Consequences of multiple paternity for female fitness in an Ontario population of northern map turtles, *Graptemys geographica*

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Table of Contents

List of Tables

List of Figures

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Abstract

Although sexual stereotypes paint males as being promiscuous and females as being choosy in order to increase their reproductive success, multiple mating by females is widespread and females of many taxa often produce progeny sired by multiple males – but why? In species in which there are no direct benefits associated with mating, females may adopt promiscuous mating strategies to increase their fitness through the acquisition of genetic benefits. Here, I examine the genetic mating system of map turtles, *Graptemys geographica* in Lake Opinicon. Based on the most conservative estimate, at least 71% of clutches in this population are sired by multiple males. There did not appear to be any relationship between female body size and frequency of multiple paternity. There was a marginally significant effect of multiple paternity on hatching success and survival of clutches, but there was no effect on hatchling morphology or locomotor performance.

Résumé

Les stéréotypes sexuels présentent les mâles comme s'accouplant librement et les femelles comme étant sélectives afin d'augmenter leur succès reproducteur. L'accouplement multiple est pourtant répandu chez les femelles de nombreuses espèces dont la progéniture est engendrée par plusieurs mâles, mais pourquoi? Chez les espèces où il n'y a pas de bénéfices directs associés à l'accouplement, les femelles peuvent adopter des stratégies d'accouplement multiple afin d'augmenter leur aptitude par l'acquisition de bénéfices génétiques. J'ai examiné le système d'accouplement génétique de la tortue géographique, *Graptemys geographica*, au lac Opinicon. Selon l'estimation la plus prudente, au moins 71% des familles dans cette population sont engendrées par plusieurs mâles. Il ne semblait pas y avoir de relation entre la taille corporelle des femelles et la présence de paternité multiple. Il y avait un effet marginalement significatif de la paternité multiple sur le taux d'éclosion et sur la survie des jeunes, mais il n'y avait pas d'effet sur la morphologie ni la performance locomotrice des nouveau-nés.

Introduction

Background

In the traditional view of animal mating strategies originally put forth by Darwin in *The Descent of Man,* males of almost all animals have "stronger passions than the females" and are "the more active member in the courtship of the sexes," while females are "less eager than the male," "coy," and even "endeavouring for a long time to escape" (Darwin, 1871). The notorious sexual stereotypes of the promiscuous male and the choosy female can be explained by the unequal investment in gamete production. By definition, males are the microgametic sex while females are the macrogametic sex. Male gametes are small and comparatively cheap to produce in large numbers, while female gametes are much larger and require a more significant energetic investment, and subsequently are produced in far fewer numbers. Female gametes therefore are a limiting resource; it is well established that in most species, male reproductive success increases in proportion to the number of mating partners obtained while female reproductive success is limited by how many eggs she can produce (Bateman, 1948). Because a single insemination can typically deliver vastly more sperm than are needed to fertilize all a female's eggs, (Trivers, 1972) this dichotomy favours males to mate promiscuously and indiscriminately, and females to mate selectively.

Polygynous mating systems – in which males mate with multiple females – are well understood, and their prevalence in almost all taxa (Anderson, 1994) is to be expected. Polyandrous mating systems – in which females mate with multiple males – are harder to understand, and their existence in nature is puzzling: the act of mating itself can be costly to females, not only in terms of the energetic expenses of gamete

production (Parker *et al.* 1972), but also in terms of physical dangers of copulation, including the risk of injury or death (Le Boeuf and Mesnick, 1991), exposure to bacteria (Westneat and Rambo, 2000), the transmission of pathogens (Loehle, 1997), and loss of time and energy (Watson *et al.,*1998). Given these costs, females should not benefit from multiple mating, and should be choosy regarding with whom they mate, as predicted by Darwin. Nonetheless, contrary to expectations, evidence accumulated over the past few decades shows that female promiscuity seems to be the rule, rather than the exception: mating with multiple males within a single reproductive season has been observed among females of many taxa, including amphibians (Liebgold *et al.,* 2006), reptiles (Calsbeek *et al.,* 2007; Olsson and Shine, 1997), birds (Foerster *et al.,* 2003), and mammals (Hoogland, 1998; Hrdy, 1979), and it has been shown that multiple paternity within broods is quite common (Jennions and Petrie, 2000). While natural selection for multiple mating by males is easily explicable, the adaptive advantages of multiple mating by females remain poorly understood.

Female promiscuity

To account for the observed high rates of female promiscuity we should expect promiscuous females to gain benefits that are inaccessible to monogamous females (excluding instances in which mating is forced). Possible benefits to females from multiple mating fall into two broad categories: the direct/material benefit hypothesis suggests that females mate multiply to acquire resources such as food or sperm that directly enhances their fitness, while the indirect/genetic benefits hypothesis supposes that females gain good genes for their offspring, improving their fitness through increased offspring viability (Zeh and Zeh, 2001). Direct benefits to females accrue with

the number of copulation events, both with a single male and with multiple males. When a male has mated with a female, he has an increased stake in the paternity of her offspring, and thus is more likely to invest in that female. By engaging in multiple mating, females of many species can improve their own fitness directly through added male investment, e.g., through the acquisition of nuptial gifts (Lamunyon, 1997, Karlsson, 1998), increased paternal investment (Stacey, 1982), the avoidance of infanticide (Hrdy, 1979), or the assurance of insemination (Pai *et al.,* 2005).

