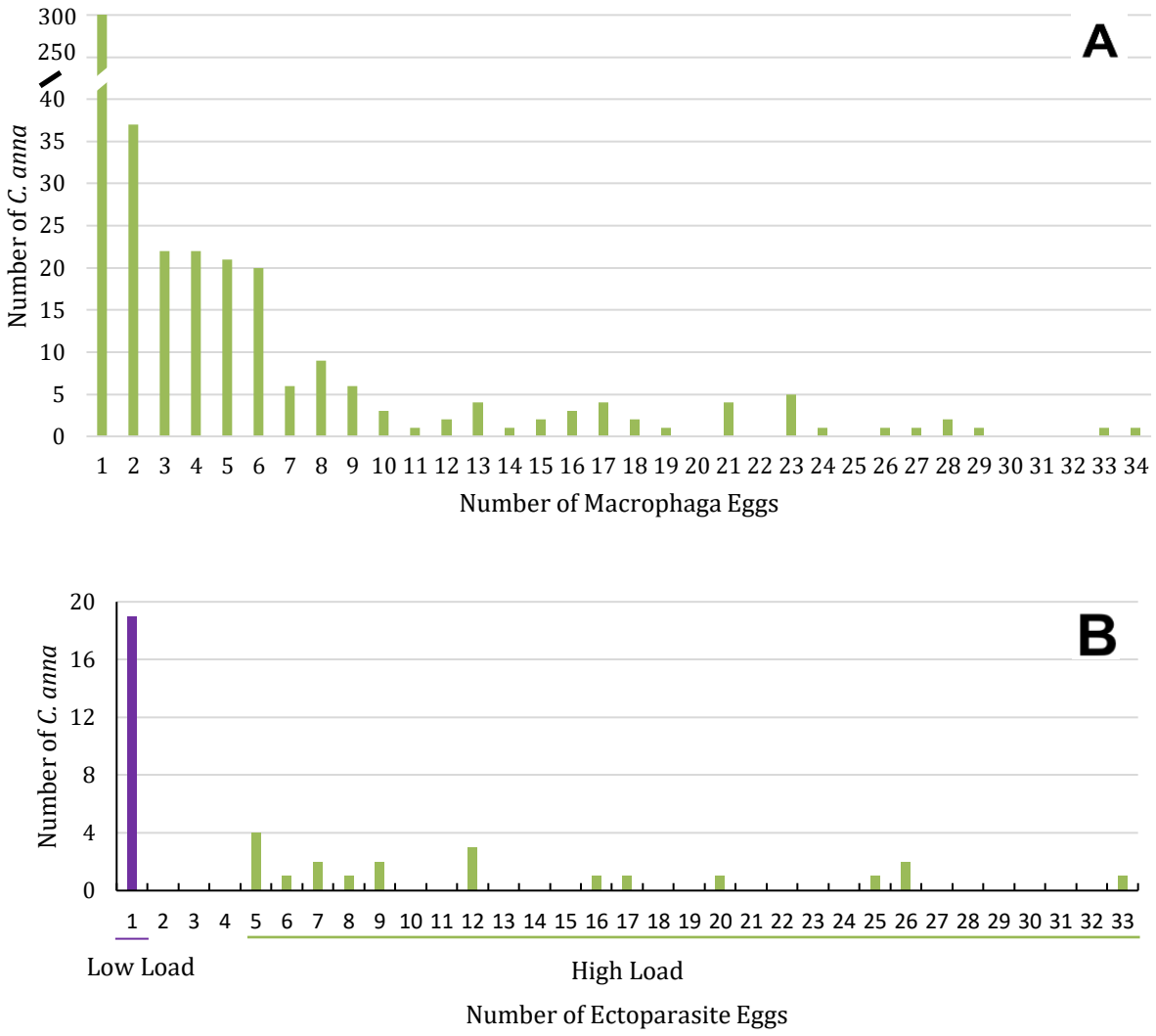


Figure 5. Sampling of *S. rufus* by *Mallophaga* tubular egg count. A. Total numbers of *S. rufus* sampled in from May 2018 to August 2019 by *Mallophaga* egg numbers recorded. **B.** Numbers of *S. rufus* used for Wilcoxon rank sum analysis by *Mallophaga* egg count.

