RESEARCH ARTICLE

Genetically derived effective population size estimates of herpetofaunal species should be used with caution

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Abstract

Effective population size (N_E) is an important parameter for conservation of threatened wildlife. Current estimators of N_E that use genetic data, however, may not be equally useful among taxa that are difficult to sample, such as cryptic, iteroparous, long-lived herpetofauna. To better understand the use of N_E estimators for such taxa, we generated and compared contemporary N_E estimates to evaluate methods, repeatability, and the effects of sample size at management scales and functional genetic population scales for the eastern box turtle (*Terrapene carolina carolina*) and eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). We chose these 2 species for their differing dispersal abilities, reproductive variances, and natural history traits, which may be useful in exploring the weaknesses and strengths of N_E estimators. We collected species-specific microsatellite genotypes from 2,712 individuals (1,133 hellbenders, 1,579 box turtles) in 2007–2011 from across their ranges in the eastern United States. We then grouped samples into range-wide genetic clusters, state-level management populations, and small regional management populations (e.g., state parks). We also randomly subsampled (rarefied) from these 3 categories to explore the effects of reduced sampling effort on N_E estimates. We used 4 popular estimators: NEESTIMATOR (linkage disequilibrium and heterozygote excess methods), LDNE, and ONESAMP. Over half of all attempts at estimating N_E failed to give complete estimates (those that included only

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non-negative and non-infinity values). There was variation between species but also among estimators. Repeated sampling of genetic populations resulted in inconsistent results, and estimates generally increased with sample size. The linkage disequilibrium version of NEESTIMATOR returned the largest percentage (95.8%) of complete estimates. Program ONESAMP obtained complete estimates for 62.0% of attempts and generally provided the numerically largest estimates. Only 19.4% of LDNE estimates and none of the heterozygote excess version of NEESTIMATOR estimates were complete. Generally, N_E estimates were higher for box turtles, perhaps because of their greater dispersal capabilities, historically larger populations, and longer lifespans. Our results suggest that estimates of N_E for long-lived herpetofauna species vary according to species, sample size, genetic cluster, management population, and estimator used. Therefore, we recommend managers of populations of long-lived herpetofaunal species use N_E estimators with caution, and consider results from multiple methods before incorporating N_E into management practice.

Effective population size (N_E) is an important parameter for quantifying genetic diversity of wildlife populations. Effective population size is equivalent to the number of individuals in a hypothetical population whose alleles are subject to the same forces as the real population (Wright 1931). It is often much smaller than the population census size (N_C), giving managers more meaningful data with which to assess populations than are available by a simple census (Frankham 1995, Hinkson and Richter 2016). Estimates of N_E can help managers prioritize conservation efforts for declining populations at risk of increased fragmentation, exploitation, demographic declines, and extinction (Shaffer et al. 2015, Bradke et al. 2018). Population declines can lead to reduced N_E and small N_E sizes can correlate with allelic loss and inbreeding (Charlesworth 2009, Frankham et al. 2014), making it an important parameter for long-term population management decisions.

Reptiles and amphibians represent the most threatened vertebrate taxa on Earth owing to habitat degradation and fragmentation, overexploitation, pollution, and emerging infectious diseases (Garber and Burger 1995, Gibbons et al. 2000, Stuart et al. 2004, Rivera-Ortiz et al. 2015), but their natural history traits can often make them difficult to study. Single-cohort sampling of species for the calculation of N_E (such as for the young of the year) are the gold standard for N_E estimates (Waples et al. 2014), but many herpetofaunal species are cryptic, long-lived, and iteroparous, making genetic sampling of wild individuals difficult and assignment of individuals to specific generations challenging. In practice, conservation managers may opportunistically sample all individuals they encounter regardless of age or cohort, but these samples are problematic for precise estimation of N_E (Waples et al. 2014). The estimation of

N_E is further complicated by specific natural history traits (e.g., dispersal ability), making it imperative to understand these effects if N_E estimates are to be used for management. Subsequently, a comparative approach to understanding natural history trait effects on N_E estimators across species could help managers improve population management strategies.

