Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles

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Most studies of genetic parentage in natural populations have been limited to a single breeding season or reproductive episode and, thus, provide only a snapshot of individuals' mating behaviours. Female turtles can store viable sperm in their reproductive tracts for as long as several years, but the extent to which this capacity is utilized in nature has remained unknown. Here, we employ microsatellite markers to assess genetic paternity in successive clutches of individually marked, free-ranging female painted turtles (*Chrysemys picta*) over a four year period. The genetic data from 113 clutches from this natural population demonstrate that most females (80.5%) remated each year and that each female generally used a single male's sperm to fertilize all clutches laid within a year. However, sperm usage among females varied considerably, and some females apparently used sperm that had been stored for up to three years to fertilize some or all eggs laid in consecutive nesting seasons. Thus, remating by females is not necessary for continued offspring production from a given sire. Furthermore, 13.2% of all clutches examined showed evidence of multiple paternity, and the genetic paternity patterns across years suggest a 'last in, first out' operation of the females' sperm storage tubules.

Keywords: *Chrysemys picta*, microsatellites, parentage, paternity, sperm competition.

Introduction

The female reproductive tract of many species is physiologically capable of storing viable sperm for varying periods of time following a copulation event (Howarth, 1974; Smith, 1984): typically a matter of days in mammals, weeks in many insects and birds, months in some salamanders, and, incredibly, several years in some snakes and turtles (Birkhead & Møller, 1993; Galbraith, 1993; Palmer *et al.*, 1998). The suspicion of long-term sperm storage by female turtles originally came from observations that captive individuals maintained in isolation may continue to produce offspring long after contact with a male (Coker, 1920; Ewing, 1943), although by hard criteria such outcomes alone cannot eliminate the possibility of parthenogenetic reproduction. The deduction that females are capable of utilizing long-stored sperm later was bolstered by the physical finding of sperm sequestered in oviduct tissue (Gist & Jones, 1989). However, such observations do not reveal whether females in nature utilize long-stored sperm to fertilize their eggs, and if so, how often or under what circumstances.

Molecular markers of genetic paternity provide a new approach for critically examining issues on the utilization of female-stored sperm (Galbraith *et al.*, 1993; Fitzsimmons, 1998). Furthermore, genetic data from successive clutches within and across breeding seasons (Kvarnemo *et al.*, 2000; Oring *et al.*, 1992) offer an extended perspective on individual reproductive behaviours and population mating systems that might differ considerably from the single-time appraisals that have characterized nearly all previous genetic parentage studies in numerous taxa (Harry & Briscoe, 1988; Barry *et al.*, 1992; Avise, 1994; Hasselquist *et al.*, 1996; Gullberg *et al.*, 1997). Here we employ microsatellite markers in conjunction with long-term field observations to assess patterns of sperm utilization within and across four breeding seasons in a natural population of the painted turtle (*Chrysemys picta*), a species in which females have the potential to store sperm for long periods of time (Gist & Jones, 1989).

Materials and methods

Samples were collected from May to July 1995 to 1998, from a population on the Mississippi River near

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Thomson, Illinois, where nesting females have been tagged and monitored for more than a decade (Janzen, 1994; Morjan & Janzen, submitted). Upon first capture, each female was notched with a unique marginal scute pattern for subsequent identification, and a blood sample was taken and stored in lysis buffer (Seutin *et al.*, 1991). Painted turtles mate in the water, and each female later comes ashore one or more times during a breeding season to dig a shallow nest and lay a clutch of about a dozen eggs (Morjan & Janzen, submitted). A subset of the offspring was sampled from each observed nest laid by a marked female (mean offspring sampled per nest = 5.51, SD = 1.66; range = 1–13). These hatchlings were taken to the lab and preserved in 95% ethanol, initially for an examination of environmental influences on gender determination (Janzen, 1994; Morjan & Janzen, submitted). The current paternity analyses include hatchlings taken from 113 clutches laid by 32 females.