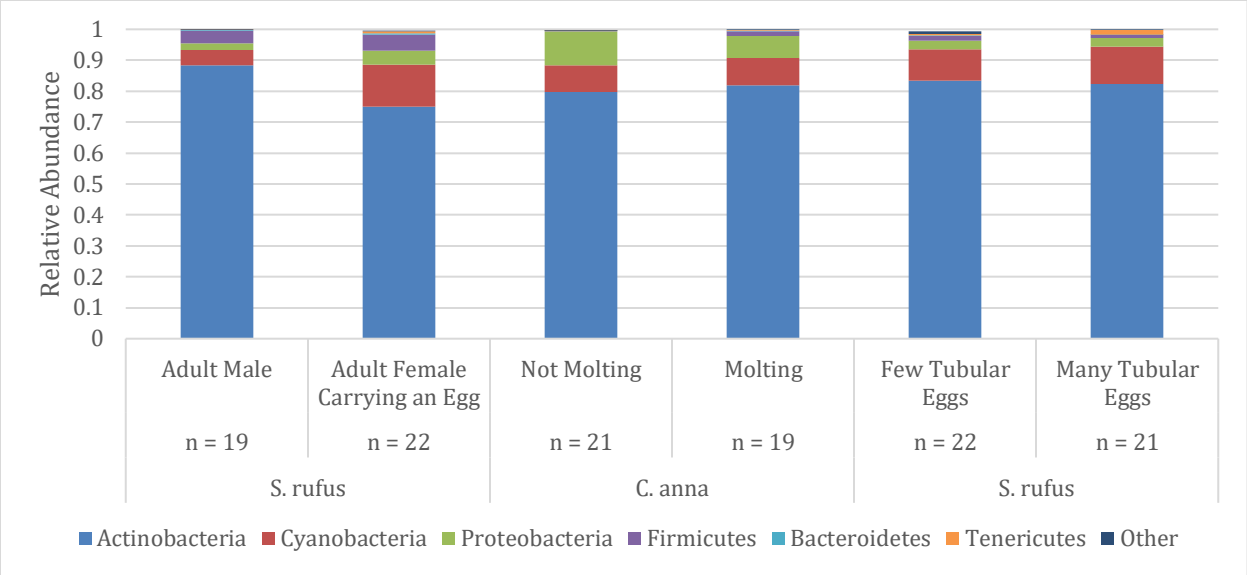
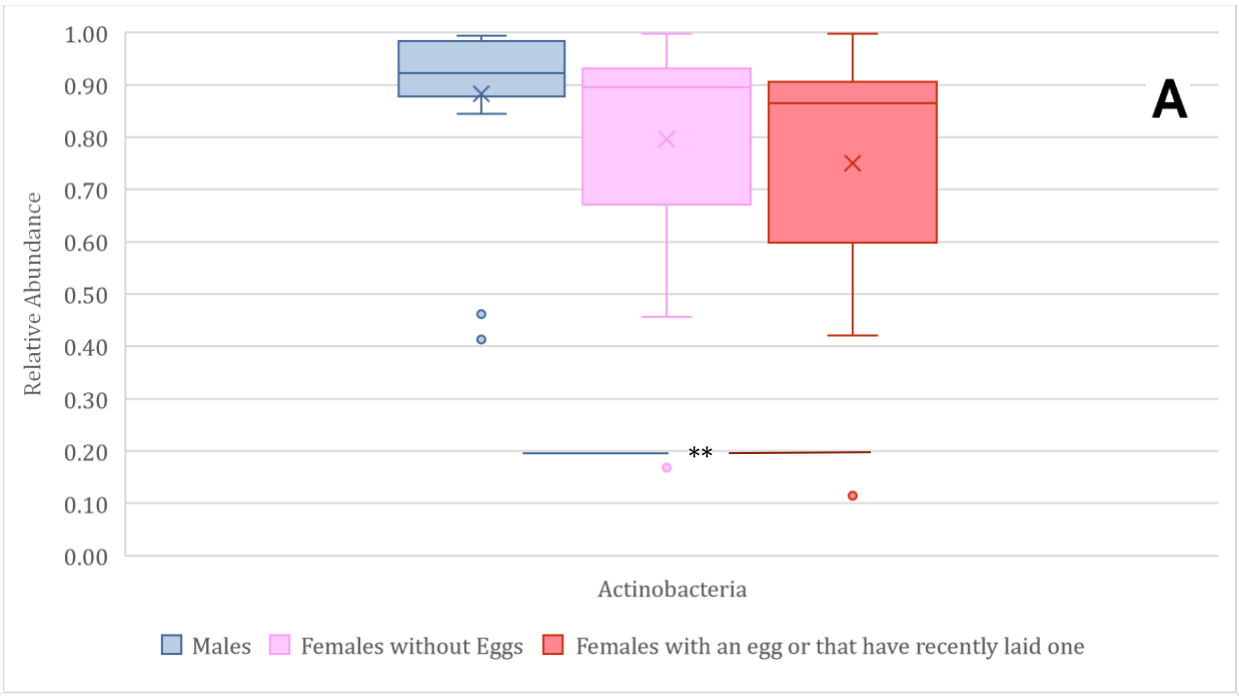
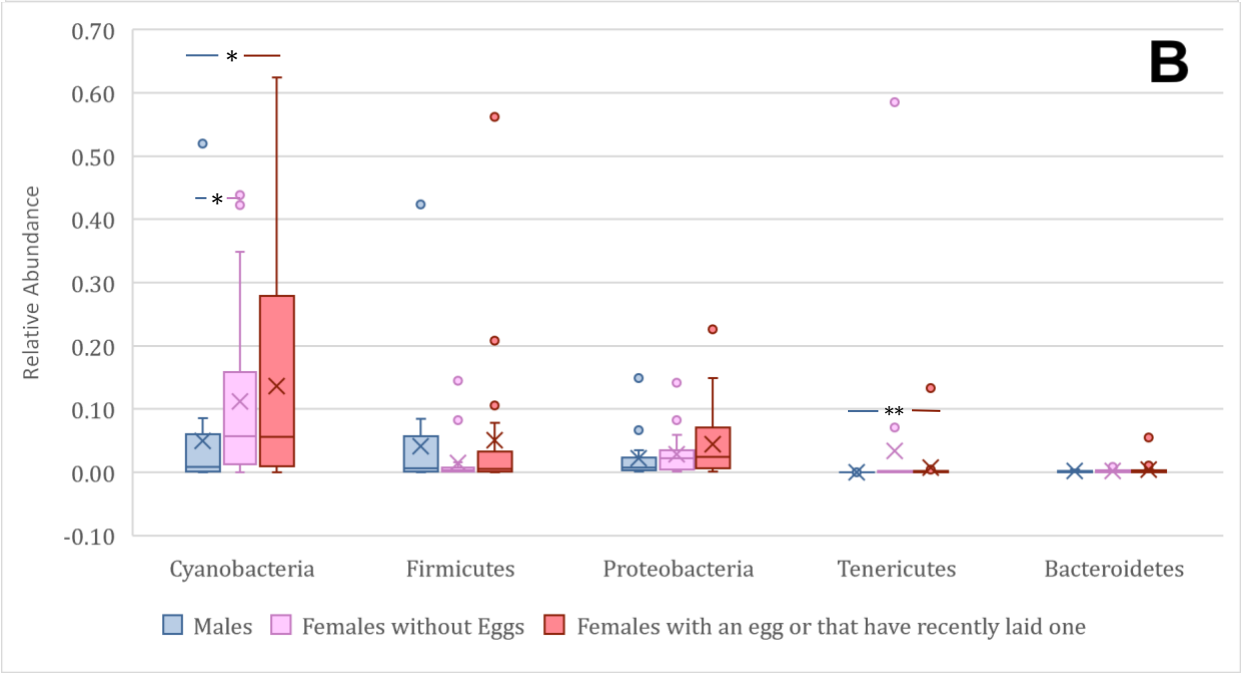


