Prevalence, Genotype Richness, and Coinfection Patterns of Hemotropic Mycoplasmas in Raccoons (Procyon lotor) in Environmentally Protected and Urbanized Barrier Islands

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Running Title: Hemoplasmas in Raccoons.
ABSTRACT

Raccoons (Procyon lotor) are successful urban adapters and hosts to a number of zoonotic and non-zoonotic pathogens, yet little is known about their hemoplasma infections and how prevalence differs across habitat types. This study identifies hemotropic Mycoplasma spp. infection in raccoons from urban and undisturbed habitats and compares hemoplasma infection in sympatric urban cats (Felis catus) from the same geographic region. We collected blood from raccoons (n=95) in an urban (n=37) and an undisturbed (n=58) coastal island and from sympatric urban cats (n=39) in Georgia, USA. Based on 16S rRNA gene amplification, 62.1% (59/95) of raccoons and 17.9% (7/39) of feral cats were positive for hemoplasma. There was a greater percentage of hemoplasma-infected raccoons on the undisturbed island (79.3%, 46/58) than on the urban island (35.1%, 13/37; X²=16.9, df=1, p=0.00004). Sequencing of the full-length 16S rRNA gene amplicons revealed six hemoplasma genotypes in raccoons, including five novel genotypes that were distinct from three known hemoplasma species identified in the sympatric cats. In addition, the hemoplasma genotypes detected in raccoons were not identified in sympatric cats or vice versa. Although all six hemoplasma genotypes were found in raccoons from urban and undisturbed islands, co-infection patterns differed between sites and among individuals, with the proportion of coinfected raccoons greater in the undisturbed site. This study shows that raccoons are hosts for several novel hemoplasmas and that habitat type influences infection patterns.

Keywords: raccoons, Procyon lotor, feral cats, Felis catus, hemoplasmas, wildlife, the 16S rRNA gene, phylogenetic analysis.
Importance

This study provides information about novel hemoplasmas identified in raccoons (*Procyon lotor*), which can be used for the assessment of the prevalence of these hemoplasmas in raccoon populations and for future studies on potential pathogenic impacts of these hemoplasmas on raccoon health. Raccoons from the undisturbed habitat had higher prevalence of hemoplasma infection than urban raccoons. There does not appear to be cross-species transmission of hemotropic mycoplasmas between urban raccoons and feral cats. Raccoons appear to be hosts for several novel hemoplasmas and habitat type influences infection patterns.
INTRODUCTION

Urbanization is a strong ecological driver that causes significant changes in the composition of wildlife communities and in various intra- and inter-species interactions (1). Ecological responses to urbanization can lead to changes in the dynamics of wildlife-parasite interactions and in patterns of pathogen transmission through mechanisms such as loss of species diversity, changes in vector abundance, and increased exposure to invasive species and their pathogens (2). Urban-adapted wildlife frequently share anthropogenic food sources with other species and can live in close proximity to other wild and feral/domestic animals (3). Resource use overlap in urbanized environments can increase direct and indirect contact within species and facilitate cross-species transmission of pathogens between wildlife and animals such as feral cats, while providing opportunities for pathogens to adapt to novel host species and expand their host range (2, 4, 5). In addition, smaller home range sizes, increased aggregation, and high population densities of some wild animals in urban areas (3) can further increase opportunities for contact between species and potentially increase cross-species pathogen transmission.

The raccoon (Procyon lotor) is well-known for its adaptability to urbanized habitats and active interaction with domestic animals such as cats and dogs (6). In urbanized habitats, raccoons and feral cats (Felis catus) are often highly abundant, frequently foraging in close proximity to one another on clumped anthropogenic food sources (e.g. garbage, intentional feeding). Raccoons also harbor diverse pathogens shared with or transmitted to other domestic and/or wild animals, such as canine distemper, parvovirus, rabies, and Leptospira (6). Here, we use raccoons from urban and undisturbed environments and urban feral cats as a study system to compare and investigate hemotropic Mycoplasma species (so-called genotypes here) composition, richness, coinfection patterns, and potential cross-species transmission.

Hemoplasmas (the common name for hemotropic Mycoplasma species) are facultative intracellular erythrocytic parasites without cell walls comprising a group of non-cultivable Mycoplasma species, including organisms formally known as Haemobartonella and Eperythrozoon species, and reclassified as Mycoplasma species based on phylogenetic analysis of their 16S rRNA gene sequences and deeper studies on cell morphologic properties (7-11). Hemoplasmas are causative agents of acute or chronic infectious anemias in several mammalian species (7, 12, 13). Human infections with hemotropic mycoplasmas and potential zoonotic transmission of these organisms have also been reported (14-16).
with hemotropic mycoplasmas is usually self-limiting and well controlled by immunocompetent animals; however, the establishment of clinically inapparent chronic bacteremia is possible in stressed, immunosuppressed, immunocompromised, and immunocompetent individuals (7, 14, 17). The main hematological observation in hemoplasma-infected animals is mild or severe anemia, and positive Coombs tests, but infections can occur with or without alterations of hematological parameters (13, 18). The intracellular life cycle of some hemotropic mycoplasmas may explain the chronicity of hemotropic mycoplasma infections in their natural hosts (19).

Several hemoplasma species have been reported from wild carnivores, including Darwin’s foxes (Lycalopex fulvipes), black bears (Ursus thibetanus japonicus), Namibian cheetahs (Acinonyx jubatus), Iriomote cats (Prionailurus bengalensis iriomotensis), California sea lions (Zalophus californianus), Japanese badgers (Meles meles anakuma), raccoon dogs (Nyctereutes procyonoides viverrinus), and Asian mongooses (Herpestes javanicus) (18, 20-25). However, hemoplasma infection of raccoons (Procyon lotor) is poorly studied (26). In 1971, Haemobartonella procyoni was described in raccoons from Maryland, USA (27). The morphology of the parasite resembled Mycoplasma haemomuris (former Haemobartonella muris), and the microorganism was found in association with the surface of host erythrocytes. The naturally infected raccoons did not have any clinical signs or hematological abnormalities, although infection prevalence was approximately 50% and parasitemia persisted for 60 days of observation (27).

