Report for Investigating bryophyte use in the nests of the Prothonotary Warbler (*Protonotaria citrea*) (NCNPS 2024 Tom and Bruce Shinn Fund)

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This report summarizes current findings from my research investigating the use of bryophytes in Prothonotary Warbler (*Protonotaria citrea*, PROW) nests collected from nest boxes in five habitats in northeastern North Carolina, supported by a Tom and Bruce Shinn award in 2024. My main research questions for this component of my research are to (**1**) determine what bryophyte and tracheophyte species Prothonotary Warblers use in varied forested wetlands habitats, and (**2**) investigate if bryophytes selected by Prothonotary Warblers produce arthropod deterrent volatile compounds, in addition to cataloging bryophyte species used in these habitats and contribute to documenting them.

There have been several challenges with this project, primarily difficulties with the gas chromatography-mass spectroscopy equipment needed for the chemical analyses; challenges identifying nonvascular plants in nests; and delays in getting molecular analyses of nest fauna communities started. As such, many of these results are still preliminary. However, this award helped me make the progress recorded in this report and has allowed me to lay groundwork to continue work once the equipment difficulties are resolved. Additionally, since receiving this award, I have transitioned from the master's program to the PhD program, so I am continuing to work on and expand this project.

Field work and sample collection summary

I collected 36 nests in 2023, and 40 nests in 2024 from nest boxes. Of these, most nest boxes were used solely by PROW and all nestlings successfully fledged (or, left the nest). In some nests, Carolina Chickadees (*Poecile carolinensis*) had previously used the nest box or at least one nestling died. This report focuses on those nests where all nestling successfully fledged, and the warblers were the only birds to use that box during that season. Results may change when nests with different histories are considered. Table 1 summarizes the nests used in this report, and Figure 1 shows field site locations.

Table 1 Summary of Prothonotary Warbler nests collected in 2023 and 2024

	Number of nests collected used only by warblers and from which all nestlings fledged	
Field site (abbreviation)	2023	2024
Alligator River National Wildlife Refuge (ARBC)	5	8
Kitty Hawk Woods Coastal Reserve (KHW)	6	8
Chowan University Meherrin River Field Site (MRFS)	4	5
Donal C. O'Brien Jr. Pine Island Audubon Sanctuary (PI)	1	1
Bertie County's Tall Glass of Water (TGOW)	3	3

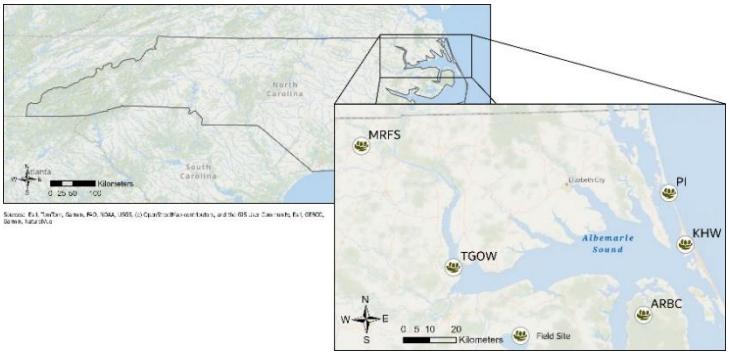

Figure 1 Map showing locations of Prothonotary Warbler field sites.

Bryophytes used

Prothonotary Warblers appear to use at least 2 different species of bryophytes in their nests. Figure 2 summarizes very rough estimates of the number of bryophyte species used in Prothonotary Warbler nests collected in 2023-2024, which, across all sites, ranges from 2 species to 10 species.

I am still in the process of identifying many of these species. Figure 3 summarizes those recovered from 2023 nests (n = 19) for which I have at least a tentative identification thus far. This figure reveals interesting trends, despite many species still needing to be identified. For example, some species are more often recovered from the more inland sites, and others from more coastal sites.