While convincing evidence supports the many direct benefits of polyandry, the indirect, genetic benefits of multiple mating remain controversial. There are several mechanisms by which multiple mating can indirectly enhance a female's fitness through the acquisition of good genes for her offspring. Multiple mating may result from a pre-copulatory "trade up": in species in which there is a second-mate advantage, females may initially mate to assure their fertility, and mate again with a genetically superior mate (i.e., in guppies, Pitcher *et al.,* 2003). Alternatively, females may benefit from the presence of genetically diverse sperm in their reproductive tract. Post-copulatory mechanisms of biasing fertilization such as male-male sperm competition or cryptic female choice may result in eggs being fertilized by genetically superior or genetically compatible sperm, leading to good offspring genotypes. Alternatively, females may be unable to predict which genes will lead to "fit" genotypes, whether due to unpredictable environments or an inability to discriminate "good genes." In this case multiple mating may then be a form of genetic "bet-hedging": by having eggs fertilized by more than one male, the average fitness of each generation of offspring should be

improved and the probability of extinction within a generation reduced (Fox and Rauter, 2003).

Model organism

Reptiles – and turtles specifically – present an ideal system for further investigation of the genetic benefits of female multiple mating among vertebrates because multiple mating and multiple paternity are well documented in many species, although at varying frequencies (Table 1; reviewed in FitzSimmons and Hart, 2007), their litter sizes are often large enough to test for multiple paternity, and because male turtles do not provide any nuptial gifts or paternal care, females do not obtain any direct benefits from mating multiply.

Among turtles, the northern map turtle *Graptemys geographica* is a good model to study the genetic benefits of female promiscuity. *G. geographica,* is an aquatic freshwater species, widespread throughout central and northeastern North America. Female-biased sexual size dimorphism in this species is extreme: the carapace lengths of mature female turtles range between 205 – 290 mm, while in mature males carapace lengths average only between 102 – 149 mm; males, therefore, measure only about half the length of females, and are approximately 20% of the females' body mass (Vogt, 1980). Because females are so much larger than males, and because males do not appear to be aggressive, multiple mating by female map turtles is not likely a side effect of male harassment, but is likely to be actively sought out. As in other reptiles, female map turtles receive no nuptial gifts or parental care from males, so if female map turtles mate with more than one male, they should derive genetic benefits, unless there are strong sexually antagonistic genes (Pischedda and Chippindale, 2006). Finally, highly

variable microsatellite loci have been identified for use in *Graptemys* species (Selman *et al.,* 2009), which can be used to assess multiple paternity in hatchling map turtles. The primer binding sites of the flanking sequences of microsatellite loci are highly conserved across turtle species (Fitzsimmons, 1995), so although no primers have been designed for the northern map turtle, existing primers designed for closely related species can be used in this analysis.

Objectives

The first objective of this study is to examine the genetic mating system of map turtles because very little is currently known about map turtle mating. Courtship and mating have been observed during spring and autumn when turtles are aggregated at communal hibernacula (Vogt, 1980) and the chance of encountering a potential mate is high. It has been shown that female turtles of many species are capable of storing sperm both within reproductive seasons and across years (Gist and Jones, 1989; reviewed by Pearse and Avise, 2001). Given that female map turtles have a high probability of mate encounter within reproductive seasons, and can use stored sperm from past matings, I expect to detect multiple paternity in map turtle clutches. In this study, I use microsatellite genotypes to determine the incidence of multiple paternity in an Eastern Ontario population of map turtles.

The second objective of this study is to investigate the role of sexual selection in the map turtle mating system. In polygynous mating systems, it is common for males to mate promiscuously and for females to mate selectively. Here, I consider whether in polyandrous mating systems the roles are reversed, and if males are more selective about mates when females are mating promiscuously. Although sperm are relatively

cheap to produce compared to eggs, recent studies have shown that the cost of sperm production cannot be completely discounted; significant costs to males associated with spermatogenesis have been documented in many species (e.g., adders, Olsson *et al.,* 1997; nematodes, Van Voorhies, 1992; and rams, Preston *et al.,* 2001). Moreover, spermatogenesis in freshwater turtles is an episodic event occurring once annually in the summer (Licht, 1984), with sperm being stored in the epididymis of the male for up to a year (Gist *et al.,* 2002). Assuming that the cost of sperm production is not negligible, and that the quantity of sperm is fixed, male map turtles would benefit from judicious allocation of sperm amongst receptive females to maximize their reproductive success. Offspring survival is positively correlated with the number and size of eggs laid, which is directly constrained by female body size (Ryan and Lindeman, 2007). Large females, therefore, have a higher potential reproductive output and success. If sperm is indeed limited, then males should mate selectively with large females, and there should be a higher frequency of multiple paternity within the clutches of larger females. I test this prediction by examining the relationship between female body size and prevalence of multiple paternity within clutches.

