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RESEARCH ARTICLE

THE ABSENCE OF NOSEMOSIS IN COMMON EASTERN BUMBLEBEES (*BOMBUS IMPATIENS* CRESSON) FROM REGIONS OF ROANOKE AND NEW RIVER VALLEY

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ABSTRACT

In a study of 220 eastern bumblebee specimens collected from the Roanoke and New River Valley areas, PCR analysis of the gut lumen revealed the absence of *Nosema* spp. infections, challenging prevailing assumptions about their prevalence in bumblebee populations. The outcome underscores the need for further research to determine the factors contributing to this absence, including the unique ecological context of the study area. These findings highlight the significance of host susceptibility and survivability, emphasizing the complexity associated with parasite-host interactions within bumblebees.

Keywords: Bee conservation, cross infection, *Nosema*, pollinator decline, southwestern Virginia.

INTRODUCTION

More than thirty percent of food for human consumption originates from plants pollinated by both commercially reared and wild bees (Klein et al., 2007; Khalifa et al., 2021). Among these bees, bumblebees are one of the most well-known pollinators and contribute heavily to sub-arctic and temperate ecosystems, thus making the species both ecologically and economically salient (Bingham & Orthner, 1998; Khalifa et al., 2021). Compared to other major pollinators, including

the prominent honeybee, bumblebees possess distinct physical advantages that are conducive to efficient pollen transference via “buzz pollination” (De Luca & Vallejo-Marin, 2013; Pritchard & Vallejo-Marin, 2020). Adaptive features such as proboscis length, larger body size and dense setal pile coupled with the unique behavior of sonication allows bumblebees to maintain the vital and distinctive symbiotic relationship between flora and fauna (Winter et al., 2006; Hines et al., 2022). As such, the recorded precipitous decline of bumblebees in North America is especially worrisome (Winter et al., 2006; Williams & Osborne, 2009; Malfi & Roulston, 2014). Among the several dozen species that are native to the continent, seven bumblebee species have been found to be in rapid decline for the past several decades (Colla & Packer, 2008; Evans et al., 2008; Grixti et al., 2009; Cameron et al., 2011). Furthermore, one species, *Bombus affinis* Cresson, otherwise known as the Rusty Patched Bumblebee, has been reported as being critically endangered after vanishing from 87% of its historic range within the last 20 years (Evans et al., 2008; Cameron et al., 2011).

This notable shift in bumblebee populations in North America have been attributed to many factors including habitat loss, pesticide use, and genetic homogeneity (Colla et al., 2006; Goulson et al., 2008; Cameron et al., 2011). However, it has been hypothesized that parasitism may have played a role in the abrupt decline of *Bombus* spp. as well (Evans et al., 2008; Martin et al., 2021). One such parasite that has emerged as a concern is *Nosema*, a microsporidian parasite that has been recently reclassified as *Vairimorpha* (Plischuk et al., 2009; Graystock et al., 2013; Tokarev et al., 2020; Martin et al., 2021). The *Nosema* species, comprising *Nosema bombi* Fanthom & Porter, *Nosema ceranae* (Fries, Feng, da Silva, Slemenda & Pieniazek), and *Nosema apis* (Zander) have been increasingly associated with detrimental effects on bumblebee colonies (Li et al., 2012; Martin et al., 2021). These intracellular parasites can weaken their hosts, potentially leading to colony failure and, in turn, disrupting the pollination services that bumblebees provide (Plischuk et al., 2009; Li et al., 2012; Skuse et al., 2019).

