A Robust Approach for New England Cottontail Abundance Estimation: Towards Reliable Assessment of Range-wide Conservation Goals

Adrienne I. Kovach and Thea V. Kristensen
University of New Hampshire, Durham, NH 03824
A Report for Partners in the Range-wide New England Cottontail Conservation Initiative
December 2017

USFWS Photo, M. Poole

Introduction

Abundance is a fundamental parameter in applied ecology because its estimation is essential for managing animal populations, including species of conservation concern. In recovery plans and delisting criteria, abundance is the most commonly used quantitative metric and it is the key parameter underlying extinction risk (Shaffer 1981, Lande 1993, Cambell et al. 2002). Robust abundance estimation is integral to prioritizing conservation resources and permits measurement of response to management actions (Nichols and Williams 2006, Johnston et al. 2015). The iterative feedback between implementation of management actions and assessment of their outcomes, which is at the core of the adaptive management process (Walters 1986, Walsh et al. 2012), is increasingly seen as valuable for species of conservation concern (Evans et al. 2016). Abundance estimation, therefore, is a critical component of monitoring programs based in an adaptive management framework.

For the New England cottontail (*Sylvilagus transitionalis*), as for any species of conservation concern, effective monitoring is essential for assessing population status and evaluating progress toward conservation goals. New England cottontails exist in small, geographically and genetically isolated populations, with low effective population size (Fenderson et al. 2011). Extensive efforts have been made over the last decade to determine range-wide occupancy patterns to identify where remnant populations occur (Litvaitis et al. 2006; Fuller and Turr 2012; Brubaker et al. 2014; New England Cottontail Range-wide Conservation Initiative, unpublished data). Additional research has focused on identifying genetic structure of range-wide and local populations (Fenderson et al. 2011, 2014, Cheeseman 2017), and connectivity across the landscape (Fenderson et al. 2014; Amaral et al. 2016). An emerging theme from this body of research is that viability is uncertain for many populations even in the short-term (Litvaitis and Villafuerte 1996; Fenderson et al. 2011, 2014, Cheeseman 2017). These findings underscore the urgent need for evaluating population status on local, regional, and range-wide scales. To do so, obtaining reliable information on abundance will be necessary for adaptively managing populations and ensuring conservation measure are effective.

Careful experimentation with sampling designs that consider imperfect detection has provided biologists with clear, highly reliable means of determining patch-specific presence of New England cottontail (Brubaker et al. 2014). Yet, knowledge of site occupancy provides an incomplete picture of population status. Evaluating population status also requires detailed knowledge of population size and density. For New England cottontail, this is critical information that is lacking for most geographic locations today, thereby hindering the ability to make carefully informed management decisions. Previous research has shown that many suitable habitat patches are unoccupied (Litvatis et al. 2006; Brubaker et al. 2014) and that occupied patches vary widely in their densities (Kovach and Brubaker 2012). This landscape-level variation in cottontail distribution suggests that extrapolating local patch-specific densities across suitable or occupied habitat patches in the landscape will lead to inaccurate abundance estimates. Consequently, evaluating the status of cottontail populations requires reliable methods for patch-specific abundance estimation. Addressing this need was the overarching goal of this project. New spatially explicit capture-recapture models applied in conjunction with noninvasive genetic sampling provided a powerful methodological framework for achieving this goal (Borchers and Efford 2008; Royle et al. 2013).

The specific objectives of this project were to:

1) **Establish an optimal sampling framework to obtain robust (precise and unbiased) population abundance estimates using spatially explicit models.**
2) **Develop a spatially explicit genetic mark-recapture analysis protocol applicable to patch-specific abundance estimation across the species' range.**
3) **Apply the newly developed methodology to select patches in Maine and Connecticut.**
4) **Provide information in an adaptive management context, allowing habitat managers to evaluate actions at local, state and regional scales.**

Methods

Site Selection: To collect data to develop the optimal sampling scheme, with the help of partners, we surveyed three patches of New England cottontail management interest in the Cape Elizabeth focus area in Cumberland County, Maine (Kettle Cove, Crescent Beach and Libby Field; Figure 1). The Libby Field site has been managed specifically for New England cottontail by the U.S. Fish & Wildlife Service for the last 10 years. To fully evaluate our approach for range-wide application, we also surveyed three patches in Windham and New London counties in eastern Connecticut, where New England cottontail occur in sympatry with eastern cottontail (Spignesi, Nichols and Hatchery). Selected sites had previously been determined to be occupied by New England cottontails. Sites comprised patches of continuous thicket habitat, which cottontails could utilize without going into areas of non-thicket, open water or roads >9 m wide. Size of study sites, which includes the surveyed transects surrounded by a 50-m buffer, ranged from 9.7 to 19.9 hectares (Table 1.)

Surveys: In the winter of 2015, we conducted intensive surveys at each of the six sites on three separate occasions in Connecticut and on two occasions in Maine. All surveys were conducted within a month interval to approximate demographic closure. Sites were surveyed 3-5 days after a snowfall event, following optimal survey conditions for New England cottontail detection outlined in Brubaker et al. (2014). Surveys focused in suitable thicket habitat, following loose transects 30 m apart, across each habitat patch. Lagomorph pellet samples were collected from distinct clusters at 20-m intervals along the transect and stored in separate 15 mL conical tubes at -20° C until used in genetic analyses. We collected geographic data on the path followed by the surveyor and pellet sample locations using handheld GPS units. The detailed protocol is available in Appendix 1.

Genotyping and Individual Identification: DNA was extracted from fecal pellets using QIAamp DNA Stool Mini Kits (Qiagen,Valencia, CA) following the methods of Kovach et al. (2003). Individuals were identified from their unique multi-locus genotypes with a suite of 12 microsatellite loci. We used 10 loci developed specifically for the New England cottontail (King et al. 2017) and two loci (Sfl011 and Sfl014) developed for eastern cottontail by Berkman et al. (2009). Primer information, Genbank accession numbers, and PCR conditions are found in Appendix 2. To ensure quality control genotyping of low copy DNA (Waits and Paetkau 2005), PCRs were performed in replicates of four, and consensus genotypes were obtained at each locus, requiring two consistent replicates in the case of a heterozygote and three consistent replicates for a homozygote (Frant et al. 2003). Samples were identified as eastern or New England cottontail based on species-diagnostic alleles. To distinguish samples that originated from the same or unique individuals, we used the multi-locus

matches option in GenAlEx (Peakall and Smouse 2012) that identifies samples with matching and nearly matching genotypes within each study site. We re-evaluated samples that differed by only 1-2 loci, including review of electropherogram profiles, to verify that these individuals were unique. We calculated P_{ID} and P_{SIBs} in GenAlEx to evaluate the discriminatory power of the loci for each study site separately. Using individual genotype, location of samples, and date that samples were collected, we constructed a capture history for individuals at each site.