Microsatellite loci were obtained from a partial genomic library constructed for *C. picta* from the blood of one turtle. This library was screened with eight radiolabelled oligonucleotide probes for specific microsatellite motifs (di-, tri-, and tetranucleotides), following standard protocols. From the ≈ 1200 colonies screened, 20 positives were identified. Sequences were obtained using the *fmol* DNA sequencing system (Promega, Madison, WI) and 32P autoradiography, and five primer-pairs were designed from these sequences. Three of these amplified microsatellite loci (consisting of complex dinucleotide repeats in each case) proved scorable and highly polymorphic. Primer sequences were as follows: *Cp2*, U(CTCTAAGGGTTGCACTTCTCAAA), L(GAGGTGGCATCAAAACATCAT); *Cp3*, U(ATCTTTAAGTCT-GTGAACTTCAGGG),L(CTGTCTCATGCAAAGCTGGTAG); *Cp10*, U(GGTGCAGCAAGTTCAGGAGAC),L(GGTGTTAATGCACTGGA-GAATCA). At least two and often all three loci were utilized to genotype a grand total of 714 offspring, mothers, and population samples (other females whose nests were not sampled).

For adults, DNA was extracted from blood samples following standard phenol : chloroform protocols, and then was resuspended in deionized water. From hatchlings, DNA was extracted from liver tissue using Chelex (Bio-Rad). Preliminary screens for allelic variation, as well as genotypes for two broods, were performed using PCR primers end-labelled with 32P-ATP. All other genotyping was performed using fluorescent dye-labelled primers (Perkin-Elmer) and Applied Biosystems 377XL automated genotypers. All loci were amplified in 10 μL reactions consisting of 1× Promega Taq buffer, 0.1 mM dNTPs, 1.25 mM MgCl$_2$, 1 picomole of each primer, and 2.0 units of Promega *Taq* polymerase. The same PCR thermal profile was used for all loci: 94°C for

2 min, then 50 s at 94°C, 50 s at 52°C, and 50 s at 72°C, for 30 cycles.

Genetic sire(s) for each clutch were deduced by subtracting the known maternal contribution from the multilocus genotype of each offspring, as exemplified in Table 1. For each clutch, single paternity was considered the null hypothesis, and multiple paternity was inferred only when concordant support existed from multiple loci. Similarly, remating between clutches was considered the null hypothesis, and sperm storage was inferred only when multiple loci and/or hatchlings indicated parentage by the same father across clutches.

Results

Background

The three loci utilized displayed an average of 24 alleles per locus and a mean heterozygosity of 0.85. The

Table 1 Deduction of paternal genotypes from genetic data on mothers and clutches of painted turtles. Representative hatchling genotypes (alleles designated by numbers) are shown for the five assayed clutches laid by female 14. A single hatchling (denoted by ***) in the second 1997 clutch is apparently the result of sperm stored from the male that was the sire of all hatchlings in 1996

Year of clutch	*Cp2*	*Cp3*
Female 14	*216/222*	*141/145*
1996	206 222	145 178
	206 222	136 141
	206 216	136 141
	206 216	141 178
Male 1996:	*206/206*	*136/178*
1997a	202 216	141 196
	216 232	145 182
	202 222	145 196
	222 232	145 196
1997b	202 216	145 182
	222 232	141 182
***	206 216	141 178
	216 232	141 182
	216 232	141 196
Male 1997:	*202/232*	*182/196*
1998a	222 222	145 196
	216 222	145 196
	222 222	141 196
	216 222	141 167
1998b	216 216	145 167
	216 216	145 196
	216 222	145 196
	222 222	145 196
Male 1998:	*216/222*	*167/196*

combined paternity exclusion probability (Jamieson & Taylor, 1997) was 0.997 when all three loci were employed; it was 0.934 for the two loci (*Cp2* and *Cp3*) that in most cases were sufficient to deduce paternity of a clutch. Each locus appeared to segregate normally within progeny arrays, and deviations from Hardy–Weinberg equilibrium were not detected in the sample of 91 presumably unrelated individuals from the adult population (Hardy–Weinberg probability test: *Cp2*, $P = 0.95$; *Cp3*, $P = 0.23$; *Cp10*, $P = 0.34$; GENEPOP 3.1c, Raymond & Rousset, 1995).

No null alleles or *de novo* mutations were detected in any of the progeny arrays. However, one hatchling displayed a genotype at locus *Cp10* in which neither allele matched either of the maternal alleles, nor the paternal alleles present in this youngster's presumed siblings (despite the fact that this hatchling's genotypes at the other two loci were consistent with its known mother and the deduced father of the clutch). The explanation for this one aberrant individual is unknown.

Paternity analyses

Examples of three patterns of paternity are highlighted in Table 2. Data on genetic parentage for all 32 painted turtle females and their 113 clutches are summarized in Table 3, from which the following conclusions derive.

