Assessing Tricolored Bat Acoustic Monitoring for Regulatory Purposes in Relation to Reproductive Period, Cover Type, and Presence of Eastern Red Bats

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Abstract: The U.S. Fish and Wildlife Service (USFWS) allows use of acoustical surveys and automated identification software to determine the presence of the proposed endangered tricolored bat (*Perimyotis subflavus*). Analytical software is required to assess the probability of species absence on a site-night basis using a maximum likelihood estimator (MLE) that accounts for interspecific misclassification rates. The current standard for occupancy determination is a returned MLE *P*-value ≤0.05 at the nightly level irrespective of the number of files identified as tricolored bats. For this species, MLE *P*-values can vary based on presence and proportion of other bat species with similar echolocation characteristics such as the eastern red bats (*Lasiurus borealis*). Large numbers of eastern red bat echolocation passes may lead to a swamping effect, causing false-negative tricolored bat determinations. Therefore, we investigated the relationship between MLE values returned by Kaleidoscope Pro automated software and nightly tricolored bat counts, along with count proportion relative to eastern red bat detections, for various summer reproductive stages (e.g., pregnancy, lactation, and volancy) and vegetation cover types. We also examined the effectiveness of using the 14-site-night level of effort (LOE) set by USFWS for the endangered northern long-eared bat (*Myotis septentrionalis*) for determining how many cumulative nights of tricolored bat presence would occur under that LOE standard. Automated identification software identified tricolored bat echolocation pass presence at the file level. However, software returned nightly MLE values that were not significant when tricolored bat passes were infrequent (<20 passes) and proportionally low relative to eastern red bat passes, suggesting an acoustic swamping effect. The probability of a significant MLE for tricolored bats was greatest during the late summer when juvenile bats were volant and actively foraging on the landscape. Cumulative nights of tricolored bat presence based on significant MLE values were greatest during the late summer and in riparian cover types.

Key words: acoustic sampling, acoustic swamping, echolocation pass, *Lasiurus borealis*, *Perimyotis subflavus*

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Prior to the advent of white-nose syndrome (WNS), the tricolored bat (*Perimyotis subflavus*), a small (4–6 g) insectivorous bat, was widely distributed throughout eastern North America south into Central America (Chapman 2007). Populations across the northern half of the species' range in the temperate forest and temperate-boreal forest tension zone hibernate in caves and mines during winter (McCoshum et al. 2023) whereas those in the southern half of the distribution, such as in the Atlantic and Gulf Coastal Plains of the southeastern U.S. can remain mostly active, day-roosting in trees, transportation structures, and culverts, with short periods of torpor and inactivity (Leivers et al. 2021, Newman et al. 2021, McCoshum et al. 2023). During the summer maternity season throughout its distribution, tricolored bats primarily use tree foliage in deciduous trees or pines (*Pinus* spp.) for day roosts (Perry and Thill 2007, Cable and Willcox 2024). Though not well-studied, tricolored bats do appear to form distinct maternity colonies that may contain <20 females during the pregnancy period (Silvis et al. 2016). Foraging occurs generally over riparian corridor and forest edges (Ford et al. 2005, Ford et al. 2006, McCoshum et al. 2023, Cable and Wilcox 2024).

For tricolored bats that hibernate during winter in caves and mines, the impacts of WNS have been severe, placing the species

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at risk of widespread extirpation (Cheng et al. 2021). In WNS-positive regions of the southeastern U.S., such as the central Appalachian Mountains of Virginia and the southern Appalachian Mountains of Georgia, declines in tricolored bats have approached 90% (Powers et al. 2015, Perea et al. 2024). Non-hibernation or short-duration hibernating populations in the Atlantic Coastal Plain appear stable as they are not exposed to *Pseudogymnoascus destructans* or if exposed do not develop WNS (Perea et al. 2022). Still, Deeley and Ford (2022) noted an overall decreasing trend in tricolored bat mist-net capture success rates in the southeastern U.S. where WNS-impacts are believed minimal. Accordingly, in 2022, the U.S. Fish and Wildlife Service (USFWS) proposed listing the tricolored bat as Federally endangered (Federal Register § 2022-18852).

