Transactions of the Illinois State Academy of Science
(2021) Volume 114, pp. 65-73

received 4/23/21
accepted 12/3/21

Analysis of Mating Patterns in a Highly Fragmented Population of the Endangered Blanding's Turtle (*Emydoidea blandingii*) in DuPage County, Illinois

Rodney J. Scott[1*], George M. Klut[2], Jean M. Dubach[3], Dan Thompson[4], Forrest Campbell[1], Kyle Mayer[1], and Timothy Chung[1]
[1]Biology Department, Wheaton College, 501 College Avenue, Wheaton, IL 60187, USA
[2]Department of Biological Sciences, Western Illinois University, 1 University Circle, Macomb, IL 61455, USA
[3]Wildlife Genetics Lab, Loyola University Chicago, 2160 S 1st Ave, Maywood, IL 60153, USA
[4]Forest Preserve District of DuPage County, 3S580 Naperville Rd, Naperville, IL 60563, USA
*Correspondence: rodney.scott@wheaton.edu

ABSTRACT

Blanding's Turtle (*Emydoidea blandingii*) is an imperiled species which has experienced a great reduction in range and occupies highly fragmented environments in many locations including DuPage County, Illinois. We assessed breeding structure and paternity in one highly fragmented environment over a three-year period. We found that mating patterns of individual turtles appeared to change, potentially leading to losses in genetic diversity. Future management strategies should consider ways to augment the genetic diversity of such fragmented populations, possibly including the relocation of offspring from head start programs to populations where they did not originate to mimic natural gene flow.

Keywords: Blanding's Turtle; multiple paternity; microsatellite markers; fragmented populations; DuPage County, Illinois

INTRODUCTION

Blanding's Turtles (*Emydoidea blandngii*) have a scattered and latitudinally compressed distribution centered within the Great Lakes region (McCoy 1973). Disjunct populations also occur east of the Appalachian Mountains from New York to Nova Scotia (Herman et al. 1995, Mockford et al. 2005). Concern for this species has grown during the past several decades (Mockford et al. 2007). Populations have declined range-wide due to habitat destruction and alteration (Congdon and Gibbons 1996), increased nest predation (Congdon et al. 2000), and road mortality (Beaudry et al. 2008, Gibbs and Shriver 2002). The International Union for Conservation of Nature now considers Blanding's Turtle as endangered (van Dijk and Rhodin 2011). In Canada, this species is listed as endangered (Nova Scotia) or threatened (Quebec and Ontario) (Herman et al. 1995). Protection status varies within the United States from endangered (Illinois, Indiana, Missouri, Nebraska, Maine, Massachusetts, and South Dakota) and threatened (Minnesota, Ohio, and New York) to special concern (Michigan and Iowa) (Congdon and Keinath 2006).

The species' range in Illinois has been greatly reduced to the northern part of the state. Using a variety of sources including publications, technical reports, museum records, and Element Occurrence Records from the Illinois Natural Heritage Database, King (2013) estimated that the species' distribution has declined from approximately 60% of the State's total area to approximately 13% and, since 2000, no observations have been made further south than Grundy County. King's report identified anthropogenic factors as the most likely explanation for the decline, including increased human population growth and a decrease of 85% in wetland areas.

Several studies within the Greater Chicago metropolitan area have focused on small, isolated populations in DuPage and Will Counties. Rubin et al. (2001 and 2004) evaluated population structure from a genetics perspective (2001) and a demographic perspective (2004) in two small forest preserves in DuPage County. They compared the DuPage County populations with less fragmented populations in Wisconsin, Michigan, and Nova Scotia and found evidence that the two DuPage County populations may be genetically depauperate compared to the Michigan population. In the demographic study, they estimated the population sizes (25 and 36 individuals respectively) and juvenile recruitment rate for the DuPage sites to be so low that local extinction of these populations in the near future could result. Anthonysamy et al. (2014) studied mating patterns in two adjoining, small, isolated forest preserves in Will County, where they estimated a population size of 33 adults and a heavily skewed sex ratio (11 males: 22 females). There was high variation in reproductive success and no evidence of gene flow with other populations, both patterns that could reduce the long-term viability of the population.

One additional genetic study (Anthonysamy et al. 2018) compared various trends among four turtle species in Lake, DuPage, Will, and Grundy Counties. Two species, Blanding's Turtle and Spotted Turtle (*Clemmys guttata*) are endangered and have restricted distributions, while the Painted Turtle (*Chrysemys picta*) and Snapping Turtle (*Chelydra serpentina*), are common widespread species. The study found that Blanding's Turtles (and Spotted Turtles) had lower levels of genetic diversity, lower levels of gene flow, and a higher risk of genetic drift than the two more common species.

Additional studies have assessed genetic diversity, connectivity, and gene flow across portions of the species' range (e.g., Mockford et al. 2005, Mockford et al. 2007, McGuire et al. 2013, Davy et al. 2014, Sethuraman et al. 2014, McCluskey et al. 2016, Anthonysamy et al. 2018, Jordan et al. 2019). Some major trends include evidence for strong genetic structure that distinguish populations centered on the Great Lakes region from those east of the Appalachian Mountains (Mockford et al. 2007), and fine-scale genetic structuring distinguishing three populations in Nova Scotia from one another (Mockford et al. 2005) and from populations in the rest of the range (Mockford et al. 2007). While other early studies (Osentoski 2001, Rubin et al. 2001) suggested a lack of fine-scale genetic structure in several locations (including the greater Chicago metropolitan area; Rubin et al. 2001), subsequent studies have revealed fine-scale structure in various locations across the Midwest (Sethuraman et al. 2014), within the greater Chicago metropolitan area in Illinois (Anthonysamy et al. 2018), in Southern Ontario (Davy et al. 2014), and in New York (McCluskey et al. 2016).

