

Female reproductive qualities affect male painted turtle (*Chrysemys picta marginata*) reproductive success

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Abstract Male reproductive success (RS) in polygamous species with minimal social systems is often determined by the number of mates. However, because male RS is translated through females, the number of offspring sired can also be influenced by female qualities. Empirically sufficient data to document how tradeoffs between mate number and quality influence male RS are seldom available for long-lived, iteroparous species. We combined long-term life history data (1983–2006) on the E. S. George Reserve (ESGR, MI, USA) with parentage data from 155 clutches of 59 female painted turtles (*Chrysemys picta marginata*) of varying reproductive frequencies (2003–2006) to determine the relative contribution of female numbers and qualities on male RS. One previously documented trait of female painted turtles that can have substantial influences on male RS is repeat paternity through the use of stored sperm to fertilize over 95 % of within-year

clutches. In addition, our study found that second-clutch producing female painted turtles on the ESGR have higher among-year reproductive frequencies than do first-clutch only females. Multiple paternity was detected in 14.1 % of clutches (min-max=6.1–30.0 % annually), and the number of mates of both sexes was low annually (males 1.0; females 1.2) and over 4 years (males 1.1; females 1.7). Among successful males, RS varied substantially (1–32 offspring) and was strongly influenced by the combination of female reproductive frequency and repeat paternity (>38 % among years), but not mate number. Low mate number for both sexes was unexpected in a species without complex mating behaviors or parental care.

Keywords Reproductive success · Painted turtles · Repeat paternity · Offspring

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Introduction

Building on Bateman's (1948) observation that reproductive success (RS) of male fruit flies (*Drosophila* spp.) increased as a function of increasing mate number, Trivers (1972) suggested that the large investment in offspring makes females a resource that males compete for. The idea that male RS is primarily mediated by the number of mates has been empirically supported in studies of mating systems of polygamous species (e.g., Weatherhead and Robertson 1977; Gibbs et al. 1990; Rodriguez-Munoz et al. 2010). However, variation in reproductive qualities of females (i.e., clutch or litter size, reproductive frequency, parental investment) can also influence male RS (Andersson 1994; Owens and Thompson 1994; Servedio and Lande 2006). For example, in some iteroparous species, the combined effects of female reproductive frequency and use of sperm from the same male (through remating or use of stored sperm) in subsequent reproductive events can have a substantial influence on male RS (Ribble 1992; Pearse

and Avise 2001; McGuire et al. 2011). Therefore, documentation of the relative contributions of mate number and mate quality to male RS is necessary to interpret mating tactics of many species.

Determining the relative importance of mate number and quality to male RS can be complicated by complex social environments (Jeswiet et al. 2012) that include factors such as parental care, costly courtship rituals, nuptial gifts, and access to high-quality resources associated with mates (Emlen and Oring 1977; Dewsbury 1982; Schubert et al. 2009a; b; Dreiss et al. 2010; Salehialavi et al. 2011; Rittschof et al. 2012). The combination of simple courtship behaviors, lack of pair bonding, and no post-ovulatory parental care found in painted turtles (*Chrysemys picta marginata*; Wilbur and Morin 1988) provides an opportunity to examine the relative importance of mate quality and number on male RS in a long-lived iteroparous species in the absence of the confounding interactions associated with complex social factors.

Female painted turtles vary substantially in reproductive qualities that can influence male RS. Reproductive qualities such as clutch size and frequency contribute directly to male RS through variation in offspring number. Indirect influences of females on male RS include (1) increased survival of hatchlings through parental investment in neonate body size and yolk reserves (Congdon and Gibbons 1987; Congdon et al. 2003), (2) the effects of nest site selection on embryo development and the probability of nest depredation (Valenzuela and Janzen 2001), and (3) the suite of environmental cues that hatchlings use during dispersal from nests to find wetlands or overwintering sites which may impact hatchling survival (Iverson et al. 2009; Pappas et al. 2009; Congdon et al. 2011). In addition, female painted turtles can apparently store viable sperm for long durations (Gist and Jones 1989; Gist et al. 2002). Gist and Congdon (1998) predicted that fertilizing sequential within-season clutches would be a primary function of stored sperm due to the narrow window of time between oviposition and ovulation of second-clutch follicles of turtles. That prediction was supported for painted turtles that use stored sperm to fertilize the majority (>95 %) of clutches produced within a season and paired clutches of some individuals among years (Pearse and Avise 2001; Pearse et al. 2001, 2002; McGuire et al. 2011). Therefore, the frequency of clutches produced within a year (intra-annual clutches) and among years (interannual clutches) and the propensity for a female to use sperm from the same male(s) to fertilize subsequent clutches (repeat paternity) can combine to potentially exert a major influence on variation in male RS. If repeat paternity also occurs in a substantial number of among-year clutches, then documenting the frequency of clutch production (reproductive frequency), repeat paternity, and their combined influence on male RS requires a long-term study to disentangle the relative influence of mate quality and mate number to male RS.

Research on the life-history traits of painted turtles on the University of Michigan's E. S. George Reserve (ESGR) near Hell in southeastern Michigan (Congdon and Gibbons 1996) was conducted over 24 consecutive years (1983–2007). Data from the study were used to quantify annual variation in the number of reproductive females and size- and age-specific reproductive qualities such as clutch size (number of eggs per clutch), egg size (mean egg width per clutch), and reproductive frequency (frequency of clutches produced both intra- and interannually; Congdon and Gibbons 1996; Congdon et al. 2003). Life-history data were combined with 4 years of genetic data on paternity of offspring collected from 2003 to 2006 to (1) determine the relative importance of mate number and mate quality to male RS, (2) test whether incidence of multiple paternity (MP) was higher in clutches of high-quality females compared to low-quality females, and (3) determine whether reductions in offspring sired per clutch resulting from MP reduced the benefits of mating with high-quality females.

Materials and methods

Field sample collection

Life history, reproductive ecology, and genetic studies were conducted using individuals from an ~4.0 ha wetland (East Marsh) on the ESGR. Turtles were captured using aquatic traps, dip nets, muddling, at a fence completely surrounding the marsh, and on land. At first capture, all individuals were marked by notching a unique set of marginal carapace scutes. The date, location, sex, and body size (straight line carapace length, CL) were recorded at every capture, and the reproductive condition of females was noted from May to mid-July. At initial capture, individuals were assigned "age 1" (i.e., age = year of life) if they were between the time of emergence from nests in spring and the end of their first activity season, and ages of older juveniles with distinct growth rings were estimated by assuming one growth ring for each year of life (Gibbons 1976). Ages of recaptured individuals were calculated from the interval between the first capture (when age was initially assigned) and each subsequent recapture. The standing crop of adult males (including subadult males) and females in East Marsh was approximately 450 and 195, respectively (Congdon and Gibbons 1996). From 1983 to 2007, all capture methods resulted in a total of 225 females and 752 males marked and 4,981 and 4,320 recaptures of each sex, respectively.