My final objective is to investigate the role of genetic benefits in map turtle mating systems. Female map turtles receive no direct benefits, and it is unlikely that they are being forced into unsolicited mating by eager males given the extreme female-biased sexual size dimorphism of the species; therefore, if female map turtles mate multiply, they should benefit indirectly by gaining desirable genes for their offspring, manifested in increased offspring fitness. Accordingly, I test the prediction that female fitness should increase with the number of sires in the clutch. I look at three aspects of

female fitness: clutch survival, hatchling morphology, and hatchling locomotory performance.

(1) Clutch survival: I look at the proportion of eggs laid that successfully hatch as a primary measure of survival, as well as the proportion of eggs laid that appear to be unfertilized. I also measure the proportion of the clutch that survives the first year; many studies of map turtles have suggested that hatchlings exhibit delayed emergence in which, post-hatching, they remain in or near their natal nest cavities throughout the fall and winter and emerge in the spring (Nagle *et al.,* 2004; Baker *et al.,* 2003). Terrestrial hibernation may be beneficial in terms of decreasing vulnerability to predation at times when resources are in decline and not conducive to rapid growth, however, it comes with the cost of increased exposure to sub-zero temperatures and frozen soil compared to under-water hibernation. I simulate terrestrial hibernation to examine the proportion of offspring from each clutch that survives the first year, as a longer-term estimate of offspring viability.

(2) Hatchling morphology: studies have suggested that larger hatchling turtles are less susceptible to predation than smaller conspecifics (Janzen, 1993; Janzen *et al.,* 2000,); therefore, size at hatching may be a reliable indicator of offspring fitness, and accordingly, I measure the size of hatchlings from each clutch. I measure hatchling body condition, an approximation of an organism's energy reserves. Finally, I take a measure of the scute anomalies of hatchlings from each clutch. Minor anomalies and asymmetries of bilateral features in bilaterally symmetrical organisms can indicate a potential flaw in the developmental stability of an organism (Polak, 2003). In a study on European pond turtles, Fernández and Rivera (2004) found that 75% of the population

had scute asymmetries, the most common being accessory and asymmetrical scutes, which may be caused by many stress-inducing factors including soil chemicals/pollution, incubation temperature or humidity, and genetic effects. Evidence from many taxa suggests that developmental asymmetry is a reliable fitness indicator, as it is related to individual quality and may affect not only survival, but also attractiveness to potential mates (Clarke, 1995).

(3) Locomotory performance: locomotory performance in hatchling turtles may be important in survival. Because the shells of juveniles are soft and cartilaginous, providing little protection from predators, the ability to escape from predators may influence hatchling survival. I measure the righting response and burst swimming speed of hatchlings from each family. In natural populations, when young aquatic turtles make their way from their nest to the water, they often fall upside-down in their haste (Burger, 1976), a phenomenon that may increase their vulnerability to predators. Righting response refers to the return to prone position after falling upside-down, and because it can be viewed as an indicator of physical strength and/or coordination (Freedberg *et al.,* 2004), it may be related to juvenile survival. Delmas *et al.* (2007) studied righting response in juvenile slider turtles, and found an effect of litter identity on both latency period and righting speed, suggesting either genetic relatedness or maternal effects may affect righting response.

Materials and Methods

General methods

Study species and study site

I collected 37 gravid female northern map turtles between May and June 2010 in Lake Opinicon (44°34'N 76°19'W) at the Queen's University Biological Station (QUBS) approximately 100 km south of Ottawa, Ontario, Canada. I captured all females by hand at the same nesting site on a small island during peak nesting times between 0600h – 0900h and 1800h – 2000h. I approached females digging nests and lightly palpated their abdomens to check for the presence and maturity of eggs. I checked nests for any eggs, and if the females had not yet begun laying, I brought them back to the laboratory; if a female had begun laying, I left her at her nest. Using a forestry caliper, I measured plastron length to the nearest 1 mm, and with an electronic scale, I measured mass to the nearest 1 g. I marked females individually by drilling small holes in their marginal scutes. I took a small (2 – 3 mm^2) clipping of skin from the lateral edge of one of the forefeet of each female using flame-sterilized surgical scissors and preserved it in 70% ethanol for use in genetic parentage analyses.

Induction of oviposition and care of eggs

I housed all females individually in clean plastic containers (120 x 80 x 50 cm) shallowly filled (approximately 10 cm deep) with lake water at ambient temperature. The morning following their capture, I induced oviposition in females by injecting them intramuscularly in the hind leg with oxytocin (20 IU/mL, 0.5 mL/kg gravid body weigh; Ewert & Legler, 1978) and monitored laying for a minimum of eight hours after the initial injection. If females had not begun to lay eggs after four hours, I re-injected them

in the opposite hind leg with the same dose of oxytocin. I removed all eggs and recorded their individual masses as they were laid. I grouped eggs by clutch in plastic containers (15 cm x 15 cm x 6 cm) with perforated lids and half-buried them in moist vermiculite (1:1 ratio by mass of water and vermiculite). I re-palpated the abdomens of all females eight hours after the initial injection of oxytocin to check for retained eggs, and if no eggs were felt I released them to the nesting site the following morning.