Figure 6. Relative abundances of six most abundant microbial phyla and other microbes in *C. anna* and *S. rufus* from Summer 2018 through Summer 2019.

Table 1. Frequency and median and IQR of relative abundances of phyla represented by amplicon sequencing in all 285 samples and controls.

Phylum	Instance of Amplicon (count of samples with nonzero relative abundance/total sample count)	Median	IQR1-IQR3
Actinobacteria	1.000	0.814	(0.458-0.935)
Cyanobacteria	0.926	0.027	(0.003-0.080)
Firmicutes	0.996	0.005	(0.001-0.076)
Proteobacteria	1.000	0.036	(0.009-0.150)
Tenericutes	0.618	0.000	(0.000-0.000)
Bacteroidetes	0.832	0.001	(0.000-0.008)
Acidobacteria	0.270	0.000	(0.000-0.000)
Other	0.986	0.001	(0.001-0.002)
Planctomycetes	0.253	0.000	(0.000-0.000)
Verrucomicrobia	0.309	0.000	(0.000-0.000)


A
Relative Abundance
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
**
Actinobacteria
Males
Females without Eggs
Females with an egg or that have recently laid one


B
Relative Abundance
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
*
*
**
Cyanobacteria
Firmicutes
Proteobacteria
Tenericutes
Bacteroidetes
Males
Females without Eggs
Females with an egg or that have recently laid one