Beginning in 2020, the USFWS and the U.S. Geological Survey began collecting acoustic data on the tricolored bats throughout the eastern U.S. concurrent with efforts to update necessary acoustic level-of-effort (LOE) metrics (site-nights sampled) for determining the presence or absence of the northern long-eared bat (*Myotis septentrionalis*) and the Indiana bat (*Myotis sodalis*). Due to declines in detection probability, surveyors have shifted from using mist-net surveys to acoustic sampling to determine the presence or probable absence of the tricolored bat, similar to efforts for northern long-eared bats and Indiana bats for regulatory clearance (Armstrong et al. 2022). Analyses reported by Thorne (2023) concluded the necessary acoustic LOE for tricolored bats ranged from 6–16 detector-nights depending on geographic region. As such, the contemporary nationwide levels for northern long-eared bat survey LOE of 14 nights (USFWS 2022) would be sufficient in most settings for tricolored bats. Nonetheless, Deeley et al. (2021) and Hauer et al. (2023) cautioned that the necessary post-WNS LOE to detect tricolored bats may require longer survey duration. These studies suggest that USFWS, as well as the North American Bat Monitoring Program standards are insufficient in part and may lead to false negatives.

Visually, among bats in eastern North America, tricolored bat acoustic echolocation characteristics are highly diagnostic (Figure 1) and to-date, approved USFWS automated bat identification software programs perform well in simulated accuracy tests using bat species assemblages sympatric with the Indiana bat (USFWS 2019). However, in some situations, the tricolored bat can be misclassified as other high frequency species, such as eastern red bats (*Lasiurus borealis*) and to a lesser degree evening bats (*Nycticeius humeralis*) and little brown bat (*Myotis lucifigus*). In less cluttered, open conditions, eastern red bats can make long

duration, low slope echolocation pass pulses that resemble those of tricolored bats. Conversely, tricolored bats foraging in and out of clutter rapidly often can change call structure to be of high slope and short duration thereby resembling eastern red bat passes. However, if both species are recorded for an extended amount of time, each species eventually will produce distinctive echolocation passes and the identity of each species becomes unambiguous.

For the reasons described above, automated echolocation identification software sometimes identifies tricolored bats as eastern red bats or assigns an echolocation pass to the correct species with low confidence whereby the proportion of tricolored bats to eastern red bats is too low to overcome built-in software misclassification rates for maximum likelihood estimator (MLE) assessment of presence. In places where the two species overlap, concerns have been raised that there is acoustic "swamping" (Ford et al. 2024) by numerous passes of eastern red bats. This results in survey results whereby programs identify tricolored bats on a site-night basis where they are known to be present but the automated identification program does not return a MLE *P*-value ≤0.05 that is the USFWS threshold (hereafter MLE threshold) for establishing presence or absence (Ford et al. 2023).

We used acoustic data from 10 states, collected during 2020–2021 (Armstrong et al. 2022) to model the relation of site-night tricolored bat MLE *P*-values with environmental cover type (e.g., edge, forest and riparian), reproductive season (e.g., pregnancy, lactation and volancy), nightly number of echolocation pass files