I held the containers of eggs in two fiberboard incubators (Blouin-Demers *et al.,* 2005) at 29°C for two weeks for the duration of field studies at QUBS, at which point I transferred them to incubators at the University of Ottawa (Constant Temperature Control Limited Model LBC 700) set at 29 °C until hatching. I weighed the containers with the eggs and vermiculite at the onset of incubation; to account for evaporation I added sufficient water every three days to maintain a constant total mass. During these manipulations I shuffled the position of the containers within the incubator to minimize potential position effects.

Hatchling collection and phenotypic measurements

Eggs (*N* = 418) began hatching after 55 days of incubation. The mean clutch size was 11 eggs (range 6 – 14); eggs averaged 11.8 g (range 7.7 – 16.0 g) and entire clutches averaged 133.9 g (range 73.4 – 190.4 g). As they emerged, I transferred each hatchling to a plastic pill bottle (12 x 5 cm dia.) containing moist vermiculite (2:1 by mass with water; Costanzo, *et al.,* 2000) to be housed individually in the incubators until each had been identified. I took small blood samples (0.03 – 0.05 mL) from all emerged hatchlings (*N* = 242) from the coccygeal vein using a 0.5 mL insulin syringe fitted with a 28 ½ gage needle (Bulté & Verly, 2006). I dissected un-hatched eggs (*N* = 177) to check for signs of fertilization and embryo development; if eggs appeared to be fertilized (*N*

= 99) I took a tissue sample. All blood and tissue samples were stored in 70% ethanol at 4°C for genetic analyses.

Once blood samples were obtained, I marked all hatchlings individually using non-toxic water-based permanent paint and placed them in randomly selected groups of 10 in clean plastic containers (30 x 20 x 10 cm) shallowly filled (approximately 10 cm deep) with damp sand to be housed over winter in an darkened environmental chamber (Constant Temperature Control Ltd. Model 600; 12L:12D) and denied them access to food and free water, following (Costanzo *et al.,* 2000). I lowered the temperature of the chambers gradually from 29°C to 20°C in September, 15°C in October, 10°C in November, and down to 4°C in December 2010, where the temperature was held constant at 4°C until April 2011.

Due to a power failure and system crash, the temperature of the environmental chambers rose rapidly to 20°C in early April 2011, where it remained constant until hatchlings were released. I transferred live hatchlings to clean plastic containers (120 x 80 x 50 cm) shallowly filled (approximately 10 cm deep) with clean water (changed daily) and small bricks for resting and basking. All containers were kept under UV lights, which were on from 0600h – 2000h each day. I fed hatchlings Tetrafauna™ *ReptoMin®baby* brand floating food pellets once a day. I released all hatchlings in late April 2011 from the small island in Lake Opinicon from which I caught their mothers.

Objective 1 – Genetic mating system

DNA extraction and PCR

I extracted DNA from the individual tissue samples of each mother and her offspring using a spin-column DNA extraction protocol modified from a glass-fiber

protocol (Ivanova *et al.,* 2006; see Appendix for extraction protocol). I assessed the purity and quantity of all isolated DNA samples using a Thermo Scientific NanoDrop 2000 spectrophotometer. I was unable to extract good quality DNA from unhatched eggs for microsatellite genotyping, so only live-hatchling blood samples could be used for genetic analysis of offspring. As I was unlikely to detect multiple paternity in clutches with very few offspring, I elected to analyze only clutches in which a minimum of eight hatchlings successfully emerged. While I could have lowered the minimum clutch size to increase the number of families in my analysis, doing so would have decreased my ability to detect mulitple paternity; thus, selecting eight hatchlings per clutch as a minimum was the best available compromise between the number of families available to analyze and the number of hatchlings per family. Due to low hatching success, only 14 clutches met this criterion, reducing my sample size significantly from the original 37 families. To ensure that the likelihood of detecting multiple paternity for all 14 clutches was the same, I randomly selected 8 offspring from each clutch for genotyping.

I ran polymerase chain reactions (PCR) to analyze fragment size at muliple microsatellite loci for use in paternity analysis. I amplified six microsatellite loci previously characterized for use in closely related species: GmuA18, GmuB08, GmuD87, GmuD51, GmuD90 (King & Julian, 2004) and TerpSH7 (Hauswaldt & Glenn, 2003) (see Appendix I for PCR protocols). All PCR products were separated and sized for use in paternity analysis using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

I obtained the multilocus genotypes at the six microsatellite loci for 14 females and their clutches for paternity analysis. I also obtained multilocus genotypes for the

remaining 23 females caught in 2010 and 42 adult males and females caught in 2006 as part of another study (Bulté *et al.*, 2008) to provide an estimate of allele frequencies of the population.

Characterization of microsatellite loci

I assessed population allele frequencies of each locus using the program CERVUS. Each locus was tested for deviations from the Hardy-Weinberg equilibrium, observed and expected heterozygosities, and mean polymorphic information content. I also used CERVUS to estimate the frequency of null alleles and the combined non-exclusion probabilities of the selected loci. Allele frequencies, observed and expected heterozygosities, and polymorphic information content for each locus and across all six loci can be found in Appendix I. There was no significant deviation from Hardy-Weinberg equilibrium at four out of the six loci. The combined non-exclusion probability (given a known maternal genotype) across all loci was 0.043. Locus GmuA18 appeared to have a null allele ($F_N > 0.05$), suggesting that there may be a mutation at a primer-binding site that may prevent successful amplification of the microsatellite allele during PCR. This is common in microsatellite loci for which the primers were designed for species other than the study species.