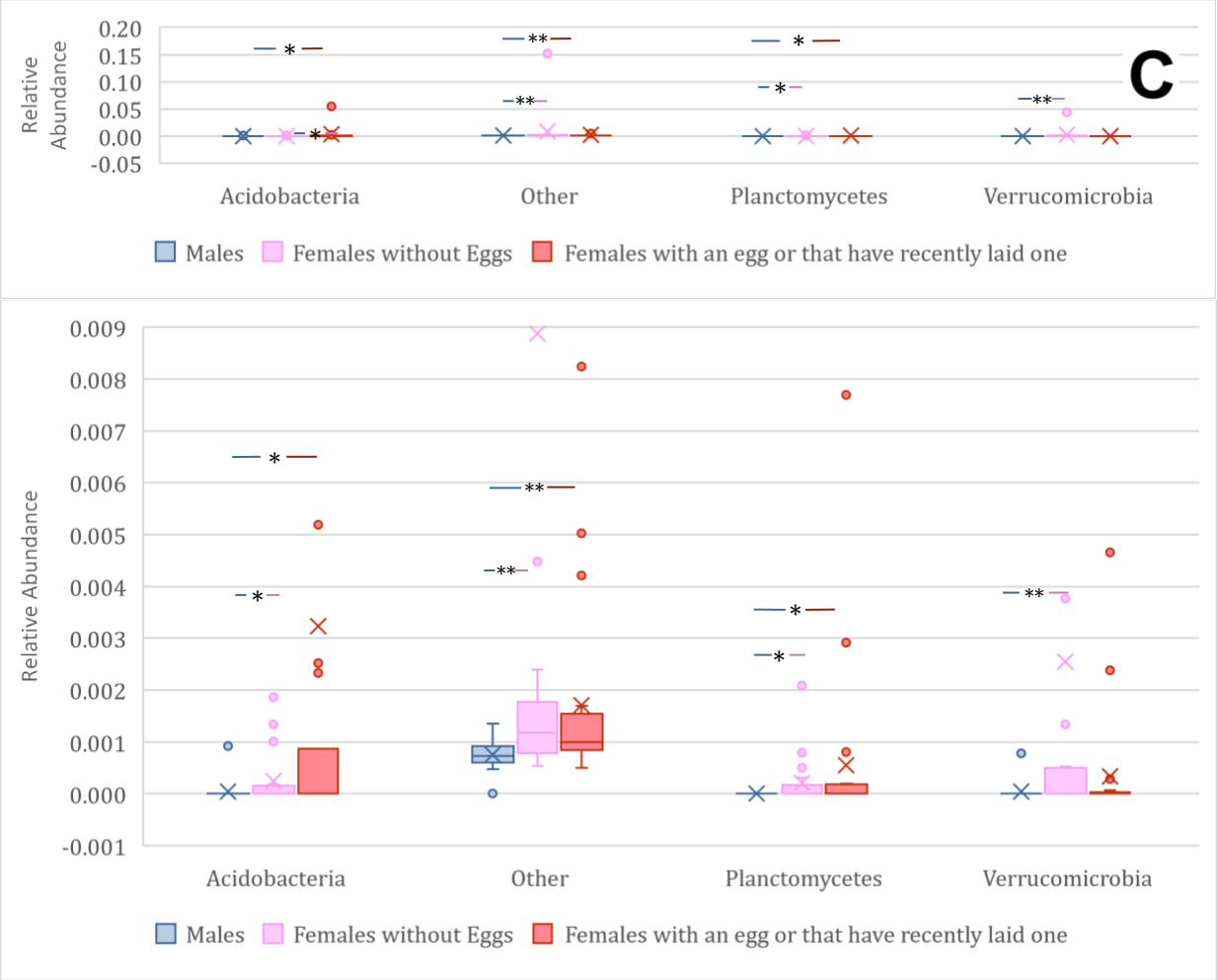


Fig. 7. Prominent phyla differ in relative abundance in adult male *S. rufus* and egg producing adult female *S. rufus* as well as female *S. rufus* not producing an egg, respectively. Relative abundance median and IQR by phylum and cohort condition. Panels, as well as the inset in (**C**) are divided to provide more clarity in relative abundances of less abundant phyla. Wilcoxon ranked sums test was applied. * - $p<0.05$, ** - $p<0.01$. All significant results have $0.37<r<0.46$, and non-significant results have $0.004<r<0.26$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range. Males: n = 19, females with egg status score two: n = 20, females with egg status score six to nine: n=22. ES is egg status score.

Table 2. Median and IQR of relative abundances of ten most abundant phyla in breeding *S. rufus.* The proportion of samples with representative taxa to all samples is included to indicate pervasiveness of the taxon in *S. rufus*.

Phylum	Statistical Parameter	Breeding Male *S. rufus*	Female *S. rufus* without an egg	Female *S. rufus* with an egg
Actinobacteria	Instance of Amplicon	1.000	1	1.000
	Median	9.23E-01	8.96E-01	8.65E-01
	1st Quartile	8.89E-01	6.71E-01	5.98E-01
	3rd Quartile	9.79E-01	9.32E-01	9.06E-01
Cyanobacteria	Instance of Amplicon	0.947368421	1	1
	Median	8.27E-03	5.66E-02	5.57E-02
	1st Quartile	1.05E-03	1.30E-02	9.87E-03
	3rd Quartile	5.98E-02	1.58E-01	2.79E-01
Firmicutes	Instance of Amplicon	1	1	1
	Median	5.83E-03	3.33E-03	4.68E-03
	1st Quartile	1.24E-03	9.38E-04	1.02E-03
	3rd Quartile	5.68E-02	7.40E-03	3.29E-02
Proteobacteria	Instance of Amplicon	1	1	1
	Median	7.28E-03	2.25E-02	2.41E-02
	1st Quartile	2.62E-03	4.08E-03	5.98E-03
	3rd Quartile	2.33E-02	3.52E-02	7.02E-02
Tenericutes	Instance of Amplicon	0.947368421	9.00E-01	9.55E-01
	Median	3.12E-05	1.23E-04	5.99E-05
	1st Quartile	1.59E-05	1.69E-05	2.97E-05
	3rd Quartile	4.51E-05	1.79E-03	1.59E-03
Bacteroidetes	Instance of Amplicon	1.000	0.85	0.818
	Median	4.18E-04	6.65E-04	8.03E-04
	1st Quartile	6.73E-05	1.11E-04	3.22E-05
	3rd Quartile	1.85E-03	3.33E-03	3.19E-03
Acidobacteria	Instance of Amplicon	0.052631579	0.4	0.363636364
	Median	0.00E+00	0.00E+00	0.00E+00
	1st Quartile	0.00E+00	0.00E+00	0.00E+00
	3rd Quartile	0.00E+00	1.53E-04	8.62E-04

Other	Instance of Amplicon	0.947368421	1	1
	Median	7.28E-04	1.18E-03	9.97E-04
	1st Quartile	6.00E-04	7.81E-04	8.44E-04
	3rd Quartile	9.14E-04	1.78E-03	1.54E-03
Planctomycetes	Instance of Amplicon	0.052631579	0.35	0.363636364
	Median	0.00E+00	0.00E+00	0.00E+00
	1st Quartile	0.00E+00	0.00E+00	0.00E+00
	3rd Quartile	0.00E+00	1.69E-04	1.78E-04
Verrucomicrobia	Instance of Amplicon	0.105263158	5.50E-01	3.18E-01
	Median	0.00E+00	8.35E-06	0.00E+00
	1st Quartile	0.00E+00	0.00E+00	0.00E+00
	3rd Quartile	0.00E+00	4.95E-04	3.11E-05

