Nesting fidelity and molecular evidence for natal homing in the freshwater turtle, *Graptemys kohnii*

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Numerous studies of sea turtle nesting ecology have revealed that females exhibit natal homing, whereby they imprint on the nesting area from which they hatch and subsequently return there to nest as adults. Because freshwater turtles comprise the majority of reptiles known to display environmental sex determination (ESD), the study of natal homing in this group may shed light on recent evolutionary models of sex allocation that are predicated on natal homing in reptiles with ESD. We examined natal homing in *Graptemys kohnii*, a freshwater turtle with ESD, using mitochondrial sequencing, microsatellite genotyping and mark and recapture of 290 nesting females. Females showed high fidelity to nesting areas, even after being transplanted several kilometres away. A Mantel test revealed significant genetic isolation by distance with respect to nesting locations ($r=0.147$; $p<0.05$), suggesting that related females nest in close proximity to one another. The patterns of fidelity and genotype distributions are consistent with homing at a scale that may affect population sex ratios.

Keywords: natal homing; turtle; sex ratio; environmental sex determination; microsatellite genotyping

1. INTRODUCTION

In oviparous reptiles, nest-site selection can be an important component of fitness. Nest failure rate for turtles is often as high as 90%, as many nests succumb to flooding, desiccation and depredation (Wilbur & Morin 1988). In all but one turtle species, females abandon nests once they are laid and thus receive neither negative reinforcement if a nest site produces few hatchlings nor positive reinforcement if a nest site is successful. Natal homing, the phenomenon by which a female returns to nest at the site from which she was hatched, can be favoured by selection because it ensures that a female nests at a location that successfully hatched offspring in the previous generation (Reinhold 1998; Freedberg & Wade 2001). Selection should therefore favour natal homing when the environment is locally predictable and when there is significant variation in fitness associated with nest-site location.

Genetic evidence for natal homing in reptiles comes from sea turtles and giant river turtles, both of which migrate hundreds to thousands of kilometres from feeding grounds to nesting areas (reviewed in Bowen & Karl 1996; Valenzuela 2001). In contrast to these migratory turtles, the home ranges of most freshwater turtles are confined to lakes or small stretches of rivers. Thus, studies of nesting for migratory turtles may reveal little about the factors affecting nesting choice in many freshwater turtles. While nest-site fidelity by adult females has been observed in several freshwater turtles (e.g. *Emydoidea blandingii* (Congdon *et al.* 1983), *Chelydra serpentina* (Loncke & Obbard 1977), *Pseudemys concinna* (Jackson & Walker 1997) and *Chrysemys picta* (Valenzuela & Janzen 2001)), it

is not known whether these observations of site fidelity are associated with natal homing. Although genetic structuring with respect to nesting areas has been observed in *C. picta*, the structuring is attributed primarily to gametic correlations between offspring from successful nests prior to dispersal, not female homing (Scribner *et al.* 1993).

Two recent theoretical models of sex allocation under environmental sex determination (ESD) have been predicated on the existence of natal homing. Reinhold (1998) showed that natal homing can favour the evolution of ESD while Freedberg & Wade (2001) demonstrated that natal homing will result in female-biased sex ratios under ESD. Although the evidence supporting natal homing in sea turtles is strong, these turtles comprise a very small fraction of the reptile species known to display ESD (Janzen & Paukstis 1991). The study of natal homing in non-migratory freshwater turtles should provide valuable insight into evolutionary models of sex allocation under ESD since they comprise the majority of vertebrate species known to possess ESD (Korpelainen 1990).

Bull & Charnov (1988, 1989) initially cited the female-biased primary sex ratios observed in map turtles (55 and 76% female) as part of a trend of unexplained female biases in reptiles with ESD. While these sex ratios reported for map turtles might be due to atypically warm summers at the study site or dredging operations that increased the amount of open sand in the nesting areas, the skewed primary sex ratios are concordant with female-biased adult sex ratios observed for map turtles (Timken 1968; Vogt 1980). Although potential mechanisms explaining the female biases in other reptilian species have recently been characterized (Freedberg & Wade 2004), map turtles stand out as one group where the skewed sex ratios are still unexplained. The study of natal homing, a phenomenon

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that can theoretically lead to skewed sex ratios, can thus be particularly valuable in this group.

