Nest-Site Fidelity and Sex-Biased Dispersal Affect Spatial Genetic Structure of Eastern Box Turtles (Terrapene carolina carolina) at Their Northern Range Edge

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Nest-Site Fidelity and Sex-Biased Dispersal Affect Spatial Genetic Structure of Eastern Box Turtles (*Terrapene carolina carolina*) at Their Northern Range Edge

Jennifer A. Moore[1], Eric M. McCluskey[1], Breanna Gould[2], Patrick Laarman[1], and Jan Sapak[3]

Dispersal and nesting philopatry are two processes that affect the connectivity, evolution, and long-term viability of populations, and thus have important conservation implications for threatened and endangered species. Here we investigate dispersal, relatedness, and the fine-scale spatial genetic structure of Eastern Box Turtles (*Terrapene carolina carolina*) at the northern extreme of their geographic range in northwestern Michigan. We analyzed georeferenced microsatellite genotypes ($n = 165$) using global, sex-specific, and two-dimensional local spatial autocorrelation (2D LSA), as well as spatial principal components analysis (sPCA). Genetic diversity was low relative to Eastern Box Turtle populations in the middle of the range. We found dispersal was male-biased, as only females showed significant positive spatial genetic autocorrelation at distances less than 2 km. 2D LSA showed local genetic "hotspots" of related turtles that tended to correspond with known nesting areas. We found evidence for global genetic structure using sPCA, which we attribute to genetic clustering rather than clinal variation. Our results suggest that restricted female dispersal and fidelity to limited open-canopy nest sites result in fine-scale spatial genetic structuring in this population. We stress the importance of maintaining high quality nesting habitat and habitat corridors for transient males, which appear to be critical for functional connectivity of Eastern Box Turtles.

UNDERSTANDING patterns of fine-scale spatial genetic structure, or the non-random spatial distribution of genotypes, can provide insights into many ecological and microevolutionary processes (Epperson and Li, 1997; Smouse and Peakall, 1999). Processes affecting fine-scale spatial genetic structure include dispersal and philopatry (Hazlitt et al., 2004; Vekemans and Hardy, 2004), sociality and mating systems (Ross, 2001), population density (Vekemans and Hardy, 2004), and reproductive behavior (Bowen et al., 1993; Avise et al., 2000). Dispersal is often difficult to characterize, but it is critical that we understand this aspect of a species' biology when they are threatened by habitat loss and fragmentation. Maintaining population connectivity through gene flow and dispersal is increasingly important for maintaining adaptive potential and reducing inbreeding in changing landscapes (Lowe and Allendorf, 2010), so understanding spatial genetic structure and its underlying mechanisms can ultimately aid conservation efforts for threatened and endangered species.

Fine-scale genetic structure is not expected to be present in highly vagile, wide-ranging species with generalist habitat preferences and random mating patterns. However, species with restricted dispersal and limited mobility, like many turtles, may show fine-scale patterns of isolation by distance, whereby individuals in close proximity to one another are more related than those that are farther apart (Wright, 1943; Malécot, 1948; Mockford et al., 2005). Furthermore, many species exhibit patterns of sex-biased dispersal, which can function to reduce competition or inbreeding and often results from differential evolutionary pressures on the sexes (Greenwood, 1980; Goudet et al., 2002). Sex-biased dispersal can therefore result in stronger genetic structuring for the more philopatric sex (Dubey et al., 2008) and population connectivity that is largely driven by the dispersing sex (Fraser et al., 2004).

For egg-laying species, like turtles, the distribution of alleles across the landscape may be largely dependent on female reproductive success (Scribner et al., 1993) and the location of nest sites. Spatial genetic structure may develop over time if hatchlings disperse from nests into habitats in close proximity to their parents. Spatial genetic structuring is further strengthened when females show fidelity to nest sites (i.e., females return to the same nest sites in consecutive nesting seasons) or natal nest-site philopatry (i.e., females return to their natal sites to nest). Selection should favor nesting philopatry for species like turtles, especially under stable local environmental conditions, because it ensures that females nest in locations that successfully produced females in the previous generation (Reinhold, 1998). Long-lived, iteroparous species with nest-site fidelity and limited dispersal would be expected to exhibit strong spatial genetic structure, even in the absence of natal nest-site philopatry, as kin accrue around nest sites over time.

Although sex-biased dispersal and philopatry are well understood in mammals and birds (Greenwood, 1980; Greenwood and Harvey, 1982), comparatively few generalizations are available for reptiles. One exception is the well-known pattern of nesting philopatry and male-mediated gene flow in sea turtles. The strong natal homing ability of female sea turtles has been well studied (Allard et al., 1994; Bass et al., 1996; Lohmann et al., 2008, 2017). Because of this philopatric tendency, genetic structure in sea turtles tends to reflect the distribution of nest sites, rather than feeding or mating grounds which can be thousands of kilometers away

and contain a heterogeneous mix of turtle genotypes (Bowen et al., 1992, 1993; Peare and Parker, 1996; Shamblin et al., 2011). Some terrestrial and freshwater turtles show nest-site fidelity (e.g., *Emydoidea blandingii*, Congdon et al., 1983; *Chrysemys picta*, Valenzuela and Janzen, 2001; *Graptemys kohnii*, Freedberg et al., 2005; *Malaclemys terrapin*, Sheridan et al., 2010), although whether natal philopatry is the rule or the exception for non-marine turtles remains to be seen (but see Freedberg et al., 2005).