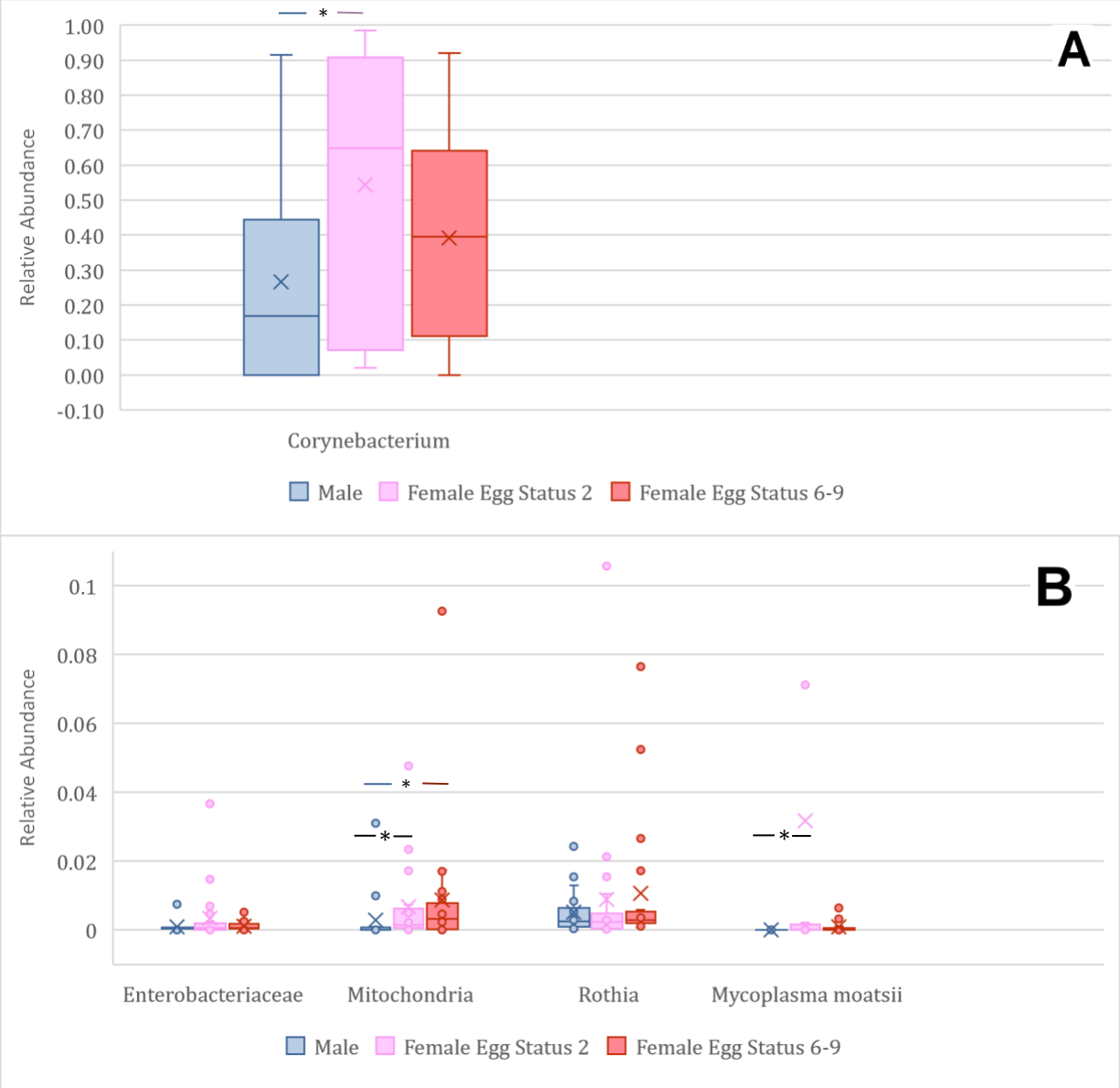


Figure 8. Unclassified Mitochondrion and *M. moatsii* were significantly more abundant in *S. rufus* females with egg status score of two in comparison with adult male *S. rufus*. Relative abundance median and IQR by genus and cohort condition. Wilcoxon ranked sums test was applied. * - $p<0.01$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range Males: n = 19, females with egg status score two: n = 20, females with egg status score six to nine: n=22.