It is worth noting that different bumblebee species exhibit varying infection rates when facing these intracellular parasites (Cordes et al., 2012), and these variations may be attributed to a range of factors. Disruptions in the composition of gut microbiota in honeybees, for instance, have been found to impact the intricate interaction between a host's gut bacteria and its ability to combat *Nosema* parasitic infections (Li et al., 2017). Similarly, investigations into the influence of climate change on *N. bombi* infections in *Bombus terrestris* (Linnaeus) have underscored the significance of host genotypes (Manlik et al., 2023). Moreover, extensive research into bee antiviral defense mechanisms across various bee species, including honeybees and bumblebees (McMenamin et al., 2018), has revealed a spectrum of crucial immune pathways. While the mechanisms of pathogenesis and the impacts of nosemosis on honeybee populations have been studied extensively, our understanding of its effects on bumblebee populations remains limited, particularly in the state of Virginia (Williams et al., 2008; Traver and Fell, 2011; Tripodi et al., 2014).

A recent microscopic study conducted in Northern Virginia has revealed the presence of *Nosema* parasitism in bumblebee populations, raising concerns about its potential impact on pollinator health (Malfi & Roulston, 2014). The discovery in Northern Virginia underscores the urgency of investigating the prevalence of *Nosema* infections in bumblebees across the broader state, especially regions such as Roanoke and the New River Valley due to their ecological and agricultural significance. Understanding the dynamics of *Nosema* infections in this context is crucial for addressing potential threats to the local bumblebee populations and, by extension, the broader ecosystem's stability.

MATERIALS AND METHODS

Field Sampling for Bumblebees

A total of 220 specimens of common eastern bumblebees (*Bombus impatiens* Cresson) were collected from 14 regions encompassing Roanoke and the New River Valley during the summer months of 2020-2022 (Table 1; Fig. 1). We opportunistically sampled the common eastern bumblebees for this study as this species is most prevalent in the regions of Roanoke and New River Valley. The samples were collected utilizing a sweep net as well as a 50ml sterile centrifuge tube. Sample collection via centrifuge tube was proven successful especially during the morning resting hours where many individuals would slumber on local flora (Fig. 2a).

Table 1. Surveyed localities representing 14 regions encompassing the area of Roanoke and New River Valley. Locations are scattered throughout Roanoke, Roanoke City, Montgomery, and Radford. Each locality has been designated a number corresponding to a specific collection site depicted in Fig.1.

Surveyed Localities	# of bees sampled	Sample #	Latitude	Longitude	Locality # on map
Roanoke					
Victoria Thomas Park[a]	25	001 - 025	37.257782	-79.95534	2
Spring Valley[a]	15	026 -040	37.24774	-80.00016	3
Mill Mountain[a]	16	041 -056	37.248821	-79.93615	4
Appalachian Trail[a]	5	085-089	37.392534	-80.03714	8
Hollins Park[a]	12	057 -068	37.344232	-79.92732	5
Shantiniketan Temple[a]	5	080-084	37.180009	-80.01419	7
Piedmont Park[a]	16	102-117	37.257782	-79.95534	10
Garst Mill Park[a]	12	090-101	37.242200	-80.01069	9
Fishburn Park[b]	22	118-139	37.246458	-79.97977	11
Elmwood Park[b]	10	140-149	37.267828	-79.93915	12
Smith Park[c]	10	180 -189	37.257782	-79.95534	1
Salem					
Salem Park[a]	11	069 -079	37.268368	-80.03755	6
Montgomery					
Kentland Farm[c]	30	150-179	37.195224	-80.58284	14
Radford					
Bisset Park[c]	31	190 -220	37.138838	-80.57099	13

Bumblebee specimens collected in [a] 2020, [b] 2021, and [c] 2022.

Gut Dissection and Extraction

The bumblebee guts were extracted either by pulling out the stinger (Fig. 2b), or by making a small incision on the ventral side of the abdomen (Fig. 2c) utilizing sterilized forceps and scalpels without damaging the abdomen unnecessarily. After each gut extraction, the dissecting tray, forceps, and scalpels were all sterilized before each subsequent extraction. The tray was wiped with 100% ethanol while the forceps and scalpels were held to a flame after dipping into 100%

ethanol. The guts were then transferred into microcentrifuge tubes, properly labeled, and placed in -80 ℃ for preservation and subsequent DNA extraction.