In this study, we use genetic data to investigate dispersal and fine-scale spatial genetic structure in a population of Eastern Box Turtles (*Terrapene carolina carolina*) in the northwestern lower peninsula of Michigan, USA. Eastern Box Turtles are terrestrially adapted, long-lived turtles that are broadly distributed across North American hardwood forests of the eastern United States (Dodd, 2001). On a regional level, box turtles have experienced drastic population declines due primarily to habitat loss, degradation, and fragmentation and the concomitant increases in disease, mortality, predation, and illegal collection (Dodd, 2001; Feldman et al., 2006). In Michigan, Eastern Box Turtles are currently listed as a state species of special concern, and their historic geographic range in the state has declined from at least 31 to 18 counties in two decades (Marsack and Swanson, 2009).

Our study site in Manistee National Forest (NW Michigan) occurs on the northernmost geographic edge of the species range. Because range-edge populations are subject to environmental extremes and are more isolated, they tend to be characterized by greater genetic differentiation and lower genetic diversity than populations closer to the core of the geographic range (Sexton et al., 2009). Eastern Box Turtles generally lack genetic population structure at broad scales (Kimble et al., 2014a); however, local spatial genetic structure could be present particularly in geographic range-edge populations where migration may be limited and individuals are faced with limiting environmental conditions. Our objectives were to examine patterns of fine-scale spatial genetic structure in a range-edge population, from which we will be able to infer the underlying ecological processes responsible for generating patterns. Because Eastern Box Turtles exhibit nest-site fidelity and dispersal may be limited, we expect 1) patterns of fine-scale spatial genetic structuring across the landscape that deviate from random, and 2) positive spatial genetic autocorrelation at short distances (i.e., less than the distance an individual is capable of dispersing) that may differ between the sexes.

MATERIALS AND METHODS

Study site and sample collection.—We collected tissue samples from Eastern Box Turtles encountered opportunistically while surveying a 20 km² area of high quality box turtle habitat in Manistee National Forest (MNF, Manistee, Mason, and Lake counties), Michigan during the active seasons (March–October) of 2012–2014. Sampling focused mainly on an approximately 25 km stretch of riparian and upland hardwood habitat that turtles are associated with in this region. Specific locality information is omitted to protect the turtles from poaching. Genetic samples were collected concurrent with radio telemetry and nesting ecology studies of this population (Altobelli, 2017; Laarman, 2017; Laarman et al., 2018). Box turtles in MNF use

predominantly upland forest habitat (mixed hardwoods dominated by oaks) and tend to be associated with lowland riparian areas (Laarman, 2017). Reproductive females use remnant patches of oak–pine barrens, pine barrens, and dry sand prairie to nest and these habitat types are limited in MNF (Laarman et al., 2018).

For each individual captured, we recorded capture location using a handheld GPS, morphometric data (size and mass), sex, and age (by counting up to 20 growth rings, after which turtles were classified as 20+; Sexton, 1959). Adult sex was determined by examination of sexually dimorphic characteristics (e.g., concave plastron in males). Carapace length and width were measured to the nearest mm using calipers (Haglöf Mantax Blue calipers or Mitutoyo stainless steel dial calipers), and mass was collected, to the nearest gram, using a Pesola spring scale. Turtles were marked by filing notches in the marginal scutes according to the method described by Ernst et al. (1974). Tissue samples were collected as tail clips (~2–3 mm) and were stored in 95% ethanol at –20°C until DNA extraction. Sampling equipment was sterilized by flaming between individuals.

Microsatellite genotyping.—We extracted genomic DNA from tissue samples using Qiagen DNeasy Blood and Tissue Extraction kits (Qiagen Inc., Valencia, CA) following standard manufacturer protocols. We amplified DNA using polymerase chain reaction (PCR) at 11 species-specific microsatellite loci (*TCC_di_045, TCC_di_082, TCC_di_189, TCC_di_300, TCC_di_318, TCC_di_352, TCC_di_366, TCC_tetra_012/342, TCC_tetra_043, TCC_tetra_070,* and *TCC_tetra_309*; Kimble et al., 2011) following the protocol outlined in Kimble et al. (2011). Amplified products were run on an ABI 3130xl genetic analyzer (Applied Biosystems) with an internal size standard (GeneScan 500 LIZ, Applied Biosystems). Alleles were visualized and scored using ABI Peak Scanner software (version 1.0, Applied Biosystems). Allele sizes were manually scored by the same observer. A random 10% of alleles were scored by two independent observers to assess scoring accuracy.

We calculated the number of alleles per locus, probability of identity, observed (H_o) and expected (H_e) heterozygosities, and tested each locus for deviations from Hardy-Weinberg equilibrium (HWE) using GenAlEx version 6.3 (Peakall and Smouse, 2006). We used a Monte Carlo chain method (1,000 dememorizations, 100 batches, 1,000 iterations) following the algorithm of Guo and Thompson (1992) and applied a Bonferroni correction for a table-wide significance level of 0.05 (adjusted *P* value = 0.0045). We calculated the inbreeding coefficient (F_{IS}) using Genepop (Raymond and Rousset, 1995). We estimated the frequency of null alleles using the Expectation Maximization (EM) algorithm (Dempster et al., 1977) as implemented in the program FreeNA (Chapuis and Estoup, 2007). We calculated pairwise relatedness between each individual pair of turtles, and averaged across all individuals, using the estimator in GenAlEx v 6.3 (Queller and Goodnight, 1989; Peakall and Smouse, 2006). We also calculated effective population size (N_e) using the linkage disequilibrium method (Waples and Do, 2010) as implemented in NeEstimator v. 2.1 (Do et al., 2014). We used a model of random mating and excluded alleles with frequencies of 0.02 as recommended by Waples and Do (2010).

