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Influences on male reproductive success in long-lived Blanding's Turtles (Emydoidea blandingii)

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Abstract

Knowing how the number and qualities of mates influence male reproductive success (RS) can help interpret mating system dynamics that are important for conservation efforts. We combined parentage data (1999-2006) with data from a long-term life history study (1953-2007) of Blanding's Turtles (Emydoidea blandingii, Holbrook 1838) on the University of Michigan's E. S. George Reserve to document the relative influence of mate number and quality on male RS. Blood samples were taken from > 92% of resident adults and tissue samples were taken from 723 hatchlings from 92 nests of 54 females over 8 nesting seasons. The incidence of multiple paternity averaged 41.6% (N = 77), was variable among years (min-max = 15.4-55.6%), and was positively associated with female age, body size, and clutch size. Repeat paternity was observed in 69.9% of sequential clutches of the same female separated by 1-7 years. Male RS was variable (1-40 offspring) and was positively associated with the number of mates and clutches sired. The youngest male to sire offspring was 22 years old. Adult movements that result in encountering different mates and/or the ability to use attributes (e.g., size or age) to identify high-quality mates have the potential to substantially increase RS.

Keywords: Emydoidea blandingii, Blanding's Turtle, life history, mating system, reproductive success, parentage
Introduction

Although males and females both contribute to offspring genotypes, females typically invest more in gametes and offspring than do males (Trivers 1972; Kodric-Brown and Brown 1987). As a result, variation in female reproductive success (RS) is primarily determined by the amount of resources allocated to reproduction, whereas male RS is typically determined by successfully competing for multiple mates (Trivers 1972; Price 1984; Rowe et al. 1994). However, in social species, complex courtship, mate guarding, pairbonding, and parental care can individually or in combination, constrain a male's ability to mate with more than one female (Weatherhead and Robertson 1977; Gibbs et al. 1990; Rodriguez-Munoz et al. 2010) and such constraints can increase the influence of female qualities on male RS.

A positive relationship between mate number and male RS has been documented in many species, particularly those with less complex social behaviors (Bateman 1948; Rodriguez-Munoz et al. 2010). However, even in species with minimal social organization, female qualities can also have a substantial influence on male RS (Altmann 1997; Craig et al. 2002; Wong and Svensson 2009; Zhang et al. 2010; McGuire et al. 2011, 2014). Spatial or temporal variation in the number and qualities of reproductive females, can alter the relative importance of mate number and quality on male RS (Emlen and Oring 1977; Gross 1996; Dreiss et al. 2010; McGuire et al. 2014). Therefore, documenting the conditions that influence the relative importance of mate number and mate quality to male RS requires detailed knowledge of reproductive histories and reproductive qualities of females that often require data from long-term life-history studies to understand mating systems of long-lived iteroparous species, such as turtles (McGuire et al. 2011).
The relatively simple social systems of freshwater turtles (e.g., adults do not form pair bonds and neither sex provides post-ovulatory parental care) provide an opportunity to study the factors influencing male RS because constraints limiting the ability of males to mate with as many females as possible should be minimal. Long-term field studies of long-lived iteroparous turtles are labor intensive and costly (Tinkle 1979); however, such studies provide empirically robust data on population size, adult sex ratios, and size- and age-specific reproductive schedules of females that are necessary to interpret how mating systems influence RS (Gibbons 1987; Gibbons 1990; Brooks et al. 1991; Iverson 1991; Iverson and Smith 1993; Roosenburg and Dunham 1997; Congdon et al. 2001, 2003).

Studies using genetic techniques to determine parentage of freshwater and marine turtles have provided a foundation for understanding turtle mating systems (for example Galbraith 1991; Fitzsimmons 1998; Crimm et al. 2002; Morre and Ball 2002; Roques et al. 2006; Lee 2008; Uller and Olsson 2008; Refsnider 2009). Mating system studies conducted in conjunction with long-term studies of freshwater turtles are rare, but the few that exist have provided new perspectives on the relative contributions of mate numbers versus mate qualities on male RS (Pearse and Avise 2001; Pearse et al. 2001, 2002; McGuire et al. 2011, 2014), and can be important for developing conservation and management plans (McGuire et al. 2013). For example, aspects of the mating system can influence the effective population size, levels of spatial-genetic structuring, and population- and cohort-specific coancestry (Nunney 1993; Scribner et al. 1993; Miller et al. 2009; Pearse and Anderson 2009).

In spite of being listed as threatened or of conservation concern throughout most of its range in the United States and as endangered in parts of Canada, Blanding's Turtle (Emydoidea blandingii) is one of the most studied turtle species in North America (Congdon et al. 2008).
Here we combined long-term data on body size- and age-specific clutch size, egg size, and reproductive frequency of Blanding's Turtle females on the University of Michigan's E. S. George Reserve (ESGR) with genetic analysis of paternity of offspring of known females over eight years (1999-2006). Our primary objective was to quantify sources of variation in the components of male RS including: 1) number of mates, 2) reproductive qualities of females (e.g., clutch size and clutch frequency), 3) multiple paternity, and 4) repeat paternity (via stored sperm or remating). Our second objective was to test for deviations from random mating related to body sizes and ages of males and females.

Methods

Life-history study

The long-term studies of turtle life history and ecology on the University of Michigan's E. S. George Reserve (ESGR) near the town of Hell in southeastern Michigan provide information on size- and age-specific reproduction of individuals that allowed us to quantify aspects of female quality. Over 43 of the 55 years from 1953-2007, the studies initiated by Owen Sexton (1953-1957) were continued by Henry Wilbur (1968-1972), Don Tinkle and Justin Congdon (1975-1980) and Justin Congdon (1981-2007). Individual turtles from the first two studies that remained in the population provided information on the oldest known age or known minimum age individuals. Data on reproduction were collected from 1977-2007 and blood samples were taken from adults and tissue samples from hatchlings from 1997-2007 and 1999-2006, respectively. All research was conducted in accordance with the University of Michigan's Animal Use and Care Committee (UCUCA #8496).
The most intensive trapping was conducted in 22 of the 33 years from 1977-2007. From April-September, turtles were captured in aquatic areas by dip netting, muddling, and using approximately 55 baited hoop traps and 30 un-baited fyke and drift sets that used net wings to direct individuals into hoop traps. Less intensive trapping was conducted during the period before and during nesting seasons (May-July) in 11 years. At first capture, all juveniles and adults were given a unique identification mark (ID) and hatchlings from nests were given unique cohort marks by notching a unique sequence of marginal carapace scutes. At each recapture the ID, body size (carapace length), and locations of individuals were recorded. At first capture, ages of hatchlings and yearlings were assigned ("age 1") and for juveniles > age 1, ages were estimated via counts of growth rings, assuming one growth ring for each year (Gibbons 1976). At subsequent captures, ages of all individuals were calculated as age at first capture plus their recapture intervals (years).