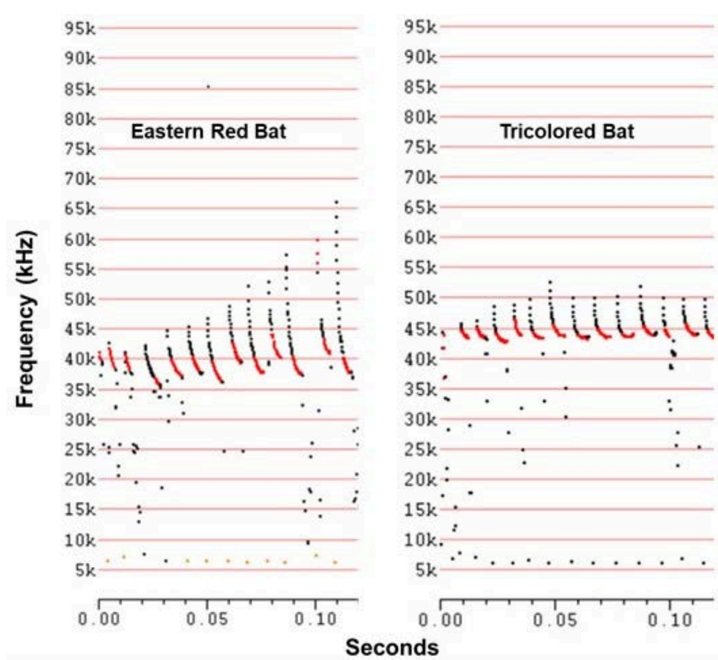

Figure 1. Representative zero-crossing/frequency-division of echolocation pulses of eastern red bat (*Lasiurus borealis*) and tricolored bat (*Perimyotis subflavus*) screen capture from AnalookW software (Titley Scientific, Columbia, Missouri). Note the consistent, longer duration and lower slope minimum echolocation pulse frequency of the tricolored bats.

identified as tricolored bats, and the proportion of tricolored bat files to identified eastern red bat echolocation pass files. Similar to Ford et al.'s (2024) observation for *Myotis* bats and because tricolored bat echolocation signatures are highly diagnostic in most situations, we predicted that frequent failure to achieve the MLE threshold ($P \leq 0.05$) would occur when few tricolored bat echolocation pass files were recorded and when these tricolored bat echolocation passes occurred in a low proportion relative to eastern red bats. To ascertain if current northern long-eared bat LOE standard (USFWS 2024) was sufficient for tricolored bats for determining presence, we also modeled the cumulative number of nights to latency of detection where the MLE threshold was reached. Owing to the tricolored bat's selection of riparian foraging areas (Silvis et al. 2016), we predicted that northern long-eared bat LOE timeframe would be sufficient to document tricolored bat presence for acoustic detectors placed near water features irrespective of reproductive condition as tricolored bat activity would be high and echolocation calls thereof would be highly diagnostic. Conversely, we expected that documenting tricolored bat presence at upland forest and edge cover types might be more likely to occur following volancy period when more individuals are on the landscape and utilizing a wider array of habitat conditions (Ford et al. 2011).

Methods

We conducted acoustic surveys at 18 sites in 10 states (Alabama, Arkansas, Georgia, Kentucky, Indiana, Illinois, Missouri, New Jersey, Tennessee, and Virginia) among locations where tricolored bats were known to occur. Sites spanned the Coastal Plain, Piedmont, Blue Ridge, Appalachian Plateau, Central Lowlands, and Interior Low Plateau physiographic provinces (Figure 2; see Ford et al. 2023, Ford et al. 2024).

We deployed acoustic detectors (n = 76 unique sites) from 15 June to 15 August 2020, and 15 May to 15 August 2021, to support the USFWS Range-wide Indiana Bat and Northern Long-eared Bat Survey Guidelines (USFWS 2022). We retained data from sites where tricolored bats were confirmed from regional mist-netting or from more southern locations where declines from WNS have not occurred. Following the methods described by Barr et al. (2021), we placed one to two detectors at every site in each of three broad cover types: forest, forest-field edge, and forested riparian following the site placement guidelines outlined by the USFWS guidelines (USFWS 2022). At each site, we cable-locked detectors to trees and mounted microphones on 3-m tall poles approximately 2–3 m from the bole of the tree. All detectors were