Spatial genetic structure.—We first tested whether a global pattern of isolation by distance (IBD) was present across the study area using a Mantel test. We created matrices of pairwise Euclidean (geographic) distance and pairwise genetic distance (a modification of Nei and Li, 1979) metric, as described in Huff et al. (1993) in GenAlEx 6.3 (Peakall and Smouse, 2006). We then performed a Mantel test for correspondence of the genetic and geographic distance matrices in GenAlEx 6.3 (Peakall and Smouse, 2006). Significance of matrix correspondence was tested by 10,000 random permutations of the data. We visualized the correlation of geographic and genetic distance with kernel density estimates implemented in the R package ADEGENET (Jombart, 2008).

We further examined patterns of fine-scale spatial genetic structure using a global multi-locus spatial autocorrelation analysis under the null hypothesis of a random spatial distribution of genotypes (GenAlEx, version 6.3; Peakall and Smouse, 2006) following the methods of Smouse and Peakall (1999). This technique calculates an autocorrelation coefficient (r) for predefined distance classes, whereby r is a measure of genetic similarity between all pairs of individuals whose geographic separation falls within each distance class. Under a model of restricted dispersal, the expectation is that genetic and geographic distance will be positively autocorrelated at short distances. Significance tests were performed using 10,000 random permutations of the data, and 95% confidence intervals for estimates of r were determined by 10,000 bootstraps. Spatial genetic autocorrelograms were created by plotting the r values as a function of distance, using eight variable distance classes (ranging from 0.5 to 11 km, to capture the relevant scale for the dispersal process while maintaining a reasonable sample size in each distance class). We also performed spatial autocorrelation analyses separately for males and females to determine whether differences in spatial genetic structuring existed based on sex.

To examine the geographic distribution of genetically similar individuals and identify spatial genetic "hotspots," we performed a two-dimensional local spatial autocorrelation analysis (2D LSA) in GenAlEx 6.3 (Peakall and Smouse, 2006) following the methods of Smouse and Peakall (1999). This method examines local patterns of spatial genetic autocorrelation across a two-dimensional landscape by comparing an individual to its n nearest neighbors. We calculated local autocorrelation coefficients (lr) for each individual and its n nearest neighbors, using 3, 5, and 10 nearest neighbors. We assessed significance by comparing each lr value to the expectation of no local spatial genetic structure, based on 10,000 random permutations of the data. We plotted individuals and their lr values using ArcGIS 10.1 to qualitatively assess the distribution of significant local spatial genetic "hotspots" across the landscape and their proximity to known nesting sites.

We incorporated an alternative method for detecting spatial genetic patterns using a spatial principal component analysis (sPCA) implemented in ADEGENET (Jombart, 2008). This method differs from Bayesian clustering programs such as STRUCTURE (Pritchard et al., 2000; Falush et al., 2003) by having no assumptions regarding population genetic models such as Hardy-Weinberg equilibrium or linkage equilibrium. sPCA uses Moran's I to identify patterns of spatial autocorrelation and is effective at detecting cryptic genetic structure (Jombart, 2008; Schwartz and McKelvey, 2008; Vergara et al.,

Table 1. Summary of locus-specific genetic diversity measures for Eastern Box Turtles ($n = 165$) in northwestern Michigan including the number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and the fixation index (F or $1-H_o/H_e$).

Locus	N_a	H_o	H_e	F
TCC_di_082	11	0.60	0.84	0.29
TCC_di_189	5	0.39	0.63	0.38
TCC_di_300	19	0.80	0.84	0.05
TCC_di_352	13	0.82	0.87	0.06
TCC_di_366	9	0.73	0.76	0.04
TCC_tetra_012/342	18	0.61	0.77	0.21
TCC_di_318	28	0.61	0.87	0.30
TCC_tetra_309	15	0.71	0.89	0.20
TCC_tetra_043	13	0.86	0.88	0.02
TCC_tetra_070	18	0.90	0.91	0.02
TCC_di_045	19	0.60	0.90	0.33
Average	15.27	0.69	0.83	0.17

2015). The components of the sPCA are separated into global (positive eigenvalues) and local (negative eigenvalues) structures. We were particularly interested in the global scores which can indicate either clusters or clines in the dataset compared to between-individual genetic differences reflected by the local scores. We assessed both patterns with the original ADEGENET Monte Carlo procedure and a newly added *spca_randtest* function recommended to increase statistical power (Montano and Jombart, 2017) using 9,999 permutations.

We applied the sPCA method to the entire dataset and a subset of individuals sampled along our focal river system because we were interested in determining if eliminating spatially isolated samples would make any genetic clines in the dataset more apparent.