Males initiating overland movements among wetlands and gravid females were also captured during frequent monitoring of drift fences located between wetlands and terrestrial areas. During all nesting seasons, fences were monitored from 0600h-2100h each day and frequent daily searches of all nesting areas were made by 4-8 people. From 1977-2007 the reproductive condition (gravid vs. non-gravid) of all captured females was determined by palpation and X-radiography ($N = 1031$) to determine clutch size and egg width (Gibbons and Greene 1979; Hinton et al. 1997). The identity of mothers and locations of nests were determined by observing females during construction of 427 nests (Congdon et al. 1983; Congdon et al. 2000). Because some females move long distances over a number of days prior to nesting and some resident and non-resident females sometimes nest on or off of the ESGR, we were unable to locate the nests of all females each year. We established relationships between
body size, clutch size, and egg widths and ages of females from a sample of 25 known-age females (17-71 years of age) with capture histories spanning from 13-28 years.

Aquatic captures over the 44 years were used to assign 83 females and 60 males as residents of the two permanent wetland areas on the ESGR. An additional 182 females were determined to be non-residents based on captures in ESGR nesting areas but no or rare captures in permanent residence wetlands on the ESGR (see Congdon et al. 2011). The previously reported estimates of an unusually female-biased population (1 male to 3.8 females, Congdon and Gibbons 1996; Congdon and Keinath 2006) included non-resident females that nest on the ESGR. Because adult sex ratios influence mating tactics, we recalculated the adult sex ratio using only ESGR residents, but used all females to quantify male RS because the breeding population includes both resident and non-resident females.

Although mating activity has been observed in most months of the active season (March-November), peak mating activity was observed in residence and ephemeral wetlands in March and April (Kinney 1999). In spite of individual fidelity to residence wetlands, both sexes make extensive terrestrial movements to ephemeral wetlands to exploit seasonally-abundant resources, find potential mates, and as temporary refuges during movements between wetlands and during nesting migrations (Congdon et al. 2011). Frequent and extensive movements by adults facilitate encounters between individuals from different residence wetlands on and off of the ESGR. Increasing encounters with different females can increase the number of mates which in turn can alter the relative importance of mate number and mate quality to male RS (Morreale et al. 1983; McGuire et al. 2013).

*Life-history information used to quantify reproductive qualities of female Blanding's Turtles*
The long-term study of the life-history and ecology of Blanding's Turtles on the ESGR provided essential information for the study of male RS. Adult Blanding's Turtles have maximum life spans of > 70 years and females have reproductive lives of more than 50 years (Congdon et al. 1983; 2001; Congdon and van Loben Sels 1991, 1993). Females mature at 14-21 years of age, produce a maximum of one clutch per year. The number of eggs (clutch size) and their widths were obtained from x-radiographs. Egg widths of females 65-75 years old were larger than those in younger females (Congdon et al. 2001). In addition, an index of minimum reproductive frequency of females captured on the ESGR (i.e., the ratio of captures of individual resident and non-resident females while gravid/total captures) of the oldest female Blanding's turtles (0.58) was 0.13 higher than the 0.45 found for younger females (Congdon et al. 2001).

The combination of age-specific increases in reproductive traits (i.e., clutch size and frequency and egg size) makes older females a valuable resource to males compared to younger females. Therefore, a propensity for males to mate with older females can increase their RS and can increase the relative importance of mate quality to male RS compared to males that mate with younger females.

**Collection of genetic samples from adults and offspring**

A blood sample (~1cc) was taken from the caudal vein of the tail from all adults captured on the ESGR from 1997-2007 (N = 68 of 74 resident males (92%), N = 64 of 69 resident females (93%), and 98 of 122 non-resident females (80%), and 17 of 18 non-resident males (94%)) to use for genetic analysis. Estimates of annual catchability of 85% for adults (Congdon et al. 1983) indicate that all resident adults would have a high probability of being captured over the 11 years blood samples were taken (1997-2007). The actual proportion of the total population of non-
resident adult females (and particularly males) that had blood samples taken could not be accurately estimated because trapping in off-ESGR wetlands was not conducted every year.

Hatchlings \((N = 723)\) from 92 nests of 54 marked females were obtained during 8 nesting seasons (1999-2006). Individual nests were protected \textit{in situ} using wire cages to exclude predators in 1999-2004, and eggs were moved to a natural nesting area within four hours of nest completion, placed in perforated plastic containers that were buried 10 cm below the soil surface, and covered with predator-proof cages in 2005-2006. At hatching, a tissue sample (< 2 mm) was taken from the tail of hatchlings collected from protected nests over the 8 year period (1999-2006) for parentage analysis.

All blood samples were stored at room temperature in blood storage buffer (100 mM TrisHCL (pH 8.0), 100 mM EDTA, 10 mM NaCl and 0.5% SDS), and tissue samples were preserved and initially stored at room temperature in 95% EtOH and then refrigerated at (4°C). The DNA was extracted from blood and tissue samples using a Qiagen DNeasy kit (Qiagen Inc., Valencia, CA) and quantified using fluorimetry.