Many of these species do not have records in Bryophytes of North Carolina (https://auth1.dpr.ncparks.gov/bryophytes/index.php) for the counties in which they were collected in my research. I intend to contribute to recording the ranges of the bryophyte species I work with my submitting voucher specimens of samples collected for chemical analyses to the University of North Carolina at Chapel Hill Herbarium, and I have received instructions for how to do so. When I identify the nonvascular plants, I also create slides of features used during identification (e.g., leaves) and am creating a catalog of photographed features of these slides that I will share to contribute to the available identification resources.

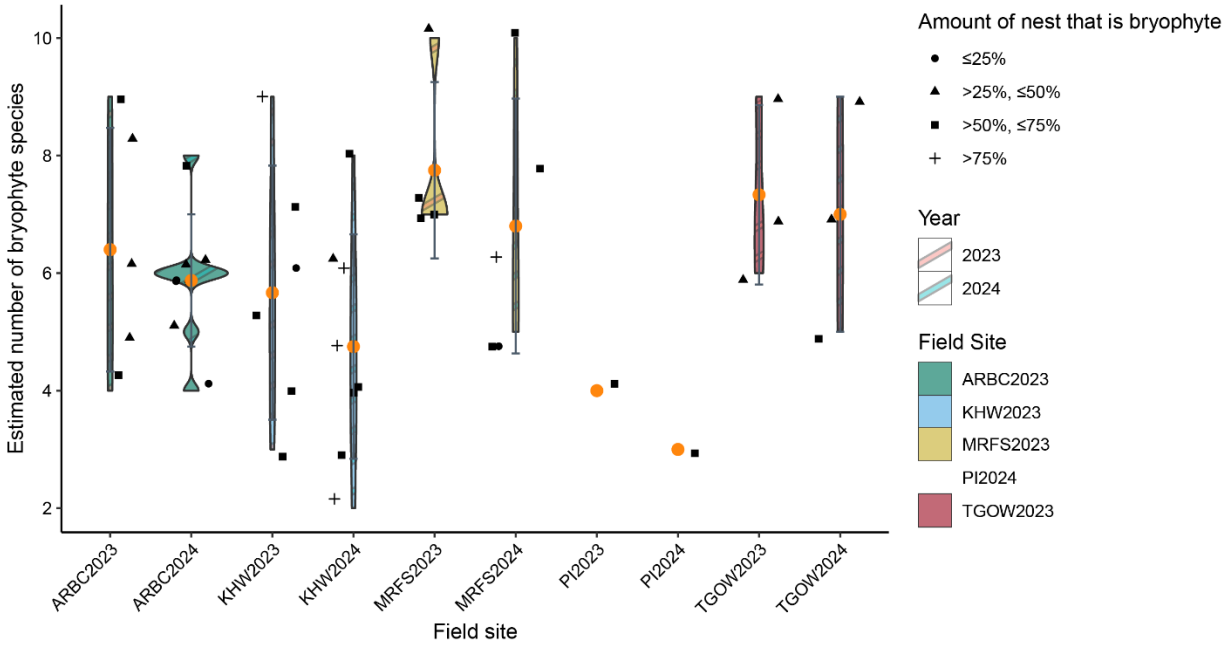

Figure 2 Rough estimates of the number of bryophyte species used in Prothonotary Warbler nests in 2023 and 2024. Error bars indicate ±1 SD about the mean, which is indicated by the orange dot for each field site. The shape of the dot indicates the percentage range of bryophytes from the nest (e.g., a circle indicates 25% or less of the nest was bryophyte, by dry weight).