A lack of genetic structure as evident, for example, in the Edwin S. George Reserve Livingston County, Michigan (McGuire et al. 2013), theoretically indicates a condition of panmixia, which occurs if there are no barriers to gene flow. Fine-scale genetic structuring may indicate the presence of Evolutionarily Significant Units (Mockford et al. 2007) if the forces driving genetic differentiation are natural. However, such structuring may also be a sign of genetic drift due to anthropogenic disturbance such as habitat fragmentation (Anthonysamy et al. 2018).

Associations between genetic diversity and changes in landscape and climate have been studied on a state-wide level in the state of Wisconsin (Reid and Peery 2014, Reid et al. 2017). Both studies sampled multiple populations of Blanding's Turtles (and two other co-occurring turtle species, the Painted Turtle (*Chrysemys picta*) and the Snapping Turtle (*Chelydra serpentina*) across the state. Increased land use intensity was associated with decreased genetic diversity in Blanding's Turtle, but recent climate change showed no such association (Reid and Peery, 2014). Patterns of genetic diversity in Blanding's Turtle also suggested that populations were genetically isolated, but that genetic divergence was not due to isolation by distance or associated with aquatic habitat type (Reid et al. 2017). Hamilton et al. (2018) used modeling to predict how future changes associated with landscape and climate might impact Blanding's Turtle populations across the state of Wisconsin by 2050. These authors predict that under most scenarios, climate change will render most habitats in Southern Wisconsin unsuitable for the species, while some possible scenarios predict the elimination of virtually all suitable habitats from the state.

Much evidence suggests that habitat fragmentation leads to reduced connectivity and gene flow which results in problems such as reduction in genetic diversity, inbreeding, and even local extinction (reviewed in Frankham 2006). While many studies have confirmed these trends, others indicate that more complex factors are at work in some situations and may obscure the effects of reduced gene flow (reviewed with meta-analyses in Keyghobadi 2007, Rivera-Ortíz et al. 2015). Two key factors include timing of events associated with habitat disruption and generation time of the organism(s) considered. Historical records and archeological findings indicate that Blanding's Turtle has experienced peripheral range reduction (McCoy 1973, Congdon and Keinath 2006) and, in many locations, populations are highly fragmented due to expanding urbanization (e.g., Rubin et al. 2001, Anthonysamy et al. 2018). Urbanization poses a unique threat to biodiversity because of its persistence and its tendency to expand over time (McKinney 2002). Blanding's Turtle is an extremely long-lived species (Ernst et al. 1994) with delayed maturation (Congdon and van Loben Sels 1991). Because survivorship increases with age, some individuals may live 70 or more years (Ernst et al. 1994). Annual survival for adults within certain populations, such as at the Edwin S. George Reserve (Livingston County, Michigan), averages 96% (Congdon et al. 2001), and cohort generation times exceed 35 years (Congdon et al. 1993). In general, females become reproductively mature between 14 and 20 years (Congdon and van Loben Sels 1991), but some populations appear to mature more rapidly. Germano et al. (2000) using growth rings on scute annuli, estimated that some female Blanding's Turtles in western Nebraska become reproductively mature at 9 to 10 years. Individual females have been documented to produce clutches into their seventies (Congdon et al. 2001). Such life history traits complicate genetic findings because results reflect past connectivity patterns and distributions (Cunningham et al. 2002, Kuo and Janzen 2004, Marsack and Swanson 2009, Sinclair et al. 2010, Pittman et al. 2011).

Other factors that may obscure the effects of reduced gene flow in Blanding's Turtle include the phenomena of multiple paternity and sperm storage. Multiple paternity, a common mating strategy in Blanding's Turtles (Refsnider 2009, McGuire et al. 2013, Anthonysamy et al. 2014, McGuire et al. 2015), as with other forms of polyandry, has the effect of increasing effective population sizes (Pearse and Anderson 2009). This helps to maximize heterozygosity levels and minimize inbreeding depression (Whittingham and Dunn 2010), thus potentially obscuring the effects of reduced gene flow. Sperm storage further amplifies this phenomenon because female Blanding's Turtles can potentially use sperm from a previous mating stored in the reproductive tract for at least four years post mating (J. Harding, pers. comm., in McGuire et al. 2015). One breeding study that incorporated microsatellite markers (Anthonysamy et al. 2014) discovered that a male who had died the previous year sired a clutch of eggs the following year.

In all the genetic studies described

above, the data used to characterize genetic diversity was gathered only from adult individuals. However, because some aspects of the life history of Blanding's Turtles may obscure effects of reduced gene flow, characterizing only adults may conceal factors that can affect the long-term status of populations. The present study was undertaken to gain perspectives on how mating patterns exhibited by Blanding's Turtles may alter genetic diversity in future generations in isolated populations. This was done by deducing specific genetic contributions of individual male and female turtles to subsequent generations. To characterize such patterns, we obtained DNA from juvenile turtles that were part of a head start program of the DuPage County Forest Preserve District (described in Thompson et al. 2020) and generated genotypes for them using seven microsatellite markers. The juvenile turtles were all offspring of known dams from a single isolated population in DuPage County, Illinois. To deduce mating patterns, we compared the genotypes of the juveniles with genotypes of adults from the same population that were sampled as part of a geographic study in northern Illinois (Klut 2011) and identified likely sires for all offspring. We hope that insights from this study will both augment understanding of the basic biology of this species and potentially lead to new approaches related to its management. Some conservation plans propose translocating individuals across geographic regions to enhance or maintain biodiversity (Weeks et al. 2011). Before initiating augmentation strategies, land managers should incorporate genetic data with ecological information to assess population structure and viability (Allendorf and Luikart 2007)

MATERIALS AND METHODS

Sample collection. Samples from a previous study of genetic diversity of Blanding's Turtles in the Chicago Metropolitan area (Klut 2011) were used to determine the genotypes of adult turtles. As part of a head start program in 2008, 2009, and 2010, females captured from one population in DuPage County laid eggs in captivity. All dams (n = 17) and adult males (n = 5) for this study were trapped within an area of not more than 5.7 kilometers radius that encompassed a single population. The offspring were reared for one to two years and, just prior to their release into the wild, we drew ca. 100 μL of blood by subcarapacial venipuncture (Rodgers and Booth 2004), once per individual, with a 1 mL syringe and 26 gauge needle. We combined blood samples with a long-term storage buffer (100 mM Tris-HCl, 100 mM EDTA, 2% SDS, pH 8.0, mixed 1:1 blood to buffer) to ensure DNA preservation. Due to limited resources, we randomly sampled a sub-set of the available clutches from 17 females and did not always analyze all offspring from a given clutch. For clutches, we sampled from 20 to 100% (average = 56%) of the total number of hatchlings.