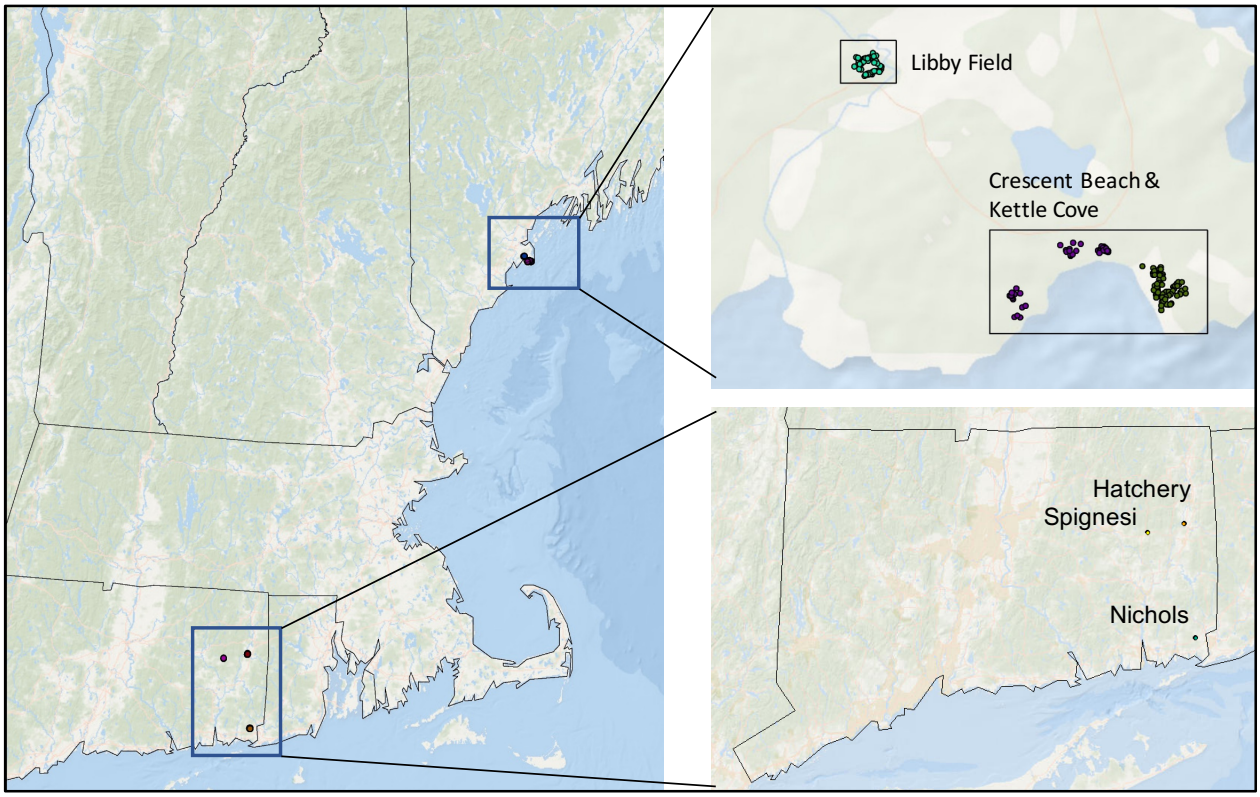

Figure 1. Locations of patches surveyed for cottontail abundance estimation in this study. Three patches – Libby field, Crescent Beach, and Kettle Cove – were located in Cape Elizabeth, Maine (top right), and three patches – Hatchery, Spignesi, and Nichols properties – were located in eastern Connecticut (bottom right).

Patch-specific Abundance Estimation: Cottontail densities were estimated for each site using capture histories from the full data collected from the replicate survey occasions and finest scale pellet sampling intervals, with a spatially explicit abundance estimation approach using the SECR package in R (Efford 2016). In areas where eastern cottontails and New England cottontails were sympatric, we separated the data for each species and estimated density independently. Our sampling design, whereby pellets were collected every 20 m along regularly spaced transects, is a modification of typical search-area approaches (Efford 2011). Because samples were only collected systematically at these designated intervals, we treated each 20-m point as a proximity detector in the model. To accommodate the patchy nature of the habitat, we used masks that set a 50-m buffer around the GPS tracks of the searched transects and excluded water and areas with high levels of human use. For each data set, we used the half-normal detection function and estimated detection probability at the activity center (g_0~1), spatial extent (σ~1), and density (D~1) using the null model. For each site, we evaluated the estimated density and abundance (based on the surveyed patch area), along with 95% confidence intervals (CI), and calculated the coefficient of variation (CV; standard error of density estimate/density estimate) as a measure of precision.

Evaluating the Optimal Survey Protocol: To evaluate the optimal survey and sampling protocol needed for precise abundance estimation, we subsampled the full data from each patch and evaluated the influence of 1) the number of sampling occasions (1-3), 2) the width of the spacing of the survey transects (30-90 m), and the spacing between subsequent pellet detections (20-60 m; Fig. 2). We ran each of these subsets of data in the SECR package, as above, then evaluated the precision, using the CV, and accuracy, using relative bias – measured as the difference in the estimate from the reduced dataset relative to the estimate from the full dataset. In this way, we used the subsampled data to explore how estimates with reduced sampling effort compared to estimates obtained with the most intensive sampling effort and to determine which survey designs (level of sampling intensity) produced acceptable levels of bias and precision, while minimizing required effort and cost.

Simulations to Evaluate Sampling Intensity Effects on Precision and Bias: To further evaluate the influence of survey effort on density estimation, beyond that achievable by subsampling of our empirical data, we used the R package *secrdesign* (Efford 2015) to simulate scenarios with different levels of cottontail density and survey intensity (pellet collection spacing and survey replication). For each combination of density and survey conditions, we ran 100 simulations. We set the area as 9 ha for all simulations; we chose this relatively small patch size because New England cottontail often occupy patches of this size and this represents a monitoring scenario relevant for management. Since precision is harder to achieve for smaller rather than large populations, scenarios on larger patches will likely be more robust than those on small patches. To most closely approximate expected field conditions, we used our observed data as a basis for selecting parameters for simulations. We selected intermediate values from our empirical data for sigma and *g0* (Table 2), setting sigma at 50 m and *g0* at 0.1. We ran scenarios with densities of 0.5, 1, 2, and 3 rabbits per hectare to approximate the range of density estimates observed in the field (0.7-3.4 rabbits per hectare; Table 2). For each level of density, we simulated combinations of 1, 2, or 3 survey occasions, pellet collection spacing of 20, 30, 40, 50, and 60 m and transect spacing of 30, 40, 50, 60, 70, 80, and 90 m. We ran all scenarios with a 50-m buffer and used proximity detectors to represent locations where pellets would be collected, as above. For each scenario, we estimated density, standard error, upper and lower 95% CIs; we also recorded whether each model converged successfully. To demonstrate how convergence, bias, and precision would be influenced by a higher *g0* (detection probability at the activity center), we

subsequently ran a full second set of simulations with *g0* at 0.2. As for the subsampled datasets above, we evaluated the precision and bias of the estimates and used the results to evaluate the influence of reduced sampling intensity on robust abundance estimation.