RESULTS

We genotyped 165 Eastern Box Turtles ($n = 104$ females, 51 males, 10 juveniles) using 11 microsatellite loci. The probability of identity across all 11 loci was 1.9×10^{-16}. Overall, genetic diversity was high with an average number of alleles per locus of 15.3 (range = 5–28). Observed heterozygosity averaged 0.69 with a per locus range of 0.39–0.86, and expected heterozygosity averaged 0.83 with a range of 0.63–0.91 (Table 1). The average inbreeding coefficient (F_{IS}) across all loci was 0.17 (range = 0.02–0.38 per locus). Eight loci showed significant deviations from HWE following Bonferroni correction, and the average frequency of null alleles was 0.076. In spite of the high proportion of loci that showed significant deviations from HWE, we attributed these deviations to either undetected genetic structuring (i.e., Wahlund effect; Sinnock, 1975) or the high allelic richness present in these microsatellite loci relative to our sample size (also noted by Kimble et al., 2011, 2014a, 2014b). Therefore, we retained all loci for further analyses. Estimated N_e was 203.4 (jackknife 95% CI = 144.4–320.2).

Average pairwise relatedness (r) was –0.0067 (0.14 SD, range = –0.41–0.84). Just over half of turtle pairs (54.57% of 13,366 pairwise comparisons) were unrelated ($r \leq 0$); however, 13.03% ($n = 1,742$ pairwise comparisons) were related at a level consistent with first cousins (r range = 0.125–0.249), 4.63% ($n = 619$ pairwise comparisons) were
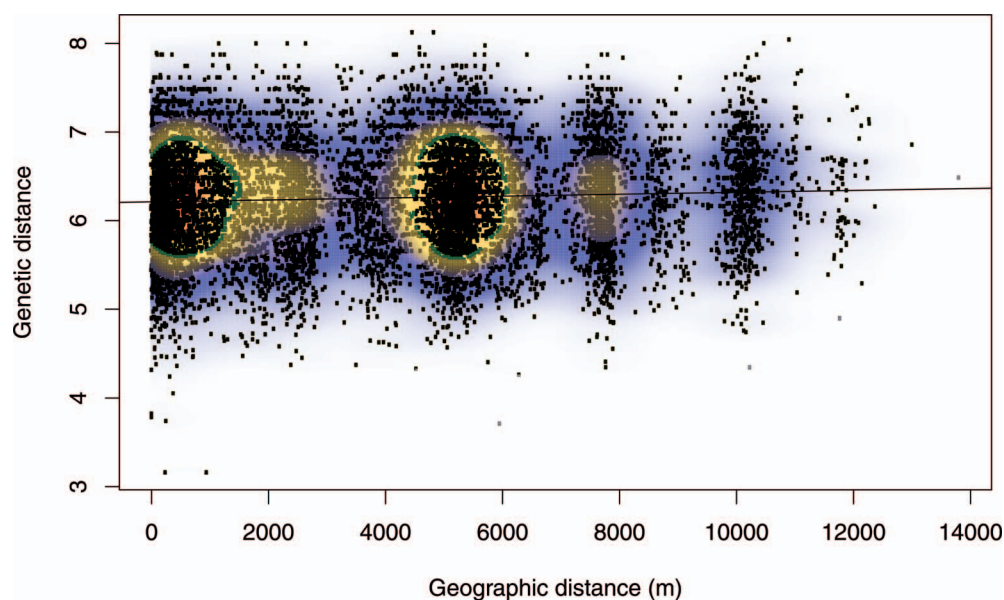
Fig. 1. Scatterplot showing the matrix of pairwise genetic distances and matrix of pairwise geographic distances for box turtles sampled along the river corridor. Warmer colors within the kernel density indicate higher densities of points. The line (slope = 1.074727e−05; R^2 = 0.002992) shows the correlation trend.

related at a level consistent with half-sibling, or aunt/uncle-niece/nephew relationships (r range = 0.25–0.5), and 0.14% of pairs ($n = 19$ pairwise comparisons) were related at a level consistent with either full-sibling or parent–offspring relationships (r $\geq$ 0.50).

We tested for a global pattern of isolation by distance, whereby geographically more distant individuals are expected to be less genetically similar than individuals that are closer to one another. We did not find a significant pattern of isolation by distance across our study site using a Mantel test (Mantel r = 0.054, $P = 0.062$; Fig. 1). We also tested for significant fine-scale spatial genetic structuring using spatial autocorrelation tests and found weak but significant positive global spatial genetic autocorrelation at distances fewer than 2 km (Fig. 2A). When separated by sex, we found no significant spatial genetic structure for males (Fig. 2C); however, females showed significant spatial genetic structure at distances less than 2 km (Fig. 2B).

We used 2D LSA to examine the geographic distribution of individuals that were significantly more related to their neighbors than expected based on permutation testing from a random distribution of genotypes. Bubble plots (Fig. 3) showed five genetic "hotspots," three of which correspond closely with the known locations of nesting sites (openings in the otherwise contiguous forest, indicated on Fig. 3 as 'NS'). Average *lr* values were 0.020 (0.090 SD), 0.021 (0.062 SD), and 0.0094 (0.050 SD) for 3, 5, and 10 nearest neighbors, respectively. When comparing each individual to its three nearest neighbors, we identified 22 individuals with significant *lr* values (range = 0.12–0.31). Of those 22 individuals, 16 (73%) were female, 3 (14%) were male, and 3 (14%) were juveniles (<12 years old) of unknown sex. Results were largely concordant when comparing 2D LSA analyses based on 3, 5, and 10 nearest neighbors.