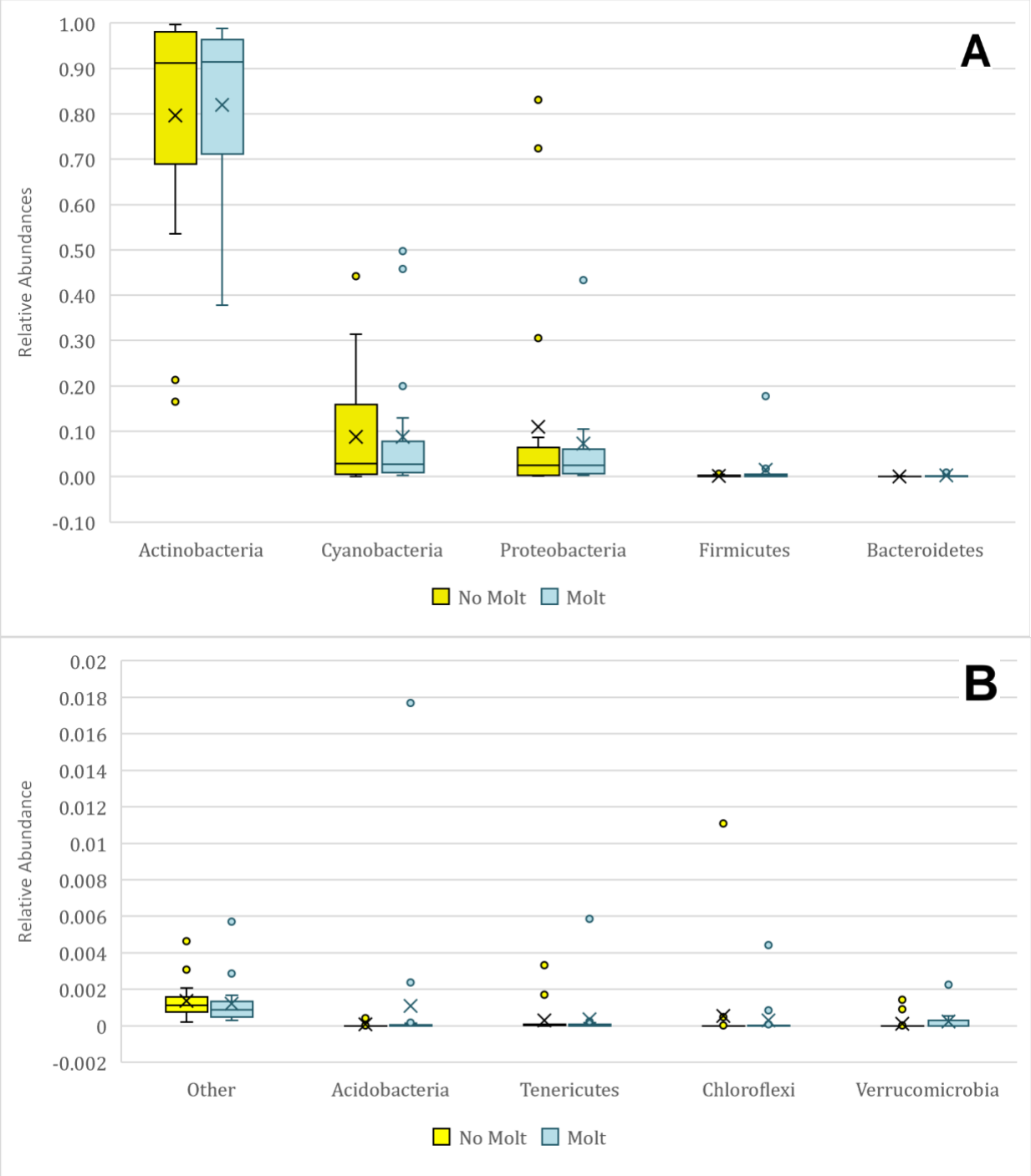
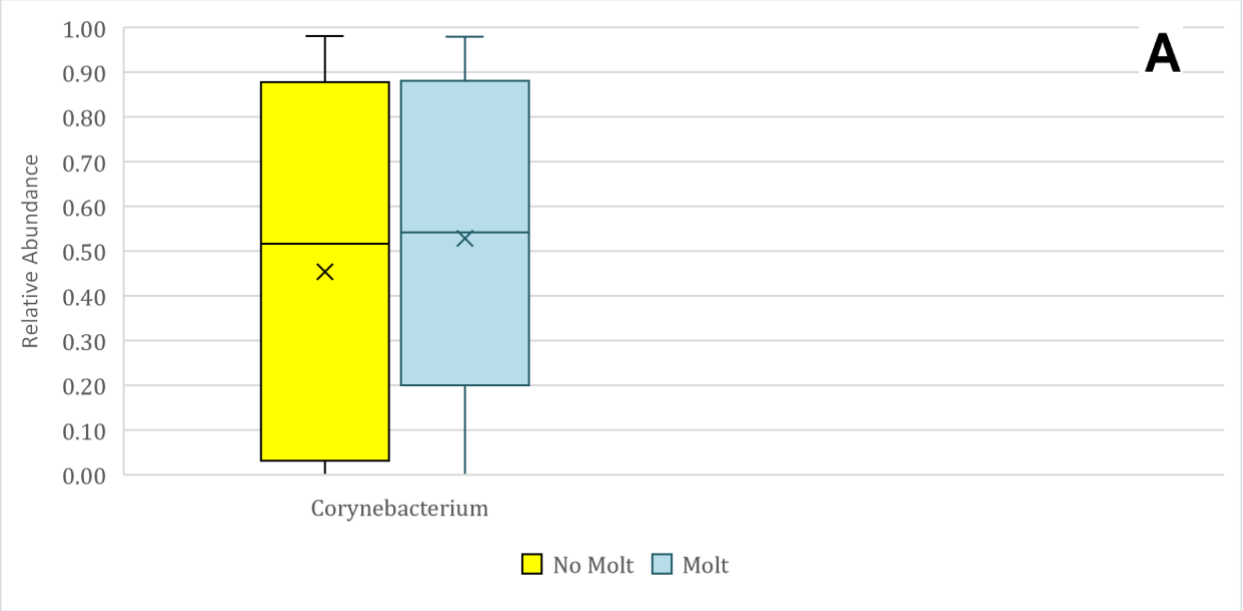


Fig. 9. Relative abundances of ten most populous phyla do not differ in molting and non-molting *C. anna*. Median and IQR boxplot of abundances of the first five (**A**) and sixth through tenth (**B**) most populous phyla in these two cohorts. Mann-Whitney ranked sums test was applied; all p-values > 0.05. $0.01<r<0.30$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range. Molting n = 21, nonmolting n = 21. Birds were placed in the molt category if they demonstrated molt on their bodies and wings. They were placed in the no molt category if they had no signs of molt on their bodies, wings, or tails.