We studied the nesting behaviour and population genetic structure of the Mississippi map turtle, *Graptemys kohnii*, a non-migratory freshwater turtle with ESD. The study was conducted at Reelfoot Lake, TN, a 15 500-acre lake created by earthquakes in the period 1811–1812. We employed mitochondrial sequencing and microsatellite genotyping on nesting females collected in different nesting areas to determine if the population genetic structure was consistent with the initial colonization of nesting habitats followed by descendants homing to these sites. We also used a transplant and recapture design to determine the extent to which females returned to the same nesting areas. If females consistently returned to their original nesting location after being transplanted to a foreign nesting area, it would indicate strong fidelity to nesting sites—a key requirement if turtles home to natal sites.

2. METHODS

(a) *Field and laboratory methods*

In June of 2000–2003, nesting female turtles were hand-collected while on land at Reelfoot Lake, TN, during all daylight hours from two areas of high nesting concentration: 4 km along the eastern shore, and 1.5 km along the western shore (figure 1). At their closest points, these areas are approximately 4.8 linear kilometres apart, with 6.5 km of shoreline separating the areas. We examined fine-scale homing in the eastern nesting area by placing markers equidistantly along the shoreline, creating 25 transects perpendicular to the shoreline, each with a width of 160 m and a length that extended to the edge of the nest area (ca. 200 m). This method was similar to one previously used to detect fine-scale homing in sea turtles (Peare & Parker 1996). In this population, we have found that females generally lay two to four clutches per nesting season. Turtles were collected while they were either digging or approaching, or returning from, the nest site. We could safely assume that females moving on land were doing so to nest because females rarely move far on land except to nest. When captured, nearly all females showed signs characteristic of approaching a nest site (cloacal expulsion of water) or returning from nesting (mud on posterior of plastron) and, when palpated, females approaching a nesting site were always gravid while females returning from nesting were not. Although the precise nesting site was not observed for many females, those females that were observed ovipositing appeared to take a relatively direct path from the water's edge to their nest site and back, suggesting that the site of capture was a good indicator of the transect in which the turtle nested. We recorded the location of each turtle's capture, clipped the tail tip for genetic analysis and preserved the tail tip in 95% ethanol.

From 2001 to 2003, nesting females were individually marked for future identification by marginal scute drilling and transplanted and released at the opposing nesting area. When marked turtles were recaptured in subsequent nesting periods, the location was noted and the turtle was released at the opposing nesting area from where it was originally collected, regardless of where it was recaptured. More specifically, turtles collected along the eastern nesting area were released at the northern tip of the western nesting area, a linear distance of 6.6 km from the middle of the eastern

Figure 1. Collection sites of nesting turtles. Collection efforts were focused on two areas of high nesting concentration. The solid bar (left) shows the western nesting area, the slashed bar shows the transected eastern nesting area. The nesting areas are separated by 4.8 linear kilometres at their closest points. Image modified from <http://www.census.gov/geo/www/maps/>.

nesting area. Turtles collected from the western area were released at a location on the eastern end of the eastern nesting area, a linear distance of 6.2 km from the middle of the western area. Most turtles were captured and released before laying their eggs, although a small fraction of turtles were not captured until they had completed their nests. Because turtles were not marked for identification in 2000, any turtle collected in the final three years of the study that already had a clipped tail but had not been scute marked was assumed to have been collected and DNA sampled in 2000. These turtles were not resampled for the 2001–2003 genetic analysis but were scute marked, transplanted and released. Occasionally, other emydid turtles (*Trachemys scripta*, *Graptemys pseudogeographica* and *P. concinna*) were observed nesting throughout the course of the study. The location of capture was recorded for a small number of these turtles and the turtles were marked and transplanted in the same manner as the nesting *G. kohnii*.