We found evidence for global structure within the full dataset ($P = 0.000$) and river corridor ($P = 0.001$) subset using the updated *spca_randtest* function. This method did not detect local structure with the full dataset ($P = 0.9$) or river corridor ($P = 0.7$). The standard sPCA global and local tests were not significant for the full dataset ($P = 0.06, 0.7$) or river corridor ($P = 0.06, 0.4$).

The discontinuities in the IBD density plot (Fig. 1) indicated that genetic clustering is more likely responsible for the significant global tests than clinal variation (Nørgaard et al., 2017). However, no clear spatial groupings are apparent from the retained first principal component for either the full dataset or river corridor (Fig. 4).

DISCUSSION

Eastern Box Turtles in the northwestern lower peninsula of Michigan generally show patterns of fine-scale spatial genetic structuring that are consistent with male-biased dispersal and female nest-site fidelity. We found that significant spatial genetic structuring was particularly evident in females, which means that dispersal is probably male-biased in *T. c. carolina*. A genetic signature of female dispersal was not detected beyond a distance of 2 km; however, restricted dispersal was not similarly detected for males. Global (site-wide) isolation by distance was approaching significance, and we detected local genetic 'hotspots' of related individuals (mostly females) that appear to correspond with the locations of nesting areas. This sex-based structuring is likely responsible for the significant global structure identified by the sPCA despite no clear geographic pattern of genotype distribution across the landscape (Fig. 4). Overall, our results suggest that restricted female dispersal and fidelity to limited open-canopy nest sites result in fine-scale spatial genetic structuring in this population.

On a broad geographic scale, Eastern Box Turtles have relatively high genetic diversity and do not show strong population genetic structuring (Hagood, 2009; Kimble et al., 2014a), even though populations have been declining range-wide (Lieberman, 1994; Gibbons et al., 2000). Many studies of threatened and endangered freshwater turtles have consistently found higher-than-expected levels of genetic diversity when considering habitat fragmentation and demographic histories (Kuo and Janzen, 2004; FitzSimmons and Hart, 2007; Bennett et al., 2010). High levels of genetic diversity and the lack of broad scale genetic structuring in Eastern Box Turtles are probably artifacts of their pre-European settlement distribution and high connectivity
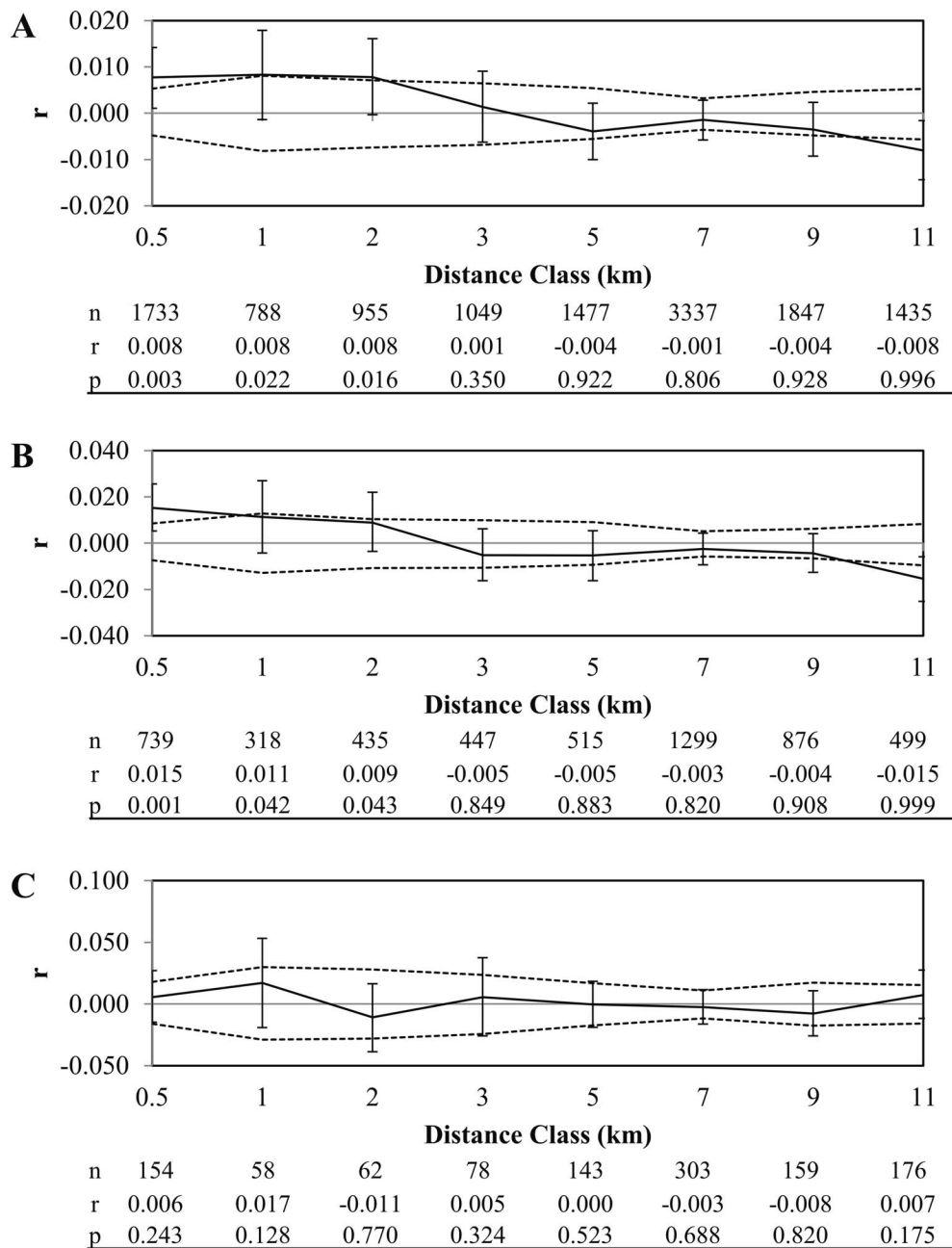
A