DNA extraction, PCR amplification and microsatellite analysis. Blood/buffer samples were incubated overnight with 5–10 U Proteinase K and DNA extracted using phenol, phenol-chloroform/isoamyl alcohol and chloroform/isoamyl alcohol washes, and precipitated using 3 M sodium acetate and 100% ethanol (Sambrook et al. 1989). We used seven microsatellite loci (Eb 09, Eb 11, Eb 12, Eb 15, Eb 17, Eb 19 and BTCA 09), previously developed for Blanding's Turtle (Osentoski et al. 2002, Libants et al. 2004) for genotyping. We amplified all loci in 12.5 µL volumes containing 1.2 µL 10X buffer, 2 mM MgCl2, 0.2 mM dNTPs, 5 pmol of each primer, 0.5 units of *Taq* polymerase (Promega, Corp) and 40-60 ng of template DNA. The program parameters were 95° C for 40 s, 55° C for 45 s and 72° C for 45 s for 40 cycles, followed by a 10 min extension at 72° C. The annealing temperature was decreased to 44° C for Eb 15 and BTCA 09. We used forward primers that were fluorescently labeled with WELLRED™ dyes for analysis on a Beckman/Coulter CEQ 8000 capillary electrophoresis system (Beckman Coulter, Fullerton, California, USA) with system software version 8.0 (Beckman Coulter, Inc.). To verify correct assignment of microsatellite alleles, allele calls for juvenile turtles from the head start program were added to a spreadsheet with the binning data from the previous study (Klut 2011). We also amplified and reran 10% of the total sample set to confirm genotype consistency and following capillary changes.

Multiple paternity determination. We used Cervus 3.0.7 (Marshall et al. 1998, Slate et al. 2000) and GERUD 2.0 (Jones 2005) to predict numbers of sires and to identify potential sire genotypes. Analysis with two programs can increase confidence in assigning potential sires (Kasumovic et al. 2003). Cervus can identify most likely sires based on genotypes of known males, and GERUD can predict likely sire genotypes in cases where male parents may not have been sampled. Since Eb12 was found to have a null allele (Klut, 2011), we removed data for that marker prior to running either program. However, knowing that Eb12 has a null allele, we were able to reconstruct possible sire genotypes for that marker as well (based on maternal genotypes and the genotypes of their offspring) and we included that information in probable sire genotypes derived from the computer analyses.

All known males whose genotypes matched any offspring in the sampled clutches as determined by Cervus, were considered to be sires for those offspring (i.e., known sires; KS). Using more stringent criteria, genotypes were generated and evaluated for hypothetical sires (HS) and considered to correspond to actual sires only if they matched offspring in two or more of the sampled clutches. In some cases, it was not possible to identify one or two specific sires for a given clutch because two or more paternal genotypes were possible. However, we were able to estimate the minimal number of sires for each clutch using a method described by Refsnider (2009) and to generate partial genotypes for these individuals, designated as unspecified sires (USS).

Two approaches were used to estimate the frequency of multiple paternity

(MP). One approach involved estimating the minimum number of sires for a clutch as described above (using the method described by Refsnider 2009). Since this approach is prone to underestimating the number of sires, we also assessed MP by identifying contributions of specific genotypes from likely sires predicted by Cervus and GERUD as described above, in the combined genotypes of the offspring. We counted a clutch as potentially being a product of multiple sires if an estimation based on number of paternal alleles alone indicated MP or if we identified two or more sires.

RESULTS

Breeding structure among clutches. We obtained genotypes for a total of 234 offspring in 34 clutches from 17 female turtles in a single, small population in DuPage County, over a span of three years. Genotypes for eight of the 234 individuals were inconsistent at one or more locus with the presumed dams for their clutches after repeated analyses and were omitted from further analysis due to possible labeling error. Among the final 226 offspring analyzed, 66 were from 10 clutches from the 2008 season, 63 were from 15 clutches from the 2009 season, and 97 were from nine clutches from the 2010 season; the number of individuals sampled per clutch ranged from 3 to 16, with an average of 7 ± 4 per clutch. Genotypes were obtained at all seven loci for 158 (69.9%) individuals, 6 loci for 58 (25.7%), five loci for nine (4%), and only four loci for one (0.4%) individual.

Assessing potential sires. We analyzed all clutches for MP (Table 1) by comparing the genotype data for each offspring to the genotypes of the known dams and to the genotypes of five adult males previously captured at the study site. Among the 34 clutches, offspring were sired by four known males (KS1–4) in 21 clutches. We constructed genotypes for five unsampled males (i.e., hypothetical sires; HS1–5) that served as sires for nine of the 21 clutches above and an additional 12 clutches. Thirteen of these 33 clutches also had offspring (n = 47) from one or two unspecified sires (USS#) that we could not unambiguously identify either because a complete genotype could not be reconstructed for the sire, and/or because multiple paternal genotypes could account for these offspring. Finally, the 34th clutch (n=3) was sired exclusively by a USS. Overall, we attributed a total of either 82 or 83 offspring to one of the four known sires, and either 93 or 94 offspring to the five hypothetical sires (uncertainty is due to one offspring in the 2010 clutch of dam F that could be attributed to either a KS or an HS male, Table 1). There were 50 offspring sired by USS (for the total of 226). The fifth known male was not identified as a possible sire for any of the 34 clutches.