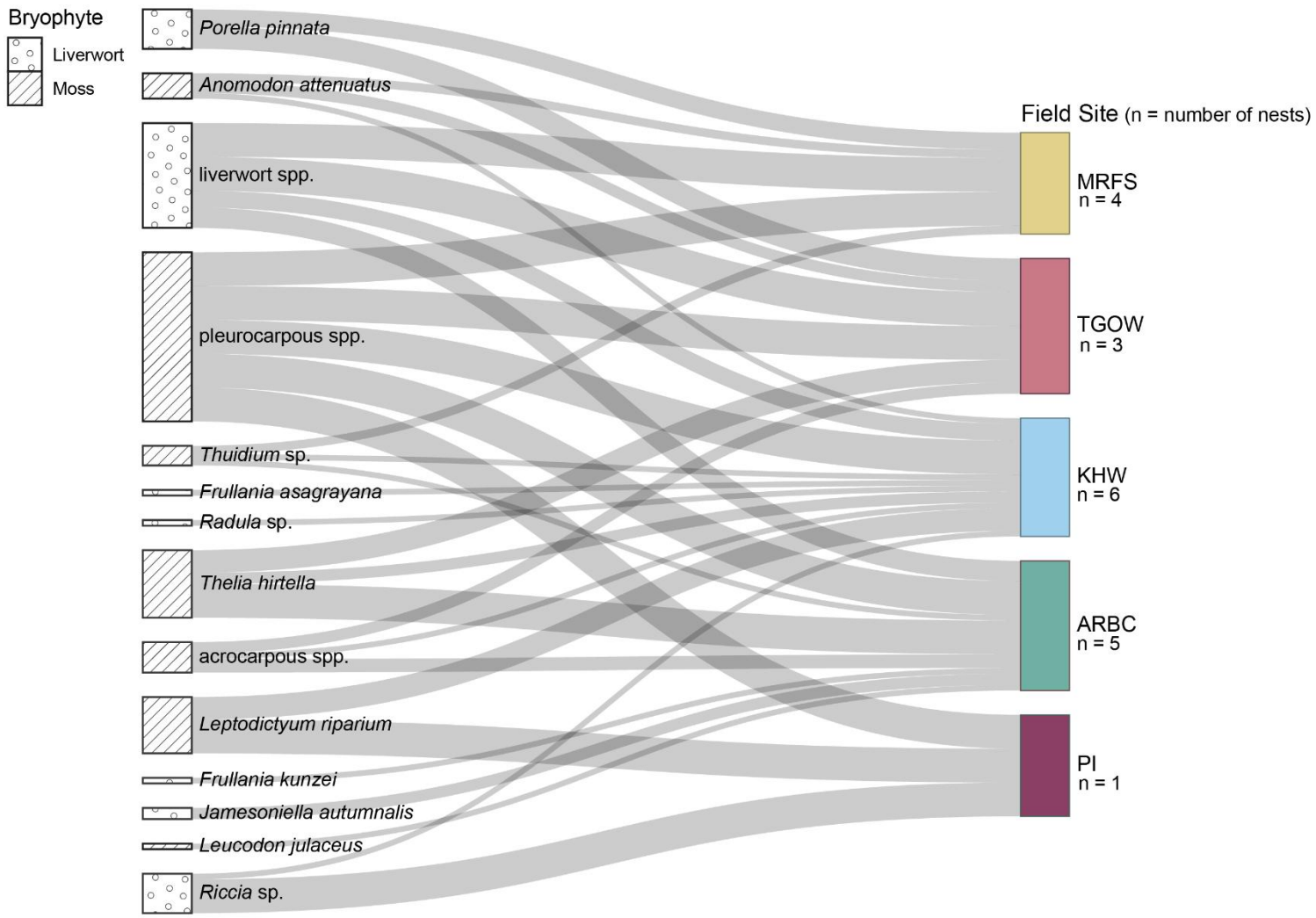

Figure 3 An alluvial plot showing where the tentatively-identified nest bryophytes were recovered in 2023 PROW nests (n = 19). The thickness of the gray lines corresponds to the fraction of nests that had that species (e.g., the thickest lines indicate all nests from that site had that species). Bryophyte species are on the left, and field sites are on the right. On the left, liverworts (Marchantiophyta) are represented by the circle pattern and mosses (Bryophyta) are represented by the striped pattern.