n	1733	788	955	1049	1477	3337	1847	1435
r	0.008	0.008	0.008	0.001	-0.004	-0.001	-0.004	-0.008
p	0.003	0.022	0.016	0.350	0.922	0.806	0.928	0.996

B

n	739	318	435	447	515	1299	876	499
r	0.015	0.011	0.009	-0.005	-0.005	-0.003	-0.004	-0.015
p	0.001	0.042	0.043	0.849	0.883	0.820	0.908	0.999

C

n	154	58	62	78	143	303	159	176
r	0.006	0.017	-0.011	0.005	0.000	-0.003	-0.008	0.007
p	0.243	0.128	0.770	0.324	0.523	0.688	0.820	0.175

Fig. 2. Spatial genetic autocorrelograms of genetic correlation coefficients (r) as a function of distance for Eastern Box Turtles in northwestern Michigan. Plots represent (A) all individuals ($n = 165$), (B) females only ($n = 104$), and (C) males only ($n = 51$). Dashed lines are permuted 95% confidence intervals across all data, and error bars are bootstrapped 95% confidence intervals within each distance class. Tables below graphs represent data for each distance class including the number of pairwise comparisons (n), the correlation coefficients (r), and the P-values (p) associated with bootstrap tests of significance for positive spatial genetic autocorrelation.

coupled with long generation times, which means that genetic diversity is not reflecting contemporary habitat loss, fragmentation, and concurrent demographic declines (Hagood, 2009; Marsack and Swanson, 2009; Kimble et al., 2014a). Understanding the effects of habitat fragmentation and disrupted connectivity for species with long generation times therefore requires the integration of ecological and evolutionary methods (e.g., for Coahuilan Box Turtles, *Terrapene coahuila*, Howeth et al., 2008). This pattern of high genetic diversity in the face of known demographic bottlenecks now appears common among freshwater turtles (Kuo and Janzen, 2004; Bennett et al., 2010; Davy and Murphy, 2014).

Northwestern Michigan represents the extreme northern edge of the species range (Powell et al., 2016). Based on the 'center-periphery hypothesis,' we would expect box turtles in Manistee National Forest to have reduced genetic diversity

and effective population size, stronger population differentiation, and be more prone to extinction because they inhabit the latitudinal range margin where populations are limited by environmental extremes like cold stress (Lawton, 1993; Vucetich and Waite, 2003; Hampe and Petit, 2005). The number of alleles present in MNF box turtles is comparable to the number of alleles present in the 26 management populations examined by Kimble et al. (2014a). However, only two of these 26 populations had higher F_{IS} values than MNF, and only one population had lower observed heterozygosity than our study population (Kimble et al., 2014a). Our moderate N_e point estimate of 204 is an order of magnitude smaller than N_e estimates from three southern Michigan populations (N_e range of 6,675–9,516 individuals; Marsack and Swanson, 2009). However, it is worth noting that the N_e estimates of Marsack and Swanson (2009) are more reflective of an evolutionary timescale, while
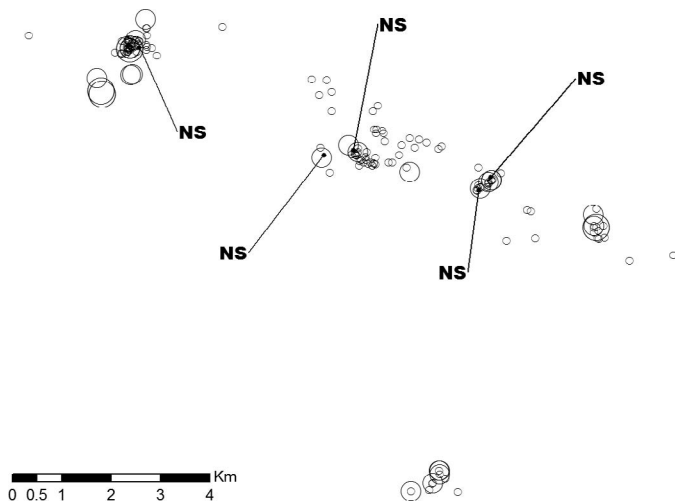
Fig. 3. Bubble plot showing the results of two-dimensional local spatial autocorrelation analysis for all Eastern Box Turtles ($n = 165$) sampled in northwestern Michigan. Circles represent individuals and the size of the circle is proportional to the *P*-values from permutation testing, with large circles representing individuals that are significantly more related to their five nearest neighbors than expected ($P < 0.05$) based on a random distribution of genotypes. Figure shows the distribution of five genetic "hotspots" in relation to known nesting sites (NS) across the study area. Some distantly sampled individuals are omitted for figure clarity.

linkage disequilibrium, the method we employed, should reflect contemporary effective population size (Waples and Do, 2010). Eastern Box Turtles are listed as a species of special concern in Michigan, but the MNF population exists in relatively unfragmented habitat. The reduced genetic diversity present in MNF box turtles, relative to other populations throughout the range, may be due in part to population declines across Michigan, but also to Manistee National Forest's location on the extreme latitudinal range margin of box turtles.

