Variation in female reproductive quality and reproductive success of male Midland Painted Turtles (Chrysemys picta marginata)

J.M. McGuire, J.D. Congdon, K.T. Scribner, and J.D. Capps

Abstract: Although mate number is perceived to be the primary factor affecting male reproductive success in polygynous systems, differences in female reproductive qualities may also influence variation in male reproductive success. We combined 32 years of data on variation in reproductive qualities (clutch size and clutch frequency) of female Midland Painted Turtles (Chrysemys picta marginata Agassiz, 1857) with genetic data on patterns of repeated paternity (i.e., stored sperm use) and multiple paternity to examine the potential influence on male reproductive success. Over 24 years (1983–2006), the number of reproductive females each year averaged 84 (minimum–maximum = 62–106) and, on average, 23% (minimum–maximum = 6%–40%) produced two clutches (intraseasonally). Among females with reproductive histories spanning 5–24 years (N = 167), 26% of individuals produced only one clutch annually, whereas 74% produced two clutches within a season. Among just intraseasonally iteroparous females, second-clutch production varied from 7% to 50%. Repeated paternity was observed in 97.5% of 40 paired clutches and 44% of 9 among-year comparisons of clutches from consecutive years. The frequent use of stored sperm to fertilize sequential clutches within and potentially among years can substantially increase a male’s reproductive success, particularly if males can base mating decisions on phenotypic characteristics correlated with female quality.

Résumé : Bien que le nombre de partenaires soit considéré comme le facteur principal à affecter le succès reproductif des mâles dans les systèmes polygynes, les différences dans les valeurs reproductives des femelles peuvent aussi affecter le succès reproductif des mâles. Nous combinons des données accumulées pendant 32 années sur la variation de la qualité reproductrice (taille et fréquence des couvées) de tortues peintes (Chrysemys picta marginata Agassiz, 1857) femelles avec des données génétiques sur les patrons de paternités répétées (c.-à-d. utilisation de sperme gardé en réserve) et de paternités multiples afin d’en évaluer l’importance sur le succès reproductif des mâles. Sur 24 ans (1983–2006), le nombre annuel moyen de femelles reproductrices était de 84 (minimale–maximale = 62–102) et 23 % d’entre elles en moyenne (minimale–maximale = 6 %–40 %) ont produit deux couvées (pendant la saison). Parmi les femelles dont l’histoire reproductrice est connue pendant 5–24 ans (N = 167), 26 % d’entre elles ont produit une seule couvée par année, alors que 74 % des femelles ont pondu deux couvées dans une même saison. Parmi le seul groupe des femelles itéropares dans une même saison, la production d’une seconde couvée variait de 7 % à 50 %. La paternité répétée pouvait s’observer dans 97,5 % de 40 couvées appariées et dans 44 % de 9 comparaisons des couvées entre années consécutives. L’utilisation fréquente de sperme gardé en réserve pour la fertilisation de couvées successives pendant une même année et possiblement sur plusieurs années peut accroître de façon appreciable le succès reproductif d’un mâle, particulièrement si les mâles basent leur décision de s’accoupler sur les caractères phénotypiques associés à la qualité de la femelle.

[Traduit par la Rédaction]

Introduction

Life-history studies have primarily focused on age-specific traits of females, but complete understanding of the evolution of life histories will require knowledge of how interactions between males and females influence the way each sex attempts to maximize fitness. Inequalities in gametic investment by males and females (anisogamy) often results in different tactics as they attempt to maximize reproductive success (Trivers 1972; Stockley 1997). For example, the use of stored sperm by females can affect male reproductive success through sexual conflict, including cryptic female choice, sperm competition, and postcopulatory sexual selection (Stockley 1997; Jennions and Petrie 2000).

Male reproductive success (RS) is thought to be primarily constrained by mate number (Bateman 1948), but male RS can also be influenced by female qualities such as reproductive frequency, number of offspring, parental investment, and...
in some species the use of stored sperm (Darwin 1871; Trivers 1972; Kirkpatrick et al. 1990; Webster et al. 1995). Variation in reproductive qualities of females can result from differences in (i) resource availability among years, (ii) the ability to acquire and utilize resources among females (Stearns 1992), and (iii) characteristics of females such as body size and age (Congdon et al. 2003). Compared with males that mate indiscriminately, individuals that have the ability to identify and mate with high-quality females can sire more offspring within and among years.