Chemical analysis update

The extraction methods for the gas chromatography-mass spectroscopy (GC-MS) analyses to investigate volatile compounds the bryophytes produce have changed since my initial proposal under advisement from those with more expertise. I am collecting fresh samples of approximately three of the most commonly used bryophyte species at each site, which are thoroughly cleaned and dried in the lab. Those samples will be extracted using an ultrasonic extraction method and then analyzed using GC-MS to characterize the terpenoids produced by those plants. Terpenoids are a category of volatile compounds plants produce, of which several have been identified as being arthropod deterrents (Asakawa et al. 2013).

Unfortunately, the GC-MS I have been intending to use is having issues, which we are in the process of troubleshooting. Prior to this difficulty, we were able to do an initial run of a liverwort collected from MRFS that was extracted using two methods: Soxhlet extraction and a more passive extraction method in which the plant was placed in a solvent for several weeks. The output of the GC-MS analysis of these extractions is shown in Figure 4. We have yet to identify the peaks (which correspond to chemical compound signatures) in these spectra, but it is promising that we were able to detect peaks with this initial analysis.

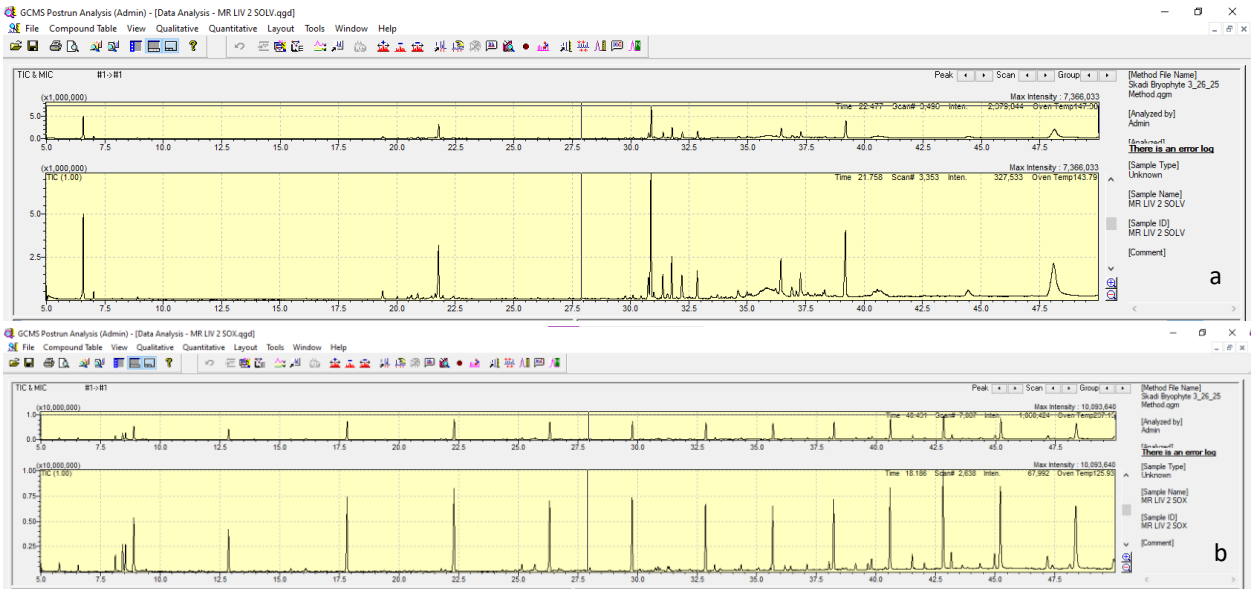

Figure 4 Rough spectra from a preliminary GC-MS analysis of a liverwort collected from MRFS using two different extraction methods. Panel a corresponds to the solvent-only extraction method, and panel b corresponds to an extraction prepared using a Soxhlet apparatus.

Tracheophytes in nests

I have yet to more formally characterize the tracheophyte species the warblers use at each of these field sites, as my focus has primarily been on the bryophytes used in nests. However, while working with nests, I have noticed differences in the tracheophytes used among sites, with nests from some sites tending to have more pine needles (likely loblolly pine, *Pinus taeda*), while bald cypress (*Taxodium distichum*) leaves, an unknown fruit stem, or broad deciduous leaves (e.g., oak (*Quercus* sp.), American hornbeam (*Carpinus caroliniana*)) tend to more commonly used at other sites.