Paternity analysis

(1) Allele Counts. I employed three analytical approaches to estimate the extent of multiple paternity in map turtles. First, I counted alleles at each locus to estimate the minimum number of fathers for each clutch. I determined maternal genotypes directly from the mothers, and I deduced paternal alleles from offspring genotypes by subtracting the known maternal alleles. Due to a relatively high expected heterozygosity of 0.65 across all loci from the general population sample, I assumed all

fathers were heterozygous for each of the six loci as a conservative estimate. I inferred the minimum number of fathers to be the smallest whole number greater than or equal to one half the number of deduced paternal alleles. For cases in which an offspring had the same heterozygous genotype as its mother, I counted the heterozygous pairing of alleles to be a single allelic contribution, and only counted this pair if neither of the contributing alleles was detected as a paternal allele in any other offspring from that clutch. As a conservative estimate (to account for mutations or mistyping that may lead to an overestimation of the number of paternal alleles), I inferred multiple paternity only when more than two paternal alleles were detected at more than one locus.

(2) DADSHARE. As a second method of analyzing paternity, I used the program DADSHARE (Version 4; www.zoo.cam.ac.uk/zoostaff/amos) to examine the degree of relatedness between offspring in a family and to identify the minimum number of males needed to father all offspring. DADSHARE takes the genotypes of the mothers and offspring and deduces potential paternal genotypes using Monte Carlo simulation that takes into account the allele frequencies of the population. The program then identifies clusters of offspring that are compatible with a single father based on evidence from at least two loci. I analyzed each family separately, identifying the maternal genotype and taking into account the allele frequencies of the population.

(3) GERUD 2.0. As a third method of paternity analysis, I used the program GERUD2.0 (Jones, 2005). GERUD2.0 deduces paternal alleles from offspring genotypes from each mother and creates progeny arrays to reconstruct all possible paternal genotypes. It analyzes the progeny arrays to estimate the most likely minimum number of fathers needed to produce each clutch.

Objective 2 – Sexual selection

I analyzed the effect of female size on minimum number of sires using ordinal logistic regression, with the minimum number of sires responsible for a clutch as the dependent variable (one, two or three sires as measured by each of the three independent methods of paternity analysis) and female plastron length as the independent variable.

Objective 3 – Genetic benefits

Clutch survival

To determine the proportion of offspring from each clutch that survived, I measured *(i) initial hatching success and (ii) first year survival.* Many eggs were either unfertilized or the embryos died at some point during development. Some hatchling turtles developed successfully to the point at which they were able to initiate pipping (using the egg tooth to break open the egg shell), but lacked the strength to successfully emerge from the egg and died either trying or shortly following emergence. I measured hatching success as the percentage of hatchlings that successfully emerged out of the total number of fertilized eggs laid in the clutch. To measure first year survival of each clutch, I recorded hatchling mortality as they awoke from hibernation, and the proportion of offspring from each clutch who survived the first winter.

Hatchling morphology

I measured three aspects of hatchling morphology*: (i) body size, (ii) body condition, and (iii) scute anomalies*. Approximately one week after hatchlings emerged from their shells (once the yolk-sac had been absorbed), I measured their plastron length (PL) to the nearest 0.1 mm with digital calipers and body mass to the nearest 0.01g with an electronic scale. I used PL as a measure of body size. I measured body condition (BC) of hatchlings as the residuals of an ordinary least square regression with

$\log_{10}$ PL as the independent variable and $\log_{10}$ mass as the dependent variable (Jakob *et al.,* 1996). This index of body condition can be used as an indirect measure of energy reserves, where hatchlings with higher residual values have higher body condition than those with lower residuals. I measured BC one week after hatching. I took photographs of the carapace of each turtle, and used *Image J* digital imagery software to analyze dorsal scute anomalies (SA). Symmetrical turtles have 12 marginal, 6 costal, and 3 vertebral scutes on both the left and the right side of the carapace. As a measure of asymmetry, I recorded the numbers of accessory scutes to obtain an overall "asymmetry score" for each hatchling.

I calculated the mean plastron length, body condition, and asymmetry score for hatchlings in each clutch, and used mean clutch values to compare families in analyses.

Hatchling performance

I performed both aquatic and terrestrial performance tests with hatchling turtles. As an aquatic measure, I recorded *(i) burst-swimming speed* at 22°C four days after hatchlings emerged from hibernation in April 2011. I placed hatchings individually in a 2-m long plastic trough filled with water at ambient temperature and encouraged them to swim at maximum speed by gently tapping their tails if they slowed down during the trial. I recorded the length of time it took each hatchling to swim at its maximum speed for one meter; I measured burst speed in this way four times for each hatchling, once a day for four days. I used the fastest trial from each hatchling in analyses.