The characteristics of turtles make them excellent models of less complex reproductive systems (Avise 2001), particularly when genetic and long-term life-history data from the same population are available (Clutton-Brock 1988; Linden and Moller 1989; Clutton-Brock and Vincent 1991). In contrast to mating systems of mammals and birds, less complex systems of poikilothermic vertebrates allow interactions between the sexes that influence reproductive success to be examined in the absence of complex social systems and parental care (Clutton-Brock 1991; Gross 1996; Avise 2001). Turtles were one of the first vertebrate groups to evolve internal fertilization (Gist et al. 2000); they do not form pair bonds (Wilbur and Morin 1988) and have little or no postovulatory parental care (Congdon and Gibbons 1990). Females of many turtle species are intraseasonally iteroparous (they produce more than one clutch of eggs within a reproductive season), with females of some species of sea turtles laying up to 10 clutches in a year (Eckert 1987). Females often fertilize clutches of eggs with sperm from more than one male (Galbraith et al. 1993; Fitzsimmons 1998;Pearse and Avise 2001; Pearse et al. 2002; Lee 2008). Female turtles are able to store sperm for longer than a year (Ewing 1943; Gist and Jones 1989) and that allows temporal asynchrony between mating and fertilization (Birkhead and Møller 1993; Shuster and Wade 2003). Because of the short interval between sequential clutches within a reproductive season, Gist and Congdon (1998) hypothesized that a primary function of stored sperm is to fertilize second clutches. Four studies (Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002; Roques et al. 2006) showed that stored sperm was used to fertilize sequential clutches within a reproductive season, suggesting that the use of stored sperm is widespread among turtles. Therefore, variation in reproductive frequency, within and among years, and a propensity for using stored sperm to fertilize sequential clutches can substantially influence male reproductive success. If males discriminate among female phenotypes that are associated with an individual’s tendency to produce single and multiple clutches seasonally, among-individual variation in male reproductive success could increase substantially.

The life history and ecology of Midland Painted Turtles (Chrysemys picta marginata Agassiz, 1857) have been studied on the University of Michigan’s Edwin S. George Reserve (ESGR) near Hell, Michigan, for 43 of the past 55 years (Sexton 1959; Wilbur 1975; Tinkle et al. 1981; Congdon et al. 2003). A summary of characteristics of ESGR Midland Painted Turtles follows. Males mature at age 4–5 years and females mature at 6–12 years (Congdon et al. 2003), and maximum age of males and females is about 40 and 50 years, respectively. Observations of mating activity of Midland Painted Turtles on the ESGR are infrequent because it occurs in highly vegetated wetlands in early spring (Sexton 1959). Females allocate the majority of resources to first- and second-clutch follicles during late summer to early fall in the year prior to egg laying (Congdon and Tinkle 1982). Although females provide no behavioral postovulatory parental care, they do provide hatchlings with substantial yolk reserves necessary to sustain juveniles during the early posthatching period (Congdon et al. 1983). Some females do not reproduce every year, but the majority oviposit one or two clutches of eggs in terrestrial nests in late May through early July (Tinkle et al. 1981). The shortest interval between first- and second-clutch nests is 10 days, but soft-shelled eggs of second clutches can be detected by palpation 6 days after the first-clutch nest (J.D. Congdon, unpublished data).

Because the size and number of eggs in first and second clutches are similar (Congdon et al. 2003; Harms et al. 2005), producing two clutches of eggs essentially doubles the annual fecundity of females. Temporal and spatial separation of first- and second-clutch nests reduces the probability that all eggs will be lost (i.e., not putting all eggs in one basket).

Detailed information on the reproductive histories of females from the long-term studies on the ESGR provides an opportunity to interpret the results from the genetic study. Data from the long-term studies include (i) numbers, body sizes, and ages of most adult males and females in the population; (ii) the number of females that reproduced annually; (iii) annual reproductive output of individual females (clutch size, clutch frequencies, egg size as egg widths from X-radiographs, and relationships of reproductive variables to body size and age); and (iv) the identities of female parents and their offspring. Genotypes of adults and hatchlings allowed us to document the frequency of first and second clutches of intraseasonally paired clutches being fertilized by the same male (or males) and thereby determine if females used stored sperm to fertilize the second clutch (Gist and Congdon 1998), whether the same male fertilized sequential clutches between years, and the frequency of multiple paternity. Because both clutch size and egg size increase with body size of females, and egg size and reproductive frequency increases with age (Congdon et al. 2003), older and larger females represent individuals of higher reproductive quality and should be sought after by males.