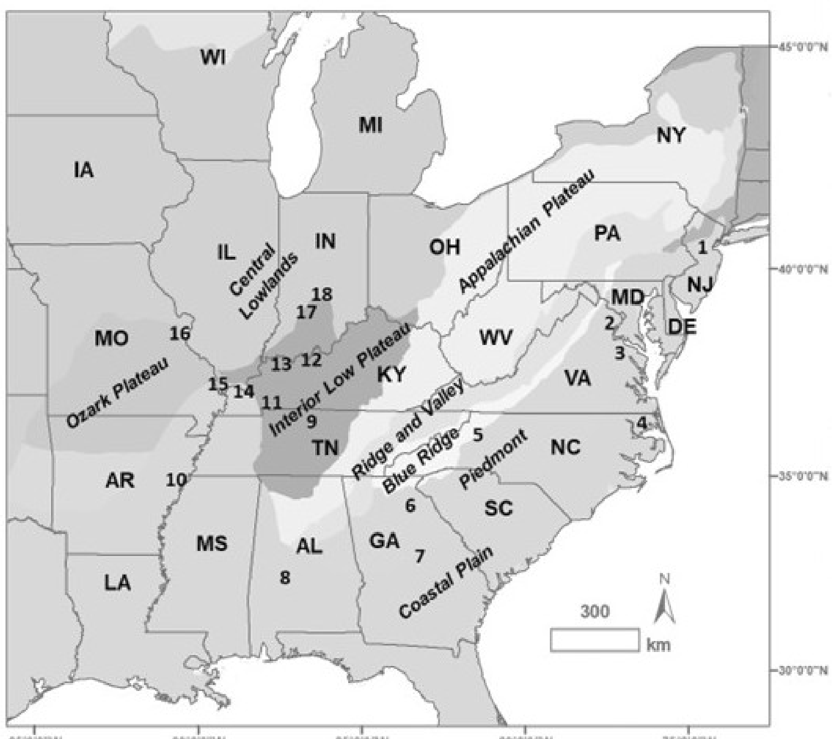

Figure 2. Acoustic survey sample sites for tricolored bats (*Perimyotis subflavus*) during 2020–2021 in the eastern U.S. including: 1. Great Swamp National Wildlife Refuge (NWR), New Jersey, 2. Prince William Forest Park/Marine Corps Base-Quantico, Virginia, 3. Fort Walker Military Reservation (MR), Virginia, 4. North River Gameland, North Carolina, 5. Pineola Bog, North Carolina, 6. Whitehall Forest, Georgia, 7. Ocmulgee Wildlife Management Area (WMA), Georgia, 8. Oakmulgee WMA, Alabama, 9. Wilson County Artificial Roost, Tennessee, 10. Ouachita-St. Francis National Forest, Arkansas, 11. Fort Campbell MR, Kentucky, 12. Fort Knox MR, Kentucky, 13. Yellowbank WMA, Kentucky, 14. Ballard WMA, Kentucky, 15. Cypress Creek NWR, Illinois, 16. Shaw Nature Reserve, Missouri, 17. Morgan-Monroe State Forest, Indiana, and 18. Beanblossom Bottoms Nature Preserve, Indiana.

the same models (Wildlife Acoustic SM4 ZC with SMM U2 omnidirectional microphones; Wildlife Acoustics Inc., Maynard, Massachusetts) except detectors used in Arkansas and Missouri (Anabat SD2s with "Stainless" directional microphones or Anabat Swift detectors; Titley Scientific, Columbia, Missouri). We replaced detector batteries and downloaded data cards at 6-wk intervals per site. Each detector was set to default settings per USFWS recommendations (USFWS 2022).

Following data collection and collation, we used classification software (Kaleidoscope version 4.2.0 classifier v. 5.1.0 Wildlife Acoustics) at the "0" sensitivity setting to identify bat passes to species, record nightly pass counts, and calculate species-specific MLE probability of presence of known or potentially present bat species at each site (USFWS 2019). We used signal detection parameters of 8–120 kHz frequency range, 2–500 ms pulse length, 500 ms inter-syllable gap, and 5 pulses for species assignment (Ford et al. 2023). We did not conduct a *post hoc* qualitative examination of echolocational passes due to the established ability of the package to identify tricolored bat presence during simulated surveys constructed from a reference call library (USFWS 2019). For analyses, we retained all nights without precipitation at each site and detector location, as determined by site-specific or nearest Meteorological Terminal Aviation Routine records (Iowa Environmental Mesonet 2021). For individual site-nights following bat species identification, we partitioned nights into those with tricolored bat presence denoted. We used a generalized linear mixed model with a binomial distribution and logit link function in SAS 9.4 (PROC GLIMMIX; SAS Institute 2020) to assess the relationships of returned nightly MLE P-values ($P > 0.05$ or $P \leq 0.05$) that serve as the USFWS standard for either absence or presence for tricolored bats with: 1) the absolute count of tricolored bat passes per detector night; 2) the ratio of tricolored bat echolocation passes to the nightly count of identified eastern red bat passes; 3) cover type (edge, forest and riparian); and 4) reproductive season. We characterized 15 May–14 June as pregnancy, 15 June–14 July as lactation and 15 July–15 August as juvenile volancy (Silvis et al. 2016). Numerical day nested within detector site location was used as a random variable. Similarly, we used a generalized linear mixed model with a Poisson distribution with a log link function to determine the cumulative number of detections of tricolored bats at the nightly MLE threshold signifying presence across cover type and reproductive period. As a cumulative response variable, we also entered day (1–30) within each reproductive period as a covariate. We checked both models for goodness-of-fit and over- and under-dispersion by examining residual quantile-quantile (Q-Q) plots. Because of the binary response variable, for our model examining the relationship between returned nightly MLE values with tricolored bat counts and their proportion to eastern red bats, we examined the area under the curve metric (AUC).