Although Eastern Box Turtles do not show strong population structuring on a broad regional scale, box turtles at our site in northwestern Michigan show fine-scale spatial genetic structuring that appears to be driven by restricted dispersal of females. Investigations of sex-biased dispersal in turtles are rare; however, the pattern of dispersal tends to be male-biased from the few studies that exist. Male-biased dispersal is known for Radiated Tortoises (*Astrochelys radiata*, Paquette et al., 2010), Diamondback Terrapins (*Malaclemys terrapin*, Sheridan et al., 2010), and Loggerhead Turtles (*Caretta caretta*, Casale et al., 2002).

Limited direct dispersal data (e.g., from mark–recapture or radio-telemetry studies) exist for Eastern Box Turtles. Laarman et al. (2018) show that neonate dispersal from nests at our study site is extremely limited within the first activity season. Mean straight-line dispersal distance from nests to the first overwintering sites was less than 20.0 m (Laarman et al., 2018). Although movement away from the nest tended to increase in the second activity season, Laarman et al. (2018) were unable to examine movement differences by sex as sexing these age classes is impossible without using invasive methods. To our knowledge, no direct data on juvenile dispersal and home range establishment (or lack thereof) exist for box turtles, which is likely a reflection of the

difficulty of collecting these data for such a long-lived species.

Male-mediated gene flow is further supported in box turtles by direct (ecological) evidence of transient adult males. The typical space use pattern for box turtles is relatively small home ranges (averaging 16.4 ha at our study site; Laarman, 2017) and limited movement, with the exception of female nesting migrations (Laarman, 2017). However, adult male box turtles occasionally exhibit transient behavior (i.e., continuous movement without traversing previously visited areas, *sensu* Kiester et al., 1982; Seibert and Belzer, 2014). Using radio telemetry, Kiester et al. (1982) found three transient male Three-Toed Box Turtles (*Terrapene carolina triungis*) that moved more or less in a straight-line pattern during the time they were monitored. One of these moved 10 km over the course of a year (Kiester et al., 1982). Likewise, in a radio-telemetry study conducted at our study site concurrent with our genetic study, Laarman (2017) radio-tracked an adult male Eastern Box Turtle that moved over 3 km in an eight-week period and was ultimately lost from radio contact. Further, in a long-term study of translocated and headstarted box turtles, Seibert and Belzer (2013, 2014) showed that males generally ranged farther, and they speculated that males expand their ranges as they age, but attenuate them as adults. Seibert and Belzer (2014) also noted the considerable variation in movement behavior, from very high site fidelity to transience. Transient males are infrequently detected using direct (ecological) methods, yet are likely to be extremely important for maintaining genetic diversity and interpopulation gene flow (Kimble et al., 2014b). This bimodal movement pattern of male box turtles, and why some males exhibit transient behavior while others maintain home ranges, is not currently understood.

We found clusters of related individuals (mostly females) that generally correspond with nesting sites, which is likely a reflection of long-term nest-site fidelity, and possibly natal philopatry. Nest-site fidelity is further supported by direct evidence of nesting behavior we observed during four years that overlapped with the genetic study. Female box turtles in MNF commonly use the same nesting areas year after year, with consecutive years' nests located as little as one meter apart (Altobelli, 2017; also observed in Maryland Box Turtles by Stickel, 1950). The observational nesting data are limited in temporal scope (Altobelli, 2017); however, the genetic data reflect longer-term processes. If females do not disperse from nests, and recruit to the same areas as their mothers, it is probable that successive generations of females use the same nest sites, resulting in the pattern of spatial genetic structure we observed across the landscape. Freedberg et al. (2005) showed that freshwater map turtles (*Graptemys kohnii*) have the ability to home to nesting beaches after being displaced and that related females also tend to nest in close proximity to one another. Freedberg et al. (2005) concluded that freshwater turtles can therefore inherit nesting beaches across successive generations. Whether or not Eastern Box Turtles have the ability to home to natal nesting sites is not currently known. Nest-site fidelity and subsequent recruitment of hatchlings therefore may influence patterns of spatial genetic structure of freshwater and terrestrial turtles (Scribner et al., 1993).