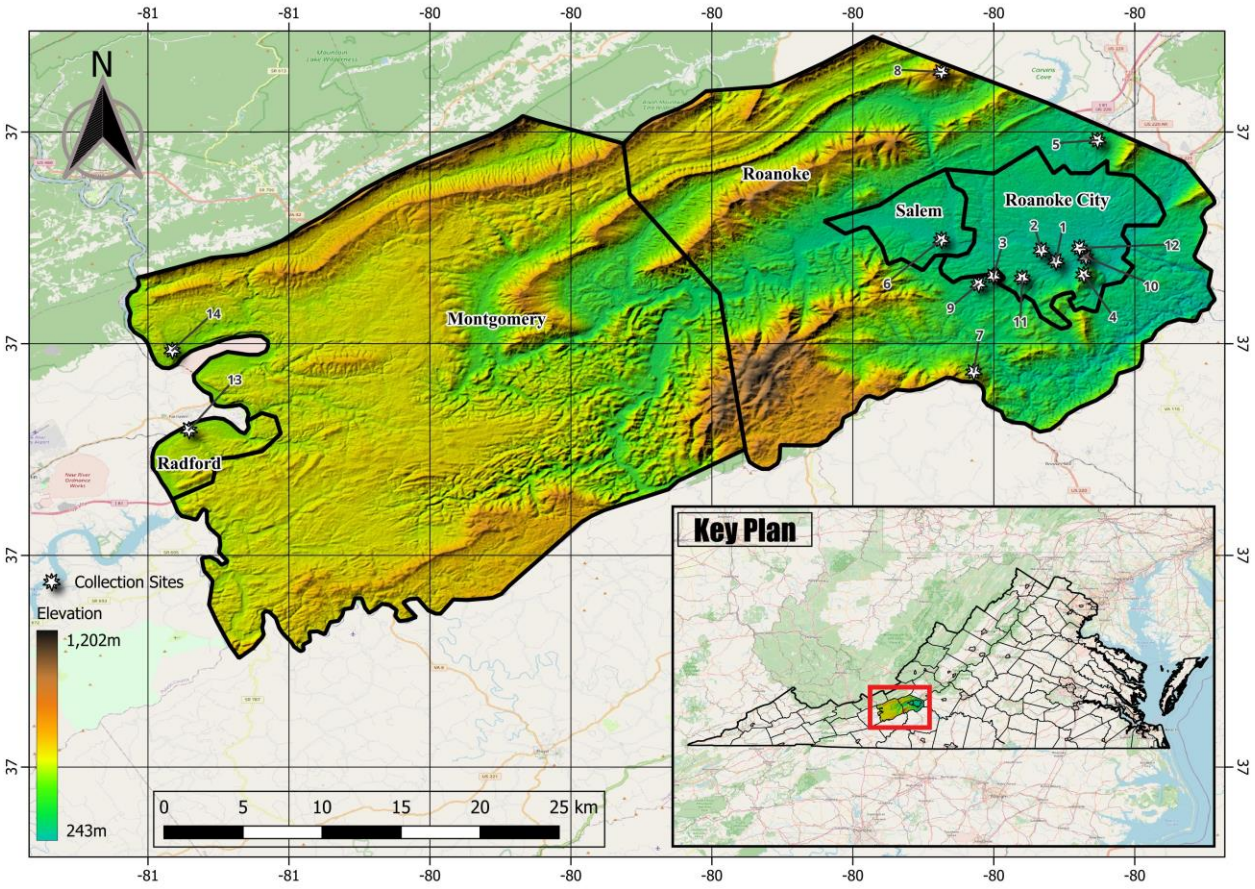

Figure 1. The map of study area showing Roanoke, Roanoke City, Montgomery, and Radford. Each white star represents the surveyed locality from where samples of eastern bumblebees (*Bombus impatiens*) were collected. Information regarding each locality is outlined in Table 1.

Pinning and Labeling

Post gut extraction, each specimen was pinned on the upper left area of the thorax. Specimen labels were provided detailing locality information, name of collecting author, date of collection, and name of the species (Fig. 2d).