The fence that encircled East Marsh from 1983 to 2006 was an important determinant of the quality of data collected during the life history study. The fence was located between the wetland and nesting areas and close to the wetland where damp organic soils precluded nesting. The fence was monitored at approximately 20 min intervals from 0600 hours until

1 h past the end of painted turtle activity in the evening on all days of 24 consecutive nesting seasons. Captures of females at the fence while they were moving to or returning from terrestrial nesting each year allowed us to document the reproductive condition of individuals (i.e., not gravid, gravid with first or second clutches) and thus determine their within- and among-year reproductive frequencies.

Just prior to and during all nesting seasons, females captured at the East Marsh fence or on land were identified and X-radiographed ($N=1,872$) to determine clutch size and egg widths (Gibbons and Greene 1979; Hinton et al. 1997). At that time, a unique alpha numeric temporary code was attached to the carapace to allow identification from a distance without disturbing females on land. Assignment of a second clutch was based on captures of gravid females that were subsequent to recorded first clutches, differences between the number or position of eggs between sequential within-season X-radiographs, or when the post first-clutch duration of captures of gravid females was greater than 15 days.

Quantification of female reproductive qualities

We quantified four major components of female quality: (1) clutch size (number of eggs or offspring per clutch), (2) sizes (widths) of eggs (as an index of parental investment), (3) reproductive frequency, and (4) relationships of reproductive traits to body sizes and ages of females (ages of 80.3 % of all reproductive females from 1983 to 2006 were known). Annual reproductive categories of females at East Marsh included primiparous females (females reproducing for the first time and that are typically of low reproductive quality) and multiparous females that generally have higher reproductive qualities than primiparous females. In addition, we used long-term recapture and reproductive data (mean annual captures=9.0 years, min-max=5–24 years, $SD=3.08$, $N=1,168$ captures over 24 years) to assign 167 individual multiparous females to two additional categories. First-clutch only (FCO) females were those that produced a maximum of one clutch per year ($N=44$) and second-clutch producing (SCP) females were those that produced two within-season clutches during some of the years they were reproductive ($N=123$). We calculated the frequency of second-clutch production over each individual's total years of capture (e.g., FCO females=0) and compared clutch size and egg widths of first clutches and annual reproductive intervals of females in both categories. Females within the two groups averaged 64.2 % (min-max=28–86 %, $SD=14.5$) of the annual numbers of reproductive females at East Marsh over 24 years.

Genetic data collection

From 2003 to 2006, 179 adult females and 288 adult males were captured in East Marsh and 1,054 hatchlings from 155

clutches of eggs of 59 females were sampled for genetic analysis. In 2003–2004, nests were protected in situ with a wire cage; however, variation in slopes and soil types at nest sites resulted in some protected nests being destroyed by burrowing mammals. Therefore, in 2005–2006, eggs were moved to a relatively level section of a natural nesting area that had been enclosed by aluminum flashing buried 20 cm in the ground. Clutches of eggs were (1) moved to the protected area within 4 h of nest completion, (2) placed in a perforated plastic container, (3) buried approximately 6 cm below the surface of the ground, and (4) covered with a wire cage. The combination of protections resulted in no losses due to surface or burrowing predators.

At the end of incubation, a <2 mm tail tip sample was taken from all hatchlings and preserved in 95 % ethanol. Long-term nest survivorships on the ESGR were used to determine the numbers of hatchlings to be released on the ESGR, and all other individuals were released in wetlands close to the ESGR.

Approximately 1 cc of blood was obtained from the caudal vein of 179 females, 288 males, and 147 subadult males (>2 years of age that would likely mature during the study). Blood samples were preserved in a buffered solution (100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 10 mM NaCl, and 0.5 % SDS), and both blood (adults) and tissue samples (hatchlings) were temporarily stored at ambient temperature and then moved to long-term storage at -20°C . The DNA was extracted from blood and tissue samples using Qiagen DNeasy kits (Qiagen Inc, Valencia, CA) and then quantified using fluorimetry.

Samples of all males ($N=435$), 59 known mothers, and all hatchlings ($N=1,054$) were genotyped at seven microsatellite loci [Cp2, Cp3, Cp10 (Pearse et al. 2001); EB11 (Osentoski et al. 2002); and BTGA2, BTGA3, BTCA7 (Libants et al. 2004)] using published conditions for polymerase chain reaction (PCR) amplification. The resulting PCR products were separated using gel electrophoresis on a 6 % polyacrylamide gel. Products were visualized using an FMBIOII scanner (Hitachi Inc., Tokyo, Japan). All gels were independently scored by two experienced laboratory personnel. To estimate genotyping error, 10 % of adults were randomly selected and re-genotyped for all loci. Estimates of expected heterozygosity, number of alleles, the exclusion probability with one parent known, and tests for Hardy-Weinberg equilibrium were performed using the program CERVUS 3.0.1 (Kalinowski et al. 2007).

Paternity assignment

The female parent of each clutch was known by observing identified individuals during nest construction. To assign paternity, we relied on concordance of assignment by multiple software programs that rely on different statistical methods.

Parentage programs that treat each offspring as an independent event, such as CERVUS 3.0.1 (Kalinowski et al. 2007) or partial likelihood approaches implemented in the program COLONY (Wang 2004), can overestimate the number of contributing sires (Fiumera et al. 2002; Jones et al. 2007), particularly when offspring are nonindependent (e.g., produced as a clutch). Therefore, we used the program NEST, which uses a Bayesian approach to analyze groups of offspring such as clutches (Jones et al. 2007), and compared results with output from CERVUS 3.0.1 comparing all candidate males to all offspring (Kalinowski et al. 2007). A father was assigned to an offspring when the male(s) assigned to a clutch by the NEST program was also determined to have high probability of paternity based on a positive log odds (LOD) score and with no sire-offspring genotype mismatches as determined by the program CERVUS. To confirm the results, we also reconstructed male genotypes from offspring genotypes for each clutch using the program GERUD 2.0 (Jones 2005) and compared them to the genotypes of candidate males using the program GENEAP (Wilberg and Dreher 2004). Paternal assignments from the program NEST, the combined GENEAP and GERUD 2.0 approach, and visual assignments (Fiumera et al. 2002) were then compared to ensure consistency in paternal assignment. Offspring were excluded from further analyses when paternity was assigned inconsistently among programs. Repeat paternity was identified when the same male sired offspring in different clutches of the same female.