Results

In 2020–2021, for tricolored bats, we retained 4533 rain-free detector site nights with at least one tricolored bat identified at the individual file level across 18 study areas and 78 detector sites (Table 1). Presence of tricolored bats at detector sites where at least one survey night met the MLE threshold for the pregnancy, lactation and volancy period was 58%, 70% and 83%, respectively. The probability of meeting the MLE threshold for accepting tricolored bat presence decreased when nightly counts of tricolored bats were <20 and the proportion of eastern red bats increased. This was particularly apparent once the proportion was equitable or skewed towards eastern red bats (Table 2, Figure 3). The

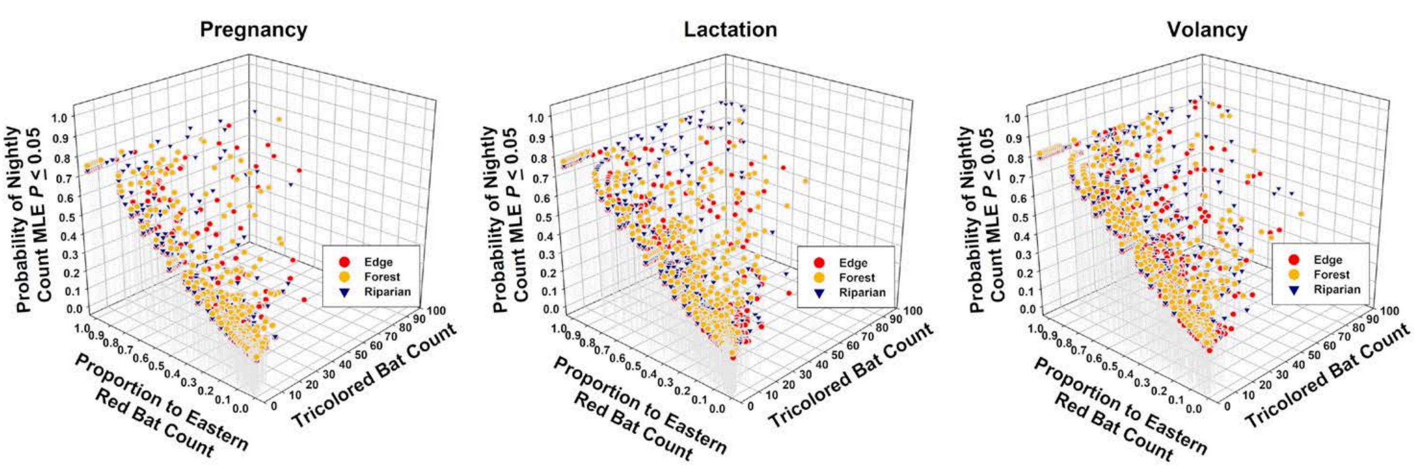

Figure 3. Predicted probability of site-nights ($n = 4533$) reaching the U.S. Fish and Wildlife Service maximum likelihood estimator (MLE) P-value for tricolored bats (*Perimyotis subflavus*) relative to nightly counts and the proportion of those counts to eastern red bat (*Lasiurus borealis*) site-night counts and cover-type by the pregnancy, lactation and volancy reproductive periods (see text) from automated identification software in the eastern U.S., 2020–2021.

Table 1. Mean echolocation passes of tricolored bats (*Perimyotis subflavus*) per acoustic detector site-night and proportion of those pass counts to eastern red bat (*Lasiurus borealis*) counts in the eastern U.S., 2020–2021 by reproductive period (pregnancy, lactation and volancy), cover type (edge, forest, and riparian), and nightly maximum likelihood estimator (MLE) *P*-value (≤0.05 or >0.05) of pass confidence from automated identification software.