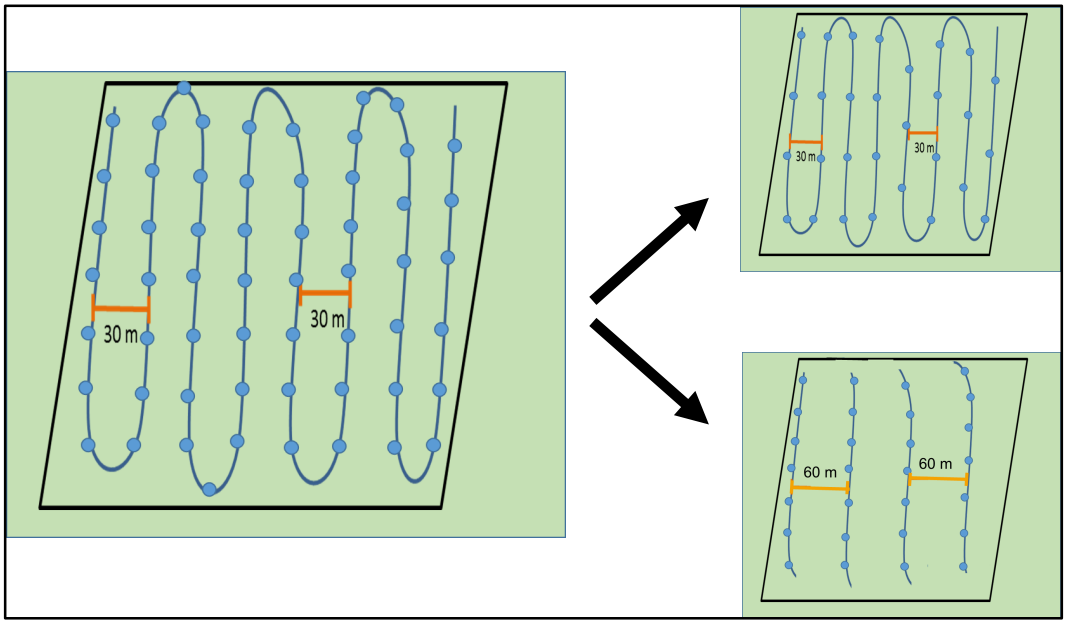

Figure 2. Sampling scheme used for patch-specific population surveys of New England cottontails. Left panel shows the intensive sampling scheme whereby pellets are collected every 20 m, along transects spaced 30 m apart. Right panels show subsampling of the individual pellet spacing (top) and the transect spacing (bottom); these subsampled datasets were evaluated to determine effects on accuracy and precision of abundance estimates relative to the full dataset.

Results & Interpretation

Across all sites, we collected 483 pellets, successfully genotyped 375 samples, and identified 122 individuals (Table 1). During each survey occasion, we collected between 3-59 pellet samples for a total of 18 – 102 pellet samples per site. Only New England cottontail occupied Maine sites; we detected eastern cottontail at all three Connecticut sites, but only detected New England cottontail at Nichols and Spignesi.

Genotyping success ranged from 63% on Crescent Beach to 100% on Spignesi, and likely was a function of the environmental conditions of the survey period (Table 1). The probability of identity (P_{ID}) ranged from 4.3×10^{-6} to 9.4×10^{-7} (P_{SIB} 1.7×10^{-3} to 3.0×10^{-3}) for New England cottontail and 8.9×10^{-4} to 8.9×10^{-9} (P_{SIB} 2.8×10^{-2} to 3.7×10^{-4}) for eastern cottontail (Table 1). The Spignesi site in Connecticut had the lowest discriminatory power (highest P_{ID} and P_{SIB} values for each species) and we detected only 3 eastern cottontails and 2 New England cottontails there, despite a high capture rate; we therefore omitted that site from density analyses. For the remaining sites, discriminatory power of the microsatellite markers was high, indicating that there was a 1 in 232,558 to 1 in 2.7 billion chance that 2 randomly selected individuals from the population shared a genotype and a 1 in 333 to 1 in 6667 chance that two siblings shared a genotype at those 12 loci. We detected 5 to 28 unique individuals per site, with the average number of captures per individual ranging 1.7 on Crescent Beach to 9.5 on Spignesi.

Table 1. Site description and patch size, number of repeat surveys conducted, genotyping success, probability of individuals sharing a genotype (P_{ID}), probability of siblings sharing a genotype (P_{Sib}), number of unique individuals detected, and average captures per individual for mark-recapture pellet surveys conducted in winter of 2015-2016 in Maine and Connecticut. Note that in sites where New England cottontail and eastern cottontail co-occur, results are listed separately by species. Area includes sampled habitat plus a 50-m buffer.

Site	State	area (ha)	species	#surveys	pellets	% genotype success	P_{ID}	P_{SIBs}	individuals detected	average captures
Crescent Beach	ME	19.9	NEC	2	73	63	9.4×10^{-7}	1.7×10^{-3}	28	1.7
Kettle Cove	ME	18.7	NEC	2	91	67	3.5×10^{-6}	2.7×10^{-3}	20	3.1
Libby Field	ME	12.0	NEC	2	63	84	4.3×10^{-6}	3.0×10^{-3}	18	3.2
Hatchery	CT	9.7	EC	3	87	78	3.7×10^{-10}	1.5×10^{-4}	25	2.7
Nichols	CT	10.7	EC	3	102	86	8.9×10^{-9}	3.7×10^{-4}	20	4.4
Nichols	CT	10.7	NEC	3	30	93	7.0×10^{-7}	1.2×10^{-3}	6	4.7
Spignesi	CT	15	EC	3	18	72	5.1×10^{-5}	8.0×10^{-3}	3	4.3
Spignesi	CT	15	NEC	3	19	100	8.9×10^{-4}	2.8×10^{-2}	2	9.5

Patch-specific Abundance Estimates: Cottontail densities ranged from 0.68 to 2.57 rabbits per hectare (7 – 52 individuals per patch; Table 2). The lowest density was on the one site with sympatric New England and eastern cottontail (Nichols). Densities on Maine sites occupied only by New England cottontail were all >1 rabbit/ha. Density for eastern cottontail was 1.99 on the sympatric Nichols site and 3.43 on the Hatchery site, from which New England cottontail were absent. The coefficient of variation (CV), a measure of the precision of the estimate, was 0.21 – 0.25 for 5 of the 6 estimates and 0.43 for the New England cottontail estimate on Nichols. These findings suggest that reasonably precise estimates (CV≤25%; following Pollock et al. 1990) can be obtained for intensive datasets collected from two to three surveys, when cottontail densities are >1 rabbit/ha. At low

densities and small population sizes, obtaining precise estimates may be challenging, and a minimum number alive may be the most robust information obtainable under such scenarios.