Statistical analyses

We tested whether within-season and among-year reproductive intervals were associated with female age and body size using mixed model analysis with a Poisson distribution based on data from the subset of 167 females with extensive reproductive and recapture histories. The intervals between clutches were calculated as the number of years between the current and next reproductive event (within-season intervals = 0). Because each female was captured from 5 to 30 times over the period 1983–2006, we included female as a random effect with a random slope and intercept (Schielzeth and Forstmeier 2009). Mixed model analyses were conducted using the lme4 and language R packages in the R statistical software (R Core Development Team 2007).

For just SCP females, we tested whether producing a second clutch during a given year increased the time until the next clutch was produced, compared to years when only one clutch was produced. Producing two clutches within a year may limit resources available for future reproduction and increase interannual intervals (Callow 2008), which may decrease the disparity between the reproductive output of FCO and SCP females, thereby limiting gains in RS of males mating with SCP females. Clutch intervals were compared

using a generalized linear mixed effect model (GLMM) with a binomial parameter (0,1) indicating whether the preceding reproductive event was characterized by production of one or two clutches and including female as a random effect for both slope and intercept.

We used linear mixed models to test whether attributes of successful males were significantly associated with the number of offspring sired. We included female as a random effect and tested the fixed effects of male age and body size, total number of mates (defined as the number of females a male sired offspring with), total number of clutches sired per year, number of clutches sired in all years, number of offspring per clutch, and MP of clutches sired on male RS (defined as the total number of offspring sired).

Sizes and ages of individual males varied over the 9 years of genetic sampling and the 4 years of assignment of paternity. Therefore, to investigate differences between the body sizes and ages of successful males and those of all sampled males, we standardized the variables for both groups. For size, we used the carapace length at last capture, and for age, we calculated the age of each male in 2003. The standardized values do not directly reflect the age or size at reproduction, but allow comparisons between the distributions of all sampled males and successful males and avoid issues of a male being represented in different bins between the two groups. We compared size and age distributions of all sampled males to successful males using the standardized estimates and tested for differences between the distributions using a chi-square test.

We used the GLMM to test whether the presence or absence of MP within a clutch was associated with female size or age, number of offspring within a clutch, or male body size or age. To reduce structuring in the data, females and males were analyzed in separate models. For example, associations between the occurrence of MP and female attributes were tested in models where each clutch was represented once. Associations between male attributes and MP were tested in models where a clutch could be represented multiple times (once for each male).

Assuming that all males are reproductive each year, variation in the total number of reproductive females changes the annual operational sex ratios in East Marsh, which can alter mating tactics (Emlen and Oring 1977; Dreiss et al. 2010). Because the number of reproductive females was low in 2003–2004 and high in 2005–2006 (Fig. 1), we tested whether the incidence of MP varied between years characterized by high or low numbers of reproductive females using chi-square tests. We also tested whether associations between the presence or absence of MP and female attributes varied between years characterized by relatively high or low numbers of reproductive females using GLMM.

Deviations from random mating provide support for mating preferences by males or females. We tested for deviations

from random mating using two approaches. First, to test for assortative mating, we used a mixed model to determine whether the ages and sizes of male–female mated pairs were correlated, including female as a random effect (intercept) in the models. Males were not included as random effects because males were not frequently repeated in the sample (i.e., only four males (6.3 %) mated with more than one female over 4 years; only one male mated with more than one female within a year). Second, for each mating pair, we calculated female/male ratios of body sizes and ages as described by Rowe et al. (2007) and tested whether ages or sizes of mated pairs deviated from random pairs of males and females. Because not all females reproduce in every year, and most females reproduced in more than 1 year of the study, we randomly sampled (with replacement) males and females to produce 155 random mating pairs (the number of clutches with genetic samples). Frequency distributions of ratios for the randomly generated mating pairs were then compared with the actual mating pairs using a G test. All statistical analyses were implemented in the statistical software R (R Core Development Team 2007).

Results

Numbers and attributes of reproductive females

The number of reproductive females in East Marsh varied substantially over the 24 years of study (1983–2006; mean=81.3, min-max=55–104, SD=12.48; Fig. 1), and over the years genetic data were collected (2003–2006; mean=91.2, min-max=77–104, SD=11.12). Annual samples of reproductive females were composed of multiparous (post first reproduction) and primiparous (first lifetime reproduction and generally of low reproductive quality) individuals. A total of 90 reproductive females were captured in 2002 and 78 different gravid individuals were captured over the next 4 years (2003–

2006). Of those 78 new females, 38 were <16 years old and predominantly primiparous (mean=48.7 %, min-max=33.3–76.4 %) and 40 were multiparous females >15 years of age (mean=51.3 %, min-max=23.5–66.7 %) with higher reproductive qualities than found in primiparous females.

Reproductive traits of females were quantified from the long-term data on female reproductive history (1983–2007). An average of 26.0 % (min-max 13.0–45.0 %, SD=0.89, $N=24$ years) of all reproductive females captured each year produced second clutches, and the number and sizes of eggs in their first and second clutches were similar. First clutches averaged 6.9 eggs (min-max=3–12, SD=1.48, $N=1,479$) and egg widths averaged 17.6 mm (min-max=13.6–22.0, SD=1.03, $N=1,607$), whereas second clutches averaged 6.7 eggs (min-max=3–13, SD=1.55, $N=364$) and egg widths averaged 17.8 mm (min-max=15.6–17.9, SD=1.03, $N=359$). Clutch sizes and egg widths were positively correlated with female body size (Fig. 2a, b). Body sizes and ages of adult females were positively correlated ($r^2=0.33$, $P<0.001$; Fig. 2c) and reproductive qualities increase with body size (Congdon and Gibbons 1987) and age Congdon et al. 2003). Females reached maturity from 6 to 12 years of age, and the mean frequency of second-clutch production of females within the age range of primiparous females was 15 %, whereas >30 % of the two older groups of females produced second clutches in years they were reproductive (Fig. 2d).