The primary goals of this study were to test the hypothesis that an important use of stored sperm is to fertilize sequential clutches within a season and quantify age- and body-size-specific reproductive traits of females that may substantially influence male reproductive success.

Materials and methods

Life history and population study

Research was conducted in accordance with the University Committee on Use and Care of Animals (UCUCA #8496) of the University of Michigan. Midland Painted Turtles from East Marsh on the ESGR were intensively trapped from 1976 to 2007. All individuals were uniquely marked by notching marginal carapace scutes at first capture in aquatic traps, on land, or at drift fences completely surrounding East Marsh or located between wetlands and potential nesting areas. At each capture, the date, individual identification,
body size (carapace length, mass), reproductive condition (e.g., gravid or not gravid) were recorded. Hatchlings and yearlings were assigned “age 1,” and at first capture, the ages of juveniles with distinct growth rings were estimated by assuming one growth ring for each year of life (Gibbons and Greene 1979; Hinton et al. 1997). Thereafter, ages of individuals were calculated from the interval between first and each subsequent recapture.

Data on clutch size and reproductive frequency were collected over 26 consecutive years (1983–2006), when East Marsh was completely enclosed by a 1.3 km fence during all nesting seasons. The fence was monitored all days of each nesting season at approximately 20 min intervals from 0600 until the end of activity of Midland Painted Turtles in the evening. Almost all females leaving the marsh to nest (with first or second clutches) were captured each year, identified, measured, and weighed. Females were also X-radiographed to determine clutch size and widths of eggs (Gibbons and Greene 1979; Hinton et al. 1997). We associated nests with females by observing them in the act of nesting during extensive searches of nesting areas.

Over the 33 years spanning the entire study at East Marsh (1975–2007), 2,796 individuals were marked, 13,917 captures were made, and females were detected with 4033 and 893 first and second clutches, respectively. The total resident adult population at East Marsh between 2001 and 2006 was calculated from catchability data (Congdon and Gibbons 1996) based on all captures and all methods (aquatic traps, on land, or at the fence) over six overlapping 3-year periods (2000–2002, 2001–2003, 2002–2004, 2003–2005, 2004–2006, 2005–2007). We used data from extensive capture and reproductive records from East Marsh (mean = 9.5 years; minimum–maximum = 5–24 years) to assign 167 females to the categories of those that had produced “one clutch only” and those that produced “second clutches”.

Paired clutches

Within-year pairs of clutches (N = 44 pairs; 88 nests) of eggs were obtained from nests of 35 marked females from 2003 to 2006. Nests were protected in situ in 2003–2004. During 2005 and 2006, eggs were transferred to a common protected natural nesting area within 4 h of nest completion. Transferred eggs were incubated in perforated plastic containers to exclude burrowing mammal predation and were buried approximately 6 cm below the surface of the ground. Different methods of nest protection resulted in similar hatching successes (χ²[1] = 0.293, P = 0.587), so samples were combined.

Genetic samples

A sample of tail tissue (<2 mm) was taken from each hatchling and preserved in 95% ethanol until DNA extraction. Approximately 0.1 cc (1 cc = 1 mL) of blood was collected from each female parent and was stored in a blood storage buffer (100 mmol/L Tris-HCL (pH 8.0), 100 mmol/L EDTA, 10 mmol/L NaCl, and 0.5% SDS). DNA was extracted from blood and tissue samples using a Qiagen DNeasy kit (Qiagen, Valencia, California, USA), and quantified using fluorimetry.

Hatchlings from all clutches were genotyped at seven polymorphic microsatellite loci: Cpf2, Cpf10, Cpf3 (Pearse et al. 2001); BTGA2, BTGA3, BTCA7 (Libants et al. 2004); and EBII (Osentoski et al. 2002). DNA was amplified using the published conditions for polymerase chain reaction (PCR), separated using gel electrophoresis on a 6% polyacrylamide gel, and visualized using an FMBIOII scanner (Hitachi Inc., Tokyo, Japan). Gels were hand-scored independently by two experienced laboratory personnel and over 10% of PCR products from hatchlings were randomly selected and reanalyzed electrophoretically and rescored independently to minimize scoring errors. Estimates of genetic variability and multilocus exclusion probability were quantified using the program GERUD version 2.0 (Jones 2005) and are based on the equations presented in Dodds et al. (1996). Analysis of stored sperm and multiple paternity were restricted to clutches with three or more offspring, reducing the sample size to 40 pairs (80 nests) from 31 females. All 44 pairs were used for analyses of hatching success (described below).