All sampled individuals were genotyped at 8 microsatellite loci including BTGA5, BTCA11, BTCA9, (Libants et al. 2004), EB11, EB19, EB17, EB09 (Osentoski et al. 2002), and GmuD70 (King and Julian 2004). The DNA was amplified using published polymerase chain reaction (PCR) conditions for each locus. The PCR products were separated electrophoretically using a 6% polyacrylamide gel and visualized using an FMBIOII scanner (Hitachi Inc., Kanagawa, Japan). Two experienced laboratory personnel independently scored all gel images and a minimum of ten percent of adults and hatchlings were randomly selected and re-genotyped to estimate the rate of scoring error. All loci were tested for deviations from Hardy-Weinberg
equilibrium, independence, and presence of null alleles. Measures of genetic diversity including average number of alleles, expected heterozygosity, summary statistics, and the multi-locus paternal exclusion probabilities were estimated using program CERVUS v3.0.1 (Kalinowski et al. 2007).

Paternity assignment

We used parentage information to quantify variation in male reproductive success. Briefly, we relied on concordance between paternity assigned by the parentage programs NEST (Jones et al. 2007) and CERVUS v3.0.1 (Kalinowski et al. 2007) that rely on different methods of statistical inference. Turtle offspring are non-independent (produced as a group or clutch), therefore programs that utilize prior probabilities derived from clutch-level data (e.g. the program NEST; Jones et al. 2007) can enhance the accuracy of paternity assignment and reduce over-estimation of the number of contributing sires (Fiumera et al. 2002; Jones et al. 2007). We then confirmed assignment based on the program CERVUS v3.0.1 that uses individually-based assignment for each offspring compared to all males with genetic samples collected (Kalinowski et al. 2007). Only in cases where the male(s) assigned by NEST also had a positive log odds (LOD) score estimated in CERVUS (based on 80% criteria) was a male assigned as a sire (McGuire et al. 2013). When a sire was selected by one program but not confirmed with the second program, the sire was assigned as "undetermined" and was excluded from further analyses. Multiple paternity (MP) was positively identified when two or more males were determined to have sired offspring in a clutch or when a minimum of three paternal alleles were detected in offspring from the clutch in a minimum of two loci, or confirmed by the same alleles in more than one offspring. Repeat paternity was positively identified when the same male sired offspring in two or more clutches of the same female. In cases where a sire could not be
identified, the offspring in all sampled clutches from the same mother were combined and re-
alyzed using the program NEST to determine whether the offspring from different clutches
were sired by the same male(s). If the program NEST assigned the offspring to the same un-
sampled male(s), the clutch was also considered to be consistent with repeat paternity. Based on
the very low probability of no captures during the 11 years genetic samples were taken from
adults, unknown male genotypes identified in hatchling genetic samples were assumed to be non-
residents (i.e., males that occasionally occupy temporary wetlands or residence wetlands on the
ESGR, but are not frequently or consistently captured in the permanent wetlands on the ESGR;
Congdon et al. 2011). The majority of candidate males (males who had blood samples taken and
analyzed for paternity analysis) were ESGR residents because trapping conducted on the ESGR
was more frequent and more intense than trapping conducted off-reserve.

Statistical analyses

Because multiple clutches from the same female were sampled during the 8 years of the
genetic study, and are therefore not independent, we used general linear mixed models (GLMM)
to test for factors that influenced male RS. Analyses were conducted using the lme4 and
languageR packages in the R statistical software (R Core Development Team 2007). Male RS
was defined as the total number of offspring sired over the 8 years of the parentage study. Fixed
effects in statistical models included male age and body size (for the years in which offspring
were sired), total number of mates, total number of clutches sired, and whether the clutch was
sired by multiple males (i.e., MP). Because adult growth rates of Blanding’s Turtles are low
(Congdon et al. 2013), when an adult male was not captured in the year offspring were sired, we
used carapace length at the previous capture and calculated age based on the year in which the
male sired offspring. Each fixed effect (male size, age, number of mates, number of clutches, or
MP) was tested independently in a mixed model that included female as a random effect.

Because some males mated with more than one female, and were therefore represented multiple times in the dataset, we also tested all models for male RS using average values for male age, male body size, number of mates (per year and all years), number of clutches (per year and all years), and average number of offspring sired for each individual male; therefore each male was only represented once within the dataset.

To test for deviations from random mating, we used two approaches. First, we tested for differences between distributions of all sampled males and males that successfully mated with sampled females that nested on the ESGR for both body size and age using a Chi-square test. Second, we used a mixed model to identify whether the age of the female predicted the age of the male for each mating pair. Female was included as a random effect, with male age, male body size included as fixed effects in separate models with female age or size included as the dependent variable respectively.

Results

During the 8 years prior to the male RS study (1990-1997), 69 female and 59 male residents and 113 non-resident females were identified. In the following years of genetic sampling (1998-2007), 49 resident females (71%) and 58 resident males (98%), and 98 non-resident females (87%) were recaptured and they represent the documented pool of potential parents during the years of male RS study. The numbers of resident and non-resident females captured each year were similar (Fig. 1a) and were not significantly correlated (Spearman Rho = 0.517, \( P = 0.132, N = 10 \); Fig. 1a).
Body size of resident females were significantly larger (Mann-Whitney U = 9010, $P = 0.001$) than for non-resident females captured each year from 1998-2007 (residents, mean = 199.5 mm, min-max = 180.0-218.0 mm CL; non-resident, mean = 195.2 mm, min-max = 168-217 mm CL; Fig. 1b). Clutch sizes and egg-widths of resident females were both significantly larger than for non-resident females (Mann-Whitney U = 154417, $P = 0.001$ and 148882, $P = 0.001$, respectively). Clutch size of resident females averaged 10.1 eggs (min-max = 3-16, $SE = 0.094$) and egg widths averaged 24.5 mm (min-max = 21.6-28.2, $SE = 0.044$) and clutch sizes of non-resident females averaged = 9.6 eggs (min-max = 4-19, $SE = 0.106$) and egg widths averaged 24.1 mm (min-max = 20.7-28.4, $SE = 0.060$). The relationships between clutch size and egg size with body size of all females were positive and significant (Fig. 2a,b). The relationships between body size and age of 25 known age females (ages 17-71 years) with capture histories spanning from 13-28 years was positive and significant (Fig. 3a; $F=4.293, P=0.039$). Clutch size was positively and significantly related to the age (Fig. 3b, $F= 4.663, P = 0.032$); whereas egg width was negatively and significantly related to ages of the 25 females (Fig. 3c, $F= 6.928, P = 0.009$). Body sizes and ages at first capture of 288 adult females and 114 adult males, were significantly correlated (Spearman Rho = 0.536, $P < 0.001$ and Rho = 0.745, $P < 0.0001$, respectively).