Of the 167 East Marsh females with extensive recapture and reproductive histories (min-max=5–24 years), 44 produced only first clutches and 123 produced second clutches during 25 % (min-max=7–49 %) of the years they reproduced. The FCO females were present in the population in all years (1983–2006) and the annual proportions averaged 19.0 % (min-max=9.2–33.3 %). The number of eggs in clutches of FCO females averaged 6.7 (min-max=2–11, SD=1.58, $N=263$) and in first clutches of SCP females averaged 7.0 (min-max=2–13, SD=1.46, $N=1,032$). Egg widths of FCO females averaged 17.6 mm (min-max=15.1–19.5 mm, SD=0.87, $N=263$) and first clutches of SCP females averaged 17.8 mm (min-max=14.4–20.8 mm, SD=0.93, $N=1,034$).

Reproductive frequencies and reproductive intervals of females with different qualities are important components of the overall variation in the range of reproductive qualities of females. Over the years from 2002 to 2006, an average of 11.0 % of reproductive females in East Marsh was not detected as reproductive in at least 1 year. First-clutch only females had longer interannual reproductive intervals (mean=1.39 years, SD=0.73, min-max=1–4, $N=44$) than did SCP females (mean=1.13 years, SD=0.41, min-max=1–4, $N=123$; GLMM; $Z=-3.34$, $P<0.001$). Second-clutch producing females producing a smaller proportion of within-season clutches had longer interannual reproductive intervals than SCP females producing a larger proportion of within-season clutches (GLMM, $t=-3.50$, $P<0.001$).

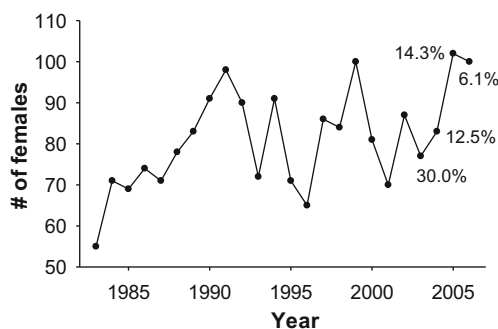
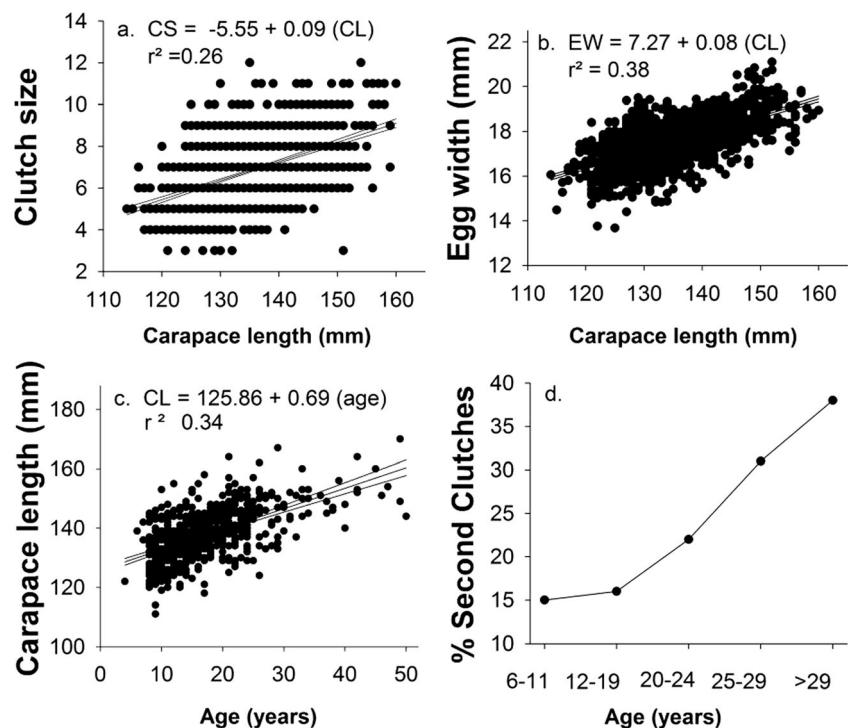


Fig. 1 The number of reproductively active painted turtle (*Chrysemys picta marginata*) females per year (1983–2006) and yearly percentage of clutches with evidence of multiple paternity (2003–2006)

Fig. 2 Linear relationships with predicted line and 95 % confidence limits between **a** clutch size and **b** egg width and body size of females, **c** female body size and age, and **d** the mean proportion of second-clutch production and female age groups



Age and size distributions of sampled females

Because the numbers of reproductive females and their qualities vary annually, we examined the relative proportions of body sizes and ages of females present during the years of the life history study and the genetics study. Body sizes (CL) of females producing clutches sampled to determine paternity ($N=59$ females from 2003 to 2006) were not significantly different from the body sizes of females sampled during the years of the life history study (1983–2006; chi-square, $\chi^2=2.85$, $df=6$ (number of bins–1), $P=0.827$); however, age distributions differed significantly (chi-square, $\chi^2=48.81$, $df=6$, $P<0.001$). Age distributions of individuals changed as the study progressed and the proportion of known age individuals in the population increased. Over the years of genetic study, the size (CL) and age distributions of mothers of genetically sampled clutches in years with low (2003–2004) and high (2005–2006) numbers of reproductive females were not significantly different ($\chi^2=3.29$, $df=6$, $P=0.772$; $\chi^2=7$, $df=6$, $P=0.321$, respectively).

Genetic data and patterns of variation in multiple paternity

Adult painted turtles on the ESGR were genetically diverse at the loci assayed, allowing a statistically robust estimate of paternity. Estimates of genetic variability which included the mean number of alleles per locus over the seven loci (mean=17; across loci min-max=7–34) and mean expected heterozygosity (0.793; min-max among loci=0.682–0.888) were high. The expected paternal exclusion probability with the female parent known was 0.998. All loci were polymorphic and in

Hardy-Weinberg equilibrium, and no evidence of gametic disequilibrium was detected ($P>0.05$). Empirically estimated multilocus scoring error rate was less than 1 %. Of the 155 nests sampled to determine paternity, males were assigned to offspring in 108 nests (70 %). Concordance in paternity assignment among the programs (NEST, CERVUS, and GERUD with GENECAAP) was 94 %.