As a terrestrial measure of performance, I looked at the righting response of the hatchlings eight days after they awoke from hibernation. I placed hatchlings on a flat

surface lined with felt and turned them upside-down and recorded the *(ii) latency period*—the length of time until the hatchling's first attempt at righting itself, and *(iii) righting response*—the length of time from the hatchling's first righting attempt to successful righting. I took two measures of righting response for each hatchling, the second trial one day following the first trial. I used the fastest trial of each measure for my analyses.

I calculated the mean burst speed, latency period, and righting response from hatchlings of each clutch, and used mean clutch values in analyses.

Statistical analyses

I performed multivariate analysis of variance (MANOVA) for my measures of fitness. I ran one MANOVA for *hatchling survival* (with hatching success and first year survival as dependent variables), one MANOVA for *hatchling body condition* (with mean plastron length, mean body condition, and mean asymmetry score as dependent variables) and one MANOVA for *hatchling performance* (with mean burst speed, mean latency period, and mean righting response as dependent variables), testing the prediction that these measures of fitness would be affected by the minimum number of sires detected in each clutch. I ran these analyses for each method of paternity analysis (allele counts, DADSHARE, and GERUD), but because the results were qualitatively the same I only present results from the allele counts method.

I inspected distributions and residual plots to verify assumptions of normality and homogeneity of variance. For all statistical analyses, I used JMP version 5 (SAS Institute, 2002). I considered statistical results to be significant at $P = 0.05$ and marginally significant at $P < 0.10$.

Results

Genetic mating system

Characterization of microsatellite loci

Despite numerous attempts, I was unable to successfully amplify the DNA template of several individuals for one or more loci. Overall, 90.1% of the individuals were genotyped at all six loci. Based on the sample population of adult map turtles (the 37 adult females from 2010 and the 42 adults from the 2006 study), I detected between 4-12 alleles per locus across all six loci (mean = 6.33 alleles per locus).

Paternity analysis

(1) Allele Counts. Based on the number of paternal alleles deduced from the known maternal genotypes in the clutches, I detected at least a single sire in four of the 14 clutches (28.6%), at least two sires in eight clutches (57.1%), and at least three sires in the remaining two clutches (14.3%; Figure 1). Therefore, this method indicated that 71.4% of clutches were multiply sired. Multiple paternity was supported by at least three paternal alleles at two loci for ten females, and at three loci for six females, indicating that mutation is unlikely to explain many of the multiple paternal alleles observed in this study.

(2) DADSHARE. I grouped together clusters of full-sib offspring compatible with a single father using DADSHARE. DADSHARE detected one family made up of one cluster (7.1%), seven families made up of two clusters (50%), and six families made up of three clusters (42.9%), indicating one, two, or three sires, respectively. Therefore, this method indicated that 92.9% of clutches were multiply sired.

(3) GERUD 2.0. Using GERUD2.0, I did not detect any clutches sired by a single male; I detected at least two sires in eight clutches (57.1%) and at least three sires in six clutches (42.9%). Therefore, this method indicated that 100% of clutches were multiply sired.

All three methods produced the same results for five of the clutches (35.7%). Two of the methods were in agreement about paternity for eight clutches (57.1%), and the three methods produced three different results for the remaining clutch (7.1%).

Sexual selection

The number of sires did not increase with female plastron length (Figure 2; ordinal logistic regression: Wald $\chi^2 = 0.097$, $N = 14$, $P = 0.755$ – allele counts; Wald $\chi^2 = 1.628$, $N = 14$, $P = 0.202$ – DADSHARE; Wald $\chi^2 = 1.275$, $N = 14$, $P = 0.259$ – GERUD2.0)

Genetic benefits

Hatchling survival

After dissecting unhatched eggs from the 14 families in this study, I found between 0 – 3 unfertilized eggs per clutch (mean number of unfertilized eggs = 1.14 per clutch). Clutches sired by more males had fewer unfertilized eggs, but this relationship was only marginally significant (ANOVA: $R^2 = 0.349$, $F_{2,11} = 2.950$, $P = 0.09$). Hatching success ranged from 69% - 100% (mean success was 84.3%), and first year survival ranged from 42.9% - 100% (mean survival was 69%). Hatching success and first year survival increased with the minimum number of sires, but this was only marginally significant (MANOVA: Wilk's $\lambda = 0.448$, $F_{2,11} = 2.47$, $P = 0.077$). Based on allele counts, clutches sired by at least three males had a higher percentage of hatchlings emerge (96.7%) than did clutches sired by one (81.8%) or two males (82.9%), but this difference was only marginally significant (ANOVA: $R^2 = 0.369$, $F_{2,11} = 3.22$, $P = 0.080$).

Clutches sired by at least three males had higher offspring survival (86.8%) than clutches sired by only one (57.7%) or two males (68.8%), but this difference was only marginally significant (ANOVA: $R^2 = 0.402$, $F_{2,11} = 3.69$, $P = 0.059$).

Hatchling morphology

Hatchling plastron length ranged from 24.3 – 32.0mm (mean = 28.4mm), body conditions ranged from -0.110 – 0.080 (mean = 0), and asymmetry scores ranged from 0 – 6 (mean = 0.810). The number of sires had no significant effect of on hatchling morphology (as measured by PL, BC and SA; MANOVA: Wilk's $\lambda = 0.586$, $F_{2,11} = 0.919$, $P = 0.504$).