The objectives of this study were (i) to identify hemotropic mycoplasmas in raccoons and compare infection prevalence, genotype richness, and co-infection patterns in raccoons in an urban and an undisturbed barrier island, and (ii) to evaluate the possibility of cross-species transmission of hemoplasmas between urban raccoons and sympatric feral cats.

RESULTS

PCR amplification of hemoplasma sequences from blood samples

Primary screening using the HBT-F and HBT-R primers for amplification of the 16S rRNA gene sequences demonstrated that 62.1% (59/95) of raccoons were PCR-positive for hemoplasma DNA. These previously published universal primers amplified the partial 16S rRNA gene of Mycoplasma spp. and were successfully used for differentiating hemoplasma-positive and hemoplasma-negative animals. In 2012 the ICSP Subcommittee on the taxonomy of Mollicutes agreed the
recommendation to require the full-length 16S rRNA gene sequences in papers describing new hemoplasmas (45). Based on this recommendation, we generated the full-length 16S rRNA gene sequences and used them for construction of our phylogenetic trees. To amplify the full-length 16S rRNA genes of hemoplasmas present in blood samples of raccoons and cats, we designed new primers based on available sequences in GenBank (see Materials and Methods). Using these new primers for eight individual 16S rRNA-based PCRs (16S-PCR-1 through 16S-PCR-8), we successfully amplified the individual full-length rRNA genes of each hemoplasma present in the raccoon blood DNA samples (see Table 1). Five of these eight primer pairs produced the full-length 16S rRNA amplicons from hemoplasma-positive raccoons (16S-PCR sets 1, 2, 3, 5, and 8; see Table 1). In contrast, only three primer pairs amplified the full-length 16S rRNA amplicons from hemoplasma-positive cats (16S-PCR sets 5, 6 and 7), i.e., the primers designed for *M. haemofelis*, *Candidatus* *M. turicensis*, and *Candidatus* *M. haemominutum*, respectively. The 16S primer pairs 6 and 7 did not generate any amplicons from hemoplasma-positive raccoons. The 16S primer pair 5, which was designed to amplify the full-length 16S rRNA genes of both *M. haemofelis* and *M. haemocanis*, was the only set that worked with both raccoons and cats. The 16S primer pair 4, which was designed to amplify the full-length 16S rRNA gene of *M. suis*, did not generate any amplicons from either raccoons or cats.

Based on our full-length 16S rRNA PCR analyses, only 54.7% of raccoons (vs. 62.1% with the HBT-F and HBT-R primers) were hemoplasma-positive. The difference (54.7% vs. 62.1%) between our new primers and HBT-F/R primers can be attributed to (i) the difference in the length of amplicons (1400-1460 bp vs. 595-620 bp), (ii) the possible difference in hemoplasma DNA load in blood among the raccoons which could affect the efficiency of amplification of the full-length 16S rRNA sequences, (iii) the degree of optimization of our PCR conditions for these new primers, and/or a combination of these factors.

Six hemotropic mycoplasma sequences (genotypes) with partial identity with the 16S rRNA gene sequences of known hemoplasma sequences (available in GenBank) were detected in raccoon populations (see Table 1 and Table S2). Except for two *M. haemocanis/M. haemofelis*-like raccoon hemoplasmas (called raccoon hemoplasma genotypes 1 and 5), which had 96-97% nucleotide sequence identity with each other in their 16S rRNA genes, the other hemoplasma sequences...
(genotypes 2-4, 6) detected in raccoons demonstrated lower levels of genetic similarity (≤86-96%) among these genotypes and to other known hemoplasma species (Table 1 and Table S2).

Raccoon hemoplasma genotypes 1 and 5 were amplified from independent raccoon blood samples using the primers designed to amplify the full-length 16S rRNA gene of *M. haemocanis/M. haemofelis*. Genotype 5 (GenBank accession reference number KF743706) had only 96-97% nucleotide sequence identity with *M. haemocanis/M. haemofelis* and genotype 1, whereas, genotype 1 (GenBank accession reference number KF743705) had 99% nucleotide similarity to *M. haemocanis/M. haemofelis* (Table 1 and Table S2). When amplification from the *rpoB* and *gyrB* genes was attempted on all the raccoon samples positive for these two *M. haemocanis/M. haemofelis*-like hemoplasma genotypes (see Table S1; rpoB-PCR-A, rpoB-PCR-B, and rpoB-PCR-D primers sets), the amplification from the *rpoB* gene was unsuccessful for the both genotypes of *M. haemocanis/M. haemofelis*-like raccoon hemoplasmas and amplification from the *gyrB* gene was only possible for genotype 1 but not from genotype 5. Sequence analysis of the amplified *gyrB* genes (GenBank accession reference numbers KF743740-KF743744) and their deduced protein sequences demonstrated low (73-75% nucleotide and 87-88% amino acid) identity with both *M. haemocanis* and *M. haemofelis*. Thus, these *M. haemocanis/M. haemofelis*-like spp. (genotypes 1 and 5) detected in raccoon blood samples were unlikely to belong to the known species *M. haemocanis* or *M. haemofelis* but were closely related to them phylogenetically. All attempts to amplify *rpoB* gene sequences from the other raccoon hemoplasma-positive samples using previously published primers designed for *rpoB* of *Mycoplasma* spp. (36) failed to yield amplicons.

In feral cats on St. Simons island, *M. haemofelis* (n=1), *Candidatus* M. haemominutum (n=5), and *Candidatus* M. turicensis (n=1) were identified by the full-length 16S rRNA gene amplification and sequencing; however, these species were not detected in raccoons. The presence of *M. haemofelis* and *Candidatus* M. haemominutum in cat blood was also confirmed by amplification and sequencing of their partial *rpoB* genes (GenBank accession reference numbers KF743746-KF743751).