Two such long-lived, iteroparous, cryptic species are the often-sympatric eastern box turtle (*Terrapene carolina carolina*) and the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*; Figure 1). The eastern box turtle is a long-lived (>75 yr; Hall et al. 1999) mostly terrestrial reptile that historically inhabited hardwood forests and associated communities over much of the eastern United States but is generally in decline range-wide (Stickel 1978, Williams and Parker 1987, Hall et al. 1999, Dodd 2002). Genetic structure exists in this species, with 2 range-wide populations that are roughly divided by the Appalachian Mountains and each spanning multiple states (Kimble et al. 2014a; Figure 2), and there is mixed evidence for structuring at finer geographic scales (Kimble et al. 2014b, Moore et al. 2020). The eastern hellbender has a shorter lifespan (~30 yr; Nickerson and Mayes 1973) and inhabits clear, cool, highly oxygenated, and fast-flowing rivers (Nickerson and Mayes 1973) across much of the same range as box turtles. Hellbenders are also experiencing severe demographic shifts, with many populations consisting mostly of older individuals and low juvenile recruitment (Mayasich et al. 2003). The eastern hellbender has 2 genetically differentiated range-wide populations divided primarily into the Ohio River drainage and Tennessee River drainage (Unger et al. 2013a; Figure 2). Moreover, many of the remaining viable
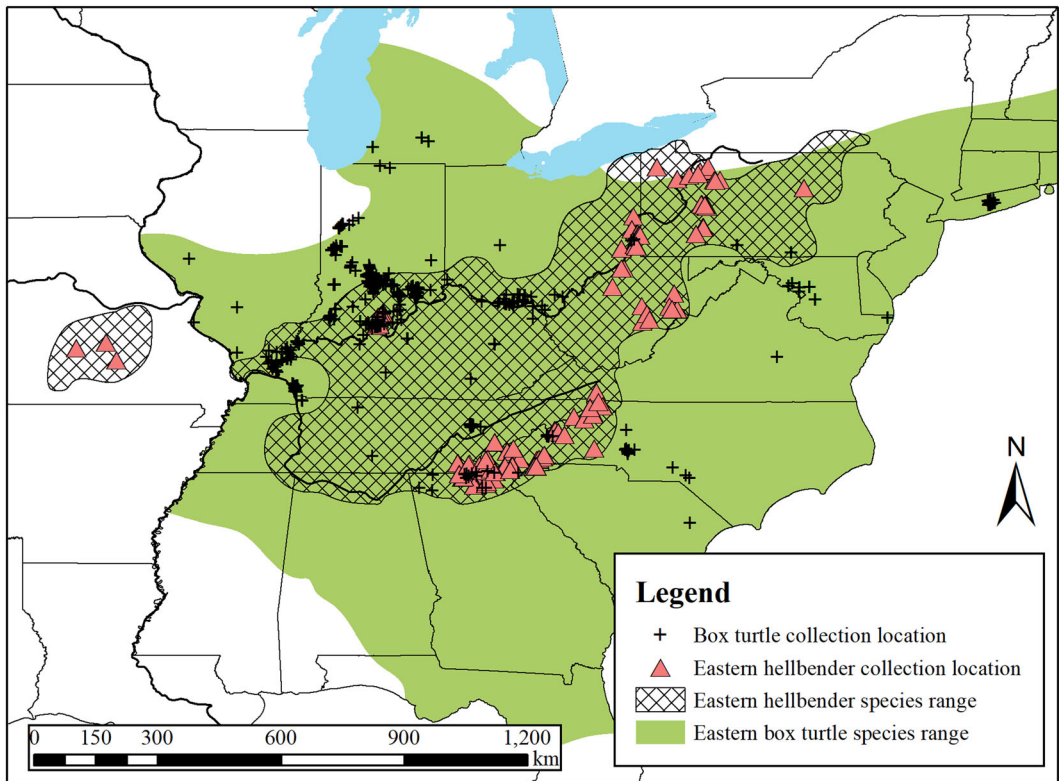
FIGURE 1 Species ranges and encounter locations of eastern box turtle and eastern hellbender samples used for this study in the eastern United States, 2007–2011. The eastern box turtle approximate range is indicated by green shading (Dodd 2002) and sample locations are indicated by black crosses (+). Eastern hellbender approximate range is indicated by black cross-hatching (Petranka 1998) and sample locations are indicated with red triangles (▲).
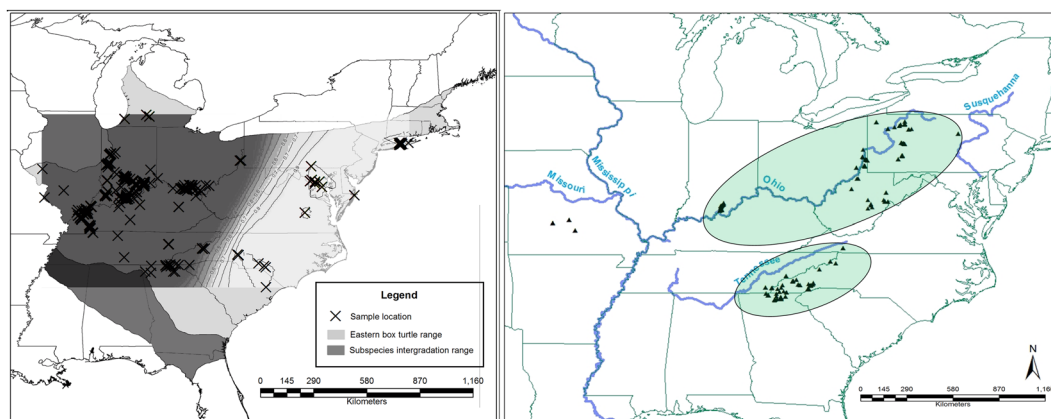
FIGURE 2 Rangewide genetic population maps for the eastern box turtle (left; Kimble et al. 2014a) and the eastern hellbender (right; Unger et al. 2013b) in the eastern United States, 2007–2011. The shading on the box turtle map indicates the probability of individuals in those areas belonging to the western population, increasing with darker shading, which decreases substantially along a line roughly congruent with the Appalachian Mountains. The oval polygons on the hellbender map indicate the approximate bounds of each genetic population. For both species, genetic population boundaries were resolved with the use of location-agnostic BAYESIAN methods that attempt to group individual genotypes into pools that minimize linkage disequilibrium and departures from Hardy-Weinberg equilibrium (Pritchard et al. 2000).

populations are within the Tennessee River drainage, and may represent genetically distinct populations associated within major watersheds (Freake et al. 2018).

Our main goal for this study was to compare and contrast microsatellite-derived N_E estimators in 2 herpetofaunal species of conservation concern that are difficult to study (long-lived, iteroparous, cryptic) and have differing natural history traits. One natural history trait in which the 2 species differ in ways salient to the calculation of N_E is reproductive variance. High variance in reproductive success has a negative relationship with N_E (Palstra and Ruzzante 2008). Reproductive variance may be expected to be relatively higher in hellbenders because salamanders often exhibit mate selection and parental care investment (Houck et al. 1985, Verrell 1989, Howard et al. 1997) and sperm limitation (Unger et al. 2013a). By contrast, there is typically a minimal role for mate selection (Dodd 2002, Belzer and Seibert 2009) or parental care (Shine 1988) in turtles. This suggests that box turtle populations have larger N_E estimate values than do hellbender populations.

Dispersal ability also influences N_E (Whitlock and Barton 1997, Nunney 1999). Limited dispersal ability generally increases population structure and decreases N_E (Nunney 2016). The geographic distance between first-degree box turtle relatives (full siblings or parent-offspring pairs) can be ≥33 km (Kimble et al. 2014a), a measure that may serve as a proxy for dispersal ability in this species. This may help explain the lack of genetic isolation by geographic distance up to 300–500 km in this species (Kimble et al. 2014a). Hellbenders typically disperse fewer than 1,200 m (McCallen et al. 2018) but have been observed dispersing up to 2.34 km (Gates et al. 1985). Hellbender populations show significant genetic isolation by geographic distance at the drainage level (<300 km, Unger et al. 2013b; <322.5 km, Unger et al. 2016). This may suggest that box turtle populations have larger N_E estimate values than do hellbender populations.

Differential estimates of N_E may be affected by other natural history traits (e.g., home range size, age at maturity, unequal sex ratios), but most of these are poorly understood for these species and so are not considered here. Against this backdrop of contrasting natural history traits, we were particularly interested in 4 objectives related to the use of using genetically derived N_E estimators in long-lived, iteroparous, cryptic herpetofauna species.