Because females do not reproduce every year, we estimated the mean frequency of reproduction as (years captured while gravid / total years captured per individual) for resident females. Over four, 5-year periods from 1986-2006, the minimum estimated frequency of reproduction for resident females averaged 0.74 (min-max = 0.61-0.87, $N = 4$). The reproductive frequency of young resident females with fewer than 15 reproductive years ($\leq 29$ years old) was
0.48, of middle-aged females with approximately 15-30 reproductive years (30-45 years old) was 0.58, and for the oldest females with >30 reproductive years (> 45 years old) was 0.71.

Measures of Genetic Diversity

All loci were polymorphic and in Hardy-Weinberg Equilibrium \((P > 0.10)\), based on all adult samples (both sexes; resident and non-resident females). There was no evidence of genetic disequilibrium (i.e., loci were independent). The number of alleles per locus averaged 7.4 (min-max = 4-15). Multi-locus expected heterozygosity was 0.69 (min - max across loci 0.54 - 0.85), and multi-locus paternal non-exclusion probability with the female parent known was 0.004. No null alleles were detected at any locus \((P > 0.10)\). The multi-locus scoring-error rate was empirically estimated to be less than 1% across all loci. We obtained multi-locus genotypes (> 4 loci amplified) from 77 clutches sampled from 1999-2006 (611 hatchlings of 49 marked females). Single and MP estimates were determined for all 77 clutches and we were able to assign paternity to offspring from 63 clutches (81.8%).

Multiple Paternity

Multiple paternity was detected in 41.6% of the 77 clutches and was higher among clutches of ESGR resident females (54.8%, \(N = 31\)) compared to non-resident females (32.6%, \(N = 46\)); however, residency was not a significant predictor of MP (GLMM; \(Z = 1.82, P = 0.069\)). The incidence of MP in clutches was positively and significantly associated with female age (GLMM; \(Z = 2.037; P = 0.042\)), body size (carapace length; GLMM; \(Z = 2.19; P = 0.03\)), and clutch size (GLMM; \(Z = 1.998; P = 0.046\)).

Among-year variation in MP was substantial (min-max = 15.4-55.6% of clutches; Fig. 1a). Rank order correlation of annual numbers of females captured and the percent MP were significant when 1999, the year with the lowest percent MP and highest number of females, was
included (Spearman Rho = -0.826, P = 0.005, N = 8 and when 1999 was removed (Spearman Rho = -0.739, P = 0.038, N = 7). Clutches of offspring of female Blanding's turtles were sired by more than one male within and among years, and the number of sires per clutch (over all years) averaged 1.6 (SD = 0.67, min-max = 1-3). Over 8 years of the genetic study, the total number of mates per female averaged 2.0 (SD = 1.05, min-max = 1-5). However, because some females skip reproduction in some years and some individuals were sampled more frequently than others (and therefore have a higher probability of having more total mates detected siring offspring), we adjusted the total number of males per female by the number of clutches sampled (adjusted number of mates per female averaged 1.4, SD = 0.70, min-max = 1-3).

Repeat Paternity

Genetic samples were collected from offspring from 28 of the 49 females (57%) that produced more than one clutch over the 8 years of the parentage study. We made paired comparisons between all clutches of each of the 28 females and found that 70% of the interannual comparisons showed evidence of repeat paternity. Repeat paternity was highest in clutches produced by females in consecutive years (85.7%, N = 7). The longest interval between clutches exhibiting repeat paternity was 7 years (the longest interval possible for an 8 year study). A substantial proportion of clutches exhibited repeat paternity regardless of durations of intervals (min-max = 50-87.5%; Fig. 4). Neither female body size (carapace length; CL) nor age was associated with repeat paternity (GLM; CL, Z = 0.698, P = 0.485; Age, Z = 0.958, P = 0.338).

Male reproductive success
Resident ESGR males that successfully mated with females sampled averaged 1.56 mates \((SD = 0.91, N = 32, \text{min-max} = 1-4)\) over the 8 years of study. The number of mates detected per male represents a minimum because we could not sample offspring of both resident and non-resident females that nested off of the ESGR and some nests on the ESGR were depredated before being protected. The occurrence of repeat paternity resulted in the average number of clutches sired per male over the 8 years of study being higher than the number of female mates (mean = 1.97, \(SD = 1.53, N = 32, \text{min-max} = 1-7\)). The average number of offspring sired among successful, ESGR resident males over 8 years of study was 11.22 (Fig. 5; \(\text{min-max} = 1-40, SD = 8.62, N = 32\)) and the average number of offspring sired per clutch for all successful males was 5.62 (min-max = 1-11, \(SD = 2.52, N = 61\) clutches).

Male RS was significantly positively associated with the number of mates (GLMM; \(t = 6.042; P < 0.001\)) and the number of clutches sired (GLMM; \(t = 13.091; P < 0.001\)). Male RS was not significantly associated with male body size (carapace length; GLMM; \(t = 0.618; P = 0.54\)), male age (GLMM; \(t = 0.150; P = 0.88\)), offspring sired per clutch (GLMM; \(t = 0.928\)), or whether a male shared paternity of a clutch (MP; GLMM; \(t = -1.846; P = 0.08\)).