Tissue samples were preserved in 95% ethanol and refrigerated at approximately 4 °C until DNA extraction. DNA was extracted with a Puregene DNA extraction kit for cells and tissue (Gentra Corporation, Minneapolis, MN). We used the microsatellite primers SH2 (5'–3': GGAAACAGCTATGACCATGGCCAG-CAGGAGTAATG; CTATTAGGGCAGAGACGAG), SH5 (TTGCTGCTATATGCTTAAT; GGAAACAGC-TATGACCATCCTCCCTGCCTATTGA) and SH7 (CAGTCGGGCGTCATCCACACACACTGTATTTTG-ATA; CTATGCCCTTTCTAGTTTG) developed for the closely related *Malaclemys terrapin* (Hauswaldt & Glenn 2003). Polymerase chain reaction (PCR) amplification was performed in 10 µl volumes consisting of 10 mM Tris pH 8.4, 50 mM KCl, 0.2 µM unlabelled primer, 0.02 µM fluorescently labelled primer, 1.5 mM MgCl₂, 0.15 mM dNTP, 0.5 units *Taq* DNA polymerase and 50 ng DNA template. For primers SH2 and SH7, an initial amplification cycle (4 min at 95 °C) was followed by 30 cycles of

40 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C, and a final 8 min extension at 72 °C. For primer SH5, the annealing temperature was lowered to 51 °C. The samples were genotyped on the Indiana University Molecular Biology Institute's ABI 3730 capillary sequencer using GENEMAPPER v. 3.5 software (Applied BioSystems, Foster City, CA, USA).

Sequences for the mitochondrial primer MS1, a 387 bp fragment of the mitochondrial control region, were obtained from M. Sorenson at the University of Massachusetts (CAAGGGTGGATCGGGCATAAC; GTGCCTGAAA-AAACAACCACAGG). PCR amplification was performed in 25 µl volumes consisting of approximately 50 ng DNA, 2.5 µl 10× tricine taq buffer, 1.5 mM MgCl₂, 0.005 µg of each primer, 100 µM of each dNTP and 0.5 unit *Taq* polymerase. An initial amplification cycle (4 min at 95 °C and 5 min at 72 °C) was followed by 40 cycles of 40 s at 95 °C, 1 min at 55 °C and 2 min at 72 °C, and a final 8 min extension at 72 °C. PCR products were purified with QIA quick PCR purification kits (Qiagen, Valencia, CA) using a microcentrifuge extraction protocol. The purified PCR product was amplified with an initial cycle (2 min at 96 °C, 15 s at 50 °C and 4 min at 60 °C) followed by 25 cycles of 30 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C. Sequencing was conducted on the Indiana University Molecular Biology Institute's ABI 3700 capillary sequencer. Sequences were processed, aligned and analysed with SEQUENCHER 3.1.

(b) Statistical analysis

A χ^2 test was performed to compare the proportion of the within-year recaptures that returned to their original capture area with the proportion of across-year recaptures that returned to their original capture area. For the three turtles that were recaptured more than once within a year, only the first recapture was included in the analysis. Similarly, only the first across-year recapture was included in the analysis for the one turtle that was recaptured multiple times across years. For each recaptured individual from the eastern nesting area, the distance between the original transect of capture and the transect of recapture was calculated. Mean distances (δ) were calculated for the 11 within-year and 20 across-year recaptures from the eastern nesting area (i.e. for a turtle recaptured in a transect adjacent to its original transect, $\delta=1$). We then created null models by computing 50 000 randomly chosen distances from 11 to 20 pairs, respectively, of individuals from the underlying distribution of captures. To test whether females showed fidelity to their original transect, the actual observed mean distances for within and across-year recapture were compared with the mean distances from the null models.

Pairwise F_{ST} values between the eastern and western nesting areas were estimated for both the microsatellite (F_{ST}) and mitochondrial DNA sequence data (Φ_{ST}) using ARLEQUIN (Schneider *et al.* 2000). Because this population is less than 200 years old, any genetic structuring observed should be due to the initial colonization of nesting areas followed by homing by descendants to these locations, and not *in situ* mutation accumulation. We thus used an infinite allele model to calculate F_{ST} values, because it assumes no relation between different allelic states.

We used F_{ST} and Φ_{ST} to calculate pairwise F_{ST} values for each pair of transects in the eastern nesting area and performed a Mantel test examining the correlation between pairwise transect F_{ST} and the geographical distance between

transects. Because we were testing for structuring created by females sharing alleles within the same transects, the three transects containing only one nesting female were not included in either Mantel test. We tested for departures from the Hardy–Weinberg equilibrium by comparing expected versus observed heterozygosity after binning rare alleles at all three microsatellite loci (Gottelli *et al.* 1994).