The first objective (objective 1) was to explore estimates across methods and species. There are multiple programs available for estimating N_E with genetic data, but because assumptions and parameters vary, a single most

appropriate choice may not be clear for any given system. We expected that there would be variation among the estimators (Ryman et al. 2019) and that populations of turtles, with lower reproductive variance and higher dispersal rates, would generally have higher N_E estimate values.

Our second objective (objective 2) was to assess the repeatability of N_E estimates across subsamples of the same genetic population. Given the relatively patchy distribution of hellbender populations (Keitzer et al. 2007, Quinn et al. 2013, Pugh et al. 2016) compared to the (historically) widely distributed box turtle (Dodd 2002, Ernst and Lovich 2009), we expected the repeatability of N_E to be more stable for hellbenders. This is because the sampling locations of hellbenders (e.g., stream reaches) are more likely to constitute a genetic neighborhood (local area in which most matings actually occur; Wright 1946) than the more politically bounded turtle sampling locations (e.g., state parks), reducing the chance of the hellbender samples actually being collected from multiple genetic neighborhoods (Wahlund 1928). This Wahlund effect (i.e., reduction in heterozygosity in a population caused by subpopulation structure) would introduce higher variance into box turtle N_E estimates (Neel et al. 2013).

Our third objective (objective 3) was to determine if the number of samples typically collected for assessment of managed populations is sufficient to estimate the N_E of the entire genetic population. Estimates of N_E made at the local and state management scale are often made with 20–25 individuals (Leberg 2002). We expected that this sample size was sufficient for hellbenders but insufficient for box turtles because the latter estimates are expected to be larger (see objective 1) and in this case some N_E estimation methods are expected to be ineffective at large N_E sizes (Gilbert and Whitlock 2015).

Our final objective (objective 4) was to quantify the effects of sample size on estimating N_E for genetic populations. In both species used in this study, genetic populations span multiple states, but management typically occurs only at the state or local level. We were interested in exploring if the typical sample size collected across the genetic population would suffice if genetic population boundaries were known a priori. We expected that rarefaction would have little effect on N_E estimates for hellbenders and would demonstrate undersampling for box turtles.

In summary, our objectives were to explore estimates across methods and species, assess the repeatability of N_E estimates, determine if the standard sample size is sufficient, and quantify the effects of sample size on N_E estimates. We predicted that there would be high variation between methods and species, low repeatability, and strong effects of sample size on N_E estimates.

STUDY AREA

We obtained data from eastern box turtle samples at multiple geographic scales from May–October in 2008–2011 in the United States from local management sites (e.g., National Wildlife Refuges) to range-wide (Figure 1) locations, including from Illinois, Michigan, Indiana, Ohio, West Virginia, Pennsylvania, Maryland, New York, North Carolina, South Carolina, Georgia, Tennessee, and Kentucky. We did not obtain samples from intergrade areas with other subspecies. Sampling locations ranged from 21–760 m in elevation and were typically mature eastern deciduous hardwood forest (dominated by oaks [Quercus spp.], hickory [Carya spp.], beech [Fagus spp.], tulip tree [Liriodendron tulipifera], and maple [Acer spp.]) and environs with low slope, a primary habitat characteristic for eastern box turtles (Dodd 2002). Prominent fauna included white-tailed deer (Odocoileus virginianus), eastern gray squirrels (Sciurus carolinesis), eastern wood-pewees (Contopus virens), and black rat snakes (Pantherophis alleghaniensis). Land use in the areas was typically for recreation and silviculture. Study areas ranged from 82–35,000 ha. Weather was typically humid and mild. Climate was typical of the eastern United States, with 4 distinct seasons.

We obtained data collected from eastern hellbenders from multiple geographic scales from June–September in 2007–2010 in the United States, from single rivers to watersheds (Figure 1), including sample sites in Indiana, Ohio, West Virginia, Pennsylvania, North Carolina, Georgia, Tennessee, and Missouri. Sampling locations were typically fast, cold, clear, well-aerated streams and rivers typified by interspersed long runs, pools, and riffles. Streams and

rivers were characterized by large boulders, bedrock, gravel, and cobble bottoms. Prominent fauna included darters (Etheostomatinae), sunfish (Centrarchidae), trout (Salmoninae), and crayfish (*Orconectes* spp.). Land and water use in the areas was typically for recreation, drinking water, and silviculture. Study areas ranged from 177–709 linear meters of river at elevations from 136–709 m. Weather was typically humid; climate was typical of the eastern United States, with 4 distinct seasons.

METHODS

Sample collection, DNA extraction, and genotyping

We sampled both species across their geographic ranges (Figure 1) between June 2007 and August 2011. We sampled box turtles when we encountered them during visual surveys on foot, road cruising (Dodd 2002), incidental encounters, and with the use of dogs (Kapfer et al. 2012). We obtained genetic samples for box turtles by blood draw from the subcarapacial sinus or muscle tissue in the case of roadkill. We sampled hellbenders during stream visual encounter surveys (Nickerson and Krysko 2003). We sampled hellbenders primarily by collecting small tail clips (~3 mm) or blood extracted from the caudal vein.

We extracted genomic DNA from all samples using a modified proteinase K protocol with standard phenol-chloroform extraction (Sambrook and Russell 2001) to ensure high quality DNA. We then amplified template DNA and multiplexed DNA across 11 (box turtles; Kimble et al. 2011) or 12 (hellbenders; Unger et al. 2010) species-specific microsatellite markers because simulations suggest that using ≥10 microsatellite loci can adequately detect population declines (Antao et al. 2011). The polymerase chain reaction (PCR) thermoprofile and reaction conditions are detailed in Kimble et al. (2014a) and Unger et al. (2013a). We analyzed all PCR products on an ABI 3739XL automatic sequencer (Applied Biosystems, Waltham, MA, USA) and genotyped manually using GENEMAPPER version 3.7 (Applied Biosystems). We randomly assessed approximately 20% of genotypes for quality control to ensure proper genotyping and to identify potential genotype errors (Unger et al. 2013a, Kimble et al. 2014b).