Amounts of bryophytes used in nests

When looking at the nests collected across all years combined, a one-way ANOVA indicates there is a significantly different amount of bryophytes used in nests ($F_{4,39} = 6.337$, $p < 0.001$). A Tukey-Kramer *post hoc* test indicated that difference was between ARBC and KHW ($p = 0.385$, 95% CI = [-61.57, -1.15]), and did not identify differences among any other field sites (Figure 5). This test was chosen due to the use of uneven sample sizes, but it may decrease the ability to detect differences among sites.

Despite both being located in Dare County, NC, ARBC and KHW are fairly different habitats, and are facing different stressors, which may contribute to this apparent difference. KHW is a maritime forest with depressional wetlands used by the PROW, and, anecdotally and superficially, has the impression of being more vegetated than ARBC. ARBC has pocosin-type wetlands, but many areas are transitioning to ghost forest, which is changing the plant communities (Ury et al. 2021).

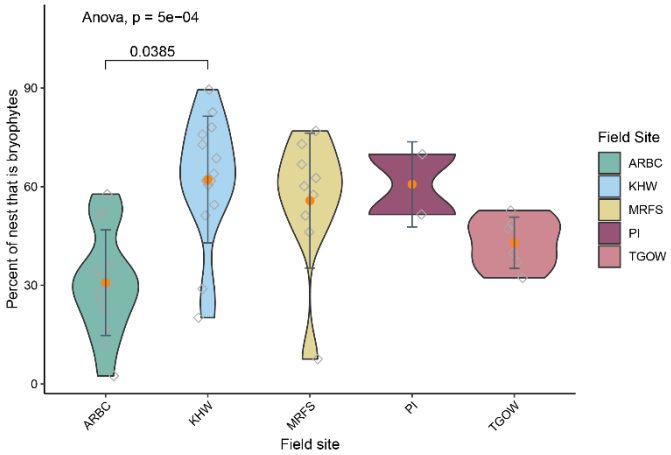

Figure 5 Percent of PROW nests that are made up of bryophytes, for 2023-2024 combined. Error bars show ±1 SD about the mean, which is indicated by the orange dot for each field site. A Tukey-Kramer *post hoc* test indicated a significant difference between ARBC and KHW ($p = 0.0385$, 95% CI = [-61.57, -1.15]).

Separating the years, a one-way ANOVA indicates there may be differences among sites ($F_{9,34} = 3.071$, $p < 0.01$), but a Tukey-Kramer test did not identify any significant differences (Figure 6).

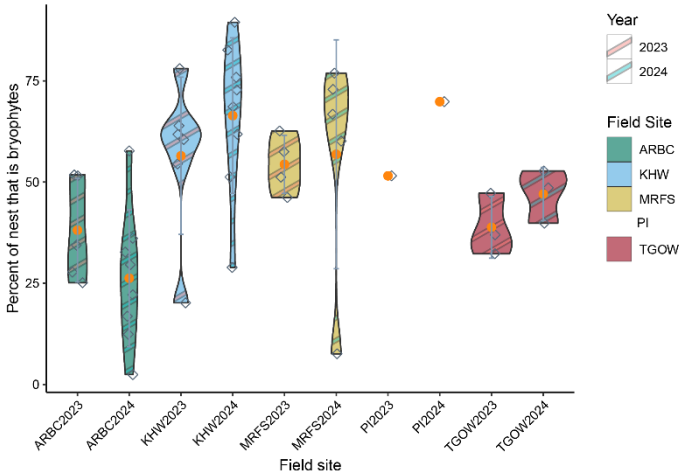

Figure 6 Percent of PROW nests that are made up of bryophytes with each year separated. Error bars show ±1 SD about the mean, which is indicated by the orange dot for each field site.