Paternal genotype reconstruction

Paternal genotype reconstruction was performed on 40 pairs of nests (80 clutches) using the program GERUD version 2.0 (Jones 2005) based on one known (female) parent. We performed three stepwise analyses involving paternal genotype reconstruction: (1) each nest independently, (2) offspring combined for paired clutches within years, and (3) offspring combined for each female among all years. Reconstructed paternal genotypes of males contributing to nests were also compared visually (Fiumera et al. 2002; Jones et al. 2007) for consistency between first and second clutches that were produced within years and among years to detect use of stored sperm (below). In cases of multiple paternity, GERUD version 2.0 provides potential paternal reconstructions along with estimates of the number of most likely male contributors based on paternal allele counts. Because the female parent was known for all clutches of eggs, we could identify paternal alleles and thus detect multiple paternity when three or more distinct paternal alleles were present. Results from the visual comparison and stepwise GERUD version 2.0 analyses were compared for consistency to ensure the most accurate interpretation. Finally, we compared reconstructed paternal genotypes of all contributing males, using the program GENECAP (Wilberg and Dreher 2004), for evidence that any male sired offspring with more than a single female within and among years.

Detecting repeat paternity (the use of stored sperm)

When paternal genotype reconstructions (GERUD version 2.0; Jones 2005) indicated that the same male(s) sired sequential clutches, we identified the second nest as having “repeat paternity”. Because of the short interval between first and second clutches within a season, repeat paternity in intraseasonally paired clutches was considered evidence of the use of stored sperm to fertilize the second clutch (Gist and Congdon 1998; Pearse et al. 2002). Because there are ample opportunities to remate between years, we refer only to repeat paternity among years. However, the male-biased adult sex ratio in the ESGR population (two males per female) reduces the probability of remating with the same male within or between years.

Previous research described the use of stored sperm based on inferences from observations of paternal genotypes being “consistent” or “inconsistent” with respect to the first clutch.
(Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002). Because of our focus on the influence of female traits on male reproductive success, we expanded the category of “consistent” to include patterns of repeat paternity that differentially influence male reproductive success (Table 1). When hatchlings from first and second clutches of an individual female had identical paternal alleles, we categorized the condition as “complete” repeat paternity. Complete repeat paternity was then subdivided into “complete type 1” (paternal alleles were consistent with one male) or “complete type 2” (paternal alleles were consistent with more than one male). When the composition of paternal alleles differed between first and second clutches because of an addition or deletion of paternal alleles, we categorized the condition as “incomplete” repeat paternity. Incomplete repeat paternity was then divided into “inclusive incomplete” (when an allele(s) from a new male was observed only in the second clutch) and “exclusive incomplete” (when an allele(s) from a male siring offspring in the first clutch was not observed in the second clutch). The terminology of inclusive and exclusive incomplete refers to the composition of paternal genotypes present and not whether paternity resulted from rematings or stored sperm from previous years.

Statistical analyses

Summary statistics of female attributes and values of life-history traits such as reproductive frequency, age, body size, clutch size, and egg size (width) among females, and demographic parameters such as the number of reproductive females for each year, and correlations between traits were performed using Spearman’s rank correlations (SAS Institute Inc. 1998).

Relationships between female attributes and incidence of multiple paternity were examined using a generalized linear mixed effect, logistic regression model (GLMM), with “paternity” (a binary output with 0 equal to single paternity and 1 equal to multiple paternity) as the dependent variable (R version 2.5 with the lme4 package for mixed models; R Development Core Team 2007). Because all females contributed at least one pair of nests and paired clutches for some females were sampled in more than 1 year, we accounted for structuring in the data by including both female and year as random effects. Fixed effects included female age, female body size (carapace length), clutch sequence (first or second), and clutch size.

When female turtles are isolated from males over several years, use of stored sperm to fertilize subsequent clutches can result in decreased fertility and hatching success (Cuellar 1966; Jun-yi 1982), via sperm depletions or a decline in sperm viability (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998). We tested whether second clutches had a reduced probability of hatching using a general linear model (GLM) with hatching percentage as a dependent variable (rather than a linear mixed effect model) because nests are independent events with respect to hatching percentage and the increased power allowed a more accurate test of fixed effects. We also independently tested additional factors proposed to influence hatching success such as female age (Blen et al. 1999), female body size (Ban et al. 2000), and whether a clutch was multiply or singly sired (Zeh and Zeh 1996; Byrne and Robert 2000; Pearse et al. 2002; Garner and Schmidt 2003; Roques et al. 2006).