Adjusted averages for each male that sired more than one clutch yielded similar results as to those above (GLMM; average mates per year, \(t = 4.40, P < 0.001\); average clutches per mate, \(t = 4.57, P < 0.001\), average offspring per mate \(t = 4.05, P < 0.001\)). In a separate analysis, where males were assigned into the same three age-classes as females, (young = under 30, middle = between 30 and 40, old = over 40) the average number of offspring sired by males was 10.1 (\(N = 10\)), 12.7 (\(N = 6\)), and 12.8 (\(N = 8\)), respectively. Because male RS is a single value that reflects the input (total offspring sired) from siring offspring with one or with multiple females of different body sizes, ages, and clutch sizes, we also analyzed RS data as the average value per
male. Male RS (total offspring sired) was not significantly associated with body size, age, number of offspring sired per clutch, or incidence of MP (GLMM; average age, $t = 0.44, P = 0.67$; average CL, $t = 1.02, P = 0.32$; average offspring per clutch, $t = 1.16, P = 0.26$, average MP; $t = 0.04, P = 0.97$). Therefore, whether sampled as raw values or as collapsed, averaged values, the results for male RS were consistent.

Body size distributions of all genetically sampled males (mean = 206.4 mm, SD = 9.6, min-max = 187-222) and males that successfully sired offspring (mean = 199.1 mm, SD = 15.6, min-max = 161-226) were similar ($\chi^2 = 6.44$, df = 18, $P = 0.994$; Fig. 6a). However, age distributions of all sampled adult males (mean = 29.4, SD = 11.8, min-max = 15-69) and successful males (mean = 36.2, SD = 10.9, min-max = 22-67) differed significantly (Fig. 6b; $\chi^2 = 11.42$, df = 5, $P = 0.044$). Although there were 27 juvenile males or adult male Blanding’s Turtles in the population from 1998-2007 that were between 14-21 years of age (Fig. 6c) the youngest male to sire offspring was 22 years old and the next youngest was 25 years old. There was no evidence of assortative mating by size or age (GLMM; size (CL), $t = -0.04, P = 0.968$; age, $t = 0.127, P = 0.90$).

Case study of individual movements and male RS

The importance of movement by males in relation to RS is illustrated by the extensive and detailed capture history of one male resident of East Marsh from 1975-2007, radio-tracked in 1997 (Kinney 1999), and for whom paternity data from 1999-2006 were available. The male was first captured in East Marsh in 1975 at age 11 and in 1981 began frequent movements to a series of ephemeral wetlands located from 0.2 to 1.2 km to the north of East Marsh at age 17 (Kinney 1999; Congdon et al. 2011). From 1981-2007 the male was captured either exiting East Marsh heading North in April or returning to East Marsh from the North six different times. In
the spring and summer of 1997 he was captured in two ephemeral wetlands approximately 0.3
km and 1.2 km to the northwest of East Marsh. During one 2-week period in 1997, the male was
captured eight times in a single baited hoop trap with five different females and was last captured
on land north of East Marsh in 2007 at 43 years of age. From 2003 and 2006 the male sired 10
offspring from clutches of one non-resident female and in 2004 and 2006 he sired 18 offspring
from clutches of one resident female from East Marsh, for a minimum estimate of 28 offspring
over 8 years (Fig. 5, gray bar). The number of females he mated with and the overall number of
offspring sired illustrate how important movements associated with ephemeral wetlands are for
increasing the probability of males encountering females from different residences and how
important such encounters are to male RS.

From 1998-2007, only one other East Marsh resident male and 12 non-resident females
were captured in the ephemeral wetland and pond complex used by the male from East Marsh.
Over the same period 26 female and 21 male residents were captured in East Marsh. The
potential ratios of adult females to males in East Marsh and the ephemeral wetland and pond
complex was 1.2 and 6.0, respectively. Therefore males that visit wetlands other than their
residence wetland have the potential to mate with different females and/or with more females
compared to those that remain in the residence wetland.

Discussion

Variation in the number of reproductive females:

The relative importance of mate number and qualities on male success is influenced by
the total number of females in the population, the number that are reproductive each year, and
variation in reproductive qualities of those females. The extended ESGR population (residents and non-residents) is relatively stable, and is comprised of long-lived individuals that remain in the population over many years because they show high fidelity to residence wetlands despite making long migratory movements. An average of 85.3% resident and non-resident females captured during the 8 years prior to the male RS study were recaptured during the following 10 years and they represent a substantial portion of the pool of potential mothers. Although many females were present over the entire period of the genetic study, the annual number of reproductive resident females captured each year varied by 15 (18.1% of the 62 total resident females; Fig. 1a). Variation in the potential number of mates available to males was somewhat buffered by the number of non-resident females occupying ephemeral wetlands on or adjacent to the ESGR and essentially doubled the potential number of mates a resident male could encounter each year (Fig. 1a).

Variation in female reproductive qualities

Reproductive output and parental investment in eggs increased with body size of female Blanding's Turtles. Over the range of body sizes of all females, average clutch size increased by approximately 6 eggs (Fig. 2a) and egg width increased 1.2 mm (Fig. 2b). At average annual indeterminate growth rates (0.6 mm/yr), it would require 17 years for ESGR females to increase body size enough add one egg to a clutch (Congdon et al. 2013). Therefore, increases in clutch size that are entirely due to adult growth are negligible, but may be important in Blandings's Turtles because they have long reproductive lives and reproductive frequency increases with age (Congdon et al. 2001; this study).
Although there are differences in characteristics of resident and non-resident females, the differences were non-substantial. Average body sizes (Fig. 1b), clutch sizes, and egg widths of resident females were significantly larger than for non-resident females. Results from regressions of clutch size and egg width on body size of all females (Fig. 2a,b) indicated that a 4 mm larger average body size of non-resident females would translate into an average increase of 0.16 mm in egg width and 0.46 of an offspring. Therefore, differences in reproductive characteristics of resident or non-resident females are not great enough to substantially influence the success of males mating differentially with either group of females.