For sequential pairs of clutches within a nesting season ($N = 29$ pairs), no assayed female utilized sperm from an additional mating between nesting events. Thus, each year, most of the females apparently copulated with only one male and utilized his sperm to fertilize all clutches laid in that season. [Alternatively, she might

have had additional mates, but did not employ their sperm to fertilize her eggs (Fitzsimmons, 1998)]. In contrast, for clutches laid by a given female in sequential years, a new male sired the second year's clutch(es) on 34 occasions (77.3% of $N = 44$ such across-year clutch-pairs). In the remaining cases (22.7%), a female's offspring in successive years were sired by the same male. Many females used a single male's sperm for multiple clutches, so it appears likely that from each mating, fathers often fertilized more than one clutch (mean = 1.57, SD = 0.65; Fig. 1).

Multiple paternity was detected in only 15 nests (13.2%). Therefore, a single male usually fathered all of the hatchlings in a clutch. However, two distinct modes of multiple paternity were evident for the occasional instances of multiple sires within a clutch. In the first mode, a female appears to have acquired sperm from two males within a year, both of whom then sired some progeny in all nests sampled that year (see, for example, female 27 in Table 3). In these cases, the relative contributions to a clutch by the two males ranged from highly skewed to nearly equal (Pearse, Janzen, and Avise, manuscript in preparation).

The second mode of multiple paternity, found in six clutches, involved apparent low-level sperm storage for one or more years coupled with female remating (e.g. female 14 in years 1996 and 1997; Table 3). In such cases, some proportion (mean = 21% per clutch) of a female's offspring in the second year were fathered by a male who was the sole sire of her previous year's clutch(es). The modest percentages of hatchlings sired by the earlier-year male do not appear to be due solely to depletion or death of stored sperm, because in cases when the female did not

Table 2 Inferred paternal genotypes for the clutches of three female painted turtles, each of which displays a different pattern of remating behaviour: female 12 apparently remated each year but stored sperm within 1998; female 18 remated each year; and female 24 used a single male's sperm for three consecutive years

| Female | Maternal genotype | | | Clutch | Inferred paternal genotypes | | |
	Cp2	*Cp3*	*Cp10*		*Cp2*	*Cp3*	*Cp10*
12	*202/206*	*145/157*	*214/230*	1996	*202/206*	*161/182*	
				1997	*206/210*	*178/145*	
				1998a	*206/222*	*141/157*	
				1998b	*206/222*	*141/145* or *157**	
18	*206/252*	*145*	*235/240*	1995	*220/228*	*161*	
				1996	*206/226*	*136/192*	
				1997	*206/214*	*136/174*	
				1998	*200/206*	*138/145*	
24	*216/222*	*141/145*	*214/238*	1996	*206/220*	*141* or *145/170**	*218*
				1997a	*206/220*	*145/170*	*218*
				1997b	*206/220*	*145/170*	*218/256*
				1998	*206*	*145/170*	*218/256*

*When the putative male shares one or both alleles at a given locus with the female, the exact genotype cannot always be determined.

Table 3 Clutch data from each mother and inferred paternity for her clutches within and across nesting seasons. Clutches within the same year are separated by a comma; an ampersand indicates that two males shared paternity within a clutch. Based on inferred three-locus genotypes, males 9, 31, and 34 each appear to have fathered offspring of two different sampled females

Mother ID	Clutches sampled per year				No. of hatchlings assayed	Father ID				Total no. of fathers
	1995	1996	1997	1998		1995	1996	1997	1998	
1	—	—	2	1	16	—	—	1, 1	2	2
2	—	1	2	2	29	—	3	3, 3	4, 4	2
3	1	1	1	—	12	5	6 & 7	8	—	4
4	—	—	1	2	12	—	—	9	9, 9	1
5	1	—	1	1	17	10	—	11	12	3
6	—	2	2	1	30	—	13, 13	14, 14	15	3
7	—	—	2	1	17	—	—	16, 16	16 & 17	2
8	2	—	1	—	17	18, 18	—	19 & 20	—	3
9	1	—	1	2	25	21 & 22	—	22	23 & 24, 23 & 24	4
10	1	—	1	1	25	25	—	26	26	2
11	1	—	2	—	16	27	—	28, 28	—	2
12	—	1	1	2	21	—	29	30	31, 31	3
13	—	—	2	1	16	—	—	32, 32	33	2
14	—	1	2	2	30	—	34	35, 34 & 35	36, 36	3
15	1	1	1	1	21	37	38	39	40	4
16	—	—	1	2	16	—	—	41	42, 42	2
17	1	—	1	1	14	43	—	44	44 & 45	3
18	1	1	1	1	27	46	47	48	49	4
19	—	—	1	2	18	—	—	50	9, 9	2
20	—	1	2	1	18	—	51	51, 51	52	2
21	—	—	1	2	17	—	—	53	54, 54	2
22	—	1	2	1	21	—	55	56, 56	56	2
23	—	—	2	1	18	—	—	57, 57	58	2
24	—	1	2	1	22	—	59	59, 59	59	1
25	1	1	1	—	11	60	34	34*	—	2
26	—	1	1	1	6	—	61	62	62*	2
27	—	1	2	—	18	—	63	64 & 65, 64 & 65	—	3
28	1	—	2	—	15	66	—	67, 67	—	2
29	—	1	1	2	21	—	68	68 & 69	69, 69	2
30	—	—	2	1	19	—	—	70 & 31, 31	31 & 71	3
31	—	—	2	2	24	—	—	72, 72	73, 72 & 73	2
32	1	—	2	2	34	74 & 75	—	76, 76	77, 77	4