N_E estimators

We tested 4 models with the assumption that if estimates are robust to assumption violations, they should be similar in value. We sampled from multiple management populations (e.g., states or state parks) within the geographic scopes of genetically defined populations, which encompass multiple states for both species. This amounts to repeated subsampling of the same genetic population (Neel et al. 2013), with the assumption that these should result in similar estimates of N_E. For example, box turtles from Hovey Lake Fish and Wildlife Area and Patoka River National Wildlife Refuge, both in Indiana, are members of the same western genetic population (Kimble et al. 2014a) and should therefore yield similar estimates of N_E. To assess the use of these N_E estimators at various scales, we grouped samples 4 ways into populations (Table 1), depending on political boundaries and species.

First, we grouped samples by genetic populations that we determined from previous work (Unger et al. 2013b, Kimble et al. 2014a) by grouping genotypes according to genetic similarity and regardless of geography. These represent the functional genetic populations of these species and both span multiple states (Figure 2). Second, we grouped samples by state because this is the highest level at which these species are currently afforded any government protection or cohesive management. For example, we pooled box turtle samples from multiple state parks in Indiana into a single state management population. Third, we established local management populations only for eastern box turtles because we did not sample hellbenders at this scale. We originally collected field samples without *a priori* knowledge of the geographic scale at which box turtle genetic populations exist. Samples were generally from management sites such as wildlife refuges and state parks that consisted of mostly intact but

TABLE 1 Descriptions and sample sizes used for estimating the effective population sizes (N_E) of eastern box turtle and eastern hellbender sampling populations in the eastern United States, 2007–2011, using 4 estimators: ONESAMP (na indicates not available), LDNE, and NEESTIMATOR (linkage disequilibrium [LD] and heterozygote excess [HE] methods). We determined genetic sampling populations using Bayesian estimates of genetically defined populations for box turtles (Kimble et al. 2014a) and hellbenders (Unger et al. 2013a). These typically spanned multiple states. State and local sampling populations were designated to reflect the scale at which these populations are often managed (state, wildlife refuge, river).

Species	Population	Sample	n	ONESAMP	LDNE	NEESTIMATOR LD	HE
Eastern box turtle							
	Western genetic population pooled	Full	499	865.8	10,692.7	1,159.1	∞
		Rarefied	24	1,594.8	−300.3	173.8	∞
	Indiana pooled	Full	941	na	16,261.9	3,448.7	∞
		Rarefied	24	na	−231.2	157.4	∞
	Seymour, IN	Full	20	na	−475.2	63.7	∞
	Tippecanoe and Warren counties, IN	Full	31	na	−2,063.9	259.2	∞
		Rarefied	24	na	15,492.7	131.3	∞
	Blue River, IN	Full	74	−68,007.1	−25,163	856.9	∞
		Rarefied	24	1,997.2	−203.3	260.5	∞
	Big Oaks National Wildlife Refuge, IN	Full	31	646.9	447.3	158.1	∞
		Rarefied	24	2,038.7	237.4	96.3	∞
	Hardwood Ecosystem Experiment, IN	Full	627	869.0	9353	∞	∞
		Rarefied	24	741.4	−687.1	124.5	∞
	Hovey Lake Fish and Wildlife Area, IN	Full	44	383,208.7	717.1	265.5	∞
		Rarefied	24	1,016.8	−282.4	218.4	∞
	Parke County, IN	Full	25	521.1	1,078.9	135	∞
		Rarefied	24	521.2	662.8	115.3	∞
	Posey County, IN	Full	20	na	−682.3	107.8	∞
	Patoka River National Wildlife Refuge, IN	Full	69	142.7	542.3	486.4	∞
		Rarefied	24	na	−1,657	152.4	∞
	Knoxville, TN	Full	182	−313.5	98.5	152.1	∞
		Rarefied	24	1,956.7	681	96.6	∞
	Land Between the Lakes National Recreation Area, KY	Full	36	4,767.6	−536.5	901.8	∞
		Rarefied	24	1,506.7	−257.2	303.5	∞
	Shawnee National Forest, IL	Full	29	1,401.2	−393.6	341	∞
		Rarefied	24	608.3	−424.5	175.2	∞
	Southwestern Michigan	Full	35	128.6	−830.9	402.8	∞
		Rarefied	24	na	−17,371	147	∞
	Shawnee State Park, OH	Full	30	453.6	558.3	96.8	∞

(Continues)

TABLE 1 (Continued)

						NEESTIMATOR	
Species	Population		Sample	*n*	ONESAMP	LDNE	LD	HE
			Rarefied	24	616.6	−1,476.2	69.1	∞
	Eastern genetic population pooled		Full	249	−2,023.5	1,812.4	792.1	∞
			Rarefied	24	608.4	−111.2	60.6	∞
	Chattahoochee National Forest, GA		Full	15	94.1	−74.2	57.2	∞
	Gaithersburg, MD		Full	12	na	−29.9	34.1	766.3
	Gettysburg National Military Park, PA		Full	28	408.6	205.7	39.2	∞
			Rarefied	24	929.0	237.5	25	∞
	Isle of Wight, MD		Full	10	na	121.4	17.9	∞
	Jug Bay, MD		Full	26	na	1,216.4	146	∞
			Rarefied	24	na	−619.1	168.6	∞
	Long Island, NY		Full	25	100.7	17,169.3	129.8	∞
			Rarefied	24	399.9	−439.4	149.9	∞
	Madison, NC		Full	9	23.5	−15.9	15.1	∞
	Maryland pooled		Full	143	−52,612.3	−1173	584.5	∞
			Rarefied	24	58.8	−3,005.8	104.9	∞
		Muddy Branch Park, MD	Full	22	na	−102.6	56.5	∞
		Patuxent Wildlife Research Center, MD	Full	47	na	704.6	260.2	∞
			Rarefied	24	992.0	−608.4	171.4	∞
		Richmond, VA	Full	24	441.8	90.5	59.7	∞
		Rock Creek, DC	Full	9	na	−20.7	6.5	∞
		Wheaton Regional Park, MD	Full	39	501.9	−36.5	52.4	10.7
			Rarefied	24	na	−75.9	69.4	∞
Eastern hellbender								
	Tennessee River watershed genetic population pooled		Full	671	−19,262.2	294.1	427.9	∞
			Rarefied	24	na	952.8	123.9	∞
		Virginia pooled	Full	77	na	2,276.3	1,192.8	∞
			Rarefied	24	34.6	326.5	277.2	4,018.5
		North Carolina pooled	Full	172	5,917.8	435	440.4	∞
			Rarefied	24	84.5	791	225.4	∞
		Little River, TN	Full	95	na	151.7	124.4	∞
			Rarefied	24	72.2	232	91.8	∞
		Georgia pooled	Full	199	692.1	180.7	210.7	∞
			Rarefied	24	321.1	161.4	180.4	∞