Results

Frequency of second-clutch production

Over the 24 years after East Marsh was fenced (1983–2006), the number of reproductive females each year averaged 84 (SD = 12.5; minimum–maximum = 62–106; Fig. 1a) and varied by 46 individuals (43.4%; minimum–maximum = 58.5%–100% of the maximum number). The proportion of reproductive females each year that produced second clutches averaged 23% (minimum–maximum = 6%–40%; Fig. 1b). Variation in numbers of reproductive females among years was reflected in changes in both first-clutch-producing and second-clutch-producing individuals (Fig. 2a).

Among 167 females with extensive individual reproductive histories, individual frequencies of second-clutch production varied widely (minimum–maximum = 0%–50%; Fig. 2a). No second clutches were produced by 44 females (26%), whereas 123 females (74%) produced second clutches in some of the years sampled (Fig. 2a). Second-clutch production averaged 25% (minimum–maximum = 7%–50%; Fig. 2a) for the 123 females that produced second clutches. Carapace length was positively correlated with age within all annual samples (1983–2006) of reproductive females (all Spearman’s \( \rho = 0.400–0.773 \), \( P \) values <0.006), as well as among just those females that produced second clutches (all Spearman’s \( \rho = 0.441–0.852 \), \( P \) values <0.004). In addition, for just second-clutch-producing females over all years combined, the frequency of second-clutch production was correlated with carapace length (Spearman’s \( \rho = 0.235, P = 0.01 \) and age (Spearman’s \( \rho = 0.206, P = 0.02 \)). The annual percentage of second clutches produced in the population was positively correlated with the total number of reproductive females documented each year from 1983 to 2006 (Spearman’s \( \rho = 0.287, P < 0.001 \); Figs. 1a, 1b).

Measures of genetic diversity

The loci \( C p2 \) and \( C p10 \) (Pearse et al. 2001) showed evidence of null alleles and were excluded from further analyses. For the remaining five loci, estimates of the measures of genetic variability included the mean number of alleles per locus over five loci (17; minimum–maximum = 7–34), mean expected heterozygosity (0.793; minimum–maximum = 0.682–0.888), and the expected exclusion probability with one parent known (all loci = 0.994; minimum–maximum = 0.437–0.772).

Proportions of the total populations of adult males and females contributing to the paired clutches

Over the 4 years of genetic sampling (2003–2006), the number of males and females in the East Marsh population averaged 335 (minimum–maximum = 321–348) and 180 (minimum–maximum = 169–190), respectively (adult sex ratio is approximately 2 males to 1 female). The proportions of high-quality females that were genotyped (those that produced the paired clutches) and males that contributed to hatchings in those clutches were 6.8% and 9.6% of the estimated total population of each sex, respectively. Within the sample of hatchlings from 40 pairs of nests, re-
Demonstration of the use of inferred paternal alleles at a polymorphic locus to distinguish among four categories of repeat paternity (stored sperm use) that can have direct influence on male reproductive success (RS) of Midland Painted Turtles (*Chrysemys picta marginata*).

<table>
<thead>
<tr>
<th>Categories of repeat paternity</th>
<th>Paternal alleles</th>
<th>Observed incidence (%)</th>
<th>Implication of repeat paternity for male RS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First clutch</td>
<td>Second clutch</td>
<td>Within years (N = 40 pairs)</td>
</tr>
<tr>
<td>Complete</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>A A</td>
<td>A A</td>
<td>77.50 (N = 31)</td>
</tr>
<tr>
<td>Type 2</td>
<td>A A</td>
<td>B B</td>
<td>7.50 (N = 3)</td>
</tr>
<tr>
<td>Inclusive incomplete</td>
<td>A A</td>
<td>C C</td>
<td>7.50 (N = 3)</td>
</tr>
<tr>
<td>Exclusive incomplete</td>
<td>A A</td>
<td>B C</td>
<td>5.00 (N = 2)</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>A B</td>
<td>D C</td>
<td>2.50 (N = 1)</td>
</tr>
<tr>
<td>No evidence</td>
<td>A D</td>
<td>B E</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Nine among-year comparisons with one female sampled in 3 years (two comparisons).

constructed male genotypes indicated that no male sired offspring with more than one female that produced two clutches within a reproductive season or among years. However, some males sired offspring with the same female among consecutive years (over 2 years by two males and 3 years by one male). In total, 32 males contributed to offspring from 24 females (operational sex ratio is equal to 1.3 males to 1 female contributing to paired clutches).