For the microsatellite data, a post-hoc χ^2 test was performed to determine whether the frequency of between-transect F_{ST} values with significant deviations from zero ($p<0.05$) increased with geographical distance. Specifically, we compared the proportion of significant F_{ST} values among transects that were located within five transects of one another with the proportion of significant F_{ST} values among transects located more than five transects apart from one another.

3. RESULTS

A total of 290 nesting map turtles were collected in the two nesting areas. Twenty-six turtles were recaptured within the same year they were originally caught and 39 turtles were recaptured in subsequent years. Seven mitochondrial haplotypes were observed and 90% of the population was characterized by two common haplotypes, which represented 165 and 97 individuals, respectively. The remaining five haplotypes were found in nine, seven, seven, three and two individuals, respectively. Haplotype diversity was low (i.e. gene diversity = 0.56). Microsatellite loci SH2, SH5 and SH7 yielded 14, 11 and 11 alleles, respectively, producing over 200 distinct genotypes. All three loci conformed to Hardy–Weinberg expectations. The high gene diversity of the microsatellite markers (i.e. expected heterozygosity = 0.84) relative to the mitochondrial sequence data indicates that the microsatellite data offer substantially greater power to detect population genetic structure.

Fidelity to the original nesting area was significantly greater across years than within years ($\chi^2=11.1931$, d.f. = 1, $p<0.001$). Thirty-eight of 39 turtles returned to the same nesting area across different years, while only 14 of 26 turtles returned to their original nesting areas within the same year (Appendix A). The across-year fine-scale fidelity (transect fidelity) was significant ($\delta=1$ transect; $p<0.0001$); 18 of 20 turtles from the eastern nesting area were recaptured either in the same 160 m transect as the original capture (11 of 20) or in a transect immediately adjacent to the transect of their initial capture (7 of 20). Within-year fine-scale fidelity was also significant ($\delta=0.73$ transects, $p<0.0001$); when turtles from the eastern area were recaptured in the same year, 9 of 11 turtles were found either in the same transect (6 of 11) or in an adjacent transect (3 of 11; figure 2). The two *P. concinna*, two *T. scripta* and one *G. pseudogeographica* from the western nesting area that were later recaptured had returned to the western nesting area.

The pairwise F_{ST} analysis of the microsatellite data revealed significant but weak genetic structure between the eastern and western nesting area ($F_{ST}=0.0026$, $p<0.05$). The pairwise F_{ST} analysis of mitochondrial haplotypes showed marginally significant differentiation between the eastern and western nesting areas ($F_{ST}=0.0173$, $p=0.056$).

The Mantel test of microsatellite data revealed a positive correlation between pairwise transect F_{ST} and

Figure 2. Transect fidelity for females recaptured along the eastern nesting area. Females returning to the eastern nesting area were not spaced randomly and were more likely to be recaptured in a transect close to their original transect of capture both across years ($p < 0.0001$) and within years ($p < 0.0001$).

geographical distance along the eastern nesting area ($r = 0.147$, $p < 0.05$; figure 3). Statistically significant F_{ST} values were less likely to occur among transects located within five transects of one another than in transects located more than five transects apart (0/95 versus 10/136, d.f. = 1, $\chi^2 = 7.30$, $p < 0.01$). No significant relationship was detected between mitochondrial haplotype and the transect of capture within the eastern nesting area ($r = -0.002$, $p = 0.66$).

4. DISCUSSION

Graptemys kohnii showed high fidelity to capture locations across years after being transplanted to a nesting area more than 6 km away. We found evidence of genetic structure between the western and eastern nesting areas with both nuclear and mitochondrial markers. Further, we found genetic isolation by distance among nesting females within the transected eastern nesting area, indicating that closely related females tended to nest in close proximity to one another. It is unlikely that low dispersal of males from the natal site is contributing to this pattern because map turtles take several years to mature and there are no barriers to movement along the lake. These findings suggest that nesting non-migratory freshwater turtles, which constitute the largest group of animals with ESD, can inherit nesting areas across generations.