		Tricolored Bats			Proportion to Eastern Red Bats		
	n	**Mean**	**SE**	**Range**	**Mean**	**SE**	**Range**
MLE ≤ 0.05							
Pregnancy							
Edge	104	34.00	4.29	1–233	0.63	0.03	0.15–1.00
Forest	139	63.12	13.43	1–959	0.64	0.02	0.09–1.00
Riparian	196	67.37	9.54	1–759	0.71	0.02	0.20–1.00
Lactation							
Edge	182	42.59	7.42	1–769	0.58	0.02	0.06–1.00
Forest	266	67.52	11.04	1–1050	0.62	0.02	0.15–1.00
Riparian	243	31.37	2.62	1–251	0.70	0.02	0.16–1.00
Volancy							
Edge	340	30.99	4.67	1–955	0.66	0.01	0.11–1.00
Forest	279	43.59	6.43	1–709	0.67	0.01	0.17–1.00
Riparian	434	47.63	4.72	1–899	0.75	0.01	0.08–1.00
MLE > 0.05							
Pregnancy							
Edge	271	4.04	0.28	1–41	0.22	0.01	0.01–1.00
Forest	214	4.04	0.32	1–32	0.28	0.02	0.01–1.00
Riparian	218	3.50	0.24	1–25	0.38	0.02	0.01–1.00
Lactation							
Edge	362	5.58	0.33	1–59	0.24	0.01	0.01–1.00
Forest	238	3.51	0.26	1–21	0.33	0.02	0.01–1.00
Riparian	321	5.70	0.42	1–83	0.38	0.02	0.01–1.00
Volancy							
Edge	287	4.52	0.30	1–36	0.38	0.02	0.01–1.00
Forest	220	4.73	0.44	1–33	0.41	0.02	0.01–1.00
Riparian	219	5.69	0.52	1–47	0.49	0.02	0.02–1.00

Table 2. Binomial generalized linear mixed model parameter estimates for probability of a maximum likelihood estimator *P*-value ≤ 0.05 for tricolored bat (*Perimyotis subflavus*) site-night identification confidence from automated identification software in the eastern U.S., 2020–2021 ($n = 4533$ site-nights) by nightly count of tricolored bat echolocation passes (Count), proportion of tricolored bats to eastern red bats (*Lasiurus borealis*) in nightly counts (Proportion), reproductive period (Period) and survey site cover types (Cover). Volancy and Riparian were used as the reference conditions for Period and Cover, respectively.

Parameter	Estimate	SE	*t*	df	*P*
Intercept	–1.45	0.09	–16.13	4526	<0.001
Count	<0.01	<0.01	4.79	4526	<0.001
Proportion	2.88	<0.01	27.15	4526	<0.001
Period					
Pregnancy	–0.39	0.08	–4.92	4526	0.003
Lactation	–0.25	0.07	–3.63	4526	<0.001
Cover					
Edge	–0.03	0.07	–0.43	4526	0.666
Forest	–0.12	0.07	1.63	4526	0.103

probability of detecting a tricolored bat at the USFWS threshold was greater during the volancy period than during pregnancy or lactation periods (Table 2, Figure 3). However, this detection probability did not differ among edge, forest and riparian cover classes (Table 2, Figure 3). Examination of Q-Q plots indicated no over- or under-dispersion and AUC = 0.92.

For predicted latency to detection, riparian cover types exhibited more nights of likely tricolored bat presence at the MLE threshold than edge or forest cover sites, as did the volancy period relative to pregnancy and lactation (Table 3, Figure 4). As would be expected, regardless of reproductive period or cover type, the cumulative number of nights reaching the MLE threshold was positively related to sampling effort within a reproductive period (Table 3, Figure 4). However, model fit was moderate as analysis of Q-Q plots suggested the data were over-dispersed.