DNA Extraction, Quantification, PCR amplification, and Gel Electrophoresis

DNA was extracted from each specimen utilizing the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturers recommendations. The DNA from each abdominal content was eluted in 100 µL and was placed in -20 degrees Celsius for preservation. The extracted DNA from each specimen was then quantified utilizing a NanoDrop (ND-1000 Spectrophotometer). The quantification analysis of all 220 samples revealed DNA concentrations varying between 45.34 and 276.65 ng/µl. We opted to employ two primer sets for PCR amplification in each sample, targeting both *Bombus* spp. and *Nosema* spp. We designed a primer set comprising Sequence 1-

forward (5'-ATTTCATTCATCACCCTCAGTAGA-3') and Sequence 1-reverse (5'-TGCTCGAGTATCAACATCTAATCC-3') to specifically target a segment of the cytochrome c oxidase (COI) gene in *B. impatiens* (Han et al., 2019). This segment results in an amplicon of 502 base pairs. To amplify a 545-base-pair coding region within the small subunit rRNA (ssrRNA) gene of *Nosema*, we adopted the primer set, ssrRNA-f1 (5'-CACCAGGTTGATTCTGCCT-3') and ssrRNA-r1 (5' TGTTCGTCCAGTCAGGGTCGTCA-3') from Li et al. (2012).