TABLE 1 (Continued)

Species	Population	Sample	n	ONESAMP	LDNE	NEESTIMATOR LD	NEESTIMATOR HE
	Ohio River watershed genetic population pooled	Full	462	8,993.0	623.6	452.7	∞
		Rarefied	24	124.8	−1,052.7	156.2	∞
	Blue River, IN	Full	103	176.6	622.8	361.5	∞
		Rarefied	24	30.3	708.5	119.8	∞
	West Virginia pooled	Full	62	96.6	87.3	89.7	∞
		Rarefied	24	22.5	178.5	55.6	∞
	Pennsylvania pooled	Full	301	na	282.4	243.3	∞
		Rarefied	24	na	−707.5	117.7	∞
	Missouri pooled	Full	41	115.9	53.3	51.4	∞
		Rarefied	24	17.0	35.6	56.6	∞

often isolated habitat. We considered samples from these local management populations to be separate populations except when pooled for state management populations and genetic populations as described above. Fourth, we rarefied both state and local management populations to a sample size of 24. We chose a sample size of 24 because it is standard in population genetic studies of non-model organisms (Leberg 2002).

We collected genotypes from 2,712 individuals, including 1,133 hellbenders and 1,579 box turtles, and divided them into 71 (51 box turtle, 20 hellbender) sampling populations (Table 1) for estimation of N_E; 2.97% of box turtle genotypes and 0.70% of hellbender genotypes per locus were missing. The genotypes of individual samples were included in >1 population (e.g., a box turtle from Indiana might have been included in the Seymour, IN population, the Indiana pooled population, and the western genetic population).

We obtained estimates of N_E using 4 single-sample estimators: ONESAMP (Tallmon et al. 2008), LDNE (version 1.31; Waples and Do 2008), and both the linkage disequilibrium (LD) and heterozygote excesses (HE) methods in NEESTIMATOR (version 2.1; Peel et al. 2004). Program ONESAMP implements approximate Bayesian computation; the parameters we used were an N_E lower bound of 4 and 9,998 as the upper bound to account for the range of our sampling regime. Program LDNE uses linkage disequilibrium or the nonrandom association of alleles at different loci arising from random genetic drift (Waples and Do 2008); we used the LDNE default parameters, excluding alleles rarer than 0.05, 0.02, and 0.01, and an assumption of random mating. We used the same NEESTIMATOR-LD and NEESTIMATOR-HE parameters as we did for LDNE.

To test the hypothesis that N_E estimates varied significantly among methods (objective 1), we averaged then log-transformed the N_E point estimates for each population. We then compared the resulting numbers between species for each method with either analysis of variance or paired t-tests (depending on the number of comparisons). We did this separately for all 4 population types (genetic, state management, local management, rarefied management; α = 0.05). To compare the repeatability of N_E estimates using subsamples of the same genetic populations (objective 2), we used a Bartlett's test of homogeneity of variance within genetic populations on log-transformed estimates of N_E for unrarefied management populations only (α = 0.05). We tested ONESAMP and NEESTIMATOR-LD box turtles estimates only for objective 2 (there were not sufficient hellbender sample locations). To compare rarefied and unrarefied estimates of N_E (objective 3) we used paired t-tests on management populations (α = 0.05) for both species for ONESAMP and NEESTIMATOR-LD estimates only. To test the effects of sample size on genetic population N_E estimates (objective 4), we used a self-starting non-linear least squares asymptotic regression model method in the R package stats (R Core Team 2017). This method fits a non-linear

accumulation curve to estimate the number of samples necessary to sufficiently estimate N_E. We log-transformed the N_E data to normalize the data.

RESULTS

As expected, there was large variation across estimators for most sampling populations at all levels (genetic, state, local, and rarefied) in both species. For example, for the Big Oaks National Wildlife Refuge in Indiana, we estimated the rarefied management box turtle population to have point values of 2,038.69 (ONESAMP), 237.40 (LDNE), and 96.30 (NEESTIMATOR-LD) for the 3 working methods (Table S1, available in Supporting Information). Point estimates varied by an average of 1.69 ± 0.57 (SD) orders of magnitude for sampling populations with multiple complete estimates, and in only 66.20% did they agree in order of magnitude for point estimates. We assessed the means of point estimates for ONESAMP and NEESTIMATOR-LD for both species (LDNE gave too few complete estimates for box turtles) with t-tests. They differed for box turtles ($P = 0.022$) but there was little evidence for a difference in hellbenders ($P = 0.18$). There was mixed evidence that the average point estimates were higher for box turtles than hellbenders (ONESAMP, $P = 0.52$; NEESTIMATOR-LD, $P = 0.80$; LDNE, $P = 0.014$). The data did not indicate a difference in average number of orders of magnitude in estimates between box turtles (1.39) and hellbenders (1.30; $P = 0.36$).

For both species, we collected samples from multiple management populations within genetic populations. We treated these as subsampling of the genetic populations. Estimates of N_E ranged up to 3 orders of magnitude within genetic populations (Table S1) and this high variation was evident across sampling population size, species, and both rarefied and unrarefied populations. There was high variation across subsamples for hellbenders for ONESAMP, LDNE and NEESTIMATOR-LD ($P = 0.031$). The variation among box turtles was also high, but the data did not indicate a difference (only ONESAMP and NEESTIMATOR-LD results used; $P = 0.68$). For example, an estimate of N_E calculated from 36 box turtles collected at the Land Between the Lakes National Recreation Area in Kentucky should be similar to an estimate of N_E calculated from 35 individuals collected in southwestern Michigan because they both belong to the same genetic population (Kimble et al. 2014a) if all confounding variables were held even (age class, sex). The NEESTIMATOR-LD point value estimates were at least of the same order of magnitude for these 2 sites (901.8 and 402.8, respectively), but the ONESAMP estimates differed by 37-fold (4,767.6 and 128.6, respectively). Both are in the western genetic population, for which the NEESTIMATOR-LD estimate is 1,159.1. Of the 29 estimates made for subsamples from this population, only 5 had confidence intervals that overlapped with those of the genetic population and only 2 agreed in order of magnitude with the population-wide NEESTIMATOR-LD point estimate.