Table 3. Poisson generalized linear mixed model parameter estimates for sample site mean predicted cumulative number of nights with maximum likelihood estimator *P*-values ≤ 0.05 for tricolored bat (*Perimyotis subflavus*) echolocation passes from automated identification software in the eastern U.S., 2020–2021 ($n = 4533$ site-nights) by reproductive period (Period), day in Period (Day) and survey site cover types (Cover). Volancy and Riparian were used as the reference conditions for Period and Cover, respectively.

Parameter	Estimate	SE	*t*	df	*P*
Intercept	0.83	0.05	17.47	5454	<0.001
Day	0.06	<0.00	31.92	5454	<0.001
Period					
Pregnancy	−1.12	0.04	−26.44	5454	<0.001
Lactation	−0.48	0.04	−13.92	5454	<0.001
Cover					
Edge	−0.24	0.04	−6.33	5454	<0.001
Forest	−0.09	0.04	−2.63	5454	0.009

Discussion

Sites where tricolored bats were identified by automated software and reached the MLE threshold tended to have nightly counts >20 and eastern red bats that comprised less than a third of the proportion of the nightly count to tricolored bat counts. Our results for tricolored bats suggest acoustical swamping occurs in some instances, unlike Ford et al.'s (2024) conclusions for northern long-eared bat and Indiana bats that suggested acoustic swamping occurred only at low (<5) nightly echolocation passes of target bats and in extremely small proportions to counts of other bat species. Moreover, the nightly presence threshold was met more often during the late summer volancy period, potentially reflecting population increases due to recently volant young (Ford et al. 2011). Similarly, using within reproductive season cumulative number of nights of reaching the MLE threshold, latency to regulatory detection was reached sooner during the volancy period and within riparian cover types compared to other reproductive periods or cover types.

Because tricolored bats forage primarily near or along water features (Ford et al. 2005, Silvis et al. 2016, Cable and Wilcox 2024), we did not expect the lack of difference among predicted nights of reaching the MLE threshold but recognize the benefits of this outcome for current survey guidance that do not specify differential levels of survey effort by cover type or across the reproductive periods. However, at most survey sites, edge or forest cover survey sites were within 1000 m of the matched riparian detector site and water feature, an area easily encompassed by the home range of an adult female, adult male, or volant juvenile tricolored bat (Thames 2020, Cable and Wilcox 2024). That said, the overarching purpose of the USFWS acoustic survey guidance is to efficiently determine, at the MLE threshold, presence or absence of tricolored bats. Our results suggest riparian cover types should be