**Phylogenetic analysis of the 16S rRNA genes**
We used sequences of the 16S rRNA genes to determine the phylogenetic relatedness of the hemoplasmas (genotypes) detected in raccoons with those of other known hemotropic Mycoplasma species available in GenBank (Figure 1). No chimeras were detected from all 16S rRNA gene sequences generated in this study. The dendrogram in Figure 1 shows the inferred phylogenetic position of the hemoplasma sequences identified in raccoons among known hemotropic Mycoplasma species. The interspecies similarity of the 16S rRNA genes among the species in the phylogenetic tree was also assessed (Table S2). The 16S rRNA-based phylogenetic analysis and the interspecies similarity data showed that the raccoon hemoplasma genotypes were phylogenetically related to known hemoplasma species, i.e. *M. haemocanis, M. haemofelis, Candidatus M. haemobos, Candidatus M. wenyonii, M. ovis, Candidatus M. haemocervae, Candidatus M. erythrocervae, Candidatus M. erythrodidelphis, Candidatus M. haemolamae, Candidatus M. haemozalophi, and Candidatus M. kahanei* (see Figure 1). However, based on the low levels (i.e. ≤97%) of sequence identity (36, 46) for five of the six raccoon hemoplasma genotypes (genotypes 2-6), we believe these five genotypes represent novel hemoplasma genotypes or putatively new hemoplasma species (see Figure 1 and Table 1) not yet described in other animal species.

The presence of regions of the low interspecies sequence similarity in the 16S rRNA genes among these five novel raccoon hemoplasma genotypes (genotypes 2-6) and the 16S rRNA genes of other hemoplasmas available in GenBank allowed us to design the species-specific PCR primers (Figures S3-S7 show detailed sequence comparisons) that can be used for qualitative detection of each hemoplasma genotype. In the current study, we used these species-specific 16S rRNA primers to demonstrate selective amplification of each hemoplasma genotype in raccoon samples that were coinfected with different hemoplasma genotypes. No cross-amplification between raccoon hemoplasma genotypes, false-positive, or false-negative amplifications were observed for these species-specific primers when used in PCR assays on DNA from all hemoplasma-positive and hemoplasma-negative raccoon blood DNA samples.

**Prevalence of hemoplasmas in the studied animals: urban vs undisturbed ecosystem and coinfection**

The proportion of raccoons infected with hemotropic Mycoplasma spp. was 62.1% (95% CI; 51.5, 71.7, n=95) overall, 35.1% (95% CI; 20.7, 52.6, N=37) on St. Simons Island (developed habitat), and 79.3% (95% CI; 66.3, 88.4, n=58) on St. Catherines Island (undisturbed habitat). Hemoplasma infection prevalence was 17.9% (95% CI; 8.1, 34.1, n=39) in feral
cats from St. Simons. Overall, infection rates with hemoplasma were 44.4% (95% CI; 28.3, 61.7, n=36) in female raccoons and 72.9% (95% CI; 59.5, 83.3, n=59) in male raccoons.

In univariate analyses, habitat and sex were significantly associated with hemoplasma infection in raccoons (habitat type: $X^2=16.9$, df=1, $p=0.00004$; sex: $X^2=6.52$, df=1, $p=0.01$); hemoplasma infection prevalence was greater in male raccoons and on the undisturbed island. We also found a significant association between hemoplasma infection and body mass (Mann-Whitney U test, $W=732$, $p=0.01$), although habitat type and body mass were not correlated (Mann-Whitney U test, $W=927.5$, $p=0.27$). We found no significant association between host species (raccoon or feral cat) and hemoplasma infection on the urbanized island ($X^2=2.89$, df=1, $p=0.09$). The best-fit GLM associated with raccoon hemoplasma infection included weight, habitat, and the interaction between weight and habitat (Table S3). When the weight*urbanized habitat interaction was accounted for, the urbanized habitat was less likely to be associated with hemoplasma infection (OR=-0.34, $p=0.02$) than the undisturbed habitat. Specifically, heavier raccoons had a greater odds of hemoplasma infection on the undisturbed island (OR=1.3) but had a lower odds of infection on the urban island (OR=0.21) (Figure S1).

Coinfection of raccoons with multiple hemoplasma genotypes was observed at both sampling sites: undisturbed habitat coinfection prevalence was 87% (40/46) and in the urban habitat was 53.8% (7/13). Raccoons from the undisturbed habitat had a significantly higher number of individuals coinfected with more than one hemoplasma genotype ($X^2=52.989$, df=1, $p<0.00001$). The single infection to co-infection ratio for the urban habitat was 1/1.2 and was 1/5.7 for the undisturbed habitat. Hemoplasma genotype richness and infection patterns in raccoons are shown in Table 2. At the population level, there was no difference in overall hemoplasma genotype richness (both sites had a total richness of 6 hemoplasma genotypes identified). However, at the individual level, raccoons on St. Simons Island (urban) had lower hemoplasma genotype richness than those on St. Catherines Island (undisturbed) (Kruskal-Wallace test, $X^2=24.03$, df=1, $p<0.0001$; GLM, $z=-5.88$, df=1, $p<0.0001$).

The mean number of hemoplasma genotypes identified per infected raccoon was 1.85 (range 1-4) on the urbanized island and 2.93 (range 1-6) on the undisturbed island. There was a slight positive but non-significant correlation between hemoplasma genotype richness and individual raccoon body weight (Spearman rank correlation, $\rho=0.25$, $p=0.06$). Although raccoons at both sites had co-infections ranging from 2-4 hemoplasma genotypes, co-infection with 5-6 genotypes...
was only seen in raccoons from the undisturbed site (Figure S2). We also observed variable composition of hemoplasma genotypes among raccoons; coinfections with two to three hemoplasma genotypes were relatively evenly distributed. An association plot (Figure 2) showed positive associations for coinfection for raccoon hemoplasma genotypes 2 and 3, 2 and 4, 2 and 5, and 3 and 4. Negative associations of co-infection were seen between genotypes 2 and 6, as well as *M. haemocanis/M. haemofelis-like sp.* (or genotype 1) and genotype 2, and between *M. haemocanis/M. haemofelis-like sp.* (or genotype 1) and genotype 3.