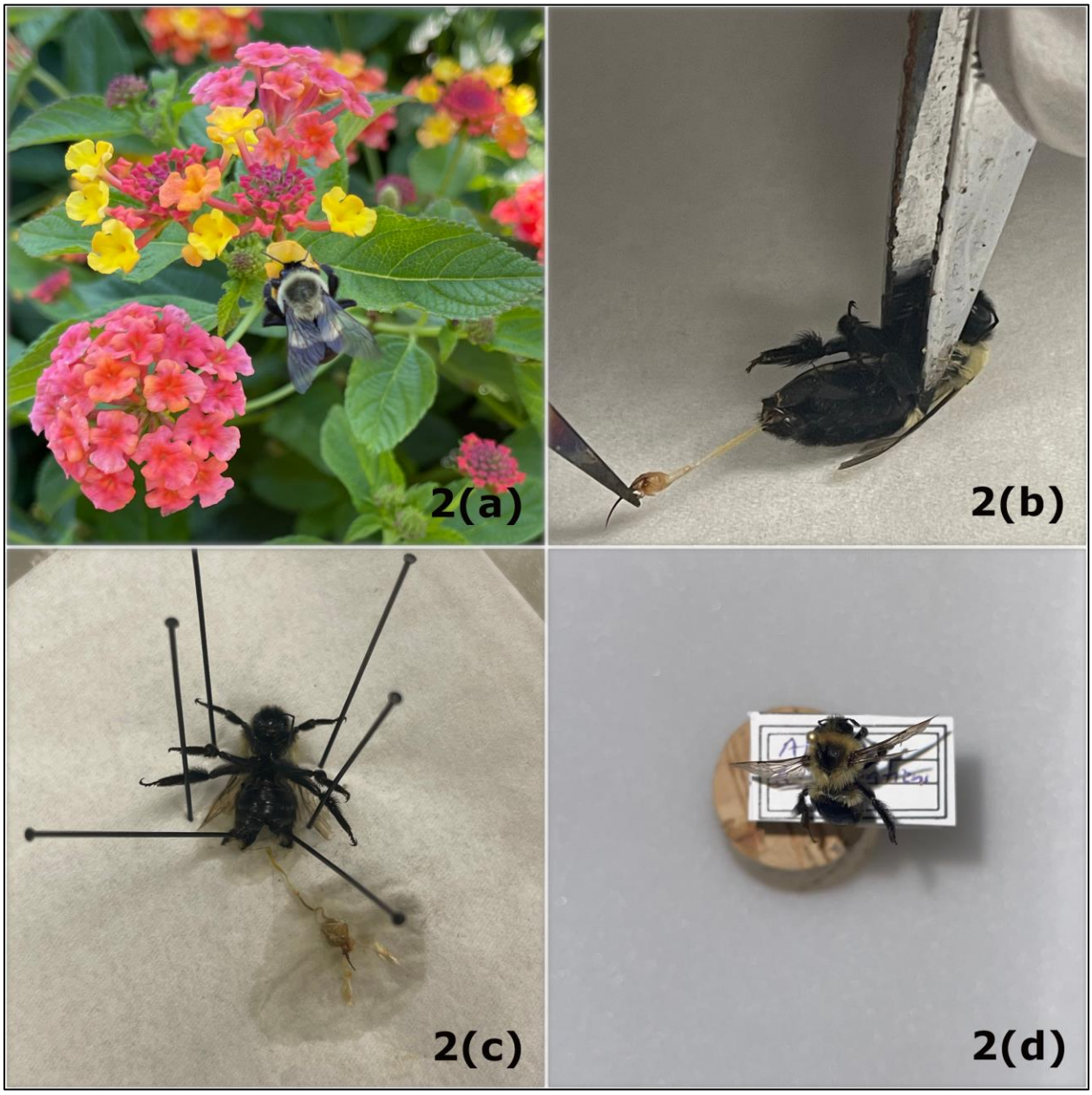

Figure 2. Illustrative pictures depicting the processes involved in gut extraction as well as pinning and labeling of a bumblebee specimen. (a) The eastern bumblebee (*Bombus impatiens)* foraging on native flowers. (b) Gut extraction via pulling out the stinger. (c) Gut extraction via dissection. (d) Pinned and labeled specimen of *Bombus impatiens* after gut extraction.

PCR analysis was conducted in a total volume of 50 µL reaction (Table 2). For each run, a previously confirmed CO I gene in *Bombus* DNA and ssrRNA gene for *Nosema* in honeybee gut DNA served as positive controls, and sterile water without DNA template served as a negative control. PCR reactions for both the detection of *Bombus* spp. and *Nosema* spp. were conducted separately and the thermocycling conditions for both were as follows: an initial denaturation step of 95 °C for 5 min, subsequently 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 60 s, and extension at 72 °C for 60 s, followed by a final extension step of 72 °C for 5 min. All PCR products were electrophoresed in 1.4% agarose gels (Figs. 3a-c).

Table 1. PCR reaction mix and final concentrations of reagents.

Component	Volume/reaction	Final concentration
Taq PCR Master Mix, 2x	25µl	2.5 units *Taq* DNA Polymerase 1x QIAGEN PCR Buffer (contains 1.5µM $MgCl_2$) 200 µM of each dNTP
10µM primer mix (of each primer)	2µl	0.2µM of each primer
RNAse-free water	21µl	---
Template DNA	2µl	90.7-553.3 ng/reaction
Total reaction Volume	50µl	

RESULTS AND DISCUSSION

Gel electrophoresis analysis revealed the absence of *Nosema* spp. in all 220 common eastern bumblebee specimens (Figs. 3a-c). The gel analysis results in Figure 3a-c represent a subset of samples and do not encompass the entirety of the samples examined. Our findings revealed a notable absence of *Nosema* infections in common eastern bumblebees, despite the documented prevalence of *Nosema* infections in honeybee colonies in Virginia, including the regions of Roanoke, Montgomery, and Pulaski (Traver & Fell, 2011). These results deviate from expectations, particularly due to a prior study that detected *Nosema* infections in bumblebees from Northern Virginia at a prevalence rate of 7.3%, consistent with previous reports for bumblebees in the eastern United States (Malfi & Roulston, 2014). However, it is worth emphasizing that Malfi and Roulston in 2014 also observed a lower occurrence and less severity of *Nosema* infections in more common bumblebee species including *Bombus impatiens*, when compared to their less common counterparts, *Bombus auricomus* (Robertson), *Bombus fervidus* (Fabricius), and *Bombus perplexus* Cresson. Furthermore, to provide additional context, findings by Averill et al. (2021) unveiled a significant disparity in parasite prevalence between *B. impatiens* and three other bumblebee species in New England bumblebee communities, namely *Bombus bimaculatus* Cresson, *B. perplexus*, and *Bombus vagans* Smith. Notably, an impressive 69.2% of *B. impatiens* individuals were found to be completely devoid of parasites, while in contrast, the collective group of the three aforementioned species exhibited a considerably lower parasite-free rate of 43.4%.