Table 2. Cottontail SECR density estimates for six sites sampled in Maine and Connecticut during winter 2015-2016. Density estimates are per hectare, CV is the coefficient of variation (SE of density estimate/density estimate), g0 indicates the probability of capture at home range center, σ indicates spatial extent in meters; N is abundance over the study area (sampled habitat patch).

	species	D (95% CI)	CV	g0 (95%CI)	σ (m)	N (95%CI)
Crescent Beach	NEC	2.57 (1.58-4.18)	0.25	0.02 (0.01-0.04)	102 (64-161)	52 (32-84)
Kettle Cove	NEC	1.22 (0.78-1.90)	0.23	0.15 (0.10-0.22)	55 (45-66)	22 (21-30)
Libby Field	NEC	1.69 (1.10-2.70)	0.24	0.08 (0.05-0.13)	67 (53-85)	20 (13-32)
Hatchery	EC	3.43 (2.27-5.17)	0.21	0.12 (0.08-0.18)	33 (28-40)	34 (29-47)
Nichols	EC	1.99 (1.28-3.08)	0.23	0.14 (0.10-0.19)	47 (41-55)	21 (20-27)
Nichols	NEC	0.68 (0.30-1.52)	0.43	0.17 (0.09-0.28)	31 (24-40)	7 (6-14)

Our results also suggest that obtaining precise estimates for New England cottontail on sites where they are sympatric with eastern cottontail may be more challenging than doing so for sites that contain exclusively New England cottontail, and the former may require multiple survey visits. In the case of the Nichols site, two independent, intensive survey efforts only identified six unique New England cottontails from 30 pellet samples. High genotyping success of 93% and 4.7 captures per individuals suggest that this sampling was quite robust and the low CV of the population estimate was due to a small population size rather than an insufficient sampling effort. Interestingly, the two species were spatially segregated on the Nichols site, with New England cottontails occupying a small portion of the central part of the patch, surrounded by eastern cottontails on the periphery (Figure 3). On the Hatchery site, although New England cottontails had been detected previously, we did not detect them during the surveys of this study. It is possible that New England cottontail no longer occupy this patch; however, it is also possible that the portion of the patch selected for this study did not include that area occupied by New England cottontail. These findings highlight the need for intensive and systematic survey protocols on these sites and for a clear definition of the site/patch for which inference is being made. For these reasons, the trap array should encompass the entirety of the habitat patch to maximize detectability.

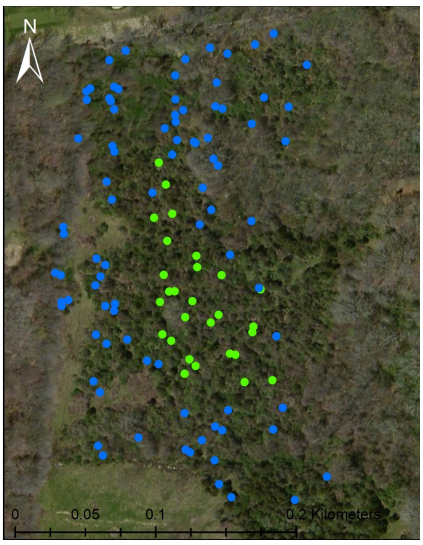

Figure 3. Locations of cottontail fecal pellets collected during winter surveys in 2015 on Nichols site in Connecticut. Spatial segregation of the species within the patch is evident; blue circles represent eastern cottontail pellet locations and green circles represent New England cottontail pellet locations.

Detection probability (*g0*) ranged from 0.02 on Crescent Beach to 0.17 on the Nichols site, and it was >10% for four of the six sites. Notably, the Crescent Beach site, with the lowest detection probability, also had the highest genotyping error and the fewest recaptures per individual. Despite these challenges, the sampling effort across the full three survey visits yielded a density estimate with a reasonable level of precision (CV = 0.25).

Spatial extent (σ) for most sites was between 31-67 m, although σ for the Crescent Beach site in Maine was estimated at 102 m. This parameter can be converted to a 95% home range radius estimate, following Repucci et al. (2011). Doing so yields circular cottontail home range estimates of 1.8 – 19.6 ha. Excluding Crescent Beach, which had the largest sigma of 102 m and the lowest recapture rate, σ ranged 31 – 67 m, with corresponding home range estimates of 1.8 – 8.5 ha, similar to prior home range estimates from radiotelemetry (H. Kilpatrick, CTDEEP, personal communication and unpublished report).

Optimal Survey Protocol: Analysis of the subsampled datasets revealed that CV and relative bias both increased with increasing transect spacing and fewer number of occasions. CV also increased with increasing spacing between pellet collections. Relative bias of 10-15% could be achieved with 20 m pellet spacing and 30-60 m transect spacing for 2-3 survey occasions. For a single survey occasion, the lowest relative bias was 25% with 20 m pellet spacing and 30-60 m transect spacing. Further subsampling led to biases of 30-50%. Similarly, CVs <25% could only be obtained for the most intensive sampling (20 m pellet spacing and 30-60 m transect spacing) with 2-3 survey occasions. For single surveys, it was possible to achieve CVs <30% on a few sites with the most intensive sampling. Precision was lower with less intensive sampling; CVs reached 40-75% for single surveys with >40 m pellet spacing and >60 m transect spacing (Fig. 4).

Similar to our findings from subsampling empirical data, number of survey occasions, pellet collection spacing, and transect spacing were all important predictors of bias and precision for the simulated datasets (Fig. 5). True density had a clear impact on measures of bias and precision, with error decreasing as density increased (Fig. 6). As pellet collection and transect spacing increased, relative bias increased (Fig. 5). Density estimates tended to be biased increasingly low as sample spacing increased for one sampling occasion, and biased high with three sampling occasions, while two sampling occasions produced a less pronounced pattern. RB < 0.15 was achieved with only one sampling occasion, though only with high sampling intensity at low densities (Table 3).
When we replicated models with a higher g0 of 0.2, both RB and CV improved, allowing less intensive sampling (Table 4).

In sum, these findings show that reasonably precise (CV<30%) and accurate (RB <15%) abundance estimates can be obtained with an intensive sampling scheme, whereby pellets are collected every 20-40 m, along transects spaced 30-60 m, under optimal environmental conditions. Typically, two independent surveys will be required to obtain the most precise and accurate estimates. Under some circumstances, one survey with the most intensive sampling (20 m pellet spacing, 30 m transect spacing) may yield an estimate with reasonable precision and accuracy, particularly if on a moderate to high density site. Low density sites with small sample sizes will require the most intensive sampling for robust estimation.