**DISCUSSION**

The genus *Mycoplasma* currently comprises twenty hemotropic mycoplasma species ([https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=2093](https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=2093)), and except the well-established species *M. haemocanis*, *M. haemofelis*, and *M. haemomuris*, all other 17 hemoplasmas have the provisional taxonomic status “*Candidatus*” as they are incompletely yet described prokaryotes (36, 46, 47). Hemoplasmas infect different hosts and are able to attach to, and sometimes intracellularly invade, their erythrocytes (7, 10, 19). Hemoplasmas have not been cultured *in vitro*, and detection of hemotropic mycoplasmas using Romanowsky-Giemsa and/or acridine orange stained blood smears in combination with the PCR amplification of target hemoplasma genes is common laboratory practice for detection of these infections in animals and humans (7, 10, 13, 14, 48-50). However, the examination of stained blood films for hemoplasmas (not performed in our study) has low sensitivity and specificity, and the absence of hemoplasma-like bodies on blood film often does not correlate with PCR results. Hemoplasma concentrations in the blood of animals may also fluctuate during the course of a hemoplasma infection, and the low concentrations may not be detected by microscopy (51), especially in immunocompetent, chronically hemoplasma-infected animals. Therefore, microscopic blood film evaluation is occasionally omitted in multiple published studies on the investigation of hemoplasma infections in domestic or wild animals (20, 21, 52). The reported prevalence of hemoplasma infected animals in a variety of species ranges from 0.5 to 56.7% (13, 18, 20, 31, 50). In this study, the PCR screening using the HBT-F and HBT-R primers revealed that 62.1% of raccoons were hemoplasma infected. The use of these published universal primers allowed us to successfully identify hemoplasma-positive animals, however, the direct sequencing (i.e., without cloning into a plasmid vector prior to the sequencing) of PCR
products from animals coinfected with different mycoplasma genotypes was complicated or impossible due to the presence of the mixed PCR amplicons generated by these universal primers. The primers amplify the partial 16S rRNA of 595-620 bp in size, and the difference in 25 nt was indistinguishable on electrophoresis in 1% agarose gel. Thus, if the primers amplified the 16S fragment from animals coinfected with different mycoplasma genotypes, it was impossible to discriminate them on gel and to obtain a clear sequence of them using the same HBT-F/R primers. To amplify all hemoplasmas (genotypes) we designed and used new primers that allowed us to amplify all genotypes separately and produce clear sequences from direct DNA sequencing of PCR amplicons. The new primers were able to amplify full-length 16S rRNA gene sequences of these hemoplasmas in raccoons in compliance with the recommendation of the Subcommittee on the taxonomy of Mollicutes that in 2012 agreed to require the full-length 16S rRNA gene sequences in papers describing new hemoplasmas (45). The use of full-length 16S rRNA gene sequences for further phylogenetic analysis may be especially necessary to distinguish between closely-related bacterial species, and thus the sequencing of the full-length 16S rRNA gene is desirable and usually required when describing new species (53-55).

After the full-length 16S rRNA sequences for raccoons’ hemoplasmas were determined, HBT-F/R primers were retrospectively analyzed for their matching to the hemoplasma sequences (against all 6 detected genotypes). The reverse primer (HBT-R) matched all genotypes with 100% identity. The forward primer (HBT-F) matched genotypes 1, 5, and 6 with 100% identity; however, one mismatch was present in this region for all sequences of genotype 3 [HBT-F primer sequence: atata\text{G}gcccatatctacg versus the sequence of genotype 3: atata\text{T}ggcccatatctacg]. Two mismatches were present in this region for all sequences of genotype 4 [HBT-F: atata\text{G}gcccatat\text{T}ctacg versus the sequence in genotype 4: atata\text{T}ggcccatat\text{C}ctacg]. Despite the presence of these mismatches between the forward primer and the 16S rRNA sequences of genotypes 3 and 4, we did not observe any negative impact on the results of our qualitative PCR with these universal primers. However, for future studies of hemoplasma infections in raccoons using HBT-F/R primers, we recommend to introducing an ambiguous base (Y = C/T) at position 4 and 14 of HBT-F, which may improve a sensitivity and yield of amplification of target hemoplasmas, especially at low DNA concentrations in tested samples.

New primers for amplification of the full-length 16S rRNA genes allowed us to detect six hemoplasmas (genotypes) in raccoons with partial identity to the 16S rRNA gene sequences of known hemoplasma species. Based on low levels (i.e.
≤97%) of sequence similarity of these hemoplasma genotypes to other described hemoplasmas and the mammalian host in which these genotypes were detected, we believe that five of these six genotypes represent novel hemoplasma genotypes or putatively novel hemoplasma species not yet described in other animal species.

Except for two raccoon hemoplasma genotypes 1 and 5 (M. haemocanis/M. haemofelis-like), which had 96-97% nucleotide sequence identity with each other in their 16S rRNA genes, the other genotypes 2-4, 6 demonstrated genetic similarity of ≤86-96% among these genotypes and to other known hemoplasma species. Only the 16S rRNA gene sequences of genotype 1 demonstrated 99% sequence identity to the 16S rRNA gene of M. haemocanis and M. haemofelis. M. haemocanis and M. haemofelis by themselves also have 99% sequence identity with each other in their 16S rRNA genes and are indistinguishable by the 16S rRNA gene analysis, without the sequencing of additional housekeeping genes, e.g. rpoB, gyrB or others. To deeply identify genotype 1 as either M. haemocanis or M. haemofelis we performed amplification of two housekeeping genes (rpoB and gyrB) for this genotype. The amplification from the rpoB gene was unsuccessful using different M. haemocanis/M. haemofelis rpoB-specific primers, and only gyrB gene was amplified for genotype 1, however, nucleotide sequence analysis of the gyrB gene and the deduced protein sequences demonstrated low similarities to both M. haemocanis and M. haemofelis. Thus, based on our results, we decided that the genotype 1 detected in raccoons is unlikely to belong to the known species M. haemocanis or M. haemofelis, but were closely-related to them phylogenetically.