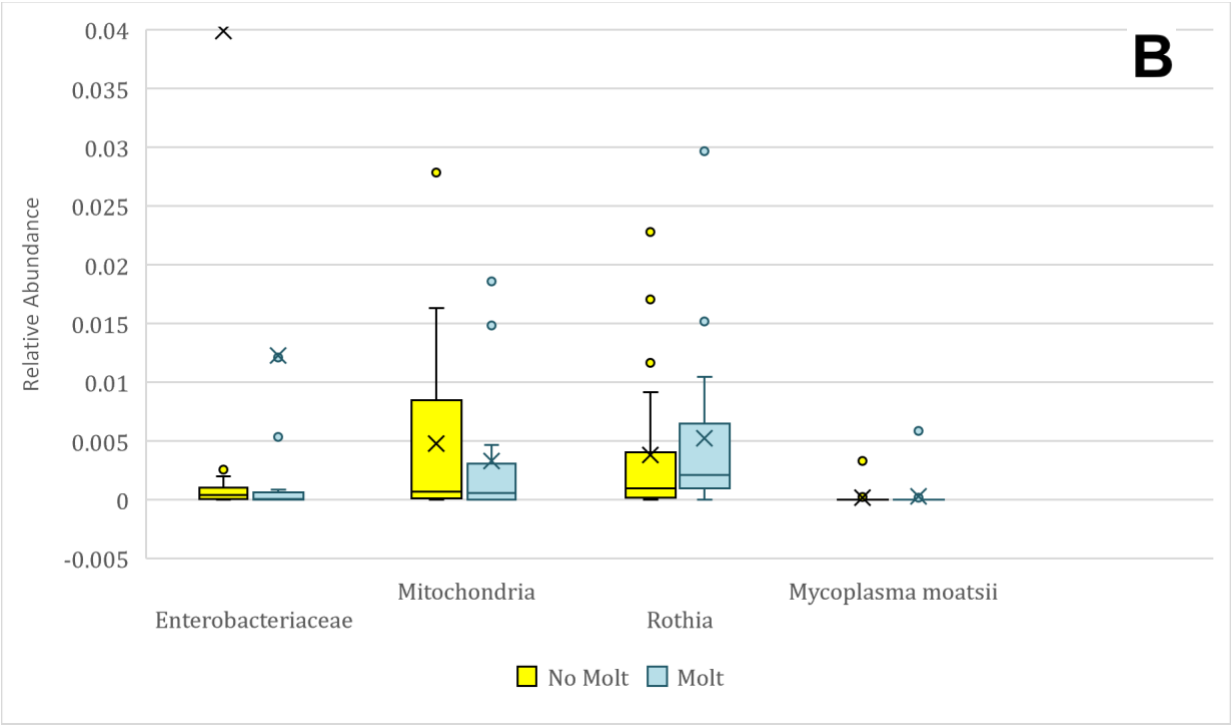


Figure 10. No genus was significantly different in abundance in comparisons of molting *C. anna* microbiota with nonmolting *C. anna*. Relative abundance median and IQR by genus and cohort condition. Wilcoxon ranked sums test was applied. * - $p<0.01$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range. Molting *C. anna*: n = 21, nonmolting *C. anna*: n = 21. Birds were placed in the molt category if they demonstrated molt on their bodies and wings. They were placed in the no molt category if they had no signs of molt on their bodies, wings, or tails.