Reproductive frequency is a major determinant of reproductive output (Gibbons 1982) and variation in reproductive output of female turtles can be an important source of variation in male RS. Female reproductive frequency varied substantially among resident females (min-max = 0.61-0.87) and increased with female age in Blanding's Turtles (average = 0.71 in the oldest age class compared to 0.48 in the youngest age class). The current overall estimate of reproductive frequency (0.74) differed from previous estimates based on resident and non-resident females (0.48, Congdon et al. 1983; 0.49, Congdon et al. 2000). Even though the 0.74 reproductive frequency is substantially higher than the previous estimates, it also represents a minimum value because approximately half of resident females may be undetected if nesting occurs beyond the ESGR boundaries (Congdon et al. 2011; McGuire et al. 2013). Regardless of the differences in the sample of individuals used in the estimates, a positive relationship between age and reproductive frequency was observed in all three cases (Congdon et al. 1983, 2001; this study) and provides support that older females represent a potential mechanism for increasing male RS particularly when coupled with repeat paternity.
Female Blanding's turtles mate with multiple males and that results in many clutches being sired by more than one male (i.e., MP). Although MP results in more males siring offspring, it can also reduce the number of offspring sired by a given male. The 41.6% overall incidence of MP in clutches of female Blanding's Turtles on the ESGR was similar to one other population of Blanding's Turtles in Scott County, Minnesota (56.3%, Refsnider et al. 2009), but higher than the 8% observed in a four year study of a small population in northern Illinois (Anthonysamy 2012). The proportion of clutches with MP in the ESGR and Minnesota populations of Blanding's Turtles was higher than found in other freshwater turtle populations (Painted turtles, *Chrysemys picta belli*, 10.7-33%, Pearse et al. 2002, and *C. p. marginata*, 6.0-30.0%, McGuire et al. 2014). In the European pond turtle (*Emys obicularis*; Roques et al. 2006) the incidence of MP was 10%, however their estimate of MP was more conservative (i.e., it was based on three or more paternal alleles in >4 loci) than the methods used to quantify MP in Painted turtles (based on three or more paternal alleles in any locus (Pearse et al. 2002, McGuire et al. 2014) or more than one male identified through parentage assignment (McGuire et al. 2014).

Multiple paternity is often positively associated with clutch size and can be influenced by biological factors such as fertility assurance and encounter probabilities, but can also be the result of non-biological factors such as detection bias (reviewed in Uller and Olsson 2008). Because clutches of Blanding's turtle females on the ESGR are sired by multiple males at higher frequencies than can be explained by detection bias, other mechanisms such as mate searching behaviors, rates of encounters with potential mates, and use of stored sperm from previous matings (residual paternity) appear to be involved (Uller and Olsson 2008). Additionally, the
The incidence of MP in ESGR Blanding's turtles was negatively correlated with the annual number of reproductive females. Results suggest that variation in the numbers of reproductive female Blanding's Turtles available each year (and the resulting operational sex ratio) is sufficient to influence encounters with potential mates. Relationships between estimated breeding population sizes and densities and levels of MP have been documented among species of sea turtles (Ireland et al. 2003; Jensen et al. 2006). However, the 44% difference among populations is within the range of variation found among populations of a single species (in Table 1 of Jensen et al. 2006). The maximum among-year difference in MP in the ESGR Blanding's turtle population was 40.2% (min-max = 15.4- 55.6%). The variation observed within the ESGR population indicates that comparisons of MP among populations, whether sampled in the same or in different years, can be confounded by temporal and spatial sources of within-population variation.

The incidence of MP increased with both body size and age of female Blanding's Turtles, but increases in reproductive qualities (i.e. clutch size and egg size; Fig. 2a,b) were more strongly associated with body size than with ages of females (Fig. 3b,c). In contrast, reproductive frequency was higher in older females than in younger females (this paper; Congdon et al. 2001). Greater numbers of males attempting to mate with higher-quality females may contribute to comparatively higher MP in their clutches compared to clutches of lower quality (i.e. smaller or younger) females. In addition, more frequent and longer-distance movements of older adult Blanding's Turtles (Congdon et al. 2011) and the increased home range size associated with age class in three-toed box turtles (Terrapene carolina triunguis; Schwartz et al. 1984) can increase rates of encounter with different mates that may result in a higher incidence of MP in older compared to younger females (McGuire et al. 2013). For example,
older female Blanding's turtles on the ESGR were more likely to sire offspring with non-resident males than younger females (McGuire et al. 2013).

Whereas the number of successful sires may increase with higher incidences of MP, the number of offspring sired per male in a clutch will be reduced. Therefore, mating with an older female may decrease male RS because there is an increased probability paternity will be shared within a clutch (MP); however, in many cases that decrease would be offset by increased reproductive qualities of females (i.e., primarily higher reproductive frequency), particularly when females use sperm from the same male to sire offspring among years (repeat paternity).

Repeat paternity

Repeat paternity in ESGR Blanding's turtles is an important component of variation in male RS because it was observed in: 1) inter-annual clutches of the same female with intervals spanning the entire duration of the study (8 years), 2) a high proportion (57%) of sequential clutches sampled, and 3) 70% of all paired comparisons of clutches from the same female regardless of interval. The high level of repeat paternity had a major influence on male RS on the ESGR by increasing the number of clutches sired, regardless of single or MP in those clutches. Even though male RS in freshwater turtles is thought to be primarily influenced by mating with more females, the average number of females mated with by ESGR males over 8 years was lower than the number of clutches sired (1.56 females vs. 1.97 clutches, respectively; Table 1), due to repeat paternity from use of stored sperm or remating.

Sperm storage by females is common in many reptile species, including turtles (reviewed in Ollson and Madsen 1998; Sever and Hamlet 2002). Female turtles use stored sperm to fertilize sequential clutches produced within years, and probably among years (Ewing 1943;
The influence of stored sperm use on male RS declines in clutches separated by > 1 year due to decreased fertility (Cuellar 1966; Jun-Yi 1982) that results from depletion or declining viability of sperm (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998). For example, captive female Eastern Box Turtles (*Terrapene carolina carolina*) that were isolated from males produced offspring for up to 4 years; however, one of four females produced infertile eggs after two years and one after 3 years (Ewing 1943). Similar results have been observed in Blanding's turtles in captivity, where females isolated from males produced fertile eggs (of declining number with time) up to, but not beyond 4 years (J. Harding, personal communication, 2014).