Phylogenetic studies of the 16S rRNA gene of closely related Mycoplasma species propose to use the arbitrary interspecies sequence similarity value of ≤97% as a minimum level indicating a separate, genetically distant, species (36, 46, 56). Data based on the expanded analysis of the 16S rRNA gene sequences of the species within the family Mycoplasmataceae generally support this proposition (36). Nevertheless, at least 20 pairs of closely related, well-established Mycoplasma species with 16S rRNA gene similarity greater than 97% demonstrated serological, genetic, and ecological features that defined them as individual species, despite the high percentage similarity of their 16S rRNA genes (for details see(36)). Thus, the 16S rRNA sequence identity of any new isolate of ≥98-99% may not be a clear indication that the Mycoplasma species is the same or different. Similar examples exist and are well-known for some other closely related species with identical or nearly identical 16S rRNA sequences (57), e.g. Bacillus and Listeria species (58, 59).
Although about 62.1% of the raccoons in this study were infected with hemoplasmas, they appeared normal upon physical examination. The pathogenicity of the detected hemoplasmas for the raccoons is unknown and should be investigated in the future. From a taxonomic point of view, similar to cases of *Eperythrozoon teganodes* in cattle (45) and *Haemobartonella* sp. in horses (49), the genetic relationship between the previously studied hemoplasma-like species *Haemobartonella procyoni* in raccoons of Maryland (27) and the hemoplasmas detected in raccoons this study is unknown, because there is no known *H. procyoni* genetic material in any national or international collection of microorganisms.

The three species of hemoplasma identified in feral cats on St. Simons Island have been previously detected in blood samples from cats throughout the world, and the overall proportion of infected cats (17.9%) was within the reported prevalence in cats seen worldwide (16, 60-66). The most common hemoplasma species found in cats in our study, *Candidatus* *M. haemominutum*, also appears to be the most common hemoplasma species in cats across many different studies (65, 66). Our study detected no evidence of cross-species hemoplasma transmission between feral cats and raccoons, despite the close proximity of feral cats and raccoons on St. Simons Island.

Interactions between pathogen prevalence, diversity, and anthropogenic disturbance, such as urbanization, can be positive, negative, or neutral, depending on the type of environmental change and how it affects abundance, density, and/or contact within and between host species, other co-infecting pathogens, and environmental influences on host immunity and pathogen susceptibility (67-69). The proportion of hemoplasma-infected raccoons was greater on the undisturbed than the urbanized island, and the proportion of hemoplasma-infected raccoons in both locations was higher than in urban cats. If hemoplasma transmission is density-dependent, an increase in hemoplasma infection in urbanized habitats is expected (70, 71). We did not see evidence of this, because trapping success on the urban island (0.41 animals/trap night) was higher than on the protected island (0.24 animals/trap night). However, we did not perform mark-recapture studies for density estimation. Regardless, in urbanized areas, raccoons may be more likely to enter traps due to greater habituation and differing food preferences. Alternatively, hemoplasma transmission may be frequency dependent, due to bites, scratches, licking (65), and potential vector-borne (flea/tick) transmission. Assuming that hemoplasma infection can be vector-borne in wild raccoons, as has been found in cats (10, 61), habitat related differences in microclimate could influence hemoplasma transmission. The prevalence of tick-borne diseases and tick infestation rates in host species is often higher in natural
habitats than in urban environments (72, 73), related to insufficient host diversity to maintain complex tick life cycles in urbanized areas (74).

The best-fit GLM showed a positive relationship between heavier animals and hemoplasma infection in undisturbed habitats, whereas heavier animals in urban habitats had lower odds of infection. Although associations between sex and hemoplasma infection were male-biased and marginally significant in univariate analyses, sex was not a significant predictor of hemoplasma infection in our best fit GLMs. The positive association between body weight and hemoplasma infection in undisturbed habitat could be related to host age; although we did not determine the age of captured raccoons, larger individuals were likely to be older and may have had increased exposure to ectoparasites (if arthropod-borne transmission is a dominant mode of transmission) or higher ectoparasite infestation. The observation of a positive relationship between weight and hemoplasma positivity in the undisturbed environment could suggest that heavier animals may have greater contact with other infected animals or vectors. For the opposite result in urban habitat, supplemental feeding (garbage and deliberate feral animal feeding by residents) on St. Simons Island could have led to higher tolerance by raccoons toward conspecific animals which may reduce aggressive (75) behavior, reducing the likelihood of fighting and hemoplasma transmission through infected blood or saliva. Higher food availability or quality in urban habitats (if raccoons were being fed nutritionally rich supplements such as cat food) could allow heavier raccoons to mount a more successful immune defense against hemoplasma (76). Additional routes of transmission for hemoplasmas, such as transplacental and transmammary transmission (48), may also influence differences in hemoplasma prevalence, particularly if one population has a different population age structure.