The number of males represented in clutches of females averaged 1.2 per year ($SD=0.38$) and 1.7 ($SD=0.86$) over the 4-year period. Multiple paternity was observed in 14.1 % of all clutches of eggs sampled and varied annually for the years 2003–2006 (30.0 %, $N=21$; 12.5 %, $N=40$; 14.3 %, $N=63$; and 6.1 %, $N=33$, respectively). The incidence of MP was similar in clutches of females that produced only a single clutch in the year sampled (13.5 %), compared to a sample of either first or second clutches of females that produced two clutches in the year sampled (13.8 %; $\chi^2=0.048$, $df=1$, $P=0.83$). In the years when the number of reproductive females was low, the incidence of MP was 12.5 % among clutches of all females that reproduced once in the year the clutch was sampled, but MP was 20.9 % among clutches of females that reproduced twice in the year the clutch was sampled. In contrast, in the years when the number of females was high, the incidence of MP was 14.3 % among clutches of all females that produced only one clutch in the year the clutch was sampled and was 9.2 % among clutches of females that produced two clutches in the year the clutch was sampled. The interaction of MP (single vs. MP), abundance of reproductive females (high vs. low years), and whether the clutch sampled was part of a pair of nests for that year (single vs.

pair) was significant ($\chi^2=292.55$, $df=11$, $P<0.001$). However, a comparison of proportional occurrence of MP between single clutches and pairs of clutches was not significant in either low or high years (GLMM: low, $Z=0.75$, $P=0.484$; high, $Z=-0.46$, $P=0.648$). Clutches of females that reproduced in the year following a nonreproductive year and clutches that were produced in consecutive years had similar incidences of MP ($\chi^2=0.14$, $df=1$, $P=0.706$).

Multiple paternity was positively associated with female age over 4 years (GLMM; age, $Z=2.01$, $P=0.044$) and in the two years with low numbers of reproductive females (GLMM; $Z=2.65$, $P=0.008$), but not in the 2 years with high numbers of reproductive females (GLMM; $Z=0.31$, $P=0.760$). Multiple paternity was not associated with female body size (CL) or clutch size (GLMM; $Z=-0.23$, $P=0.819$ and $Z=-0.32$, $P=0.752$, respectively).

Variation in repeat paternity

Repeat paternity (the same male-female pairs) with interclutch intervals from 1 to 4 years occurred in 38 % of the clutches sampled (Fig. 3). There were no significant relationships between the presence or absence of repeat paternity and the age or size of females (GLMM; age, $Z=0.04$, $P=0.966$; size, $Z=0.79$, $P=0.428$) or males (GLMM; age, $Z=1.04$, $P=0.300$; size, $Z=1.03$, $P>0.301$). A female's history of producing clutches sired by a single male or more than one male did not affect the probability of repeat paternity in subsequent clutches (single male 38.1 %, $N=42$ comparisons; multiple males=42.8 %, $N=14$ comparisons; GLMM; $Z=0.35$, $P=0.725$).

Components of male reproductive success

Of the 435 candidate males, 65 successfully mated with the 59 females whose clutches were sampled and offspring genotyped during 2003–2006. Among the successful males, the total number of offspring sired within years averaged 7.28 (min-max=1–19 offspring, $SD=3.92$; Table 1) and over the

4 years of study averaged 8.64 (min-max=1–32 offspring, $SD=5.81$). Male RS (number of offspring sired from 2003 to 2006) was not associated with the number of mates (GLMM, $t=1.47$, $P=0.144$).

We calculated the number of mates per male both annually and total over the 4 years. The majority of males did not have more than one mate annually and did not change mates between years. The maximum number of mates for males both within and among years was 2 (Table 1), and the average number of mates per year was 1.05 ($SD=0.23$). Deviation from one mate per year occurred only in 2005 (mean=1.15 mates), when the number of reproductive females was relatively high and four males (6.3 %) sired offspring with more than one female. The average of the total number of mates per male over 4 years (2003–2006) was 1.09 ($SD=0.30$) with six males (9.5 %) siring offspring with more than one female.

Male RS was significantly and positively associated with the number of clutches sired (GLMM, $t=10.11$, $P<0.001$) and the number of offspring sired per clutch (average=5.17, min-max=1–10; $SD=2.19$; GLMM, $t=3.41$, $P=0.002$). The average number of clutches sired was 1.4 ($SD=0.57$) per year and 1.7 ($SD=0.87$) over the 4 years of study. After we removed intra-annual clutch variation by randomly selecting and removing one of each paired clutch within the total sample, among-year repeat paternity was still sufficient to result in a positive association between male RS and the number of clutches sired (GLMM, $t=11.71$, $P<0.001$).

Age distributions of successful males did not differ from all sampled males (Fig. 4b, d; successful males' mean age=8.5; all sampled males' mean age=10.9; $\chi^2=6.97$, $df=8$, $P=0.540$). All sampled males and successful males had similar body sizes ($\chi^2=19.23$, $df=13$, $P=0.116$). The statistical difference between the distributions was driven mainly by a higher proportion than expected in both the largest size class and one small-medium size class (Fig. 4 arrows), but may also reflect substantial differences in sample sizes between the two groups. Among successful males, the total number of offspring sired was not associated with male body size or age (GLMM; size: $t=1.70$, $P=0.095$; GLMM; age: $t=-0.94$, $P=0.361$), but was marginally negatively influenced by MP (GLMM; $t=-1.89$, $P=0.06$).

Evidence of assortative mating

Frequency distributions of genetically identified mating pairs differed substantially from randomly generated mating pairs with respect to age ($G=20.17$, $df=7$, $P=0.005$), but not body size ($G=1.39$, $df=7$, $P=0.986$). The age of the female parent was positively correlated with the age of the male mate or mates (GLMM, $t=2.67$, $P=0.01$), whereas body sizes of female and male parents were not correlated (GLMM, $t=1.23$, $P=0.20$).