Assessing the frequency of multiple paternity and other trends in reproductive behavior. Fourteen clutches (41.2%) were single-sired clutches (SSC) and 20 (58.8%) showed evidence of MP. We also noted an apparent trend of reduced frequency of MP over three years, with MP equaling 80%, 60%, and 33.3% in 2008, 2009 and 2010 respec-

Table 1. Assessment of number, and identity of possible sires, for clutches from a population of Blanding's Turtles over a 3-year period. Number sampled for each clutch (n), minimum number of sires (Sn) based on a given number of loci that indicated multiple paternity (Ln), and possible sires representing known sires (KS#), hypothetical sires (HS#) and unspecified sires (USS#).

Year:	2008				2009				2010			
Dam	n	Sn	Ln	possible sires	n	Sn	Ln	possilbe sires	n	Sn	Ln	possible sires
A	-	-	-	-	5	1	na	KS4 (5)	-	-	-	-
B	5	2	3	KS1 (4) HS1 (1)	3	2	1	KS1 (2) USS10	-	-	-	-
C	5	2	4	HS2 (1) HS3 (2) USS1	5	2	2	KS1 (1) HS1 (2) USS11	10	1	na	HS1 (10)
D	-	-	-	-	3	2	2	KS2 (2) HS5 (1)	-	-	-	-
E	-	-	-	-	3	1	na	USS12	-	-	-	-
F	4	1	na	HS4 (1) USS2	-	-	-	-	9	2	1	KS1 (6-7[a]) HS4 (1-2[a]) USS16
G					5	1	na	HS4 (5)	11	1	na	HS4 (11)
H	6	1	na	KS2 (6)	4	1	na	KS2 (4)	-	-	-	-
I	6	2	3	KS1 (4) HS1 (1) USS3	4	2	1	KS1 (3) HS1 (1)	-	-	-	-
J	-	-	-	-	5	2	1	KS1 (2) USS13 USS14	14	1	na	HS1 (14)
K	5	1	na	KS1 (5)	3	1	na	KS1 (3)	8	1	na	HS1 (8)
L	3	1	na	HS4 (1) USS4	5	2	1	HS4 (4) HS5 (1)	12	1	na	HS1 (12)
M	-	-	-	-	5	2	3	HS5 (2) USS15	-	-	-	-
N	-	-	-	-	4	2	1	HS1 (3) HS3 (1)	-	-	-	-
O	16	2	1	KS3 (5) USS5 USS6	3	1	na	KS3 (3)	11	1	na	KS3 (11)
P	10	4	6	KS3 (3) HS2 (2) USS7 USS8	6	2	3	KS3 (2) HS1 (4)	13	2	4	KS3 (9) HS1 (4)
Q	6	1	na	KS3 (1) USS9	-	-	-	-	9	1	na	KS4 (1) USS17

[a] one individual in this clutch has a genotype that is compatible with either KS1 or HS4.

tively. While these means are not statistically different (2008:2009 p = 0.572, 2009:2010 p = 0.399, and 2008:2010 p = 0.105; analyzed via ANOVA), the possible trend described above is worth noting given that it is associated with increased participation in matings for one male that we did not sample in our study, HS1. While the majority of MP clutches had two sires, six had three sires, and one had four sires; the average number of sires for MP clutches was 2.4 ± 0.6.

Other patterns are associated with the mating success of individual males. For example, three out of the nine identified sires, KS1, KS3, and HS1, were the most frequently detected sires, being represented nine, seven and 11 times respectively out of the 34 clutches. They were also the most prolific, with 30 or 31, 34, and 60 total offspring respectively out of 176 offspring for which specific sires could be determined. Another trend associated with male reproductive success is frequency with which males produced SSC. While some males (HS2, HS3, and HS5) never produced a SSC, two (KS4 and an unspecified sire USS12) produced one such clutch each, four (KS1, KS2, KS3, and HS4) produced two SSCs, and one, (HS1) produced four SSCs. Some males showed repeat paternity with a specific female in two or more seasons. There were 12 dams from which we collected offspring in two or three years (two years for seven dams and three years for five dams). Nine of these 12 (75%) showed evidence of repeated paternity, with various patterns of males participating, sometimes with one male being the only sire, sometimes with multiple males mating with one dam, but with one or more males showing repeat paternity.

Patterns of genetic diversity in parents and offspring, and mating contributions of specific males in each generation. There were 20 alleles that were present in the offspring of at least one season but absent in the offspring of one or two years (Table 2). Twelve of these 20 alleles were contributed by sires alone and not by dams. Among these 12 alleles, only five were absent among the offspring of the 2008 season and five were absent in 2009 (one was the same as an absent allele from 2008). In contrast, 10 of the 12 alleles contributed by sires alone were absent in the offspring from 2010. This reduction in the number of alleles contributed by males in the third year correlates with a shift from random inseminations in 2008, with each male being as likely as any other to be a sire (p value based on chi square equals 0.56), to non-random patterns in 2009 (p = 0.01) and 2010 (p = 7.6 x 10^{-5}) with some males being much more likely to sire offspring, especially in the 2010 season.

Table 2. Alleles that were present ("yes") in the offspring of at least one season, but absent ("no") from the offspring of at least one other season. Entries in bold type indicate alleles that were contributed to the offspring by sires alone.