Differences in population management of raccoons between islands may influence the prevalence of hemoplasma infection. Although there are no published data available on the relative population densities of raccoons on these two islands, raccoons are routinely culled on St. Catherines Island to protect damage to sea turtle nests. Culling may inadvertently increase transmission of pathogens with frequency dependent transmission in part by increasing the birth rate, leading to an increase in susceptible individuals in a population (77). However, further study is required to thoroughly understand the epidemiology and mode of hemotropic mycoplasmas in raccoons in order to pinpoint the causes for differences in infection rates between undisturbed and disturbed habitats.
Coinfection with multiple hemoplasmas has been described in humans (16), domestic animals (60, 78, 79), and wildlife (52). However, it remains unknown why patterns of hemoplasma coinfection vary among raccoons and habitats. For instance, negative co-occurrence of some hemoplasmas (Figure S2) may be due to cross-immunity, ecological interference, or differing contact networks (80) between raccoons in different habitat types. One possible explanation for our results is that the protected island offers a greater opportunity for within and cross-species hemoplasma transmission due to contacts with a more diverse host community.

Horizontal transmission of hemotropic mycoplasmas in species other than raccoons has been hypothesized to be potentially associated with blood-feeding arthropod vectors as well as direct transmission via infected blood (e.g., aggressive interactions and injuries related to animal-to-animal contact, and contact with blood) (10, 81-83). All these transmission routes may account for the widespread occurrence of hemoplasmas in the studied raccoon populations and require additional investigation.

To conclude, this study identified novel hemoplasma genotypes in raccoons and provided new molecular tools to detect these species. We identified six hemoplasma genotypes in raccoons that were phylogenetically related to hemoplasma species previously reported in other mammalian hosts (see Figure 1). Five of these six hemoplasmas appear to be novel hemoplasma genotypes (i.e., never previously reported or deposited in GenBank). Future studies should (i) explore the probability of cross-species transmission with additional samples from various sympatric host species and (ii) evaluate the pathogenicity of hemoplasmas in raccoons, particularly using hematological and immunological assays. The potential mechanism of intra- and interspecies transmission of hemoplasmas, and the drivers of its species composition in sympatric host species remain to be elucidated.

**Conclusion:** This study provides information about novel hemoplasmas in raccoons (*Procyon lotor*), which can be used for the assessment of the prevalence of these hemoplasmas in raccoon populations. Raccoons from the undisturbed habitat had higher hemoplasma infection rates than raccoons in a rural habitat. There does not appear to be cross-species transmission of hemotropic mycoplasmas between urban raccoons and feral cats.
MATERIALS AND METHODS

Field Sites and Study Populations

Raccoons were live-trapped on two Georgia coast barrier islands, St. Simons Island (31°9'40"N 81°23'13"W) and St. Catherines Island (31°37'50"N 81°9'36.5"W), which is approximately 50 km north of St. Simons Island. St. Simons Island has complex ecosystems including ocean beach, salt marsh, maritime forest, and freshwater slough. In addition, St. Simons Island is one of the most urbanized Georgia Barrier Islands, with a large resident human population and rapidly increasing residential developments (28). St. Catherines Island also has diverse habitats, including marsh, deciduous, and evergreen forest, palmetto scrub, and open savannah (29). St. Catherines is a protected barrier island for scientific research, with no human development, residential areas, or domestic animals. Adult feral cats were live-trapped on St. Simons Island as a part of spay/neuter program and the physical examination by local veterinarians; no information on sex of these feral cats were recorded.

Sample Collection

Raccoons (n=95) were trapped in spring and summer of 2012 along trapping transects using 20 Tomahawk traps (Tomahawk, Live Trap Company, Tomahawk, Wisconsin, USA). Raccoons were anesthetized by intramuscular injection of ketamine (20 mg/kg, Aveco Co., Fort Dodge, Iowa, USA) mixed with xylazine (4 mg/kg, Mobay Corp., Animal Health Division, Shawnee, Kansas, USA). The body weight of each animal was measured and their general physical health was evaluated by local veterinarians. Approximately 3 ml of blood was collected from all animals (raccoons and cats) by jugular venipuncture into vacuum tubes containing anticoagulant (EDTA) tubes. Blood samples from feral urban cats (n=39) was collected by local veterinarians as part of the physical examination for the St. Simons Island spay/neuter program. Whole blood samples in the EDTA tubes were stored at -20°C until laboratory analysis. In total, we collected 37 and 58 raccoon blood samples from St. Simons Island and St. Catherines Island, respectively. Institutional Animal Care and Use Committee (A2011 03-042-Y2-A2) and Georgia Department of Natural Resources wildlife permits (29-WBH-12-100) were obtained before sampling (30).
DNA Extraction, PCR Amplification, and Sequencing of Amplicons

Total DNA was extracted from 200 µl of blood from each individual using the DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA) or the Quick-gDNA™ MiniPrep kit (Zymo Research Corporation, Orange, California USA) according to the manufacturers’ protocols and with standard clinical PCR laboratory precautions to avoid cross-contamination. DNA samples were stored at −80°C until use.

The primary screening for the presence of hemoplasmas was performed by PCR using previously published HBT-F and HBT-R universal primers for amplification of the partial 16S rRNA hemoplasma genes (31). These primers amplify the 16S rRNA gene region from positions 313–332 to positions 889–908 based on the 16S rRNA gene reference sequence of *M. haemofelis* (AF178677) (31, 32). Based on our *in silico* PCR analysis (32) of these universal primers against the different mycoplasmal 16S rRNA gene sequences available in the GenBank database, it was demonstrated that depending on the target *Mycoplasma* spp., these primers produce PCR fragments with size of 595-620 bp. In addition, these universal primers were successfully used for amplification of the partial 16S rRNA hemoplasma genes in a few published studies (33-35).

A second aliquot of whole blood from each of the hemoplasma positive samples was used to isolate additional DNA for amplification of the full-length 16S rRNA gene, the RNA polymerase beta subunit gene (*rpoB*), and the DNA gyrase subunit B gene (*gyrB*) using PCR primers designed in this study (Table S1). Eight primer pairs were designed to amplify the full-length 16S rRNA genes, three primer sets were designed to amplify part of *rpoB*, and one primer set was designed to amplify part of *gyrB* based on sequences of other known hemoplasma species available at GenBank (Table S1, 16S-PCR-1 through 16S-PCR-8, and PCR-A through PCR-D).