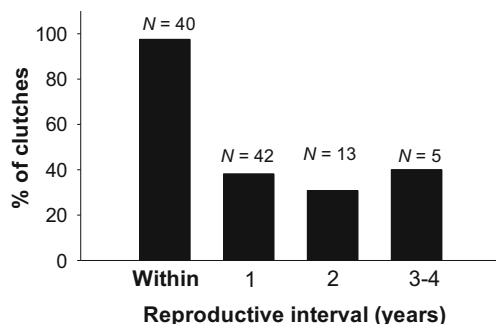


Fig. 3 Percent of clutches with genetic evidence of the same male and female parents (repeat paternity) within and among years

Table 1 Summary of the number of offspring, clutches, and mates per year and over 4 years (including repeat paternity) for successful male painted turtles (*Chrysemys picta*); the average number of mates was greater than 1 only in 2005 (1.15 mates/male)

Fixed effects	Per year	4 years			
	Average per male (min-max; SD)	Average per male (min-max; SD)	<i>t</i>	<i>P</i>	
Number of mates	1.05 (1–2; 0.23)	1.09 (1–2; 0.30)	1.47	0.144	NS
Number of clutches	1.41 (1–3; 0.57)	1.68 (1–5; 0.87)	10.11	<0.001	***
Offspring per clutch	5.13 (1–10; 2.18)	5.17 (1–10; 2.19)	3.41	0.002	**
Multiple paternity	–	–	–1.89	0.06	NS
Number of offspring sired	7.18 (1–19; 3.99)	8.64 (1–32; 5.81)	–	–	–

NS not significant

** significant at alpha <0.01; *** significant at alpha <0.001

Discussion

Variation in the number and qualities of reproductive females each year

We identified three major factors that influenced the relative importance of mate number and mate quality to variation in male RS: (1) the number and (2) qualities of reproductive females per year and (3) the overall variation in reproductive qualities among females in the population. Even though painted turtles occupy a highly productive permanent wetland on the ESGR (Fiala and Congdon 1983) and females store and allocate lipids to subsequent reproductions (Congdon and Tinkle 1982),

the number of reproductive females captured in East Marsh each year from 1983 to 2006 varied from 55 to 104. Resource levels and processing constraints during the summer activity period are likely major sources of variation in the number of reproductive females among years (Congdon 1989). Assuming that all males were reproductively active each year, variation in the number of reproductive painted turtle females appears to be high enough to substantially alter operational sex ratios. Annual variation in the number and qualities of reproductive females can alter male mating tactics by altering the costs and benefits of males and females attempting to mate multiply (Emlen and Oring 1977) and has been demonstrated in other poikilothermic vertebrates, such as lizards (*Lacerta vivipara*, Dreiss et al. 2010).

Variation in the proportion of East Marsh SCP females that produced two within-season clutches was positively correlated with the total number of reproductive females each year from 1983 to 2006 (McGuire et al. 2011). However, increases in overall reproductive quality due to a greater number of SCP females present in a year can be diluted by recruitment of the young reproductive females with low reproductive qualities (that represented from 33.3 to 76.4 % of new females in annual samples over the 4 years of the genetic study, 2003–2006). From 2003 to 2006, the lowest level of MP in clutches of painted turtles occurred in 2006 when the number of reproductive females was high (Fig. 1) and the proportion of young females was the highest: a combination suggesting that variation in the recruitment of young, low-quality reproductive females altered the relative importance of mate number and mate quality to males. The documented annual variation in the number and qualities of reproductive females within a population indicates that data collected from a single year can substantially misrepresent multiyear patterns of variation that are important to male reproductive tactics and success.

The benefits of producing two clutches in a season can be diminished if there are associated costs that reduce future reproductive output. Because the number and size of eggs in first and second clutches are similar, female painted turtles that produce two clutches of eggs within a season allocate two times the resources to reproduction compared to individuals

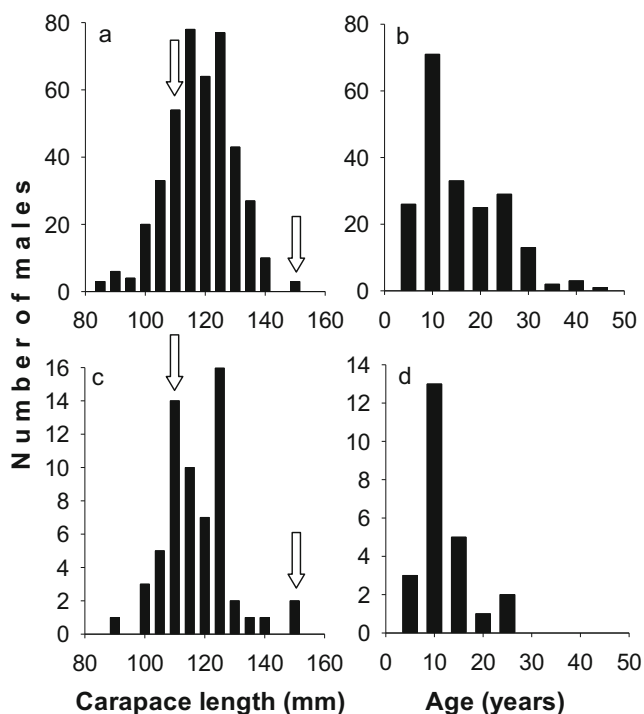


Fig. 4 Body sizes and age distributions of **a, b** all males sampled and of **c, d** males that mated successfully over the period of the genetic study (2003–2006); arrows indicate bins where the largest proportional differences occur between the two groups

that produce a single clutch. Resource limitations on reproductive output should result in longer interannual reproductive intervals compared to females producing only one clutch, a tradeoff that would reduce the benefits for males mating with SCP females, particularly if the likelihood of repeat paternity decreases with increasing reproductive interval. However, our results did not support resource limitation because (1) interannual reproductive intervals of SCP females were shorter than those of FCO females, (2) females producing second clutches at higher frequency had shorter interannual intervals than did females that produced second clutches less frequently, and (3) allocating resources to the second clutch did not reduce the probability of females reproducing in the following year.

In the sections below, we synthesize data demonstrating the influence of the number and qualities of reproductive females on three major components of male RS: (1) the proportion of a clutch sired (multiple paternity), (2) the reproductive qualities of the female(s) he mates with, and (3) the number of female(s) a male mates with.

Patterns of variation in multiple paternity

Male mate preference for females with traits that are reliable indicators of reproductive quality (Gwynne 1981; Amundsen 2000; Clutton-Brock 2007; Zhang et al. 2010) is expected to result in a higher incidence of MP in clutches of high-quality females (Uller and Olsson 2008). Painted turtle males should be able to use female body size as a reliable indicator of reproductive quality because female body sizes, ages, and reproductive traits were positively correlated (Fig. 2a–c; Congdon et al. 2003). Specifically, MP was expected to be higher in clutches of larger or older females than in clutches of smaller or younger individuals. Over the 4 years of genetic study, MP was positively associated with female age, but not body size, suggesting that male painted turtles rely on phenotypic traits other than body size to assess female age-specific qualities (e.g., the intensity and contrasts of red and yellow colors, Inouye et al. 2001). In contrast to increased mating attempts by males directed at higher quality females that result in higher MP, the longer reproductive intervals of lower quality females may result in increasing their opportunities to mate and store sperm between reproductive events and that may also increase the incidence of MP. We found no difference in the incidence of multiple paternity between clutches produced in the year following a nonreproductive year and those that reproduced in consecutive years. Therefore, differences in MP observed between low- and high-quality females may represent a difference in mating pressures between high- and low-quality females.