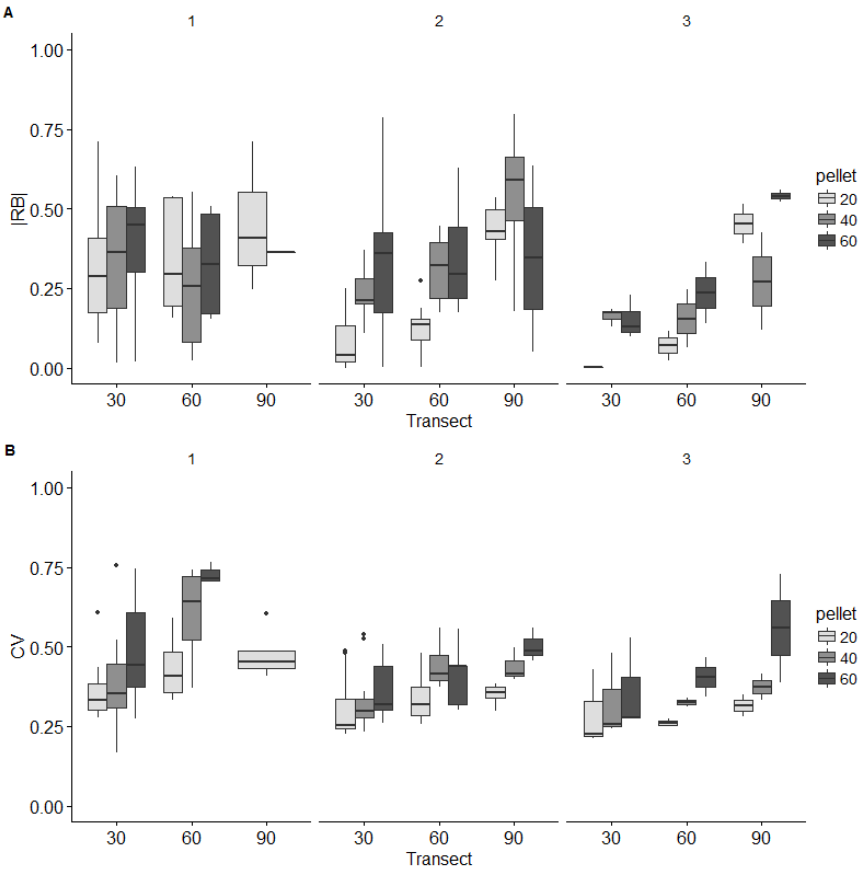

Figure 4. Measures of A) bias (absolute value of relative bias; RB) and B) precision (coefficient of variation; CV) for New England and eastern cottontail density estimates from subsampled empirical data, as a function of pellet spacing, transect spacing, and number of survey occasions.

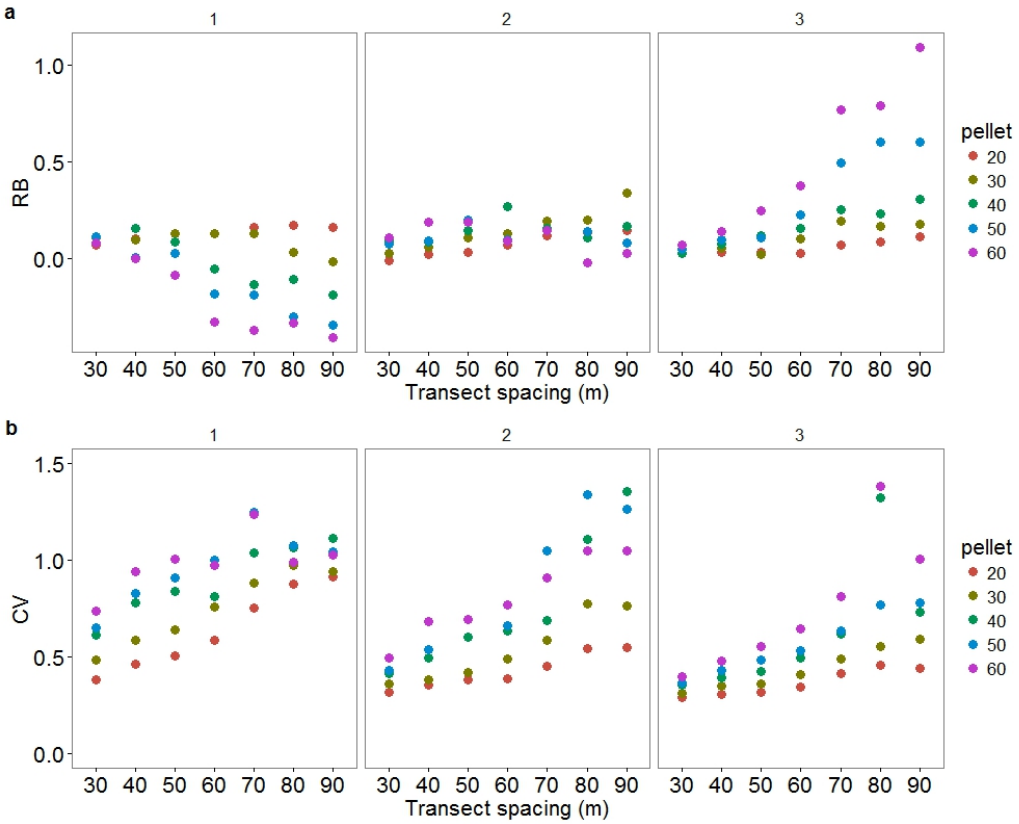

Figure 5. Average error measures (y axis) by survey occasion for cottontail simulated data, for varying transect spacing (x axis) and pellet spacing (colored circles). CV is coefficient of variation and RB is relative bias.

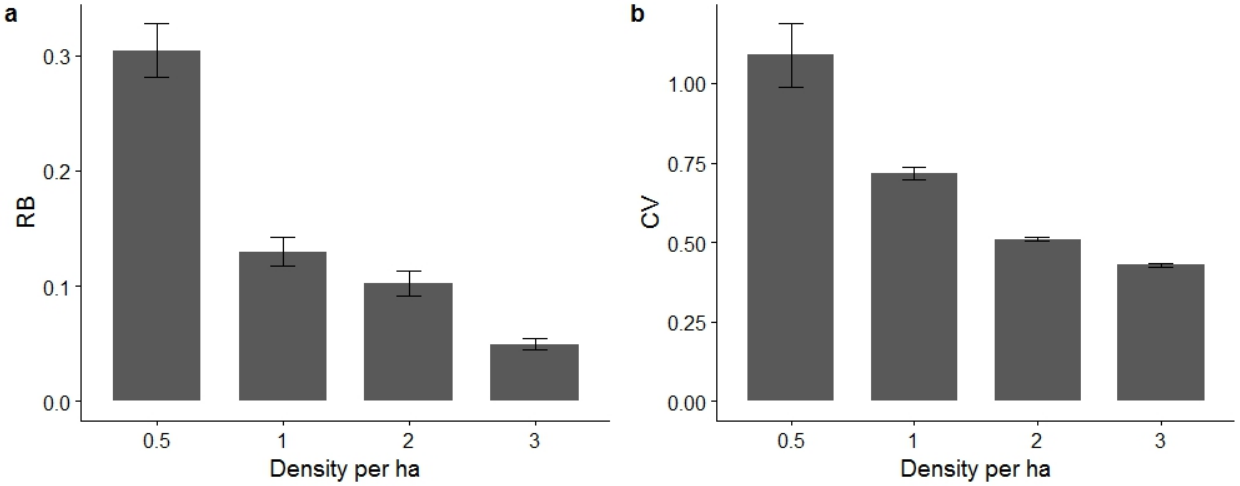

Figure 6. Variation in error (mean +/- SE) in response to density for simulated cottontail populations across all sampling scenarios. CV is coefficient of variation and RB is relative bias.

Table 3. Results from simulated scenarios for lowest effort sampling (pellet and transect spacing intervals and number of survey occasions) to obtain model convergence and desired levels of bias and precision with g0 (detection probability) of 0.1 for four levels of cottontail density on the landscape.

Density		0.5 rabbits/ha			1 rabbits/ha			2 rabbits/ha			3 rabbits/ha		
	cutoff	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions
converge	100%	20	30	2	20	30	1	20	60	1	30	50	1
RB	0.15	20	30	1	30	40	1	40	40	1	50	60	1
CV	0.2	-	-	-	-	-	-	20	30	3	30	30	2
	0.3	-	-	-	20	30	3	20	30	1	20	50	1

*Note that for 0.5 rabbits per ha, convergence was 98%.

Table 4. Results from simulated scenarios for lowest effort sampling (pellet and transect spacing intervals and number of survey occasions) to obtain model convergence and desired levels of bias and precision with g0 (detection probability) of 0.2 for four levels of cottontail density on the landscape.