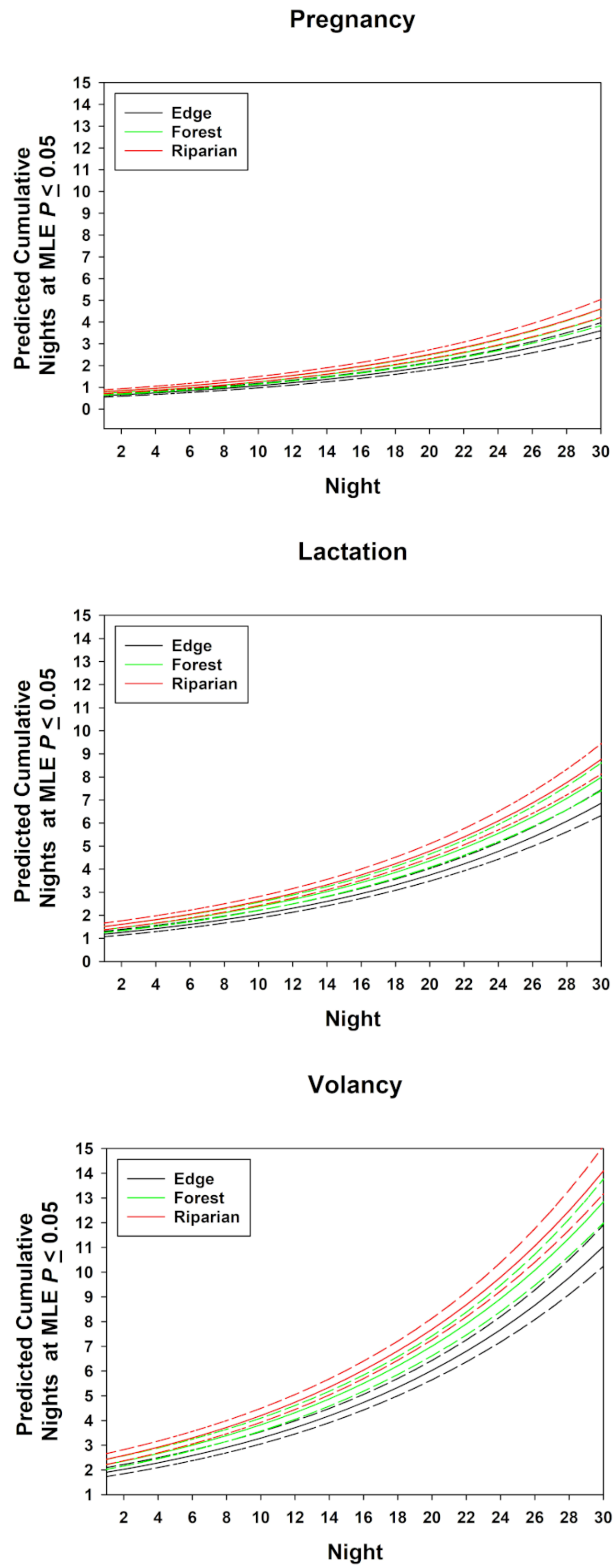

Figure 4. Predicted number of cumulative nights ($n = 4533$) modeling on a site basis reaching the U.S. Fish and Wildlife Service maximum likelihood estimator (MLE) *P*-value for tricolored bats (*Perimyotis subflavus*) relative to cover-type by the pregnancy, lactation and volancy reproductive periods (see text) from automated identification software in the eastern U.S., 2020–2021.

prioritized for surveys and that latency to detection occurs quicker during the volancy portion of the reproductive period. Although the latency to regulatory detection was low for the pregnancy period, it still was sufficient across all cover types assuming the 14 rain-free site-night northern long-eared bat level-of-effort standard (USFWS 2024) was applied.

Relative to the nightly count and proportion of nightly counts to other high echolocation frequency bats where identification of northern long-eared bats and Indiana bats was uncertain, Ford et al. (2024) suggested that visual inspection of the relatively few northern long-eared bat or Indiana bat echolocation passes would be prudent to eliminate an error of omission in presence and absence surveys. Our observations show that MLE *P*-value uncertainty could occur with relatively high nightly counts of identified tricolored bat echolocation passes, thereby presenting a daunting post-survey task for visual inspection. However, assuming individual echolocation passes are tagged with their putative identification, sorting or filtering by tricolored bat or eastern red bat for visual inspection can be performed easily as tricolored bat echolocation passes are highly diagnostic in most echolocation software viewers. Currently, for linear projects, such as tree-clearing along highway expansions, USFWS Field Offices recommend visual examination of echolocation passes to confirm tricolored bat presence when project surveyors record >10 tricolored bat passes at the individual file level concomitant to nightly MLE *P*-values >0.05 (USFWS 2024). However, our study suggests that nightly pass counts per site of tricolored bats must exceed 20 when encountered in proportion is equitable or skewed towards eastern red bats to overcome misclassification rates and reach the nightly MLE threshold for presence.

We offer three caveats to our work. First, similar to Ford et al. (2024), we conducted our study with only one of the USFWS approved automated bat identification software packages, Kaleidoscope Pro, and only using the "balanced" 0 sensitivity setting. Although Nocera et al. (2019) largely found cross-software program concurrence for *Myotis* species occupancy at the nightly level, we do not know if the relationships of ascertaining regulatory-level presence or absence relative to reproductive period and cover type would mirror those with other packages for tricolored bats. Second, our findings may only be applicable to eastern North America. In portions of the tricolored bat's distribution in western Oklahoma, western Texas and eastern New Mexico, it is sympatric with the canyon bat (*Parastrellus hesperus*; McCoshum et al. 2023) a species that shares very similar echolocation pass characteristics, making acoustic discrimination difficult (Riedle and Matlack 2013). Finally, the tricolored bat can be active in the late fall to early spring dormant season in the Southeast (Jorge et al. 2021); we suggest a similar dormant season effort to better understand how acoustic detection at the regulatory threshold varies from our summer cover type and latency to detection to inform necessary LOE guidelines (Ford et al. 2024).

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