* Remating status uncertain due to small sample size, but consistent with sperm storage.
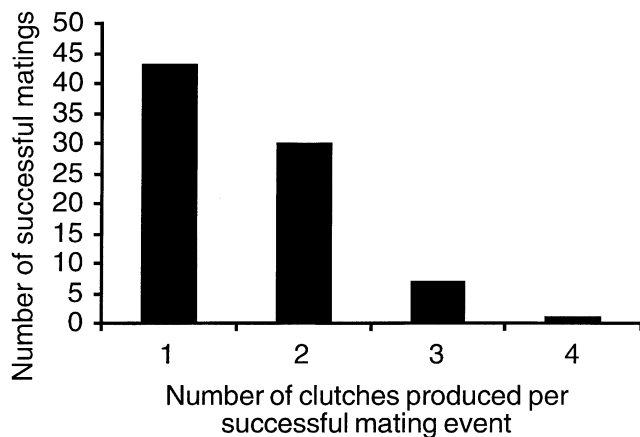
Fig. 1 Numbers of clutches sired per male per successful mating event in painted turtles. For example, female 2 (Table 3) had three clutches from a mating with male number 3 and at least two clutches from a mating with male number 4. In the absence of sperm storage, most or all males probably would have contributed to only one clutch per female. Note that this histogram underestimates the true number of clutches per successful mating because of temporal truncation effects (i.e. clutches were not sampled beyond 1998 or prior to 1995).

remate in the second year, the hatching success of her second-year clutch (mean = 0.86, SD = 0.14; Fig. 2) was not significantly different from that of her first-year clutch (mean = 0.83, SD = 0.19; Fig. 2).

Discussion

Overall, the genetic paternity analyses strongly suggest that these female painted turtles normally remate successfully only between nesting seasons, and that they frequently store and utilize sperm across multiple years. One potential caveat to the latter conclusion is that consecutive clutches sired by the same male might register remating by the female with that individual, rather than the storage of his sperm for long periods in her reproductive tract. Although we cannot rule out this possibility completely, such remating is unlikely for several reasons.

First, seldom were females found to employ a single male's sperm in a nonsequential (interrupted) sequence of clutches, yet such a dispersed pattern of paternity could be common if females often remated with particular males during their lifetimes. Second, the population size at this location is large, numbering several hundred adult males at least, and probably more than 1000 sexually mature individuals (as judged by field observations and various lines of mark–recapture evidence; Pearse, Eckerman, Janzen, and Avise, submitted). Finally, this species is highly mobile (MacCulloch & Secoy, 1983), so females probably encounter many
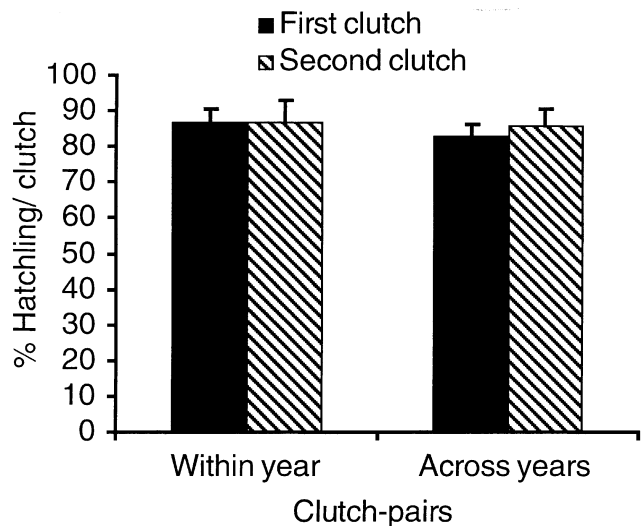