Turtles readily swam 6 km back to their original capture site and were often observed there within 1–2 days of being released. In addition, similar fidelity was observed in a small number of individuals from three other species, suggesting that this phenomenon may be common in freshwater turtles. One *G. kohnii* that was initially found nesting along the eastern area was transplanted and recaptured there three times in the next 6 days, having travelled a minimum of 20 km over

Figure 3. Mean pairwise F_{ST} values at each distance interval versus geographical distance. Negative mean F_{ST} values were converted to zero since negative values probably represent a negligible genetic structure at that interval. No F_{ST} values that deviated significantly from zero were observed between transects located within five transects of one another.

that time to return to its original nesting area (Appendix A). Females were more likely to exhibit fidelity across years than within the year that they were transplanted. While all but one of the turtles recaptured after one year had returned to their original nesting area, nearly half of the turtles recaptured within the same summer in which they were transplanted were recaptured at the nesting area where they were released. Because most turtles were captured and transported before laying their eggs, many turtles recaptured within the same year had held their eggs beyond the time they usually attempted to lay them. The failure to show strict fidelity within the same year as transplantation may be attributable to the temporal constraints of impending oviposition, which could prevent turtles from returning to their desired nesting sites. The four turtles that were recaptured at a transplanted area the same summer they were released and recaptured in a subsequent year had all returned to their original nesting area. These findings provide further evidence that nest-area selection is motivated by more than just suitable nesting habitat since transplanted turtles that had nested in viable foreign nesting areas failed to nest there in subsequent years.

The precision of fidelity observed was high; 27 of the 31 times that turtles were recaptured along the eastern nesting area (either within or across years), they were found in the same or adjacent 160 m transect. However, the remaining four that returned to the eastern shore were each captured more than 160 m away from their initial site. Thus, either fine-scale homing is not dependably exhibited by all females, or females occasionally take indirect paths on land to or from their nest site.

The significant and positive correlation observed between mean pairwise F_{ST} and geographical distance within the eastern nesting area is especially notable given that the upper limit of this correlation is constrained by the fact that males are unlikely to exhibit homing to transects, and that females from unrelated lineages may be homing to nesting sites within the same transect. The significant correlation observed indicates that natal homing is strong enough to produce genetic isolation by distance among nuclear markers, despite these factors providing noise in the dataset. Moreover, genetic

correlations between hatchlings from successful nests cannot account for the genetic structure among nesting locations because only adult females were surveyed (Scribner *et al.* 1993).

Although we detected significant isolation by distance with the microsatellite markers between transects within the eastern nesting area, we did not detect a significant difference in mitochondrial haplotype distributions among transects. The extremely low haplotype diversity observed in this study ($h=0.56$) hindered our ability to detect fine-scale homing: one haplotype was found in more than half of all turtles and 90% of the *G. kohnii* in this study exhibited one of two haplotypes, suggesting that each of these common haplotypes represent several distinct founding lineages within this population. Therefore, several transects that were genetically indistinguishable with regard to these markers are likely to have been characterized by completely unrelated maternal lineages. Conversely, all three microsatellite loci were highly variable and over 100 genotypes were observed on the eastern nesting area alone.

We can gain additional insight into the mechanisms underlying the detected patterns of genetic differentiation by considering quantitative estimates of gene flow. The ratio of total gene flow between nesting areas to maternal gene flow between the eastern and western nesting areas can be calculated with

$$(1/F_{ST \text{ nuc}} - 1)/(2/F_{ST \text{ mt}} - 2),$$

where $F_{ST \text{ nuc}}$ is the F_{ST} calculated from the microsatellite data and $F_{ST \text{ mt}}$ is the F_{ST} calculated from the mitochondrial data (McCauley 1998). The calculated m ratio of 3.32 suggests that gene flow is three times more common in the nuclear genome than in the mitochondrial genome, and thus gene flow among males may be more than six times more common than in females. This pattern is expected if the structuring between nesting areas is created by maternal homing because greater gene flow among males between nesting areas should increase paternal gene flow relative to maternal gene flow. While the finding of increased gene flow among males does not exclude the possibility that males are homing to their natal areas (FitzSimmons *et al.* 1997), it suggests that they must also be mating with females from other nesting areas.