There was significant discrepancy between rarefied and unrarefied estimates (Table 2; Figure 3). We compared rarefied and unrarefied log-transformed point estimate means with t-tests and rarefied estimates of N_E were significantly lower than those using unrarefied data for 3 (2 hellbender and 1 eastern box turtle) of 5 comparisons (Table 2; Figure 3). These results suggest that for these 2 species the typical number of samples typically collected at management scales is not sufficient to estimate the N_E of the entire genetic populations.

The N_E point estimates increased with sample size up to hundreds or thousands of samples (Table 3; Figure 4). For example, for the hellbender ONESAMP data, the accumulation curve becomes asymptotic at approximately $N_E = 14,859$ ($\log N_E = 4.17$; Figure 4A). On this curve, the minimum sample size necessary to accurately calculate N_E would be approximately 1,799 individuals, but at the standard sample size of 24, N_E would be estimated at 43.66. These results suggest that the sample size necessary across genetic populations of these 2 species is often orders of magnitude larger than standard practice.

Though we were able to meet the primary 4 objectives of the study, we also discovered that the estimators often failed; a majority (55.6%) of estimates were not complete (we defined complete estimates as those that reported non-infinity, non-negative point estimates and confidence sets). Variation in the number of complete

TABLE 2 Paired *t*-test results for comparisons of mean rarefied (subsample of *n* = 24) and unrarefied (full sample) effective population size (N_E) estimates per species per method for eastern box turtles and eastern hellbenders in the eastern United States from 2007–2011 using 3 estimators: ONESAMP, LDNE, and NEESTIMATOR with linkage disequilibrium (NEESTIMATOR-LD) methods. There were no complete estimates from LDNE for rarefied box turtle populations.

Species	Estimator	t-statistic	df	P-value
Eastern hellbender	ONESAMP	−3.9489	5	0.005
	LDNE	−1.7773	1	0.163
	NEESTIMATOR-LD	−4.0635	8	0.002
Eastern box turtle	ONESAMP	0.8226	8	0.435
	NEESTIMATOR-LD	−4.2475	19	<0.001
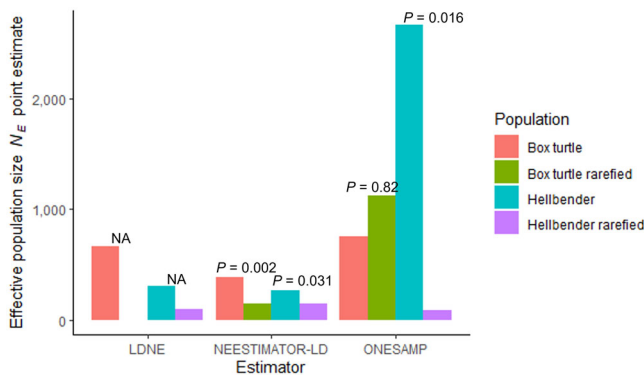
FIGURE 3 Effects of rarefaction on N_E point estimates for ONESMAP, LDNE, AND NEESTIMATOR-LD methods for eastern box turtles and eastern hellbenders in the eastern United States, 2007–2011. Bars represents the means of the effective population size (N_E) point values per species and method and *P*-values are results of paired *t*-tests. NA indicates not applicable. There were no complete estimates from LDNE for rarefied box turtle populations.

estimates given by each estimator ranged from 0.00% to 95.80% (Table S1). Program NEESTIMATOR-LD gave the most complete estimates (95.80%). Program ONESAMP gave the second most complete estimates overall (62.00%). When ONESAMP did generate confidence intervals, it always reported a non-infinity estimate. Program LDNE gave complete estimates 19.40% of the time; the remaining 80.60% of estimates either had negative point values or infinity as the upper bound of the confidence intervals. For 31.00% of all sampling populations, only a single method worked, 2 methods worked for 54.90%, and 3 methods worked for only 12.70%. The NEESTIMATOR-HE method was unable to calculate any complete estimates and we did not include this estimator's results in further analyses. Thirteen of the 14 sampling populations for which only one method worked were for box turtle populations.

DISCUSSION

The amount of variation across methods varied across 3 orders of magnitude (Table 1). Though there was a significant difference in N_E values between species for LDNE estimates only and there was no significant interspecies difference in variation. We had predicted that variation in N_E estimates would be higher in eastern box

TABLE 3 Asymptotic regression values of effective population size (N_E) estimates for eastern hellbender and eastern box turtle populations in the eastern United States from 2007–2011 using 3 different estimators: ONESAMP, LDNE, and NEESTIMATOR with linkage disequilibrium (NEESTIMATOR-LD) methods. A sample size of 24 individuals is a standard sample size in population genetics (Leberg 2002). We calculated the sample size and N_E using a self-starting non-linear least squares asymptotic regression model. Asymptote is assumed to represent the maximum N_E estimate made from increasing sample sizes. There were no complete estimates from LDNE for rarefied box turtle populations.