Costs and benefits associated with mating with high-quality females can be altered by the level of reproductive investment made by other males (Trivers 1972). If all adult males in the ESGR population are reproductive each year, the

substantial variation in the annual number of reproductive females (min-max=55–104 individuals) appears to be sufficient to alter operational sex ratios enough to influence mating tactics. Compared to 2005–2006, the numbers of reproductive females was relatively low in 2003 and 2004, and the occurrence of MP was higher (Fig. 1). However, the increase in MP in 2003–2004 was greater in clutches that were part of a pair (with one clutch of the pair randomly removed) than in clutches that were not part of a pair, whereas in 2005–2006, there was no difference between the two groups. Additionally, female age was positively associated with MP only when the number of reproductive females was low. Overall, our results suggest that as the numbers of reproductive females decrease and presumably operational sex ratios became more male-biased, more matings occurred per female and the increase occurred disproportionately among high-quality females.

Among successful males, the 14 % average occurrence of MP over 4 years was not a major source of variation in male RS. In other turtle species and in reptiles in general, the incidence of MP in clutches of females has been found to be much higher (in some cases >90 %; Jensen et al. 2006; Lee 2008; Uller and Olsson 2008) and therefore, may have a greater influence on male RS than MP does in ESGR painted turtles. However, because MP represents a double-edged sword where some males gain and others lose paternity, RS of individual males is influenced by MP even in cases when the effect of MP on population-level variation in male RS is minimal. For ESGR painted turtles, the reproductive benefits of mating with high-quality females offset the reduction in an individual male's RS that results from sharing paternity of a clutch (MP).

Importance of repeat paternity to male reproductive success

Mating with a high-quality female that uses the same male's sperm to fertilize sequential clutches within and among years is an important way for male painted turtles to increase RS. Many reptiles, including turtles, exhibit temporal asynchrony between matings and fertilization that necessitates the use of sperm storage (Birkhead and Møller 1993; Shuster and Wade 2003; Uller and Olsson 2008) and female turtles are capable of storing sperm for longer than a year (Ewing 1943; Gist and Jones 1989). On the ESGR, painted turtle females use sperm from the same male to fertilize eggs in 97.5 % of second within-season clutches (McGuire et al. 2011) and 38 % of paired clutches produced between and among years (Fig. 3). Similar frequencies of repeat paternity have been reported in other populations of painted turtles (100 % within season and ~40 % among years, Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002) and in other species of freshwater (58 % among years, Roques et al. 2006) and sea turtles (near 100 % within season, Fitzsimmons 1998; Phillips et al. 2013).

Previous work on painted turtles demonstrated that females with a history of MP (evidence of MP in any previous clutch) did not sire offspring with the same male(s) among years (0 % repeat paternity, $N=15$), whereas females with a history of producing clutches sired by a single male mated with the same male more often (22 % repeat paternity, $N=50$; Pearse et al. 2002). In contrast, ESGR female painted turtles with a history of MP had similar estimates of repeat paternity compared to females with a history of producing clutches sired by only a single male (MP females=43 % repeat paternity, $N=14$; single paternity females=38.1 % repeat paternity, $N=42$). Compared to the large and open population studied on the Mississippi River by Pearse et al. (2002), East Marsh is a relatively small (~4 ha) and more closed system (i.e., less connected to other wetlands than a site in a riverine system) and that should result in a higher probability of repeated interactions with the same males over time, even with 2.3 adult males per female in the population (Congdon and Gibbons 1996). Although repeat paternity occurred in clutches of ESGR painted turtle females produced up to 4 years apart, as inter-clutch intervals increase, the probable cause of repeat paternity shifts from the use of stored sperm from the same male to remating with the same male.

Despite the proposed benefits of multiple mating (Jennions and Petrie 2000), the relatively low occurrence of MP and the high frequency of repeat paternity among years in ESGR painted turtles beg the question: why would some females use sperm from the same male in multiple years? In the marine hawksbill turtle (*Eretmochelys imbricata*), the majority of females used sperm from the same male to fertilize successive within-season clutches, which was attributed to the use of stored sperm to assure fertility in situations of low male abundance (Phillips et al. 2013). However, in the ESGR population of painted turtles, mating opportunities are presumed to be high with 2.3 males per female and a stable population of ~450 subadults and adults within the 4.0 ha East Marsh (Congdon and Gibbons 1996). Therefore, the low number of mates per female does not appear to result from low densities of potential mates. Alternatively, the “trading up” hypothesis predicts that mating with a new individual will occur if a female subsequently encounters a higher quality mate or mates (Halliday 1983; Uller and Olsson 2008). Although iteroparity provides an opportunity for females to change mates between reproductive bouts, we found no consistent pattern of increasing or decreasing body size or age with a “new” male compared to the male who sired progeny in a previous clutch. However, even though paternity analysis indicated that the majority of clutches of a female are sired by only one male, our results do not eliminate the possibility that females were mating with multiple males and postcopulatory mechanisms are reducing the number of successful males (Zeh and Zeh 1997; Calsbeek and Bonneaud 2008; Le Roux et al. 2008).

Components of male reproductive success

Male RS (number of offspring sired) is a function of the numbers of mates, the number of clutches per mate, and (if females mate with different males) the proportion of offspring within the clutches that a male sires (Webster et al. 1995). We observed substantial variation in the total number of offspring sired among successful painted turtle males both within a year (1–19 offspring) and over 4 years (1–32 offspring; Table 1). However, variation in male RS was associated with the number of clutches sired, but not with mate number. Collectively, our results indicate that (1) repeat paternity resulting from a female’s use of stored sperm or by remating with the same male substantially contributed to male RS and (2) mate quality, particularly clutch frequency, is a more important source of variation in male RS in the ESGR population than is mate number.