(a) Natal homing and sex allocation

A recent model by Freedberg & Wade (2001) provides a theoretical underpinning for the otherwise unexplained occurrences of female-biased sex ratios in ESD reptiles (Bull & Charnov 1989). Specifically, they show that natal homing, in conjunction with ESD, will bias sex ratios towards females. A maternal lineage that produces an excess of daughters, who return to the proven, natal nesting site, will be favoured over maternal lineages producing an excess of sons when the nesting area is inherited maternally. This type of selection favouring maternal lineages that produce excess daughters is analogous to the female biases that occur when maternally inherited parasites or genetic elements distort the sex ratio (Taylor 1990). In contrast, Reinhold (1998) predicts that natal homing can favour the evolution of ESD. His model states that female fitness is more strongly affected by

Table 1. Recapture locations of nesting females.

(E and W denote the eastern and western nesting areas, respectively. Numbers denote transect locations along the eastern nesting area; zero is the westernmost transect.)

ID no.	original capture	transect	recapture location (within a year)	recapture location (across years)
B24	E	0	W	—
P63	E	2	—	1
D16	E	2	—	3
D40	E	3	4	4
P17	E	4	4	—
B15	E	4	4	4
B2	E	4	4, W	—
P16	E	4	W	—
P45	E	4	W	—
D42	E	4	W, W	4
B7	E	4	—	4
P8	E	4	—	4
D12	E	4	—	4
D44	E	4	—	13
B20	E	5	4	5
B17	E	6	6	6
P27	E	13	W	—
D5	E	13	—	13
B6	E	13	—	9
B29	E	14	14, 14	14
D10	E	14	—	13
X22	E	16	16	—
P30	E	16	13, 15, 21	—
D45	E	16	—	17
P68	E	17	—	17
P14	E	20	22	—
P48	E	21	20	—
P37	E	21	W	—
B4	E	21	—	20
B21	E	21	—	21
B11	E	22	—	21
E6	W	—	W, 13	—
A14	W	—	—	W
D32	W	—	—	W
E21	W	—	5	—
A27	W	—	8	W
A9	W	—	12	—
A28	W	—	13	W
A22	W	—	13	—
E29	W	—	16	—
A48	W	—	W	W
E30	W	—	W	—
A40	W	—	—	11
A8	W	—	—	W
A19	W	—	—	W
A12	W	—	—	W
A15	W	—	—	W
A20	W	—	—	W
A24	W	—	—	W
A41	W	—	—	W
A7	W	—	—	W
A11	W	—	—	W
A52	W	—	—	W
A6	W	—	—	W
E12	W	—	—	W
E28	W	—	—	W

nest-site quality, since females transmit the site to their female descendants. Consequently, selection favours the evolution of ESD since ESD allows for the production of females at high quality nest-sites.

Both models are founded on the assumption that females home to natal sites on a scale small enough that different inherited nesting areas are characterized by different mean sex ratios. In this study, we found evidence that females transmit nesting areas across generations. While yearly thermal variation will impact offspring sex ratios, the physical properties of nesting areas, such as solar exposure and overstory vegetation, can produce consistent differences in the distributions of offspring sex ratios (Vogt & Bull 1984; Janzen 1994), indicating that turtles with fidelity to different nesting areas are likely to produce different sex ratios over their lifetimes. Although we did not measure offspring sex ratios, there was observable heterogeneity in the amount of overstory vegetation among nesting transects, suggesting that these transects are characterized by different offspring sex ratios. Furthermore, sex ratio variation among different nesting locations and female-biased sex ratios has been observed in other map turtle populations (Timken 1968; Vogt 1980; Vogt & Bull 1984). The correlation between nesting habitat and offspring sex ratio observed in many studies indicates, in conjunction with our findings that females home to natal areas, that freshwater turtles may transmit offspring sex ratios through imprinting nesting areas. This in turn supports the conclusion that nesting location inheritance across generations could produce female-biased sex ratios in non-migratory freshwater turtles.

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APPENDIX A

Recapture locations of nesting females (see table 1).

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