Fig. 2 Hatching success of clutch-pairs that were sired by a single male either within ($n = 29$) or across years ($n = 9$). Hatching success in first and second clutches (solid and lined bars, respectively) did not differ significantly within years ($t = 0.015$, $P = 0.99$) or across years ($t = -0.39$, $P = 0.70$).

males from outside the immediate area over the course of a breeding season. All of these biological points argue that inter-year matings between the same pairs of painted turtles in this population are unlikely to underlie most of the genetically deduced instances in which a female's consecutive clutches were fathered by the same male.

In female painted turtles, stored sperm evidently can be sufficient to produce clutches over multiple seasons. Thus, these genetic data imply that females remate for reasons other than the acquisition of gametes for fertilization (Fig. 2; also see Reinhardt *et al.*, 1999 for similar findings in a grasshopper). One possibility for this long-lived species (life span more than 30 years; Ernst *et al.*, 1984) is that serial monogamy confers one or more life-long genetic benefits to a female similar to those within a multiple-paternity clutch in a short-lived species: e.g. an opportunity for 'better' paternal genes for progeny, or production of offspring with a broader collective diversity of genotypes (Parker, 1984; Gowaty & Bridges, 1991; Madsen *et al.*, 1992). In this cumulative way, a female turtle might receive possible benefits of multiple paternity over several years of reproduction, rather than within a clutch. Against such suspected benefits are the energetic or ecological costs to females that multiple matings also might entail (Birkhead & Møller, 1992).

From the male's perspective, sperm storage confers more evident fitness opportunities, as well as costs. Whereas sperm storage usually is thought of as a female adaptation that separates copulation from nesting,

encourages sperm competition, or allows for cryptic female choice (Olsson & Madsen, 1998), the production of sperm that can survive long-term storage in females clearly can benefit successful males by increasing the number of eggs that they may fertilize (Fig. 1). For rival males, the flip side of that coin is that female-stored sperm in effect can 'steal' some of the potential fertilization events otherwise available to them (Oring *et al.*, 1992). For example, in the current cases where females remated between years but also utilized some stored sperm, their second year mates lost on average about 21% of their reproductive potential to stored sperm from the previous year's male.

The pattern of stored sperm use in these cases can also be informative with respect to the mechanism of sperm storage. Two of these females (numbers 14 and 31, Table 3) produced two (rather than one) assayed clutches in the second year. In both cases, the fertilization(s) by sperm stored from the first-year father were confined to the second of her clutches in the subsequent year. One reasonable interpretation is that in her initial clutch of the second year, the second male's sperm took precedence over the first (Birkhead, 1998), perhaps by stratification, displacement, or some form of female choice. This pattern of 'last in, first out' generally follows paternity predictions based on details of the morphology of the female reproductive tract and the timing of ovulation and fertilization in turtles (Gist & Congdon, 1998).

An additional observation regarding patterns of sperm use involves cases where a female did not remate after laying a multiply sired clutch. In each of three such cases (females 9, 29 and 30, Table 3), a later clutch was singly sired by just one of the two fathers from the previous clutch. Thus, through some combination of sperm depletion, sperm competition, or cryptic female choice, one of the males gained substantial fitness by siring an additional clutch not shared with the other male.

Snapshot investigations of genetic paternity in many species have provided powerful information about parentage, mating behaviour, and the mating system in a given breeding episode. However, extended temporal analyses can offer additional perspectives. Previous studies of paternity patterns in multiple clutches within a year have indicated that female turtles in nature can store sperm throughout a nesting season (Galbraith *et al.*, 1993; Fitzsimmons, 1998). In the current study of painted turtles, a snapshot view merely would have indicated that females are primarily monogamous. However, our temporally extended genetic survey yields a richer documentary: frequent mate-switching by females across but seldom if ever within years; storage and utilization of sperm by females for long periods of time (at least three years in some cases); and a stratified pattern of sperm usage from multiple matings dispersed in time.

In the future, it would be desirable in studies of turtles or other long-lived species to extend genetic investigations of the current sort to assess life-long means and variances in reproductive success, as has been done in some cases from behavioural data (Clutton-Brock, 1988). Such studies would bring us closer to an understanding of the relationships between mating behaviours and lifetime genetic fitness.

Acknowledgements

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