Density		0.5 rabbits/ha			1 rabbits/ha			2 rabbits/ha			3 rabbits/ha		
	cutoff	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions	pellet spacing (m)	transect spacing (m)	# survey occasions
converge	100%	30	40	2	20	50	1	30	60	1	50	60	1
RB	0.15	30	40	1	40	40	1	60	60	1	60	60	1
CV	0.2	-	-	-	-	-	-	20	30	2	20	50	1
	0.3	-	-	-	20	30	2	30	50	1	20	80	1

Conclusions and Recommendations for Robust Patch-Specific Abundance Estimation

Here we provided a protocol and optimal sampling strategy for obtaining precise and unbiased abundance estimates of New England cottontail from spatially explicit mark-recapture models in conjunction with noninvasive genetic sampling (See Appendix 3 for Protocol). We demonstrated that, using our protocol, reliable and robust patch-specific abundance estimates can be consistently obtained for addressing management goals. A key finding of this work is that very intensive surveying and sample collection are required to obtain robust abundance estimates for New England cottontails. The appropriate sampling intensity may either be achieved from a single spatially intensive survey or two, independent surveys (see Table 5 for explicit guidelines).

While we demonstrated the efficacy of our abundance estimation approach, we also caution against efforts that deviate substantially from these guidelines. Surveys that deviate substantially from systematic, intensive sample collection (every 20-40 m along 30 m transects) will yield unreliable estimates that could be grossly biased and extremely imprecise; such estimates would not be useful to inform conservation management and we caution against their employment. Methodologically, this is due to the need for robust capture histories that provide representative data on multiple individuals from the patch as well as the rich recapture rates that underlie the mark-recapture framework. Further, it is critical to clearly define the "site" or "patch" and to carefully match the survey effort to the area for which inference is made. When possible, we suggest fully surveying continuous areas of suitable habitat until a boundary of non-habitat (road, development, open or forested area lacking understory habitat) is encountered. This is particularly important in areas where New England and eastern cottontail occur in sympatry, as partial or incomplete surveys of a site may fail to detect one of the two species due to their spatial segregation within the habitat patch. This will affect both abundance estimates and detection during occupancy surveys, whenever a patch is not fully surveyed. For this reason, in these areas, making landscape-level predictions of New England cottontail abundance from habitat data alone will not yield reliable information. Instead, where the two species occur in sympatry, landscape-level models of New England cottontail abundance will require accurate data on occupancy, knowledge of the distribution and density ratios of the two species, and habitat covariate associations. With careful application of these types of data, we anticipate that the spatially explicit models presented here can be extended to the landscape.

Several factors may influence the precision and bias of the estimates, including patch size, placement of the sampling transect array within the patch, cottontail density, sympatry with eastern cottontail, environmental conditions of the survey, and experience of the surveyor. The optimal survey design would consider these factors, as well as the level of precision desired for the targeted outcome. Based on these factors and the results of our study, we make the following recommendations for abundance surveys (see also the protocol in Appendix 3:

- Patches should be surveyed with an intensive sampling scheme, with pellet collections every 20-40 m along survey transects separated by 30 m.
- Patches must be defined and delineated carefully, and inference should be made only to the areas that are intensively surveyed according to this protocol.
- Surveys should focus systematically on continuous stretches of suitable habitat bounded by areas of non-habitat (roads, development, open areas, or forests lacking understory).

- For highest precision and accuracy, two independent surveys should be conducted, following the optimal survey conditions outlined in Brubaker et al. (2014).
- Repeat surveys of a site should be conducted within a narrow window of time (2-3 weeks when possible) to minimize violation of the closure assumption.
- Many factors influence the outcome of the surveys and estimation; less intensive sampling or single surveys may be appropriate under certain conditions. See Table 5 for some guidelines.

Table 5. Guidelines for decision-making about the intensity of surveys required to obtain abundance estimates for cottontails, based on factors that influence the outcome of survey and analysis efforts.

	Less Intensive/Single Survey May be Sufficient	**2 Intensive Surveys Needed**
Precision Required	Low/Moderate Precision High Precision (if high density, large patch & optimal survey conditions)	High Precision
Rabbit Density	High Density (if conditions are optimal)	Low Density
Patch size	Large Patch (if high density & optimal conditions)	Small Patch (or use minimum number alive if not need high precision/accuracy)
Sympatry with EC	No EC (if conditions are optimal)	EC present (with optimal conditions a single survey may be sufficient; may depend on the relative densities of the two species)
Survey Conditions	Optimal Conditions (high genotyping success)	Suboptimal Conditions (fewer samples, reduced genotyping success)
Surveyor	Experienced Surveyer (may lead to higher sample collection success)	Inexperienced Surveyer

Future Directions and Management Implications

The results of our study provide an important new tool for the management and monitoring of New England cottontail. To date, cottontail monitoring efforts have relied exclusively on occupancy surveys. Yet, occupancy is an inadequate surrogate for abundance (Johnston et al. 2015), and abundance is the critical metric for conservation decision-making, especially for threatened and endangered species and those with recovery plans. The methods presented here provide a framework for effective abundance estimation of New England cottontails using spatially explicit mark-recapture in conjunction with noninvasive genetic surveys. This approach can be integrated into an adaptive management framework for assessing the outcomes of management actions implemented via the species' Conservation Strategy. Specifically, abundance estimation will be needed for assessing response to alternative management actions, tracking the success of translocations and releases from the captive breeding program, considering the impact of sympatry with eastern cottontails, and evaluating population goals set by the species' recovery plan (Fuller and Tur 2012, 2017).

With careful study design and collection of associated covariate data, the patch-specific results from this approach can be used in conjunction with habitat data to develop models for predicting population density into occupied areas with unknown density (e.g., Drewry et al. 2013). With accurate occupancy data, these models can then be applied for landscape-level abundance estimation. We suggest this is a powerful application of this spatially explicit approach that, with caution, can be used to provide much needed insight into New England cottontail population status assessments on the scale of metapopulations and focus areas.

Finally, the genetic data obtained through this approach provide additional information from which population viability can be assessed. Specifically, the monitoring of genetic metrics (genetic diversity, effective population, etc.) is informative about the overall health and structure of small populations. This is particularly true for captive rearing and translocation programs where genetic monitoring can inform managers about which founders are producing offspring and how long the founder genes persist in the population gene pool through time (DeMay et al. 2017). Tracking changes in genetic diversity of augmented populations may also provide insight into population health and individual fitness, as increased levels of heterogygosity may result in increased survival (e.g., as was found in pygmy rabbits by DeMay et al. 2017). These additional genetic metrics increase the value of the assessments and inferences that can be made from the abundance estimates.

Acknowledgements

Funding for his project was funded by the Maine Department of Inland Fisheries & Wildlife, Connecticut Department of Energy and Environmental Protection, U.S. Fish and Wildlife Service Region 5, Division of Natural Resources, National Wildlife Refuge System, and the New Hampshire Agricultural Experiment Station, through a USDA National Institute of Food and Agriculture McIntire-Stennis Project # 225575. We are grateful to Howard Kilpatrick, Travis Goode, Zach Olsen, Kelly Boland, and undergraduates from the University of New England, who collected samples for this study. We also thank Kelly Boland, Walter Jakubas, Kate O'Brien, and Howard Kilpatrick for their input on site selection. Conversations with Walter Jakubas and Kate O'Brien informed our ideas about New England cottontail adaptive management and we are grateful to both of them for their feedback on a previous draft of this report. Margot Popecki, Christina DiMeo, and Alyson Duquette assisted with lab work.