Locus	Allele	2008	2009	2010	Adult(s) Contributing the Allele
Eb09	**140**	**yes**	**no**	**no**	**USS7 or USS8**
Eb09	**150**	**yes**	**no**	**no**	**HS2 and HS3**
Eb09	**156**	**no**	**yes**	**no**	**USS11**
Eb11	191	no	yes	yes	Dam J
Eb12	129	yes	yes	no	Dams H, I and N
Eb12	139	no	yes	no	Dam A
Eb12	**141**	**yes**	**no**	**yes**	**USS7 or USS8, and USS17**
Eb12	**155**	**yes**	**yes**	**no**	**KS2**
Eb15	144	no	yes	yes	Dam J and USS13 or USS14
Eb15	**156**	**yes**	**yes**	**no**	**KS2**
Eb15	162	no	yes	yes	Dam G
Eb15	170	yes	yes	no	Dam H
Eb15	**178**	**no**	**yes**	**no**	**USS12**
Eb15	**180**	**no**	**yes**	**no**	**KS4**
Eb15	**182**	**yes**	**yes**	**no**	**KS2**
Eb15	**186**	**no**	**no**	**yes**	**USS17**
Eb17	107	no	yes	no	Dam M
BTCA09	**165**	**yes**	**no**	**no**	**USS4**
BTCA09	181	no	yes	no	Dam M
BTCA09	**183**	**no**	**yes**	**no**	**USS13 or USS14**

DISCUSSION

Frequencies of multiple paternity – comparison with other studies. Previous reports addressing multiple paternity in Blanding's Turtle have been highly variable in terms of their approaches and study sites (Refsnider 2009, McGuire et al. 2013, Anthonysamy et al. 2014, McGuire et al. 2015). The study conducted by Anthonysamy et al. (2014) was the most similar to ours in its duration (four years) and in the type of population studied (small and located in a suburban setting), while that of McGuire et al. (2013 and 2015) was the most different in duration and type of population (it lasted eight years and took place in a pristine habitat). A unique feature of our study is that it occurred at a site where over 1,500 young Blanding's Turtles from a head start program (HSP) have been released into the wild since 1996 (Thompson et al. 2020), and it is possible that some could have matured sufficiently to mate during our study. If as Rubin et al. have suggested, DuPage County populations of Blanding's Turtles are genetically depauperate (2001) and juvenile recruitment rate is low (2004), such artificial population augmentation could lead to shifts away from established mating patterns that could dramatically change the genetic structure of such populations. In the case of the current study, since young turtles from the HSP were originally derived from the same population (some potentially being offspring of the dams in this study), such shifts in mating patterns might be expected to lead to reduced levels of genetic variation.

The frequencies of MP reported in other studies varied widely. Refsnider (2009), who studied a population in a nature reserve located in the outskirts of the suburbs of Minneapolis, reported the highest frequencies, estimating that between 56.3% (based on variations at two microsatellite loci) and 81.2% (based on variations at one locus) of clutches were sired by multiple males. McGuire et al. (2015) reported a relatively high frequency of MP over eight years of 41.6% (range from 15.4% to 55.6%) that is similar to our average (58.8%). And Anthonysamy et al. (2014), who studied a highly isolated, small population in the Chicago metropolitan area, reported a frequency of only 11%.

Our finding that specific males were among the most prolific sires (KS1, KS3, and HS1) is consistent with findings of two previous studies. While Anthonysamy et al. (2014), found nine of the 11 known males contributed to the offspring, one male sired 38% of the offspring. McGuire et al. (2015) reported that the average number of offspring sired by the 26 resident males was 12.1, and only four males sired 23, 25, 28, and 40 offspring. However, unlike these studies, our study also appears to show patterns of shifting dominance among several males, with one male (HS1) that sired very few offspring initially, becoming highly dominant (see below). Perhaps the head start program plays a role in this pattern with females mating more frequently with younger males that were reared in that program. Our study, like that of Anthonysamy et al. (2014) also found that not all known males served as sires inasmuch as no offspring of the fifth known male were detected in the clutches we sampled. This may have been due in part to his isolated location that was separated from most of the dams by a road and the railroad track, or he may have died prior to our study given that he was last trapped in 2007. In any case, these barriers do not seem to prohibit all movement between locations, given that KS3 and KS4 were identified as sires for clutches from dam Q, while dam Q was always captured in the isolated part of the study site.

We identified multiple cases of repeat paternity, but without having directly observed mating behaviors, we were unable to determine whether such patterns were due to repeated matings or sperm storage by females. As noted above, sperm storage for up to four years in female Blanding's Turtles in captivity has been documented (J. Harding, pers. comm., in McGuire et al. 2015). This was also observed by Anthonysamy et al. (2014) when a male that died in August of 2007 was identified as a sire for a clutch produced the next year. Though our data do not provide direct evidence for sperm storage, the patterns of repeat paternity we observed strongly suggest that it occurred. However, the observed loss of alleles among the sampled offspring over the three-year period may be due to changes in the dominance of males that served as sires and could indicate reduced use of sperm storage (Table 2). In this study, 10 of the 12 alleles contributed by sires alone were absent from offspring in the 2010 season. This loss of alleles together with an apparent decrease in MP may suggest that females in this population avoided the use of stored sperm from some males (via a yet unknown mechanism) during the latter part of the study.

Number of breeding males and females in the population in this study. Long-term capture records associated with the previous study by Klut (2011) and data from this study provide estimates of the number of adult males (n = 24) and females (n = 26) in this population. All recently captured females had been fitted with a radio transmitter as part of the HSP and could be located. While some males were last captured in the 1990s and might have died prior to this MP study, our assessment identified 26 sires (KS1–4, HS1-5 and USS1–17). The estimated numbers of adult males and females indicate a sex ratio close to 1:1. Anthonysamy et al. (2014) found a much lower frequency of MP in a population that was similar in its isolation from other surrounding populations and concluded that a low frequency of MP was due at least in part to a female-biased sex ratio.

We only had blood samples from five recently caught males (KS1-5) and not from the remaining 19 previously captured males at this study site. Therefore, we cannot definitively assess the origins of the five hypothetical sires (HS1-5) or the 17 unspecified sires (USS1-17) whose existence we deduced from our mating study. However, the possibility that some of these sires were non-residents seems unlikely since the population we studied is separated from the only other known nearby population by a distance of approximately 5 km, which includes a large housing development and a multilane highway.