Relationships between nest fauna communities and nest composition

I am also characterizing the fauna communities in the nests using environmental DNA and molecular techniques. I am currently reworking those analyses to attempt to better characterize the nest communities. However, a preliminary Pearson's correlation coefficient calculation did not indicate a relationship between the amount of the nest that is bryophytes and my current estimates of species richness of nest fauna (r_{39} = -0.044, p = 0.78; Figure 7a). Additionally, though liverworts may have a greater concentration of terpenoid compounds (Adio et al. 2004), which may deter arthropods, there also does not appear to be a correlation between my current estimates of nest fauna species richness and amounts of liverworts in the nest (r_{39} = -0.041, p = 0.8; Figure 7b). This may change as I continue to work with these metabarcoding data to improve the characterization of nest fauna communities. For example, these comparisons are considering all nest fauna; it is possible if I only select the potentially ectoparasitic arthropods (e.g., mites, blowflies) there could be a different relationship.

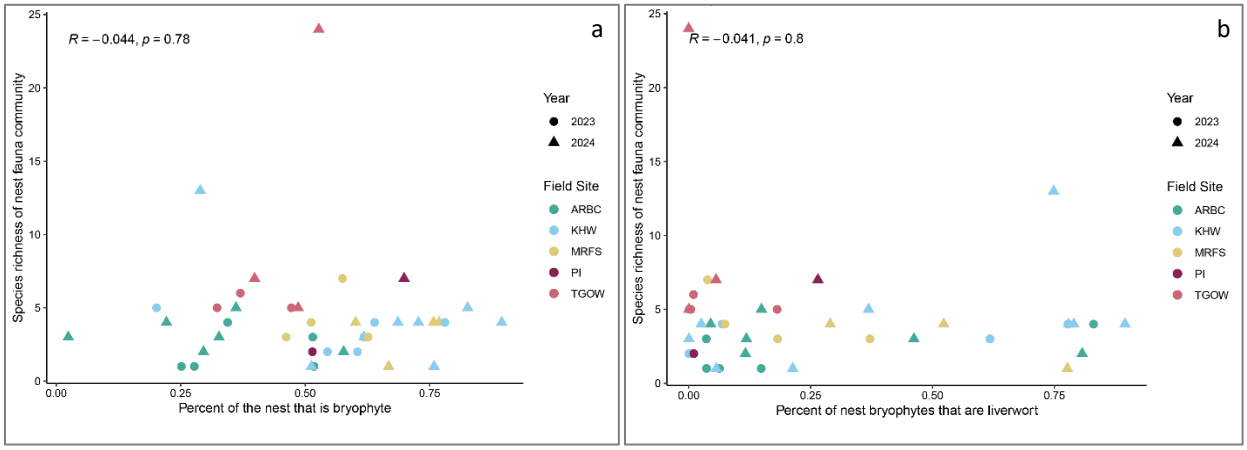

Figure 7 Scatterplots showing relationships between the initial species richness estimates of nest fauna (determined with molecular techniques) and (a) the percent of the nest that is bryophyte and (b) the percent of nest bryophytes that are liverworts. A Pearson correlation coefficient was used to evaluate these relationships, and indicated the relationships was not significant [(a) r_{39} = -0.044, p = 0.78), (b) r_{39} = -0.041, p = 0.8]. Color indicates the field site, and shape indicates the year.

Summary

- PROW in eastern North Carolina appear to use approximately 2-10 species of bryophytes in their nests, and those bryophytes sometimes differ depending on the habitat in which they nest.
- Chemical analyses have been delayed, but an initial test analysis with extracts from a liverwort indicate these analyses may yield interesting information once completed.
- There appear to be differences in tracheophyte species used in nests, but those are yet to be investigated further.
- The amount of PROW nest that is bryophyte in different habitats may differ, though this difference is not detectable with a Tukey-Kramer *post hoc* test when years are separated.
- Based on current nest fauna community characterizations, there does not appear to be a relationship between nest fauna communities and the amount of bryophytes used in nests.
- This award has contributed to better understanding bryophyte species used in different habitats by PROW (and present in these habitats) and laid the foundation for further contributing to this understanding with chemical analyses of those plants.

References

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