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Appendix 1 – Survey Protocol Used in this Study

New England Cottontail

Spatially Explicit Mark-Recapture Protocol

University of New Hampshire

Adrienne Kovach and Thea Kristensen

What sites to survey*: patches of ~20+ ha in size*

- If smaller patches are more typical of your focus area, discuss patch selection with Adrienne/Thea.

How many surveys*: 2 independent surveys at each site*

- Pellets should be collected after two separate snowfall events.
- Ideally, these snowfall events will be close in time. However, it is more important to do the 2 sampling sessions rather than be concerned about how close they are in time.

When to survey*: 3-5 days after a snowfall event*

- This allows for accumulation of pellets and tracks.
- Ideally 4 days after snowfall event, however weather is a factor, hence the 3-5 days after an event.
    - It will be important to monitor the upcoming weather conditions to be able to make optimal decisions for collection.
        - If it warms above freezing and causes snow to melt this can degrade DNA. Hence if temperatures are expected to warm in the 4th or 5th day, it would be advisable to sample on the 3rd day after snowfall.
        - Cold weather <-10° C and high winds, may limit rabbit activity. If these are the conditions, sampling 4-5 days after snowfall is advised.
    - Note that there is a limit to the benefit derived from increasing pellet deposit days after snowfall, because of the negative effects of environmental exposure on pellet DNA quality (freeze-thaw, rain, warm temperatures, and dampness in particular are known to degrade DNA). For this reason, 4 or 5 days after snowfall is considered a maximum wait period for surveys.
- Preferable to have <12 inches of snow.
    - While snow cover increases the detectability of fecal pellets and tracks, deep snow may inhibit cottontail activity or even facilitate subnivean behavior (rabbits may travel through air pockets beneath the snow, especially around dense vegetation). For this reason, surveys conducted in deep snow may be less effective than surveys conducted with <1 foot snowpack. However, it may not be possible to survey three times during these ideal conditions. Thus, it may be best to survey whenever there is snowfall to do so.

How much of patch to survey: *as much as possible, representative of landscape*

- If it is possible, it would be ideal to survey the entire patch or a large representative portion (*not just what is assumed to be suitable habitat*), allowing for inclusion of

variation in the habitat. This will let us identify features that correlate with occupancy/abundance and may help us develop future predictive models.

Spacing between samples: *every 30 m along transect*

- Aim to sample intensively, collecting pellets approximately every 30 m along the sampling transect (20-40 m is acceptable).

Spacing between transects: *30 meter spacing between transects*

- Search the patch systematically using a continuous transect that winds its way back and forth across the patch keeping approximately 30 meters between passes.
- Due to the difficulty of traversing thicket habitat, the search transects need only serve as a rough guide from which one may need to deviate to facilitate movement around denser thickets. If cottontail tracks are discovered they should be followed until either pellets are found or the tracks are lost (due to crusted snow or dense vegetation). The survey should then continue from the point where the tracks were first discovered.
- *While 30 m transect spacing is ideal, slightly greater spacing between transects may be allowed if necessary. Limit the greatest distance between transects to 60 m.*

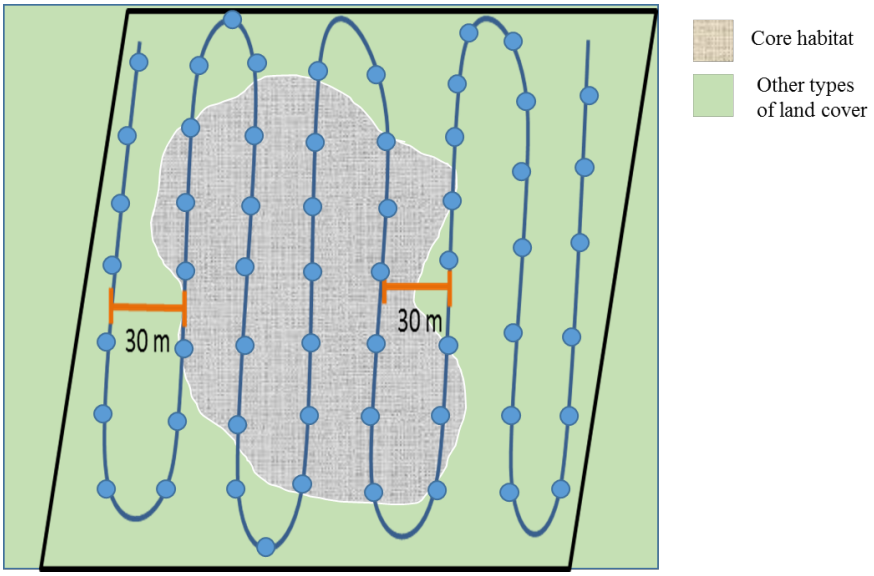


Figure shows the survey design focused on intensive sampling of pellets every 30m, along loose transects winding back and forth across the sampling area at approximately 30 m intervals. Note that surveys include the core, suitable habitat (in grey) as well as less suitable habitat areas surrounding the core.

Tracking: *Use a handheld GPS unit to track movements of surveyors during the duration of the survey and mark 4 corners of polygon sampled.*

Pellet Collection: *collect pellets from single cluster into a unique vial labeled with location coordinates*

- Pellets may be found within a distinct or slightly diffuse clump, or alternately, several pellets may be scattered individually along a short trail. The objective is to collect several pellets from the same individual rabbit (2-10); these pellets will be placed together in a unique vial. Restrict collection for each vial to an area approximately 5 x 5ft or smaller to minimize the number of individuals represented in each vial (ideally, one rabbit per vial). Use disposable sterile gloves and place the pellets in a unique vial; try to keep snow out of the vial but avoid rubbing the pellet as it may remove DNA. Do not touch the pellets with bare hands, as this may cause contamination, and use a fresh glove for each vial.
- Use a GPS to record the exact location of pellets collected. Record this in a data sheet and on the pellet vial. It may be useful to record other identifying information on the vial, such as: initials or name of person collecting samples, site name, date, survey number (1, 2 or 3) and a unique sample identification number.

Sample storage: *keep samples cold/frozen at all times to the extent possible*

- Because DNA can degrade quickly if not frozen, return the vial(s) to a cooler upon returning to your vehicle and deposit in a freezer as soon as possible. A good trick is to temporarily store your pellets in a ziplock bag filled with snow while you are in the field (e.g., to prevent them from heating up in your pocket), and transfer them to a cooler once you get to your vehicle.

Sample shipment: *on ice overnight*

Ship samples, preferably by FedEx, overnight on ice/cold packs to:
Adrienne Kovach
University of New Hampshire
46 College Road
Durham, NH 03824
603-862-1603

Data to record:

- GPS location and patch name for each set of pellets collected, and an identification number for each separate sample.
- Number of days since snowfall and weather conditions since snowfall
- GPS tracks of surveyors
- 4 points surrounding sampling area (the 4 corners of a polygon surrounding the patch)

If a technician is able to record sample IDs, patch location information, survey dates, and geographic coordinates into a spreadsheet, this is very helpful. If not, we will do so.