**Within-year use of stored sperm**

Among 40 paired clutches, 97.5% of the within-year second clutches exhibited evidence that stored sperm was used to fertilize all or a portion of the second clutch of eggs. Among clutch pairs, 77.5% exhibited “complete type 1” use of stored sperm (i.e., identical paternal alleles indicative of one male in both clutches), 7.5% exhibited “complete type 2” use of stored sperm (identical paternal alleles indicative of more than one male in both clutches), 7.5% displayed inclusive incomplete use of stored sperm (i.e., some hatchlings had paternal alleles not found in the first clutch), and 5% displayed exclusive incomplete (some hatchlings had paternal alleles not found in the second clutch; Table 1). In all cases where overall composition of paternal alleles differed between first and second clutches, the second clutch showed evidence of incomplete use of stored sperm.

**Repeat paternity among years**

For seven females, paired clutches were sampled in 2 consecutive years (14 pairs) and for one female in 3 consecutive years (3 pairs), allowing 9 among-year comparisons. Overall, 44.4% (4 out of 9 among-year comparisons, N = 8 females) of the clutches among years showed evidence of repeat paternity (3 complete type 1, 0 complete type 2, 1 inclusive incomplete, and 0 exclusive incomplete; Table 1). One female with clutches sampled over 2 years and one with clutches sampled over 3 consecutive years (3 among-year comparisons; 33.3%) had hatchlings with identical paternal alleles in all clutches. Both clutches of one female in the 2nd year (11.1%) had offspring fertilized by the same male as the previous year and an additional male (evidence of inclusive incomplete use of stored sperm) and one among-year comparison was inconclusive (Table 1). Four among-year comparisons (44%) had different compositions of paternal alleles among years (no evidence of repeat paternity). For females where relationships could be conclusively assigned, 50% of among-year comparisons (37.5% of females) showed evidence of repeat paternity among years.

**Multiple paternity**

Over 4 years, multiple paternity was evident in 16.3% of all clutches (N = 80) and varied among years (22.2% (N = 18), 30.0% (N = 20), 8.8% (N = 34), and 0% (N = 8) in 2003, 2004, 2005, and 2006, respectively; Fig. 1a). The proportion of clutches exhibiting multiple paternity was highest in the 2 years with the fewest reproductive females and lowest in the 2 years with the most reproductive females. The occurrence of multiple paternity did not differ significantly between first (15%; N = 40) and second (17.5%; N = 40) clutches (GLMM, Z = 0.339, P = 0.735). However, in 5 out of the 40 pairs (12.5%), single and multiple paternity varied between pairs of clutches, with two pairs having multiple paternity only in the first clutch and three pairs having multiple paternity only in the second clutch. Multiple paternity was positively but not significantly correlated with the number of eggs sampled from a clutch (GLMM, Z = 1.763, P = 0.08) and female age (GLMM, Z = 1.779, P = 0.08), but not female body size (GLMM, Z = 0.424, P = 0.52), suggesting
that there may be an important biological relationship between female age and probability of multiple paternity.

Comparisons of clutch size and egg size in first and second clutches

Over the 24 years (1983–2006), X-radiographs were taken of 312 pairs of clutches of eggs. Mean clutch size was significantly larger (7.2 and 6.2 eggs for first and second clutches, respectively; paired \( t = 4.39, P < 0.001 \)), and egg widths (measured from X-radiographs) were slightly and significantly wider (18.1 and 18.0 mm for first and second clutches, respectively; paired \( t = 3.40, P < 0.008 \); Table 2) in first clutches compared with second clutches. However, minimum–maximum of clutch size were identical (3–13 eggs) and egg widths were similar (15.4–20.8 and 15.9–20.5 mm) for first and second clutches, respectively (Table 2). Over the years in the study for which genetic data are available (2003–2006), the number of offspring genotyped in first and second clutches was not significantly different (GLM, \( t_{[87]} = -1.077, P = 0.285 \)). Hatching success was not associated with female age (GLM, \( t_{[80]} = 1.444, P = 0.153 \)), body size (GLM, \( t_{[87]} = -0.370, P = 0.711 \)), or the incidence of multiple paternity (GLM, \( t_{[79]} = 0.808, P = 0.421 \)).