Repeat paternity was observed in clutches produced 7 years apart (the longest interval possible given the duration of our study), however there was also evidence of MP in those clutches so it remains difficult to interpret our results from the perspective of a sperm depletion or sperm inviability hypotheses. Therefore, we considered the paternity data only from two pairs of clutches exhibiting single-paternity and produced 5 and 6 years apart; within a pair of clutches, both clutches were sired by a single male and clutch sizes were similar to the first clutch in the pair. There was no decline in the proportion of offspring sired (i.e., no loss of fertilization due to sperm depletion or inviability), suggesting that remating had occurred. Co-occupancy of small ephemeral wetlands by the same male-female pair, but infrequently visited by other individuals could result in repeated matings in different years, without requiring pair bonding or social relationships.

Because females of many turtle species use stored sperm to fertilize sequential clutches both within and among years (Gist and Congdon 1998), female reproductive frequency can have a substantial influence on male RS, particularly in species where females produce multiple
clutches per year. Results from two populations of Painted Turtles indicated that females apparently used stored sperm to fertilize almost all within-season clutches (100%, Pearse et al. 2002; 98%; McGuire et al. 2011). Repeat paternity among years in Blanding's turtles (70%) was higher than found in other species (e.g. 38% in Painted turtles, McGuire et al. 2011; 58% in the European pond turtle, Roques et al. 2006). The maximum reproductive frequency of ESGR Blanding's Turtles is less than one clutch per year and that limits the use of stored sperm to among-year clutches only. In combination, repeat paternity (whether through remating or using stored sperm) and female reproductive frequency exert substantial influence on male RS. Therefore, males that can identify females with a propensity for using sperm from the same male to fertilize multiple clutches would leave more offspring than males that cannot identify such females. However, the data from the ESGR do not support that males use body size or some other phenotypic trait as reliable indicators of a female's propensity to have repeat paternity in future clutches of eggs.

Male attributes associated with RS

Although there were 27 young resident male Blanding's Turtles in the population during 1998-2007 (Fig. 6c), only two sired offspring at ages 22 and 25 years old. If 22 represents the actual minimum age at maturity of males, it is 7 years greater than the minimum and almost equal to the maximum age at maturity of females (min-max = 14-21 years; Congdon et al. 2001a). When all males < 22 years of age were removed from the category of potential sires of offspring, proportionally more young males sired clutches than did older males relative to the potential sires in the population (Fig. 6b). However, when males were divided into ages younger than 30 years, 30-40 years, and older than 40 years, the average number of offspring for young,
middle age, and old males was 10.1, 12.7 and 12.8, respectively indicating no strong association
between male age and RS. Mixed model analysis also showed no relationship between male age
and RS. However, the small sample size of the oldest males in the population limited our ability
to detect age-based effects. Frequency histograms of body sizes of successful males were not
different from all sampled males with or without the older juveniles included and we found no
evidence of positive or negative assortative mating by size or age.

Both males and females mated with multiple mates within and among years. Females
mated on average with 1.6 males per clutch and over the 8 years of the genetic study had on
average 2 mates and was variable among females (1-5 mates). Part of the variability of the
number of mates per female is due to variation in reproductive frequencies of females and in the
number of clutches sampled per female. After adjusting for the number of clutches sampled, the
average number of mates per female was 1.4 males. Males mated with 1.6 females over the 8
years of study, and mate number was also highly variable among males (1-7 mates).

Part of the variability in the number of mates may be attributed to individual movements
and spatial distributions of both sexes, which can affect both mate number and mate preferences
(Shuster and Wade 2003; Kokko and Mappes 2005). Although both male and female Blanding's
Turtles reside in a single residence wetland for long durations (>40 years; Congdon et al. 2011),
they move to ephemeral habitats to exploit seasonally abundant food sources (Kinney 1999) and
to find mates (Kinney 1999; Anthonysamy 2012, McGuire et al. 2013). Adults that visit
ephemeral wetlands may increase their probability of repeatedly encountering the same mates
and resulting in a higher probability of repeat paternity among years. Although adult sex ratio of
ESGR residents was approximately (1:1), movements of adult males and females to ephemeral
wetlands can result in substantial differences in the number of potential mates adults of either sex
may encounter. Differences in encounter rates with potential mates can result in females becoming more or less selective among encountered males (Kokko and Mappes 2005) compared to adults that remain in residence wetlands, and could reduce selection for particular male attributes (e.g. age, size).

A high proportion of matings occurred between individuals from different residence wetlands, demonstrating that females are receptive during these migrations. For example, one particularly successful male (28 offspring; Fig. 5, gray bar) that over many years moved from his residence wetland to ephemeral areas encountered a minimum of 5 different females in one active season. Movements by individuals to ephemeral wetlands can increase encounters with potential mates and if individuals revisit the same habitats over multiple years may increase the likelihood of repeatedly encountering the same potential mates. Extensive movements of male and female Blanding's turtles have been documented on the ESGR (Kinney 1999; Congdon et al. 2011; McGuire et al. 2013) and in other populations (Ross and Anderson 1990; Rowe and Moll 1991; Grgurovic and Sievert 2005).

Factors influencing variation in male RS

Blanding's Turtles in the extended ESGR population have a polygamous mating system where both males and females frequently have more than one mate annually and both sexes had on average ~1.6 mates over the 8 years of study. Among males that successfully mated with females that nested on the ESGR, the total number of offspring sired was variable (min-max = 1-40) and was primarily associated with the number of mates and the number of clutches sired, but not with MP. Because adults make extensive movements, mate with individuals from different
residence wetlands, and some females nest outside the boundaries of the reserve, the number of
offspring sired represents a minimum estimate for individual male success.