Appendix 2 – Primer information and corresponding PCR conditions for microsatellite markers used in two multiplexes for genotyping analysis of cottontail pellet samples.

Primer	GenBank reference	Multiplex
Sfl011	GQ387171.1	B
Sfl014	GQ387173.1	A
StrQ02	KX530819.1	B
StrQ08	KX530821.1	A
StrQ15	KX530825.1	B
StrQ18	KX530827.1	B
StrQ25	KX530833.1	A
StrQ32	KX530839.1	B
StrQ41	KX530843.1	A
StrQ43	KX530845.1	A
StrQ46	KX530848.1	B
StrQ49	KX530850.1	A

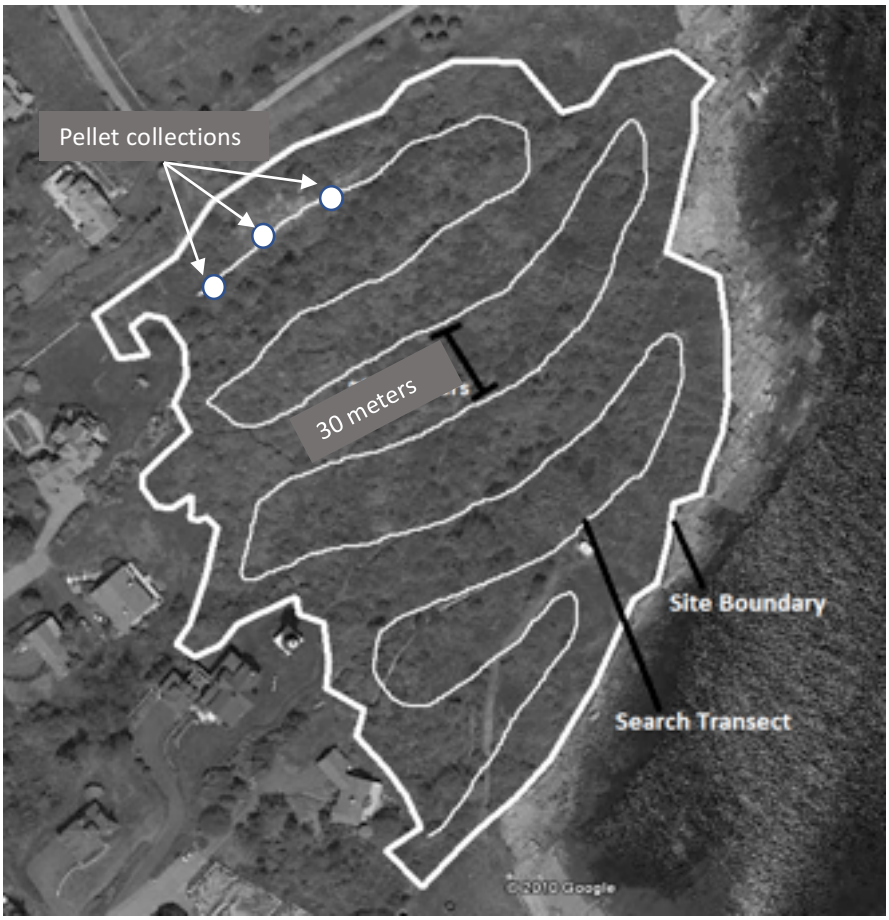
PCRs began with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 30-s denaturation at 94 °C, 30-s annealing (at 58°C for multiplex A and 59 °C for tmultiplex B) and 30-s extension at 72 °C. We used a 16 uL PCR for each multiplex with 4 μL of DNA. For both multiplexes, the PCR contained primers (between 0.2 and 0.4 μM each), 1x buffer, and 1 unit of GoTaq from Promega. For multiplex A, the PCR also contained: 1.5 mM $MgCl_2$, 0.15 mg/mL of BSA, and 0.15 mM DNTPs; for the multiplex B: 2 mM $MgCl_2$, 0.17 mg/mL of BSA, and 0.17 mM DNTPs were also included.

Appendix 3. New England Cottontail Abundance Survey Protocol

Follow this protocol to survey patches to collect cottontail fecal pellets for abundance estimation using a spatially explicit genetic mark recapture approach.

- Survey patches in their entirety, using loose transects, systematically weaving through the habitat patch.
  - Delineate the patch boundaries based on suitable cottontail habitat
  - Patch boundaries are delineated by hard boundaries (roads, waterbodies, and open areas) or areas of unsuitable vegetation (e.g., mature forest lacking an understory) of 50 m or more.
  - Transects should be spaced 30 m apart to cover the entirety of the patch
  - If the entire patch cannot be searched, survey as much as possible and delineate carefully the area surveyed.

- Collect pellets every 30 meters along the transect (see figure below).



This figure shows a loose transect used to thoroughly search a patch for pellets. Survey transects are spaced 30-m apart and pellets are collected every 30 m along the transects (as exemplified in the upper left of the patch). The patch boundary is delineated by the solid, thick, white line.

- Conduct **two independent surveys**, within 1-3 weeks of each other (see table on next page for guidelines on number and intensity of surveys).

- Conduct surveys **3-5 days after a snowfall event** (especially 3-5 days in which there have not been high winds).

- **Search the patch thoroughly**, collecting all pellets according to the search protocol on the previous page.
    - Use a handheld GPS unit to **track the search path** used by the surveyor.
    - Delineate the **patch boundaries** on a google earth or other map.

- Collect several pellets from each separate cluster of pellets into a **separate vial**.
    - **Label the vial** with the site name, date, surveyor initials, and the GPS coordinates of the pellet location (this is very important!).
    - If possible, **give each sample a unique identifying number** (e.g., site name abbreviation along with a number) and record this ID on the vial.
    - Pellets may be found within a distinct or slightly diffuse clump, or alternately, several pellets may be scattered individually along a short trail. The objective is to collect several pellets from the same individual rabbit (2-5). Restrict collection for each vial to an area approximately 5 x 5ft or smaller to minimize the number of individuals represented in each vial (ideally, one rabbit per vial). Collect pellets carefully to minimize potential for contamination. Do not touch the pellets with bare hands.

- Place the vial(s) into a cooler upon returning to your vehicle and **store them in a freezer** as soon as possible, until you are ready to ship them to the lab.

- **Record the following on a datasheet** to submit with samples:
    - Patch name and location
    - GPS coordinates and unique sample ID for each vial of pellets collected
    - Date of survey
    - Weather conditions, snow depth, and number of days since snowfall.
    - Whether there has been rain, high winds (>25 mph), cold temps <14° F or warm temps (>35° F) in the days since last snow event
    - GPS tracks of survey (save and provide a digital file).
    - Patch boundary delineation (provide on a map or as shape file) OR GPS coordinates of four corners of plot, if entire patch was not surveyed.

- If possible, provide a **spreadsheet file** that contains an entry for each unique sample (vial) collected during the survey.

Sample shipment: *on ice or cold packs overnight*, preferably by FedEx, to:

Adrienne Kovach
University of New Hampshire
46 College Road
Durham, NH 03824
603-862-1603