Discussion

Annual male reproductive success can be affected by among-year variation in (i) total number of reproductive females, (ii) the proportion of reproductive females that produce second clutches, and (iii) the probability that a given mate will produce a second clutch (Figs. 1a, 1b). That the proportion of second clutches is positively and significantly correlated with the number of reproductive females suggests that resources available during the previous year may be the source of the variation in clutch production (Stearns 1992; Kitaysky et al. 2000). Whereas it may be difficult for males to predict how many females will be reproductive and what proportion will produce two clutches in a given year, resource levels in the present year may allow males to assess the quality of the next reproductive season (e.g., the proportion of second clutches or number of reproductive females).

Among all females and within the subset of intraseasonally iteroparous females, age and carapace length (body size) were positively correlated with frequency of second-clutch
production. Males may be able use phenotypic traits associated with age (e.g., body size) to identify not only females that lay two clutches in a year, but also identify within those females, individuals with high propensity for producing intra-seasonally iteroparous clutches. Additionally, other phenotypic cues may allow males to identify females that lay two clutches. Red and yellow colors on the head, neck, and marginal scutes of adult Midland Painted Turtles (and in many other species of turtles) may vary in intensity and contrast resulting from variation in carotenoid production, which has been suggested as a reliable indicator of individual quality in other taxa (Kodric-Brown and Brown 1984; Badyaev and Hill 2000; Maan et al. 2006). If males are able to evaluate indicators of female quality to target larger (older) females to increase the probability of mating with an intraseasonally iteroparous female, the increase in reproductive success would be substantial (even more so if females use stored sperm to fertilize a substantial proportion of second clutches within a year).

Gist and Congdon (1998) hypothesized that an important function of sperm storage in turtles is to facilitate fertilization of sequential clutches within a season because of a short receptive period between clutches. In sea turtles, ovulation of sequential clutches of eggs occurs in $< 36$ h (Licht et al. 1979). Female turtles may not be receptive in the short time between nesting and ovulation of the next clutch of eggs because they must recover physiologically (e.g., from the accumulation of lactic acid during nesting; Congdon and Gatten 1989; Jessop and Hamann 2004). Furthermore, hormones involved in nesting and mating behavior (e.g., testosterone, estradiol, and progesterone) must readjust to levels suitable for mating (Rostal et al. 1998) because they also appear to provide information about current reproductive state. For example, after gravid females were induced to lay eggs with oxytocin and released back into wetlands, they moved from aquatic to terrestrial nesting areas and performed the entire sequence of nesting behaviors without having eggs to lay (Tucker et al. 1995; J.D. Congdon, unpublished data).

Comparisons of reconstructed paternal genotypes indicated that 97.5% of second clutches of East Marsh females were fertilized with stored sperm (85% complete (combined type I and type II) and 12.5% incomplete (combined exclusive and inclusive)). The high frequency of stored sperm use found in this study is similar to that in another population of Painted Turtles (Chrysemys picta (Schneider, 1783)) (100%) in an open riverine environment (Pears 2002) and in other species of turtles (Fitzsimmons 1998; Kichler et al. 1999; Roques et al. 2006). We found that sperm from the male that fertilized the first clutch was used to fertilize all or part of the second clutch 77.5% of the time, and two males shared paternity of both clutches 7.5% of the time. In 12.5% of paired clutches, one male was not represented in either the first or the second clutch (incomplete repeated paternity). Incomplete repeated paternity results in a male not siring any offspring in one of the two clutches. Overall, male reproductive success has the potential to dramatically increase without further investment in reproductive activities by mating with females that frequently produce second clutches within a year.

The variation among females in production of second clutches could result in two male tactics: (1) if there is no way to discriminate among females based on their reproductive potential (i.e., clutch frequency), males should mate with as many females as possible (Bateman 1948), or (2) if there are phenotypic cues that allow males to identify females with a high propensity to produce second clutches, males should increase efforts to mate with those females. Bateman (1948) suggested that a primary mechanism for males to increase their reproductive success is through mate number. However, tactic 2 (above) may increase a male’s reproductive success while maintaining or reducing the number of matings attempted with different females. Among the ESGR males that successfully mated with an intraseasonally iteroparous female, none were found to have sired offspring of any other intraseasonally iteroparous female during the 4 years of this study. Our results do not support the assumption that mating with as many females as possible is a primary way male Midland Painted Turtles in this population increase reproductive success.