In conclusion, the importance of female quality to male RS in turtles can have broad
implications for any iteroparous species with the potential for repeat paternity regardless of
whether through non-social mechanisms (e.g. frequent repeated encounters due to non-random
habitat use or decreasing population sizes, or use of stored sperm) or through social mechanisms
(e.g. pair-bonding). A comparison between Blanding's Turtle and Painted Turtles supports the
prediction that the effect of female quality (specifically reproductive frequency combined with
repeat paternity) would be higher in species where females produce multiple clutches per year
relative to species that produce a maximum of one clutch per season (McGuire et al. 2014).
Some female Painted Turtles produce more than one clutch within a season that are almost
always sired by the same male, resulting in substantial increases to a male's RS stemming
specifically from the increased reproductive frequency of females (Pearse and Avise 2001;
McGuire et al. 2011, 2014). In Blanding's turtles, the number of clutches sired was a substantial
source of variation in male RS, but was achieved through increasing mate number or through
increased reproductive frequencies of the females he sired offspring with (or a combination of
both). Repeat paternity was observed throughout the duration of the study (1-7 years between
clutches), and may result from use of stored sperm at shorter intervals (<5 years), or remating
(>4 years) that can be facilitated by co-occupancy of the same habitats each year. Occurrence of
repeat paternity among years in both Painted and Blanding's Turtles demonstrates the
reproductive benefits to males that mate with females that produce clutches at higher
frequencies, and suggests that the importance of female quality to male RS may be widespread in
turtles, and in other taxa where females vary widely in reproductive quality.
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patterns, and nest distributions influence gene flow among population units of Blanding's

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reproductive quality and reproductive success of male Midland Painted Turtles


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Nunney, L. 1993. The influence of mating system and overlapping generations on effective


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Fig. 1.  a) The number of female Blanding's Turtles (*Emydoidea blandingii*, Holbrook, 1838) captured on the E.S. George Reserve from 1998-2007 with the % multiple paternity documented in clutches each year of the genetic study (1999-2006) and b) average body sizes of resident and non-resident females from 1998-2007.

Fig. 2.  Relationships of a) clutch size (CS) and b) egg width (EW) measured from x-radiographs to body sizes of resident and non-resident female Blanding's Turtles combined.

Fig. 3.  Relationships of a) body size (carapace length = CL) and b) clutch size (CS), and c) egg width measured from 306 x-radiographs, and the ages of 25 female Blanding's Turtles with mean ages of 38 years (min-max – 17-71) and mean reproductive histories of 21.3 years (min-max = 13-28).

Fig. 4.  The percent of comparisons of paired clutches of individual females that were sired by the same male (i.e., repeat paternity; number of comparisons given above each bar) as a function of the annual interval between clutches with the bars indicating the probable ways that clutches were fertilized based on studies of fertility or depletion rates of stored sperm over time.

Fig. 5.  The number of offspring in clutches of females that were residents and non-residents of the E.S. George Reserve that were sired by resident males (gray bar is the male presented as a case-study of movements and reproductive success).

Fig. 6.  Frequency histograms of a) body sizes (carapace lengths) and b) ages of all males (black bars) and males that mated with females that nested on the E.S. George Reserve (gray bars), and c) number of young males present on the E.S. George Reserve beginning at the minimum age of maturity for females (14 years of age) through the age of the second oldest male to sire offspring in this study (25 years of age).
Fig. 1.

![Graph a.]: Number of females per year.

- **Non-residents**
  - 1998: 188
  - 1999: 190
  - 2000: 192
  - 2001: 194
  - 2002: 196
  - 2003: 198
  - 2004: 200
  - 2005: 202
  - 2006: 204
  - 2007: 206

- **Residents**
  - 1998: 15.4%
  - 1999: 44.4%
  - 2000: 50.0%
  - 2001: 53.8%
  - 2002: 55.6%
  - 2003: 36.4%
  - 2004: 42.9%

![Graph b.]: Carapace length (mm) per year.

- **Non-residents**
  - 1998: 190 ± 10
  - 1999: 192 ± 10
  - 2000: 194 ± 10
  - 2001: 196 ± 10
  - 2002: 198 ± 10
  - 2003: 200 ± 10
  - 2004: 202 ± 10
  - 2005: 204 ± 10
  - 2006: 206 ± 10
  - 2007: 208 ± 10

- **Residents**
  - 1998: 192 ± 10
  - 1999: 194 ± 10
  - 2000: 196 ± 10
  - 2001: 198 ± 10
  - 2002: 200 ± 10
  - 2003: 202 ± 10
  - 2004: 204 ± 10
  - 2005: 206 ± 10
  - 2006: 208 ± 10
  - 2007: 210 ± 10
Fig. 2.

a. $CS = -9.98 + (0.102 \times CL)$

$r^2 = 0.25$

$P = 0.001$

b. $EW = 20.690 + 0.0188 \times CL$

$r^2 = 0.032$

$P = 0.001$
Fig. 3.

- **Carapace length (mm)**
  - \( CL = 196.8 + 0.099 \times AGE, r^2 = 0.0139 \)
  - \( N = 25 \) females

- **Clutch size**
  - \( CS = 9.38 + 0.025 \times AGE, r^2 = 0.0151 \)

- **Egg width (mm)**
  - \( EGG WIDTH = 25.00 + (-0.013) \times AGE, r^2 = 0.022 \)
Fig. 4.

Interval between clutches (years)

% of clutches

1 2-3 4-7

- stored sperm
- mixed
- re-mating
Fig. 5.

The bar chart shows the distribution of offspring numbers. The x-axis represents the number of offspring, ranging from 0 to 40, while the y-axis represents the number of males, ranging from 0 to 5. The chart indicates that the highest number of males are associated with a certain number of offspring, with a peak around the 10-20 range.
Fig. 6.
Table 1: A summary of mean and standard deviations (SD) of major factors influencing male reproductive success (total number of offspring sired) over 8 years in Blanding's Turtles (*Emydoidea blandingii*) as determined by mixed model analysis (t and p values).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mean</th>
<th>min-max</th>
<th>SD</th>
<th>T</th>
<th>P</th>
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<tr>
<td>Number of mates per male</td>
<td>1.6</td>
<td>1-4</td>
<td>0.91</td>
<td>6.042</td>
<td>&lt;0.001</td>
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<tr>
<td>Number of clutches sired per male</td>
<td>2.0</td>
<td>1-7</td>
<td>1.53</td>
<td>13.091</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Offspring sired / clutch per male</td>
<td>5.6</td>
<td>1-11</td>
<td>2.52</td>
<td>0.091</td>
<td>0.928</td>
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<tr>
<td>Multiple paternity</td>
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<td></td>
<td></td>
<td>1.846</td>
<td>0.08</td>
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<tr>
<td>Total number of offspring sired (minimum estimate)</td>
<td>11.2</td>
<td>1-40</td>
<td>8.62</td>
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