Species	Estimator	N_E at $n = 24$	Sample size at asymptote	N_E at asymptote
Eastern hellbender	ONESAMP	43.7	1,799	14,859.3564
	LDNE	12.5	424	353.5086
	NEESTIMATOR-LD	20.5	470	360.2466
Eastern box turtle	ONESAMP	390.3	65	628.0583
	NEESTIMATOR-LD	92.1	245	755.6140
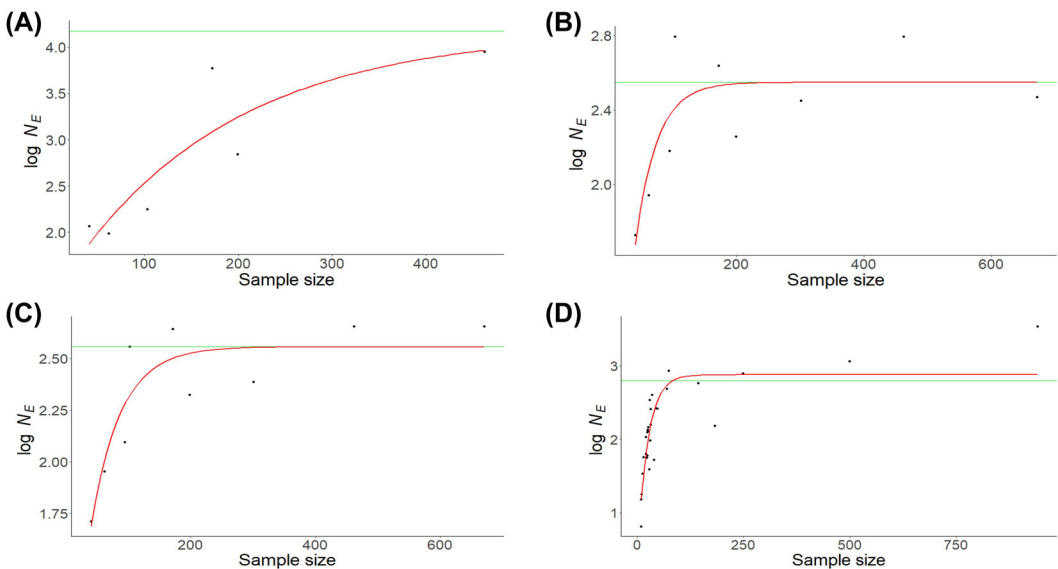
FIGURE 4 Self-starting non-linear least squares asymptotic regression model plots of effect of sample size on effective population size (N_E) estimates for eastern box turtles and eastern hellbenders in the eastern United States, 2007–2011. Best fit models are shown with red curved lines; the curves are estimated to become asymptotic at the green lines. Plots are for A) hellbender ONESAMP, B) hellbender LDNE, C) hellbender NEESTIMATOR-LD, and D) box turtle NEESTIMATOR-LD estimates.

turtle populations because of the likely higher dispersal abilities and lower reproductive variance in this terrestrial species. This may be due to higher variance across methods, as has been documented before (Wang 2005, 2016). For example, Wang (2016) reported that estimators based on heterozygote excess can underestimate N_E and linkage disequilibrium estimators can overestimate it, especially when the sample size is lower than the real N_E value. Given that the N_E values for many estimates in the current study were up to 3 orders of magnitude greater than the sample size, this may indicate that our sample sizes were insufficient to estimate N_E in these populations.

We observed several substantial differences between eastern box turtle and hellbender estimates; N_E estimates were higher for rarified and pooled estimates of box turtle populations relative to hellbender populations.

One reason for these differences might be biases in the microsatellite libraries used for comparing these species (Jarne and Lagoda 1996). Both libraries were developed simultaneously in the same laboratory using the same equipment, materials, reagents, and protocols (Unger et al. 2010, Kimble et al. 2011), however, suggesting that library construction bias should be low. They have similar numbers of loci (11 for box turtles, 12 for hellbender), both were constructed using similar numbers of individuals (35 box turtles and 31 hellbenders), both libraries had 2 loci exhibiting deviations from Hardy-Weinberg equilibrium, and neither had loci exhibiting linkage disequilibrium. These suggest that technical reasons do not account for observed differences in N_E estimates.

It is more likely that the higher N_E estimates in box turtles is attributable to differences in natural history traits, including dispersal abilities (Nunney 1991, 2016), reproductive variance (Palustra and Ruzzante 2008), or a combination of other demographic and biological factors. The observed differences were not great, however, which may suggest that dispersal abilities and reproductive variances may be more similar for these 2 species. For box turtles, most adults have temporally conserved home ranges (0.02–19.2 ha; Dodd 2002), a trait that could be expected to set up genetic population structure, decrease N_E estimates, and increase variation in N_E. Reproductive variance may also be higher than appreciated in box turtles because nest mortality is often high in turtles (Kolbe and Janzen 2002), the presence of courtship behavior (Evans 1953) suggests mate choice (Clutton-Brock 2007), and sperm storage in other turtle species can last up to 3 years (Pearse et al. 2001). As for hellbenders, they typically have smaller home ranges (0.003–0.22 ha; Peterson and Wilkinson 1996, Burgmeier et al. 2011a), but the presence of occasional transients (14.13 km; N. G. Burgmeier, Purdue University, personal communication) may suggest occasional but long-distance dispersal. Further, the likely high mortality of wild hellbender larvae (Bodinof et al. 2012, Unger et al. 2013c) may also increase reproductive variance. These results suggest that unless dispersal and reproductive variance are well understood for a population and indicate a specific estimator, managers should not rely on a single method for calculating N_E.

Comparing conspecific populations at different locations to identify abnormally genetically depleted populations is often done by comparing the ratio of effective (N_E) to census (N_C) population size (N_E/N_C). The only population for which contemporary and N_C estimates are available in our data, however, is the Blue River, Indiana hellbender population, which has experienced a steep documented demographic decline (Burgmeier et al. 2011b). Burgmeier et al. (2011b) estimated an N_C of 114 (95% CI = 94–152) and N_E estimates include similar values (e.g., NEESTIMATOR-LD, rarefied = 119.8). Because N_C is typically higher than N_E in wild vertebrate populations (Frankham 1995, Palstra and Fraser 2012), the N_C of this population may have historically been much higher than it is now. At this site estimates for N_E and N_C are similar (N_E/N_C = 1.05) which would signal to managers that this hellbender population should be considered for potential augmentation (i.e., captive breeding, translocations) given the potential for continued population declines. Another estimate of N_E for this population was 30.3 (ONESAMP), however, which gives an N_E/N_C ratio (0.266) closer to the average of 0.10–0.11 often expected for wild populations (Frankham 1995), suggesting that this population may be stable and not in need of management actions. High variation in N_E estimates may therefore mislead population managers.