Perhaps even more striking than the high incidence of repeat paternity within a year is that the same males fertilized all, or a portion of, 44% of pairs of nests among years, and a single male sired all offspring in six clutches that one female produced over 3 years. In European Pond Turtles (Emys orbicularis (L., 1758)), a similar proportion (58%) of clutches fertilized by the same male among years was observed, indicating that among-year use of stored sperm may also be important to male reproductive success (Roques et al. 2006). Because females have more time (and presumably more opportunities) to remate among years than within years, it is not possible to exclude remating as an explanation for the same male fertilizing paired clutches among years.

The use of stored sperm by ESGR females is the most parsimonious explanation, given a population size of 515 adults and a sex ratio of 1.9 males to 1 female. Our interpretation is that remating is not the primary mechanism for fertilizing sequential clutches of a female intra- or inter-seasonally. First, under the assumption of random mating, the probability of a given male mating with a female = 1 male/335 total males, and for mating to occur twice with the same male would be $1/335^2 = 8.9 \times 10^{-6}$. Second, between reproductive sea-

### Table 2

<table>
<thead>
<tr>
<th>Female category</th>
<th>N</th>
<th>Clutch size</th>
<th>Egg width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intraseasonal</td>
<td>Mean</td>
</tr>
<tr>
<td>No second clutches</td>
<td>263 clutches</td>
<td>First only</td>
<td>6.7</td>
</tr>
<tr>
<td>Second clutches</td>
<td>312 pairs</td>
<td>First</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Second</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Note: min., minimum; max., maximum.
sons, the majority of females almost certainly encounter many potential mates. Third, the use of stored sperm reduces the necessity for spatial and temporal synchrony of matings with female receptivity (Birkhead and Möller 1993; Shuster and Wade 2003), which would allow for more time (and presumably opportunity) to mate with more females. Finally, there is no evidence of pair bonding in turtles (Galbraith 1993; Pearse and Avise 2001; Roques et al. 2006).

If stored sperm is the mechanism that explains the repeat paternity among years, then the question becomes: why do females use stored sperm when males are plentiful and frequently encountered? Regardless of whether repeat paternity is the result of stored sperm or remating, the findings suggest that once a male is successful with a female, he has a high probability of continuing to be successful with that female among years. Either way, the results are not consistent with the general perception that the number of mates is the only substantial determinant of male reproductive success.

Clutch sizes and hatching success were similar in first and second clutches. Female turtles are able to store sperm over several years (Ewing 1943), and although declines in fertility have been documented in turtles (Palmer et al. 1998), the decline in fertility was observed among years, not within a season. The high probability that females use stored sperm to fertilize the second clutch and that the hatching success of first and second clutches were similar indicates that mating with intraseasonally iteroparous females has the potential to substantially increase male reproductive success.

Two major influences on male reproductive success of Midland Painted Turtles on the ESGR appear to be the potential that the females have for producing second clutches and the propensity for females to use stored sperm to fertilize sequential clutches within and among years. If males have no way of discriminating among females, then the intensity of courtship by males and the cost and benefits of courting or defending any female in the population should be equal. However, if males can recognize high-quality females (those that produce and use stored sperm to fertilize multiple clutches within a year), the costs may increase because more males would be attempting to mate with them and that could result in reducing a male’s ability to mate with other females. Even with increased costs, the benefits to males would apparently remain high for those mating with females that are intraseasonally iteroparous.

The large proportions of sequential clutches within a year that are fertilized with stored sperm in different species (Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002; Roques et al. 2006); this study) suggest that use of stored sperm within a year is widespread among turtles. In many species of sea turtles, females lay 2–10 clutches of eggs in a single season (van Buskirk and Crowder 1994). If repeat paternity is observed among clutches within a year (e.g., Fitzsimmons 1998), then the benefits to male reproductive success could be great. However, the interannual nesting intervals can extend 5 years or more (Limpus et al. 1994), a period that may be too long for stored sperm use among years. Additionally, with clutch sizes much larger (many species over 100 eggs per clutch; van Buskirk and Crowder 1994) than those observed in Midland Painted Turtles (mean of approximately 7 eggs per clutch), the issue of sperm depletion within and among years must also be considered in sea turtles. Females in other taxa (including birds, reptiles, mammals, and insects) are also known to store sperm (Birkhead and Möller 1993; Birkhead 1998), and several species are intraseasonally iteroparous (Verhulst et al. 1997). If frequent use of stored sperm to fertilize offspring in sequential reproductive bouts (within or between years) occurs in these groups, it represents a potentially important component of male reproductive success.

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