Alternately, though males from the HSP would have been quite young at the time of our study, the possibility that some matured early is supported by results of other studies. First, while delayed sexual maturation in both sexes is typical for Blanding's Turtles, the specific timing for the onset of sexual maturity appears to vary among populations (e.g., Graham and Doyle 1977, Ross 1989, Congdon and van Loben Sels 1993, McGuire et al. 2015). Perhaps the sire identified as HS1 was a HSP offspring that went from producing very few offspring in two matings in 2008, to becoming the most highly represented sire in both numbers of identified matings and numbers of offspring in 2010. Second, in Blanding's Turtle and other turtle species, it has been documented that some environmental factors may promote early maturation including enhanced nutrients (Graham and Doyle 1977, Blanding's Turtle) and warmer temperatures (Gibbons et al. 1981, the Slider Turtle, *Pseudemys scripta*, Thornhill 1982, Red-eared Turtles, *Chrysemys scripta elegans*, Frazer et al. 1993, Painted Turtles, *Chrysemys picta*). Since turtles in the DuPage Forest Preserve HSP are kept at consistently warm temperatures and fed daily, it is possible that these factors could lead to early maturation in offspring from the HSP. This possibility is also supported by the capture of a female from the HSP that was gravid at the age of 11 years old (based on a microchip that was previously implanted; data not shown). This represents the earliest age at reproduction to be directly documented for a female Blanding's Turtle, and it supports the estimates of 9 to 10 years based on growth rings made by Germano et al. (2000). It also suggests that similar accelerated maturation is possible for males reared in the HSP. Another possible explanation for the 22 unsampled males whose existence is indicated by our analysis is that at least some of them were simply resident adult males that we failed to sample. We know from our capture data that at least 19 of the 24 previously captured males were never sampled (although five of these were last captured in the 1990s).

SUMMARY AND CONCLUSIONS

Although our paternity analysis was limited both in terms of the duration of the study and the number of clutches and offspring that we analyzed, the overall trends appear valid. These include a relatively high frequency of multiple paternity, but with a shift in mating patterns that led to fewer males serving as sires, and a potential reduction in genetic diversity in the offspring as compared to the parents. We may have underestimated the occurrence of MP for clutches with low sample numbers. However, our use of seven polymorphic microsatellite markers reduces the likelihood of this kind of error due to small sample sizes (Neff and Pitcher 2002). While failure to identify contributions of specific males could obscure some aspects of mating patterns and changes in allelic diversity, the overall trends we observed in these areas are unlikely to be due to sampling limitations alone. These patterns are consistent across the three years and most evident in samples from 2010, where sample sizes were generally large compared to previous years.

Breeding patterns in this study suggest that even within a small population, levels of MP can be relatively high, similar to those found in pristine habitats. However, our study indicates that breeding patterns can be unstable and that shifts in mating dominance may lead to fewer males participating in reproduction, resulting in a loss of genetic diversity. Furthermore, although our results can be interpreted in several ways, it is possible that one or more of the unsampled males originated as juveniles from the HSP, which could lead to further losses of genetic diversity due to possible high levels of relatedness between sires and dams. If some sires did originate as juveniles from the HSP, this indicates that conditions associated with the HSP lead to early maturation, a premise that may be supported for females via an unrelated discovery of a young gravid female that originated in the HSP. Such a finding should be highly relevant for the future management of this species via population augmentation involving HSPs.

If the trends suggested by our results can be confirmed, they should have significant implications for how this species is managed. Specifically, if unstable breeding patterns can lead to declines in genetic diversity, this may argue in favor of conservation programs that involve translocation of unrelated individuals, primarily of juveniles from HSPs, to new locations with the goal of genetic augmentation of existing populations. Furthermore, our findings suggest that any augmentation should be accompanied by genetic characterization of the population to be augmented, along with candidate individuals from other populations to enhance the potential for genetic compatibility.

ACKNOWLEDGEMENTS

Fieldwork was supported by the Forest Preserve District of DuPage County. We collected blood samples for DNA extraction under the auspices of Illinois Department of Natural Resources permit 04-S6. We thank the Biology Department, Provost's Office and the Office for Alumni Affairs at Wheaton College, the Department of Biological Sciences at Western Illinois University, and All Species Consulting for providing funds to pay for lab supplies.

LITERATURE CITED

Allendorf, F.W., and G. Luikart. 2007. Conservation and the Genetics of Populations. Blackwell Publishing, Malden, Massachusetts, USA.

Anthonysamy, W.J.B., M.J. Dreslik, M.R. Douglas, N.K. Marioni, and C.A. Phillips. 2014. Reproductive ecology of an endangered turtle in a fragmented landscape. Copeia 3:437–446.

Anthonysamy, W.J.B., M.J. Dreslik, M.R. Douglas, D. Thompson, G.M. Klut, A.R. Kuhns, D. Mauger, D. Kirk, G.A. Glowacki, M.E. Douglas, and C.A. Phillips. 2018. Population genetic evaluations within a co-distributed taxonomic group: a multi-species approach to conservation planning. Animal Conservation 21:137–147.

Beaudry, F., P.G. deMaynadierb, M.L. Hunter. 2008. Identifying road mortality threat at multiple spatial scales for semi-aquatic turtles. Biological Conservation 141:2550–2563.

Congdon, J.D., A.E. Dunham, and R C. van Loben Sels. 1993. Delayed sexual maturity and demographics of Blanding's Turtles (*Emydoidea blandingii*): implications for conservation and management of long-lived organisms. Conservation Biology 7:826–833.

Congdon, J.D., and J.W. Gibbons. 1996. Structure and dynamics of a turtle community over two decades. Pp. 137–159 in Long-term studies of vertebrate communities. Cody, M.L., and J.A. Smallwood. (Eds.). Academic Press, San Diego, California, USA.

Congdon, J.D. and D.A. Keinath. 2006. Blanding's Turtle (*Emydoidea blandingii*): a technical conservation assessment. USDA Forest Service, Rocky Mountain Region. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5182075.pdf.