Within this framework, it has been reported that rare bumblebee species such as *Bombus occidentalis* (Green) and *Bombus pensylvanicus* (DeGeer), are more susceptible to *Nosema* infections than others (Cordes et al., 2012). This finding aligns with studies reporting a higher

prevalence of *Nosema* infections in midwestern states where these specific bumblebee species are more prevalent (Cordes et al., 2012; Skuse et al., 2019). Bumblebee species frequently infected with *Nosema*, such as the aforementioned *B. occidentalis* and *B. pensylvanicus*, compared to other species exhibit lower genetic diversity (Lozier et al., 2011). The varying infection rates of *Nosema* among bumblebee species of variable genetic diversity suggest a complex interplay between host genetics and the susceptibility to *Nosema* infections (King & Lively, 2012).

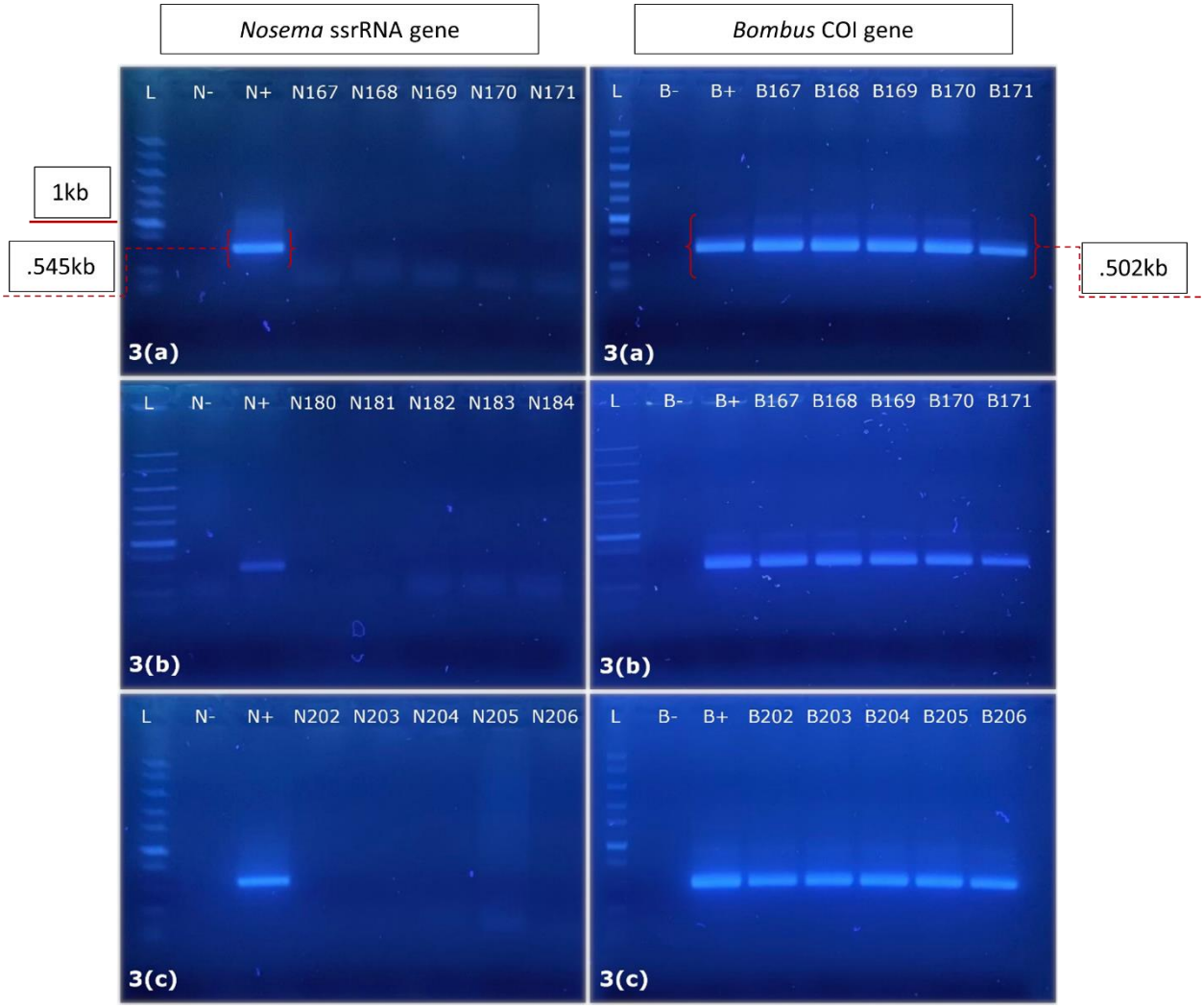

Figure 3. Representative gel electrophoresis analysis results encompassing specimens #167-171, #180-184, and #202-206. L: Ladder. N (-): *Nosema* spp. negative control. N (+): *Nosema* spp. positive control. B (-): *Bombus* spp. negative control. B (+): *Bombus* spp. positive control. a) Gel Electrophoresis results for samples collected from Blacksburg region (167-171), b) Gel Electrophoresis results for samples collected from Roanoke region (180-184), and c) Gel Electrophoresis results for samples collected from Radford Region (202-206).

Notably, climate change coupled with genetic variability may also be an important factor in determining host susceptibility to *Nosema* spp. infections. The association between climate and *Nosema bombi* infection in certain bumblebees unveiled the phenotypic plasticity displayed by specific mitochondrial DNA cytochrome oxidase I (COI) haplotypes of the host, specifically in response to climatic variation (Manlik et al., 2023). For instance, certain host haplotypes exhibited