If the first priority of males is securing any mate, and random mating occurs, all successful painted turtle males have a 74 % probability of mating with an East Marsh SCP female and a 26 % probability of mating with a FCO female. However, for those that mate with a SCP female, variation in RS would then be influenced by the rates that females produce second clutches (mean=25 %, min-max=7–49 % second-clutch production in years that they reproduce). Because age is positively associated with reproductive frequency, males that can identify and mate with older females characterized by higher within- and among-year reproductive frequencies will have substantially higher RS compared to males that mate indiscriminately. In many species, including reptiles, males that mate with high-quality females leave more offspring compared to those that mate indiscriminately (Altmann 1997; Shine et al. 2003; Zhang et al. 2010).

The genetic data on paternity provide two lines of indirect evidence of age-based preferences. First, multiple paternity is higher among high-quality (older) females, suggesting that males increase efforts to mate with older females. Second, genetically determined mating pairs deviated from randomly assigned pairs with respect to age (but not size). Specifically, we found evidence of positive assortative mating with respect to age. Because older females have higher reproductive frequencies (Congdon et al. 2003), and there is evidence of assortative mating by age (this study), we suspected that older males would have higher RS. However, neither body size (Fig. 4a, c) nor age (Fig. 4b, d) of the male parent was associated with male RS. Because variation in the ages of all males (and successful males) in the population is smaller than those of females, our ability to detect an age effect associated with male RS may have been influenced. The widespread presence of stereotypic courtship behaviors among painted turtle males (Taylor 1933; Ernst 1971) provides the opportunity for male or female choice, and the evidence of deviations from random matings associated with age found in this study

suggests that choice occurs; however, whether choice is male- or female-based requires detailed behavioral data.

Although variation in female quality can promote male mate choice (Darwin 1871; Bonduriansky 2001), simulation studies suggest that there are often costs to males attempting to mate with multiple females, which reduces the number of mates (Rittschof et al. 2012). Compared to species with complex social characteristics (e.g., male parental care, sex role reversal, or cooperative breeding), costs associated with multiple matings are often lower in species that have limited social characteristics (Trivers 1972; Clutton-Brock 1991; Clutton-Brock and Vincent 1991; Hauber and Lacey 2005). Therefore, costs associated with mating preferences based on female quality in less social taxa such as turtles should be easier to offset by benefits of mating with high-quality females. However, the low number of female mates per male (1.05 per year; 1.09 over 4 years) suggests that there are constraints on the ability of painted turtle males to successfully mate with more than one female.

At least three, nonexclusive mechanisms have the potential to limit the ability of male painted turtles on the ESGR to mate with more than a single female. First, the male-biased adult sex ratio (2.3:1) suggests that increased male competition for females may limit mate number. However, because females are able to store sperm for long durations (multiple years, Ewing 1943) from matings that presumably occur any time during the active season, temporal constraints on matings are relaxed compared to systems where mating seasons are short (Shuster and Wade 2003). Second, if male courtship behaviors or mate guarding is prolonged or energetically costly, the ability of males to mate with more than one female would be constrained. Male courtship in painted turtles involves chasing a female, followed by stroking the female with his foreclaws along the sides of her head and the area between the eyes and mouth (Taylor 1933; Ernst 1971). Males can be displaced by other males (Taylor 1933), suggesting that competition for females may be high, particularly in a population with a male-biased sex ratio. In natural settings, a single act of courting can last between 5 and 15 min, but in captive settings, males may court periodically for over 2 weeks before mating is accomplished (Ernst 1971). Therefore, successful male courtship may require a substantial investment in time and energy. However, costly courtship alone would not explain low mate numbers among years because a male should be able to successfully court different females in different years. Finally, males may attempt to mate with multiple females, but are excluded by female choice or through postcopulatory sexual selection. Postcopulatory sexual selection is widespread (Eberhard 1996; Birkhead and Pizzari 2002) and can result from mechanisms such as sperm competition or cryptic female choice (Eberhard 1996, 2009). Postcopulatory sexual selection could provide reproductive advantages to some males (e.g., via genetic compatibility, ejaculate quality, mating

order, and raffles) or exclude some males, which could reduce the number of mates of both sexes (Zeh and Zeh 1997; Calsbeek and Bonneaud 2008; Le Roux et al. 2008). Postcopulatory sexual selection could also result in a male being successful with the same female among years. In the absence of behavioral observations on mating tactics, genetic methods allow paternity to be identified, but do not allow us to disentangle pre- and postcopulatory mechanisms leading to the outcome of paternity.

Although painted turtles have been described as having a polygamous mating system (Wilbur and Morin 1988), our data suggest that ESGR painted turtles are better characterized as exhibiting limited polyandry. The majority of males successfully mated with a single female even among years, and females are seasonally monogamous with low incidences of MP and some mate switching among years. Over a 4-year period, some clutches of eggs were sired by multiple males within and among years (mean number of mates of females per year=1.2 and over 4 years=1.7), but males mated with the same female (mean number of mates of males per year=1.05 and over 4 years=1.09). The low mate numbers of both sexes within and among years contributed to female reproductive qualities having the strongest effect on male RS.

Although restrictions on mate number are often attributed to social factors, the surprisingly low mate numbers for both sexes in a less socially complex species such as painted turtles indicate that social interactions are not the only source of influence on mate number and that constraints on mate numbers may exist even when costs are not readily apparent. Any source of variation in female reproductive quality has the potential to increase the importance of female qualities to male RS. Reproductive frequency is one of the largest contributors to variation in reproductive output of female turtles (Gibbons 1982; Congdon et al. 2003), and a major function of stored sperm is to fertilize within-season clutches (Gist and Congdon 1998; Pearse and Avise 2001; Pearse et al. 2001; McGuire et al. 2011). Therefore, we predict that female quality will have the greatest influence on male RS in turtle species that produce multiple within-season clutches compared to species that produce a maximum of one clutch per season. Species of many taxa produce multiple clutches (litters) within a season including frogs, lizards, fish, birds, and mammals (Tinkle et al. 1970; Eisenberg 1981; Stearns 1983; Heins and Rabito 1986; Blomqvist et al. 2001; Morrison and Hero 2003) and, if coupled with repeat paternity (whether through rematings or use of stored sperm), can increase variation in male RS as a result of variation in female quality. Nonsocial factors such as sperm storage, declining population sizes, shifting sex ratios, and nonrandom habitat use can all increase the propensity for females to sire offspring with the same male. Collectively, the findings of this study highlight the need to consider factors other than complex social relationships when evaluating the importance of mate number and quality to variation in male RS.

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Ethical standards All research was conducted in accordance with the laws of the United States of America and the University of Michigan Animal Use and Care Committee (UCUCA #8496).

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