Congdon, J.D., R.D. Nagle, O.M. Kinney, M. Osentoski, H. Avery, R.C. van Loben Sels, and D.W. Tinkle. 2000. Nesting ecology and embryo mortality: implications for the demography of Blanding's Turtles (*Emydoidea blandingii*). Chelonian Conservation and Biology 3:569–579.

Congdon, J.D., R.A. Nagle, O.M. Kinney, and R.C. van Loben Sels. 2001. Hypotheses of aging in a long-lived vertebrate (Blanding's Turtle, *Emydoidea blandingii*). Experimental Gerontology 36:813–827.

Congdon, J.D., and R.C. van Loben Sels. 1991. Growth and body size in Blanding's Turtles (*Emydoidea blandingii*): relationships to reproduction. Canadian Journal of Zoology 69:239–245.

Congdon, J.D. and R.C. van Loben Sels. 1993. Reproductive characteristics and body size: relationships with attainment of sexual maturity and age in Blanding's Turtles (*Emydoidea blandingi*). Journal of Evolutionary Biology 6:547–557.

Cunningham, J.E., H.W. Baard, E.H. Harley, and C. O'Ryan. 2002. Investigation of genetic diversity in fragmented geometric tortoise (*Psammobates geometricus*) populations. Conservation Genetics 3:215–223.

Davy, C.M., P.H. Bernardo, and R.W. Murphy. 2014. A Bayesian approach to conservation genetics of Blanding's turtle (*Emys blandingii*) in Ontario, Canada. Conservation Genetics 15:319–330.

Ernst, C.H., J.E. Lovich, and R.W. Barbour. 1994. Turtles of the United States and Canada. Smithsonian Institution Press, Washington, D. C.

Frankham, R. 2006. Genetics and landscape connectivity. In Connectivity Conservation. K.R. Crooks, and M. Sanjayan (Eds.). Cambridge University Press, Cambridge, UK.

Frazer, N.B., J.L. Greene, and J.W. Gibbons. 1993. Temporal variation in growth rate and age at maturity of male Painted Turtles, *Chrysemys picta*. American Midland Naturalist 130:314–324.

Germano, D.J., R.B, Bury, and M. Jennings. 2000. Growth and population structure of *Emydoidea blandingii* from western Nebraska. Chelonian Conservation and Biology 3:618-625.

Gibbons, J.W., R.D. Semlitsch, J.L. Greene, and J.P. Schubauer. 1981. Variation in age and size at maturity of the Slider Turtle (*Pseudemys scripta*). American Naturalist 117:841–845.

Gibbs, J.P., and W.G. Shriver. 2002. Estimating the effects of road mortality on turtle population. Conservation Biology 16:1647–1652.

Graham, T.E., and T.S. Doyle. 1977. Growth and population characteristics of Blanding's Turtle, *Emydoidea blandingii*, in Massachusetts. Herpetologica 33:410–414.

Hamilton, C.M., B.L. Bateman, J.M. Gorzo, B. Reid, W.E. Thogmartin, M.Z. Perry, P.J. Heglund, V.C. Radeloff, and A.M. Pidgeon. 2018. Slow and steady wins the race? Future climate and land use change leaves the imperiled Blanding's turtle (*Emydoidea blandingii*) behind. Biological Conservation 222(3):75-85

Herman, T.B., T.D. Power, and B.R. Eaton. 1995. Status of Blanding's Turtles (*Emydoidea blandingii*) in Nova Scotia, Canada. Canadian Field Naturalist 109:182–191.

Jones, A.G. 2005. GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Molecular Ecology Notes 5:708–711.

Jordan, M.A., V. Mumaw, N. Millspaw, S.W. Mockford, and F.J. Janzen. 2019. Rangewide phylogeography of Blanding's Turtle [*Emys* (= *Emydoidea*) *blandingii*]. Conservation Genetics 20:419–430.

Kasumovic, M.M., L.M. Ratcliffe, and P.T. Boag. 2003. A method to improve confidence in paternity assignment in an open mating system. Canadian Journal of Zoology 81:2073–2076.

Keyghobadi, N. 2007. The genetic implications of habitat fragmentation for animals. Canadian Journal of Zoology 10:1049–1064.

King, R.B. 2013. Illinois conservation assessment for the Blanding's Turtle (*Emydoidea blandingii*). Submitted to the Illinois Species Protection Board. https://niu.edu/biology/_pdfs/rking/Illinois-Blandings-Turtle-Conservation-Assessment-FINAL-25-Feb2013.pdf.

Klut, G.M. 2011. Genetic homogeneity among Blanding's turtle (*Emydoidea blandingii*) populations across the Chicago area. MS thesis, Western Illinois University.

Kuo, C.H., and F.J. Janzen. 2004. Genetic effects of a persistent bottleneck on a natural population of Ornate Box Turtles (*Terrapene ornata*). Conservation Genetics 5:425–437.

Libants, S., A.M Kamarainen, K.T. Scribner, and J.D. Congdon. 2004. Isolation and cross species amplification of seven microsatellite loci from *Emydoidea blandingii*. Molecular Ecology Notes 4:300–302.

Marsack, K., and B.J. Swanson. 2009. A genetic analysis of the impact of generation time and road-based habitat fragmentation on Eastern Box Turtles (*Terrrapene c. carolina*). Copeia 4:647–652.

Marshall, T.C., J. Slate, L.E.B. Kruuk, and J.M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7:639–655.

McCluskey, E.M., S.W. Mockford, K. Sands, T.B. Herman, G. Johnson, and R.A. Gonser. 2016. Population genetic structure of Blanding's turtles (*Emydoidea blandingii*) in New York. Journal of Herpetology 50 (1): 70-76.

McCoy, C.J. 1973. *Emydoidea blandingii*. Catalog of American Amphibians and Reptiles 136:1–4.

McGuire J.M., J.D. Congdon, O.M. Kinney, M. Osentoski, and K.T. Scribner. 2015. Influences on male reproductive success in long-lived Blanding's Turtles (*Emydoidea blandingii*). Canadian Journal of Zoology. 93:487–497.