Hatchling performance

Hatchling burst swimming speeds ranged from 4.1 – 20.8cm/s (mean = 10.5cm/s), latency periods ranged from 0.5 – 742.2s (mean = 73.0s), and righting times ranged from 0.3 – 1000s (mean = 38.3s). The minimum number of sires had no significant effect on hatchling performance (MANOVA: Wilk's = 0.796, $F_{2,11} = 0.362$, $P = 0.894$).

Discussion

Genetic mating system

I genotyped 14 female map turtles and their offspring to determine the minimum number of males responsible for siring each clutch. Based on the multilocus genotypes from the six microsatellite loci used, I found clutches with a minimum of one, two, or three sires; my most conservative estimate of paternity indicated that at least 71% of the clutches were sired by multiple males, and my least conservative estimate indicated that 100% of clutches were sired by multiple males. Although multiple paternity is common in many species of turtles studied (reviewed by Pearse and Avise, 2001), this is the first study to date in which multiple paternity has been found in *G. geographica,* and even the more conservative estimate of 70% is one of the highest frequencies of multiple paternity documented in turtles (among other freshwater species, multiple paternity has been documented at varying frequencies, ranging from 0% to 56%, and 1% to 95% in marine species, see Table 1). This is particularly interesting given the extreme female-biased sexual size dimorphism in map turtles, indicating that this high level of multiple paternity is not likely to be a consequence of forced copulation by males. It is also important to note that the estimates of minimum number of sires are likely to be underestimations: not all offspring from each clutch were analyzed and different males may have contributed the same allele to certain offspring in a clutch, thus allowing the potential for some males to have gone undetected in analyses of minimum number of sires.

These results are consistent with my expectation that the genetic mating system of this species would consist of multiple paternity, given that turtles do not form pair

bonds, that the probability of mate encounter is high at communal hibernacula, and that in closely related species, female turtles have the ability to store and use sperm from previous mate encounters both within and across mating seasons (Pearse *et al.,* 2001), such that multiple paternity is a possibility even if multiple mating does not occur in a single reproductive season.

Sexual selection

Contrary to my prediction, my results showed no relationship between female body size and multiple paternity, suggesting that male map turtles may not mate selectively with larger females, despite their higher fecundity. This is similar to what Blouin-Demers *et al.* (2005) found in black rat snakes. In comparison, Zbinden *et al* (2007) found a positive relationship between the number of sires and female size in loggerhead sea turtles, and Lee and Hays (2004) found that females laying multiply sired clutches were larger than those laying singly sired clutches, although their results were not statistically significant. Similar evidence of male preference for larger females has been documented in many other species of fish and reptiles in which female fecundity is related to size, including painted turtles (Pearse *et al.*, 2002), garter snakes (Garner and Larsen, 2005), sand lizards (Olsonn, 1993), guppies (Dosen and Montgomerie, 2004), and redlip blenny (Côté and Hunte, 1989).

My hypothesis that males should mate selectively with larger females because of their higher potential reproductive output was based on the assumption that sperm may be a limiting factor in male reproductive success; however, this assumption was not tested. In their study on painted and slider turtles, Gist *et al.* (2002) found that although spermatogenesis occurred episodically and was over by October and despite

the fixed quantity of sperm produced, the ductus epididymis contained viable sperm throughout the year. Thus, even if the quantity of sperm is indeed fixed, the amount produced may be abundant such that it is not a limiting factor in practice, and the risk of potential sperm depletion may be so low that the benefits of mating indiscriminately outweigh the costs. Importantly, my assumption of sperm limitation was based on evidence from other species, and as of yet, no studies on sperm production in male map turtles have been performed.

Because fecundity tends to increase with female size in reptiles, I predicted that by examining paternity, I would find evidence of male preference for larger females. While my results did not support this prediction, they did not refute my hypothesis that males mate selectively with larger females. The link between multiple paternity and multiple mating is unclear, especially if fertilization of eggs is selective. For example, there are post-copulatory mechanisms such as sperm competition or cryptic female choice that may be at play; copulations do not necessarily lead to fertilizations, and paternity patterns are thus not necessarily accurate reflections of mating patterns. Although I detected no differences in paternity in the clutches of small and large females, I cannot extrapolate to corresponding differences in mating frequency between these females. It is possible either that large females may receive more male attention but due to the extreme size differences between the sexes they are able to resist mating attempts with little cost, or that they do, in fact, mate with more males but due to post-copulatory mechanisms, fertilization is biased so that not all the sperm available is actually used to fertilize their eggs, thus making male preference impossible to detect by these methods.

The limitations associated with my sampling effort of female turtles and their clutches must be taken into consideration when considering the significance of these results. Not only was my sample size small (I only had two clutches with a minimum of three sires), the range of sizes of females in my sample (most female plastron lengths ranged from 200 – 230 mm, with only a single female measuring over 250 mm) was quite low compared to the possible size range of mature map turtle females, who can grow up to 290 mm in length. Given that my observed size range was relatively narrow, the differences in fecundity of females in such a small size range could be biologically insignificant; if male map turtles actually display preference for larger females, this preference could go undetected in the current study.