The 16S rRNA amplicons produced were directly sequenced (with and without cloning into a plasmid vector) by Macrogen (https://www.macrogenusa.com), and the *rpoB* and the *gyrB* amplicons were sequenced directly without cloning. Prior to sequencing, PCR amplicons were purified by electrophoresis through 1.5% agarose gels and extracted with the QIAquick Gel Extraction kit (Qiagen). Amplicons were sequenced with the same primers used for PCR amplification and then with internal (walking) primers when needed. Cloned amplicons were produced as described elsewhere (36), and 15-20 clones of the 16S rRNA gene PCR products of each amplicon were sequenced and analyzed.
When the full-length 16S rRNA gene sequences of the hemoplasma genotypes of raccoons were determined, species-specific 16S rRNA primers to selectively amplify each hemoplasma genotype identified in raccoons were designed (see Table S1; 16S-PCR-9 through 16S-PCR-13). We used these species-specific 16S rRNA primers to selectively identify each hemoplasma genotype in blood samples from raccoons coinfected with different hemoplasma genotypes. The selectivity of these primers for each hemoplasma genotype was demonstrated by gel electrophoresis (i.e., the presence of a single amplicon band) and direct sequencing of amplicons.

The amplification mixture for all PCRs (for direct sequencing without cloning) contained 5 µl of 10^x HotStarTaq PCR buffer, 1.5 mM MgCl₂, 200mM dNTP mixture, 1 mM of each primer and 2.5 U HotStarTaq Plus DNA Polymerase (Qiagen) in a final volume of 50 µl including 3 µl of DNA template. The Vent DNA Polymerase Kit (New England Biolabs), which contains high-fidelity thermophilic Vent DNA polymerase, was used for the amplification of PCR products for subsequent cloning and sequencing using plasmid DNA. The absence of PCR inhibitors in isolated blood DNAs was confirmed by PCR amplification of the *Procyon lotor* mitochondrial gene for 16S rRNA as an extraction positive control (37) (with primers F1-Animal and R1-Animal) on each sample and negative (no DNA added) PCR control were run for each PCR assay. DNA of *M. haemocanis* and *M haemominutum* were used as positive controls for PCRs with the primers PCR-A through PCR-D.

All PCR reactions in this study were conducted under the following conditions: a polymerase activation step at 94°C for 5 min (or 15 min for HotStarTaq only) then 40 cycles of 95°C for 30s, 60°C for 60s and 72°C for 60s, and a final extension at 72°C for 10 min. PCR products were detected by electrophoresis through 1% TAE-agarose gels containing ethidium bromide concentrations followed by UV visualization.

**Nucleotide Sequence Accession Numbers**

All DNA sequences from this study were deposited in GenBank under the accession numbers KF743704-KF743751, KC920439-KC920448, and KC936280.
Phylogenetic Analysis

The 16S rRNA sequences determined in this study were compared to those available in the GenBank database using procedures, algorithms, and methods for phylogenetic tree inference as described elsewhere (18, 36). To avoid the potential presence for chimeric sequences or polymerase chain reaction-derived variants in the data set, all hemoplasma 16S rRNA PCR products for phylogenetic analyses were directly amplified from blood DNA samples of raccoons and cats with two different DNA polymerases (HotStarTaq and Vent) and were directly sequenced without cloning (38, 39). All gene sequences prior to the downstream phylogenetic analysis were subjected to the chimeric sequence analysis using DECIPHER (40) and UCHIME (41). All sequences are deposited in GenBank and are publicly available.

Ecological Data Analysis

All statistical analyses were performed using R (http://cran.r-project.org) (42). Descriptive analysis of hemoplasma infection status of animals collected in each habitat type was performed. For univariate analyses, we used Pearson’s Chi-squared tests to compare the frequency of hemoplasma infection in raccoons in urban and undisturbed environments, raccoon sex and hemoplasma infection, raccoon hemoplasma coinfection and habitat type, and hemoplasma infection in urban cats and raccoons. Additional univariate analyses included a Mann-Whitney U test to evaluate differences between habitat type and raccoon body mass, hemoplasma infection and body mass, and habitat type and body mass. A Kruskal Wallace test and a generalized linear model (GLM) with Poisson errors were used to evaluate associations between hemoplasma species richness in individual raccoons and body weight. We used a global GLM with individual hemoplasma infection status (positive or negative) as a response variable with a binomial error structure to identify factors that may affect mycoplasma prevalence (43). Sex, habitat type, and body weight were included as explanatory variables alongside biologically meaningful interactions between covariates. Using the R package “AICmodavg”, we applied a stepwise algorithm and calculated Akaike’s information criteria corrected for small sample sizes (AICc) to determine which set of covariates provided the best fit to the data. A species co-occurrence matrix was calculated to evaluate if co-infecting putative hemoplasma species were negatively, randomly, or positively associated with one another within each host using the R package “co-occur” (44).
ACKNOWLEDGEMENTS

Funding for this research was provided by St. Catherines Island Foundation, Inc. through the American Museum of Natural History and the Odum School of Ecology, UGA, through Vanessa Ezenwa and Andrew Park. H. Danaceau was supported by Grant Number 9T35OD010433-06 from the Office of the Director a component of the National Institutes of Health (NIH) and its contents are solely the responsibility of the authors and do not necessarily represent the official view of NIH. We would like to thank Joanne Messick, Andreas Santos and Michael Yabsley for advice, Mark Heth, Sea Island hospital of St. Simons Island for providing samples, Veronica Greco, Royce Hayes and on-site staff of St. Catherines Island for technical advice and help during the fieldwork on the island. We also thank Daniel Becker for critical review of the manuscript and three anonymous reviewers for providing comments on earlier draft of this manuscript.

Conflict of interest statement

The authors have no conflict of interest to declare for this research.