Our study has important conservation implications for Eastern Box Turtles. First, maintaining existing high-quality nesting areas is of the utmost importance. Box turtles, at
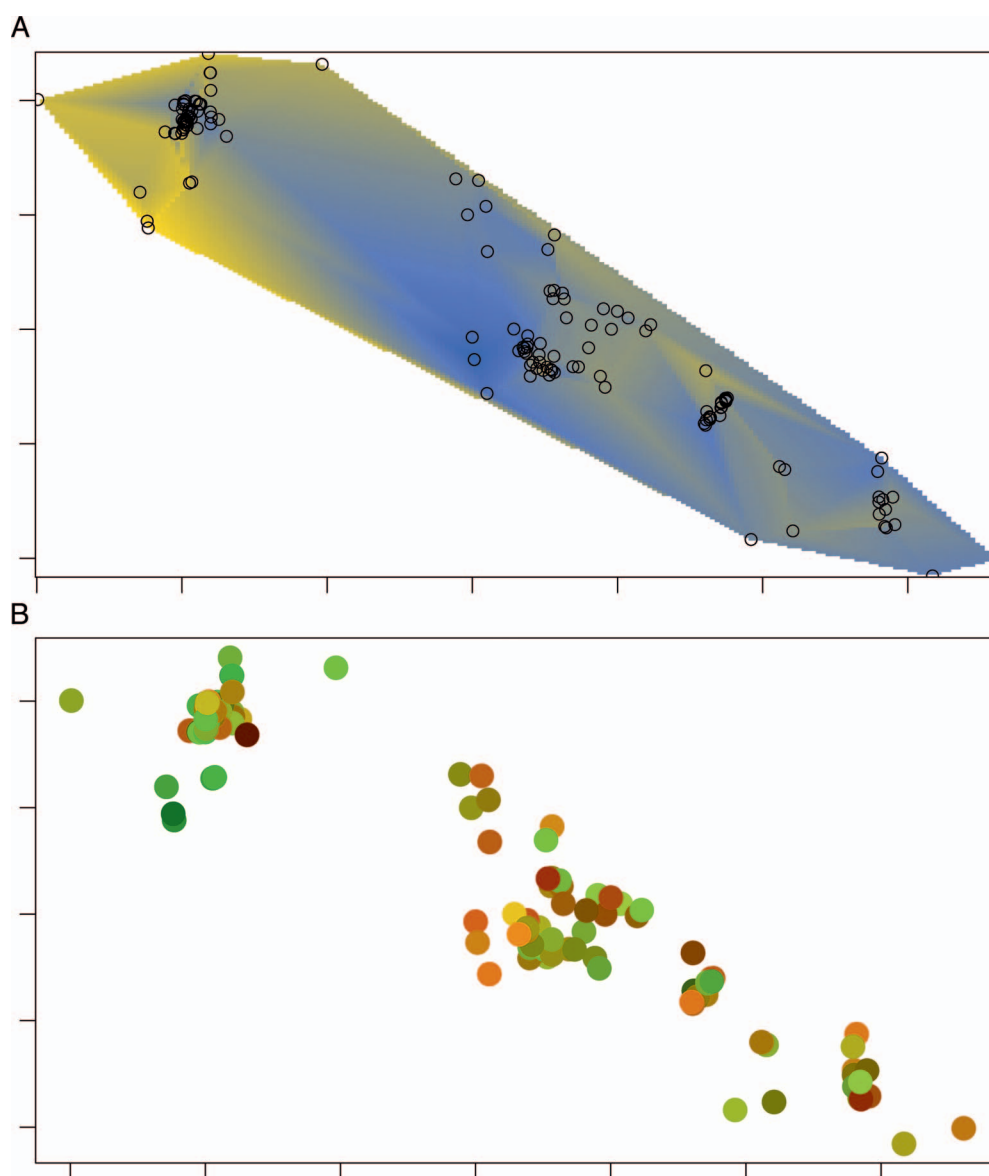
Fig. 4. Analysis of global structure along the river corridor from the first principal component of the sPCA represented by (A) interpolation of lagged principal scores showing genetic clines and (B) colors indicating individual scores. Coordinates have been deliberately omitted to deter poachers.

northern latitudes, require open-canopy nesting sites that provide specific thermal and hydrological conditions. The microclimate experienced by developing hatchlings affects their development and determines their sex (Standora and Spotila, 1985; Cagle et al., 1993). If habitats are degraded (e.g., via invasive shrubs or encroachment of woody species), females may continue to use those sites even though they no longer provide the conditions necessary for hatchlings to thrive. Over time, this would result in loss of recruitment that may go undetected for many years due to the extreme longevity of these turtles. How female box turtles respond to degradation of nest sites and changing environmental cues is unknown and should be investigated. Secondly, transient males appear to be extremely important for maintaining gene flow, yet are likely at a high risk of road mortality (Shepard et al., 2008) or poaching (Hohn, 2003). Protecting transient males and migrating nesting females by maintaining large connected areas of suitable habitat is critical for the continued maintenance of gene flow and population genetic diversity of Eastern Box Turtles. A comparative landscape genetics approach may be able to identify landscape elements important for mediating gene flow in males (e.g., riparian corridors, roads) and genetic "hotspots" in females (e.g., nesting conditions). Long generation times have thus far largely buffered box turtles from the genetic diversity losses that typically accompany human-induced demographic declines (Kuo and Janzen, 2004). Population connectivity, likely mediated by males, will be critical for maintaining this diversity into the future (Hagood, 2009; Lowe and Allendorf, 2010). Further, understanding latitudinal range-edge dynamics and protecting range-edge populations may be particularly important when considering future climate regimes (Hampe and Petit, 2005).

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