In addition to variation among methods and between species, there was also variation among multiple subsamples taken from the same genetic populations in both species. Both eastern box turtle and eastern hellbender ranges divide into 2 populations rangewide (Unger et al. 2013a, Kimble et al. 2014a) by Bayesian methods that group samples into populations by minimizing departures from Hardy-Weinberg equilibrium and linkage equilibrium (STRUCTURE, Pritchard et al. 2000; Figure 2; Table 1). In the case of box turtles, these Bayesian methods detected no smaller-scale geographical structuring (Kimble et al. 2014a, b). Theoretically, the groups of genotypes resulting from such analyses are panmictic, but this assumption could be violated by the presence of genetic neighborhoods (Neel et al. 2013) and cryptic population substructure set up by dispersal and migration (Gagne et al. 2018). As forest-dwellers, box turtles may be able to disperse farther than riverine hellbenders, and long-distant dispersal events in box turtles have been recorded (Kiester et al. 1982); however, this has also been reported for hellbenders (N. G. Burgmeier, personal communication). In our data, the average number of orders of magnitude for N_E point estimates was similar at 1.39 for box turtles and 1.30 for hellbenders. Sampling biases may

also affect repeatability, as samples were not from the same cohort, age class, or sex. These findings suggest that at least for wildlife species with geographically large genetic populations, managers should not assume that samples taken from smaller areas are representative but may rather reflect the local N_E only.

For both species, the mean N_E point estimates were generally lower for the rarefied populations. This suggests that the typical sample size (n = 24) often employed in studies of wildlife may be insufficient to gauge the effective population size. Especially for linkage disequilibrium methods (LDNE and NEESTIMATOR-LD), sample sizes smaller than the true N_E can give erroneous results (England et al. 2006, Waples and Do 2008). Wang (2016) demonstrated by simulation that N_E estimates can be inaccurate when the sample size is much less than the true N_E for several methods of estimation. Trends in our data are impossible to gauge for box turtles given the paucity of successful LDNE results and conflicting ONESAMP and NEESTIMATOR-LD results, but for hellbenders the unrarefied N_E estimates were much higher (and statistically significantly higher for ONESAMP and NEESTIMATOR-LD) than rarified estimates. These data further support the finding that for some wildlife species sample sizes in the typical range of n = 25 may be insufficient to accurately estimate N_E at the local scale.

By convention, population genetic studies in the era of microsatellite markers have used approximately 20–25 individuals (Leberg 2002), though this is insufficient for several uses (Luikart et al. 2002). Rarely do published studies justify the chosen sample size and it may be primarily determined by the number of available individuals, especially for cryptic species such as hellbenders and box turtles. At least for these 2 species, 24 was an insufficient sample size to be able to accurately estimate N_E (objective 3). Furthermore, the sufficient sample size varied (objective 4), ranging from approximately 65 to almost 1,800 (Figure 4; Table 3). These results suggest that for wildlife species with widely dispersed genetic populations, even when samples are collected across the genetic population, sample sizes in the typical range of 25 may be insufficient to accurately estimate N_E or provide biologically meaningful estimates.

Unexpectedly, a majority of estimators failed to provide complete estimates. The NEESTIMATOR-HE method failed to give any complete estimates for either species. Further, 40.8% of ONESAMP, LDNE, and NEESTIMATOR-LD attempts failed to give complete estimates. There are several potential reasons that could explain estimator failure. Deficits of heterozygotes in sampling populations with population structure (Wahlund effect) can, along with the presence of migration, upwardly bias estimates of N_E (Waples and England 2011) and many of these estimates for our data were infinity. Both ONESAMP and LDNE methods failed to give complete estimates even for the sampling populations that were previously identified as genetic populations by Bayesian approaches that putatively detect substructuring (Unger et al. 2013a, Kimble et al. 2014a), but if box turtles and hellbenders do fit the model of a Wahlund effect in the presence of migration, then perhaps the failure of so many estimates is due to this combination of migration and structure.

A second possible cause of estimator failure is that N_E estimates are sensitive to the model assumption of limited dispersal, especially in large populations capable of higher dispersal rates (Nunney 2016). For example, estimates of N_E including negative point estimates and estimates of an upper confidence interval with infinity have been reported in large population size and high dispersal systems, such as in salmonids (Harris et al. 2017). In a study of large marine fish populations with typical sample sizes of 50 individuals, negative estimates were common and often interpreted as large or infinite N_E values (Marandel et al. 2019). Box turtles, being primarily terrestrial, may have greater dispersal capabilities (e.g., 10.0 km; Kiester et al. 1982) than do the obligate aquatic hellbender (e.g., 315.88 m; Bodinof et al. 2012), and in our data we obtained a greater proportion of incomplete LDNE N_E estimates for box turtles than for hellbenders. Though in both species the typical individual adult moves relatively short distances within their home ranges (Dodd 2002, Burgmeier et al. 2011a, Currylow et al. 2012), transients may exist in populations of box turtles (Kiester et al. 1982, Schwartz and Schwartz 1991) and hellbenders (N. G. Burgmeier, personal communication). Juvenile dispersal, however, is largely undocumented for both species, highlighting a need for better understanding of the relationship between this natural history trait and estimates of N_E.

MANAGEMENT IMPLICATIONS

Conservation managers designing management plans should carefully consider software and parameters used when making genotype-based estimates of effective population size for animal populations. While estimates of N_E can provide important data on the genetic health of many species, they should be interpreted with caution for populations of cryptic long-lived species in conservation need. Based on our observations, NEESTIMATOR-LD was most likely to give complete estimates. There is high variation among estimators, up to several orders of magnitude in some cases. We recommend using several estimators and the conservative acceptance of any values they produce unless supported by additional demographic data (e.g., increased temporal sampling). In the case of unknown age or cohort of individuals sampled, larger sample sizes are likely to produce more realistic results. In the present study, this number ranged from tens to thousands. We recommend the use of an accumulation curve to gauge the sample size necessary to obtain more realistic, reliable, and biologically meaningful results for each population. Many populations of box turtles and hellbenders are facing numerous threats, which have the potential to affect genetic diversity within remaining populations. This means that while it becomes ever more important to use accurate estimators of N_E, managers should be aware of their potentially misleading results and continue to conduct long-term species-specific demographic studies with genetic studies for wildlife conservation. Therefore, we suggest incorporation of further mark-recapture and geographic-specific natural history studies for future management of each species.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

ETHICS STATEMENT
We sampled turtles and hellbenders under Purdue University Animal Care and Use Committee protocol 07-037 and protocol 08-025, respectively.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of the sensitivity of location data for these species.

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SUPPORTING INFORMATION

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