McGuire, J.M., K.T. Scribner, and J.D. Congdon. 2013. Spatial aspects of movements, mating patterns, and nest distributions influence gene flow among population subunits of Blanding's Turtles (*Emydoidea blandingii*). Conservation Genetics 14(5):1029–1042.

Mckinney, M. 2002. The impacts of urbanization on native species are poorly studied, but educating a highly urbanized human population about these impacts can greatly improve species conservation in all ecosystems. Bioscience 52(10):883–890.

Mockford, S.W., T.B. Herman, M. Snyder, and J.M. Wright. 2007. Conservation genetics of Blanding's turtle and its application in the identification of evolutionary significant units. Conservation Genetics 8:209–219.

Mockford, S.W., L. McEachern, T.B. Herman, M. Snyder, and J.M. Wright. 2005. Population genetic structure of a disjunct population of Blanding's turtle (*Emydoidea blandingii*) in Nova Scotia, Canada. Biological Conservation 123:373–380.

Neff B.D., and T.E. Pitcher. 2002. Assessing the statistical power of genetic analyses to detect multiple mating in fishes. Journal of Fish Biology 61:739–750.

Osentoski, M.F. 2001. Population genetic structure and male reproductive success of a Blanding's Turtle (*Emydoidea blandingii*) in southeastern Michigan. Ph.D. Dissertation, University of Miami, Coral Gables, Florida, USA.

Osentoski, M.F., S. Mockford, J.M Wright, T.B. Herman, and C.R. Hughes. 2002. Isolation and characterization of microsatellite loci from the Blanding's Turtle, (*Emydoidea blandingii*). Molecular Ecology Notes 2:147–149.

Pearse, D.E., and E.C. Anderson. 2009. Multiple paternity increases effective population size. Molecular Ecology 18:3124–3127.

Pittman, S.E., T.L. King, S. Faurby, and M.E. Dorcas. 2011. Demographic and genetic status of an isolated population of Bog Turtles (*Glyptemys muhlenbergii*): implications for managing populations of long-lived animals. Conservation Genetics 12:1589–1601.

Refsnider, J.M. 2009. High frequency of multiple paternity in Blanding's Turtle (*Emys blandingii*). Journal of Herpetology 43:74–81.

Reid, B.N., and M.Z. Peery. 2014. Land use patterns skew sex ratios, decrease genetic diversity and trump the effects of recent climate change in an endangered turtle. Diversity and Distributions 20:1425–1437.

Reid, B.N., D.J. Mladenoff, and M.Z. Peery. 2017. Genetic effects of landscape, habitat preference and demography on three co-occurring turtle species. Molecular Ecology 26. 781–798.

Rivera-Ortíz, F.A., R. Aguilar, M.D.C. Arizmendi, M. Quesada, and K. Oyama. 2015. Habitat fragmentation and genetic variability of tetrapod populations. Animal Conservation 18:249–258.

Rodgers, K.D., and D.T. Booth. 2004. A method of sampling blood from Australian freshwater turtles. Wildlife Research 31:93–95.

Ross, D. 1989. Population ecology of Painted and Blanding's Turtles (*Chrysemys picta* and *Emydoidea blandingii*) in central Wisconsin. Wisconsin Academy of Sciences 77:77–84.

Rubin, C.S., RE. Warner, J. L. Bouzat, and K.N. Page. 2001. Population genetic structure of Blanding's turtle (*Emydoidea blandingii*) in an urban landscape. Biological Conservation 99:323–330.

Rubin, C.S., R.E. Warner, D.R. Ludwig, and R. Thiel. 2004. Survival and population structure of Blanding's Turtle (*Emydoidea blandingii*) in two suburban Chicago forest preserves. Natural Areas Journal 24(1):44–48.

Sambrook, J., M.T. Fritsche, and T. Maniatis. 1989. Molecular Cloning: A Laboratory manual. Cold Springs Harbor Laboratory Mress, New York City, New York, USA.

Sethuraman A., S.E. McGaugh, M.L. Becker, C.H. Chandler, J.L. Christiansen, S. Hayden, A. LeClere, J. Monson-Miller, E.M. Myers, R.T. Paitz, J.M. Refsnider, T.J. VanDeWalle, and F.J. Janzen. 2014. Population genetics of Blanding's turtle (*Emys blandingii*) in the midwestern United States. Conservation Genetics 15:61–73.

Sinclair, C.S., P.J. Dawes, and R.A. Seigel. 2010. Genetic structuring of Gopher Tortoise (*Gopherus polyphemus*) populations on the Kennedy Space Center, Florida, USA. Herpetological Conservation and Biology 5:189–195.

Slate J., T. Marshall, and J. Pemberton. 2000. A retrospective assessment of the accuracy of the paternity inference program CERVUS. Molecular Ecology 9:801–808.

Thompson, D., G. Glowacki, D. Ludwig, R. Reklau, A.R. Kuhns, C.K. Golba, and R. King. 2020. Benefits of head-starting for Blanding's Turtle size distributions and recruitment. Wildlife Society Bulletin 44(1):57-67.

Thornhill, G.M. 1982. Comparative reproduction of the turtle, *Chrysemys scripta* elegans, in heated and natural lakes. Journal of Herpetology 16:47–353.

van Dijk, P.P., and A.G.J. Rhodin. 2011. *Emydoidea blandingii*. In The IUCN Red List of Threatened Species 2011. https://www.iucnredlist.org/species/7709/121707136.

Weeks, A.R., C.M. Sgro, A.G. Young, R. Frankham, N.J. Mitchell, K.A. Miller, M. Byrne, D.J. Coates, M.D.B. Eldridge, P. Sunnucks, et al. 2011. Assessing the benefits and risks of translocation in changing environments: a genetic perspective. Evolutionary Applications 4:709–725.

Whittingham, L.A., and P.O. Dunn. 2010. Fitness benefits of polyandry for experienced females. Molecular Ecology 19:2328–2335.