increased susceptibility to infection with rising temperatures, while others were more resistant during years with higher moisture levels. While our study did not delve into host genetics, it warrants further investigation into whether genetic factors within bumblebee populations in our study area contribute to their resistance to *Nosema* infections. Moreover, the elimination of gut bacteria through antibiotic treatment significantly impaired honeybee immune responses, making them more susceptible to *Nosema* infection (Li et al., 2017). This insight suggests that the absence of *Nosema* infections in our study area may be associated with the healthy gut microbiota of the sampled bumblebees, contributing to their disease resistance. Furthermore, it was observed that bumblebees consuming a diet low in protein but high in carbohydrates exhibited the highest parasite prevalence and spore levels within their guts, reaching up to 70%, as reported by Gómez-Moracho et al. (2021). This finding implies that bumblebees' immunity, susceptibility, and survivability from *Nosema* infections may be influenced by their dietary preferences as well as the diversity of native flora in the local environment.

CONCLUSIONS

We observed a significant absence of *Nosema* infections in each of the 220 eastern bumblebee specimens collected from Roanoke and the New River Valley areas. This lack of nosemosis among the local wild bumblebee population could be associated with a range of factors. Previous studies have shown variations in infection rates among different bumblebee species, with rare species being more susceptible and common species displaying greater resistance. The interplay between host genetics, susceptibility, and *Nosema* infections adds another layer of complexity to this phenomenon. Furthermore, the influence of climate change on bumblebee susceptibility and the potential role of gut microbiota and dietary choices in disease resistance emphasize the multifaceted nature of this issue and further highlights the complexity of *Nosema* infections within bumblebee populations. Understanding these factors is crucial for the conservation of regional bumblebees and the overall health of other pollinator communities in our region. Further investigations are necessary to untangle this intricate relationship between *Nosema* spp. parasitism and the abrupt decline of wild bumblebees.

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