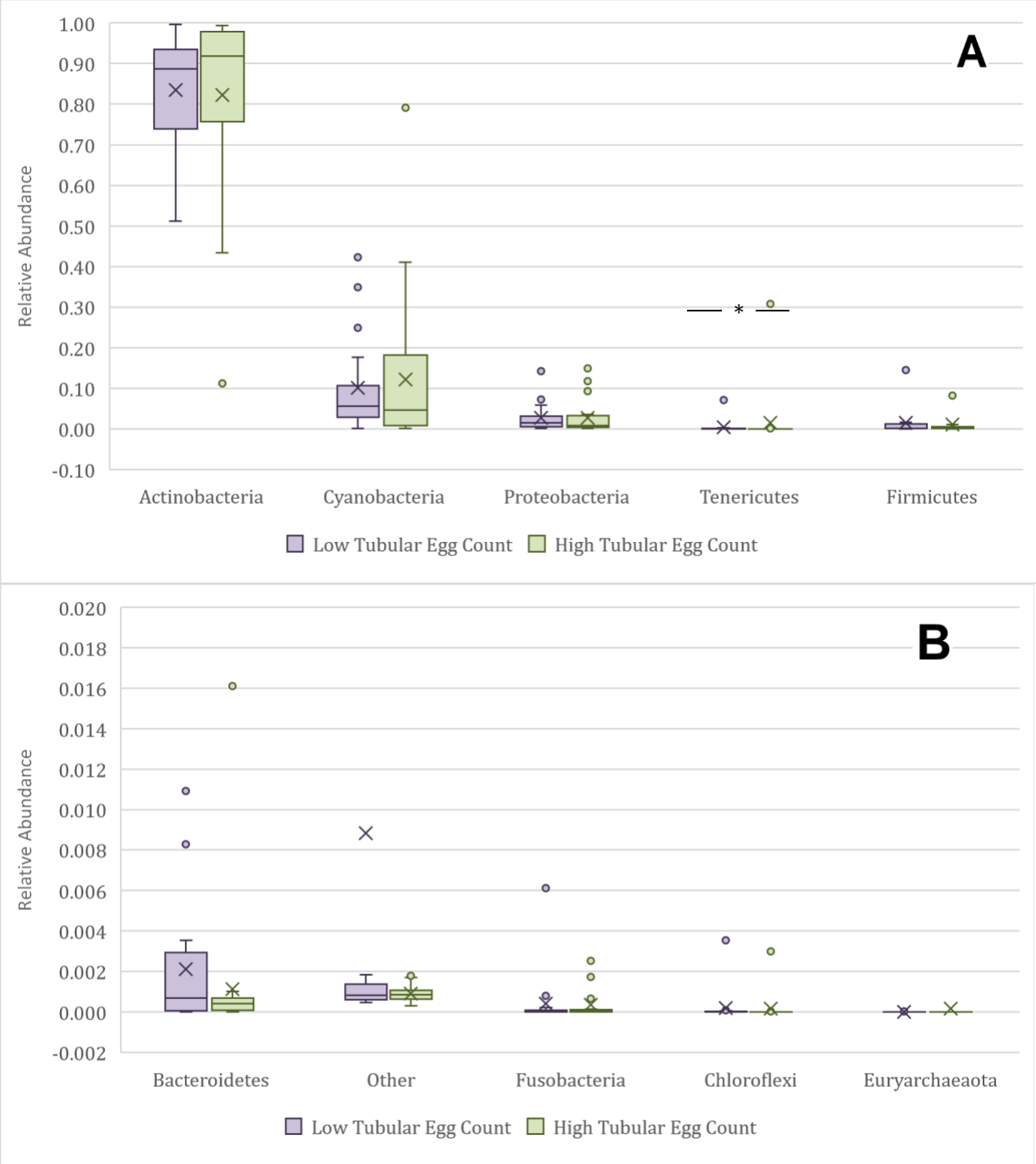


Fig. 11. Relative abundances of Tenericutes, the fourth most populous phylum in these cohorts, are distinct in *S. rufus* with low and high *Mallophaga* tubular egg counts. Median and IQR boxplot of relative abundances of the first five (**A**) and sixth through tenth (**B**) most abundant phyla by bird cohort. Mann-Whitney ranked sums test was applied. *-$p<0.05$. $r=0.32$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range. Low tubular egg *S. rufus*: n = 22, high tubular egg *S. rufus*: n = 21. Birds were binned in the "low" category if they carried one tubular egg, and birds were binned in the "high" category if they carried more than five.

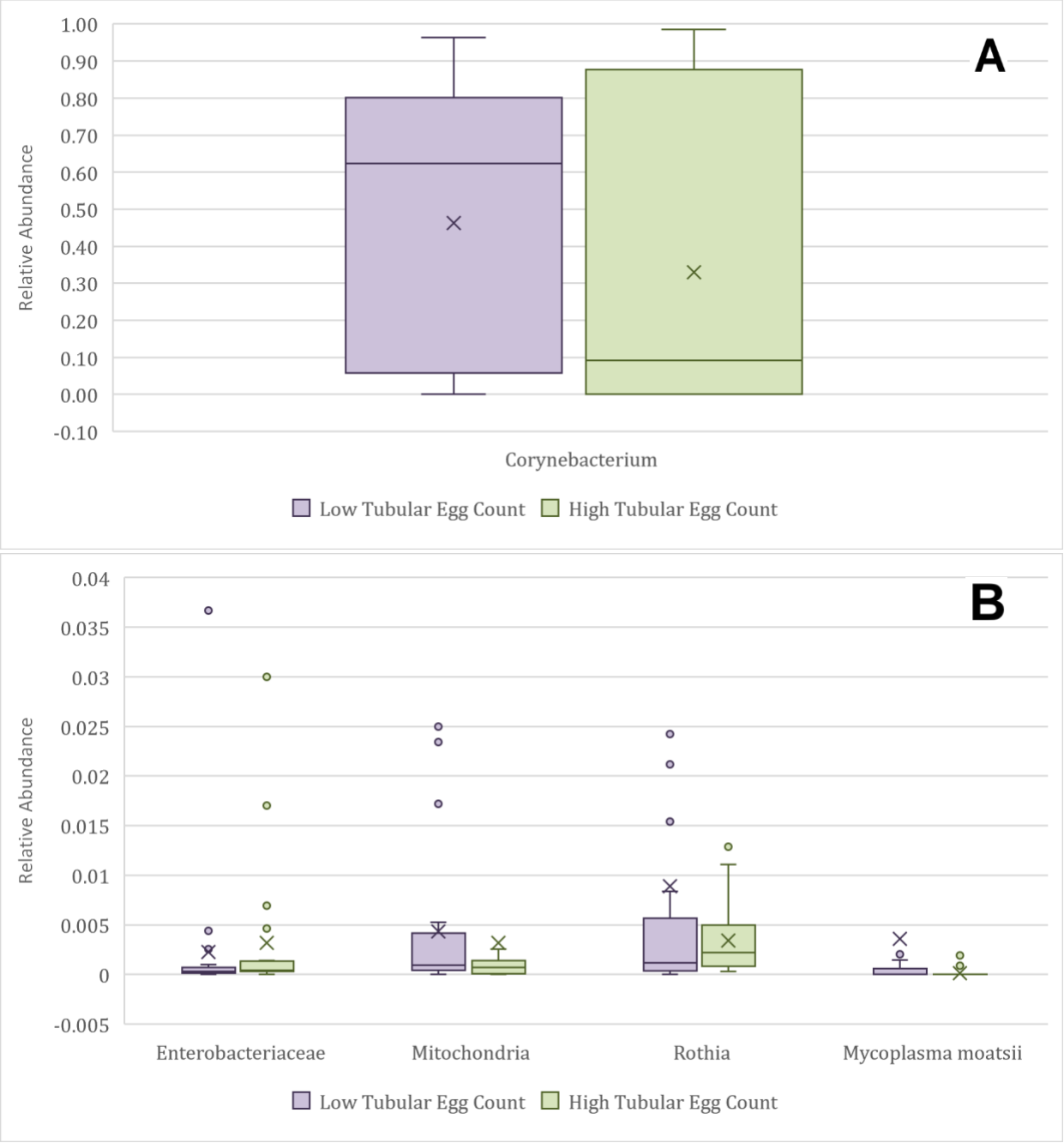


Figure 12. No genus was significantly more or less abundant in comparisons of differentially parasitized *S. rufus*. Relative abundance median and IQR by genus and cohort condition. Wilcoxon ranked sums test was applied. * - $p<0.01$. X indicates the mean, the line represents the median in each phylum, and points represent data from samples with relative abundances less than the lowest value in the first quartile range or higher than the highest datum the fourth quartile range Low tubular egg *S. rufus*: n = 22, high tubular egg *S. rufus*: n = 21. Birds were binned in the "low" category if they carried one tubular egg, and birds were binned in the "high" category if they carried more than five.