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    Identification, molecular characterization, and experimental transmission of a new hemoplasma


Table 1. Hemotropic mycoplasmas detected in raccoons

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Sample ID</th>
<th>Hemoplasma genotypes detected</th>
<th>Primer sets used for full-length 16S rRNA amplification (see Table S1)</th>
<th>Sequence homology to other hemoplasmas:</th>
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<td>KC920443</td>
<td>PRLO50</td>
<td>Racoon hemoplasma genotype 3</td>
<td>16S-PCR-1</td>
<td>96% to <em>Candidatus M. erythrodidelphis</em> (AF178676)</td>
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<td>KC920441</td>
<td>PRLO49</td>
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<td>KC920448</td>
<td>PRLO53</td>
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<td>KC920439</td>
<td>PRLO96</td>
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<tr>
<td>KF743729</td>
<td>PRLO102</td>
<td>Racoon hemoplasma genotype 2</td>
<td>16S-PCR-3</td>
<td>92-93% to <em>Candidatus M. haemozalophi</em> (GU905012) and <em>Candidatus M. haemolamae</em> (AF306346)</td>
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<tr>
<td>KF743724</td>
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<td>KF743726</td>
<td>PRLO91</td>
<td>Racoon hemoplasma genotype 4</td>
<td>16S-PCR-2</td>
<td>86-88% to raccoon hemoplasma genotypes 2 and 3, and to <em>Candidatus M. haemominutum, M. wenyonii, M. ovis</em></td>
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<td>KF743711</td>
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<td>KF743706</td>
<td>PRLO24</td>
<td>Racoon hemoplasma genotype 5</td>
<td>16S-PCR-5</td>
<td>96-97% to <em>M. haemocanis</em> (AY529641) (including <em>M. haemocanis</em> detected in Japanese raccoon dog (GenBank AB848714) and <em>M. haemocanis/M. haemofelis</em>-like sp. detected in Japanese black bear (GenBank AB725596)), to <em>M. haemofelis</em> (AF548631) and to raccoon hemoplasma genotype 1.</td>
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<td>PRLO56HC</td>
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<td>KF743710</td>
<td>PRLO44</td>
<td>Racoon hemoplasma genotype 6</td>
<td>16S-PCR-8</td>
<td>91-92% to raccoon hemoplasma genotype 5, and to <em>Candidatus M. haemobos</em> (EF460765)</td>
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<td>KF743705</td>
<td>PRLO21</td>
<td>M. haemocanis/M. haemofelis-like sp. (Raccoon hemoplasma genotype 1)</td>
<td>16S-PCR-5</td>
<td>99% to <em>M. haemocanis</em> (AY529641) and <em>M. haemofelis</em> (AF548631)</td>
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<td>KF743712</td>
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<td>KF743730</td>
<td>PRLO103</td>
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</table>

Note: The reference sequence for each hemoplasma genotype is indicated in bold. The numbering of raccoon hemoplasma genotypes (1-6) is based on their sequential discovery.
Table 2. Hemoplasma genotypes identified in developed (urban) and protected (undisturbed) islands

<table>
<thead>
<tr>
<th>Hemoplasma genotypes detected in raccoons</th>
<th>Urban (%) N=13</th>
<th>95% CI</th>
<th>Undisturbed (%) N=46</th>
<th>95% CI</th>
<th>Total (%) N=59</th>
<th>95% CI</th>
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<tr>
<td>Raccoon hemoplasma genotype 5</td>
<td>15.4</td>
<td>2.7, 46.3</td>
<td>58.7</td>
<td>43.2, 72.7</td>
<td>49.2</td>
<td>36.0, 62.4</td>
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<tr>
<td>M. haemocanis/M. haemofelis-like sp.</td>
<td>61.5</td>
<td>32.37, 84.9</td>
<td>41.3</td>
<td>27.3, 56.7</td>
<td>45.8</td>
<td>32.9, 59.2</td>
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<tr>
<td>(Raccoon hemoplasma genotype 1)</td>
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<tr>
<td>Raccoon hemoplasma genotype 2</td>
<td>7.7</td>
<td>0.4, 3.79</td>
<td>56.5</td>
<td>41.2, 70.8</td>
<td>45.8</td>
<td>32.9, 59.2</td>
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<tr>
<td>Raccoon hemoplasma genotype 3</td>
<td>30.8</td>
<td>10.4, 61.1</td>
<td>60.9</td>
<td>45.4, 74.5</td>
<td>54.2</td>
<td>40.8, 67.1</td>
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<tr>
<td>Raccoon hemoplasma genotype 6</td>
<td>46.2</td>
<td>20.4, 73.9</td>
<td>32.6</td>
<td>20.0, 48.1</td>
<td>35.6</td>
<td>23.9, 49.2</td>
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<td>Raccoon hemoplasma genotype 4</td>
<td>23.1</td>
<td>6.16, 54.0</td>
<td>43.5</td>
<td>29.2, 58.8</td>
<td>39.0</td>
<td>26.8, 52.6</td>
</tr>
</tbody>
</table>

Figure 1. Dendrogram showing phylogenetic relationships based on nucleotide sequence data for the 16S rRNA gene among the hemoplasma genotypes detected in raccoons (Procyon lotor) with other hemotropic Mycoplasma spp., and three non-hemotropic phylogenetically closely related Mycoplasma spp., (M. insons, M. fastidiosum, and M. cavipharyngis) and two not phylogenetically closely related Mycoplasma spp. The trees were constructed by the minimum evolution method in the MEGA 6 package.

Figure 2. Species co-occurrence matrix showing patterns of co-infection among the hemoplasma genotypes detected in raccoons. Results of the genotype co-occurrence matrix calculates the probability (p-value) of determining if the co-occurrence observed is greater or less than that expected due to chance. Only significant p-values (p≤0.05) are shown.
Genotype Co-occurrence Matrix