Genetic benefits

Consistent with my prediction, I found that hatching success and first-year survival tended to increase with the number of sires, although my results were only marginally significant. In addition, I found a marginally significant trend towards fewer unfertilized eggs with increasing number of sires. Contrary to my predictions, however, I found that there was no significant relationship between either the morphology or the performance of hatchlings and the number of sires. These results suggest that multiply mating females may have the advantage of increased reproductive success in terms of the number of surviving offspring produced. Increased success related to multiple mating due to either fewer unfertilized eggs or greater offspring survival has also been found in many reptiles in which females receive no direct benefits, including leopard geckos (LaDage *et al.*, 2008), black rat snakes (Blouin-Demers *et al.*, 2005), and common lizards (Uller and Olsson, 2005), although in similar studies on green turtles

(Lee and Hays, 2004) and painted turtles (Pearse *et al.* 2002) there were no differences in the success of multiply vs. singly sired clutches.

The high incidence of multiple paternity associated with increased reproductive success in this population of map turtles supports the hypothesis that female promiscuity may be associated with genetic benefits, as opposed to simply "convenience polyandry," in which females give in to male harassment, as was found in green turtles (Lee and Hays, 2004). While genetic benefit seems the most likely explanation for the high rates of polyandry in map turtles, the mechanism by which these benefits arise is less clear. Multiple paternity may simply be a byproduct of pre-copulatory mate choice in the form of a genetic trade-up, in which females mate indiscriminately with the first male encountered to assure fertility, and then re-mate if a male of superior quality is encountered (Jennions and Petrie, 2000). However, this explanation appears to be unlikely in this species for two reasons. First, if females re-mate with superior males, this favours a second-male fertilization advantage, and the clutches that are multiply sired are expected to be composed of fitter offspring. Such biasing of fertilization towards the second, superior male is commonly observed in species in which females re-mate to trade-up for good genes, (e.g., guppies, Pitcher *et al.,* 2003; fruit flies, Frentiu and Chenoweth, 2008). Although the current study did not look at the relative contribution of each sire, that the clutches with more fathers were not phenotypically superior does not support this hypothesis. Secondly, the trade-up scenario requires that the female exhibits pre-copulatory mate selection and is able to detect differences in mate quality, and such a selection ability has never been demonstrated in reptiles (reviewed by Uller and Olsson, 2008).

Given that female turtles are unlikely to actively and consciously be selective about their mates, it is more likely that post-copulatory mechanisms explain multiple mating in this species. Enhanced offspring survival could be attributed to either the intrinsic quality of genes inherited, or from the compatibility of maternal and paternal genotypes. The sperm competition hypothesis, in which multiply mating females acquire good genes for their offspring through the promotion of sperm competition posits that males with "competitive" sperm – due either to superior genetic quality or simply superior competitive abilities – will be more successful at fertilization, and will sire high quality offspring, or at least sons with competitive sperm. This hypothesis assumes that there is variance in the competitive ability of sperm and that these competitive abilities are heritable, although neither assumption has yet been tested in reptiles (reviewed by Jennions and Petrie, 2000). The sperm competition hypothesis also suggests that paternity will be biased towards one male, a possibility that this study did not test. However, the current results of increased fertility and success of offspring with increasing sires are consistent with this hypothesis, so it cannot be ruled out.

The cryptic choice hypothesis, in which multiply mating females can bias fertilization post-copulation, is associated with benefits from either good genes or compatible genes. Accumulating evidence from inbreeding avoidance studies support the hypothesis that females may "cryptically" select sperm, favouring genetic combinations that are most compatible with their own, reducing the likelihood of mating with a closely related or otherwise incompatible male (reviewed in Jennions and Petrie, 2000; Uller and Olsson, 2008). Tregenza and Wedell (2002) found that

female crickets who mated with only related males had decreased hatching success compared to females who mated with both a related male and an unrelated male, demonstrating that in this case multiply mating females were avoiding genetic incompatibility. In the current study, the main effect of multiple paternity was on egg and offspring viability, which provides indirect evidence for the genetic benefits of polyandry via the avoidance of genetic incompatibility. Similar findings of increased egg or offspring viability, as seen in black rat snakes (Blouin-Demers *et al.,* 2005), and in sand lizards and adders (Olsson and Madsen, 2001), add to the growing support of this mechanism being responsible for polyandry in reptiles.

Similar to the avoidance of incompatibility hypothesis is the genetic bet-hedging hypothesis, in which mating with multiple males increases the genetic diversity of offspring, which under uncertain environmental conditions, can lead to increased offspring viability. This can been likened to the idiom "don't put all your eggs in one basket," and has been offered as a reason for female promiscuity in some species (i.e., anoles, Calsbeek *et al.,* 2007; prairie dogs, Hoogland *et al.,* 1998). The results of the current study may indirectly support this hypothesis, however there is yet no evidence of its importance in reptilian mating systems.

Future directions

Unfortunately, I was unable to extract good quality DNA from embryonic tissue of unhatched eggs. Unless they were obviously unfertilized and completely collapsed, I did not remove any eggs from the incubators until other eggs had hatched, to avoid the possibility of discarding any potentially viable hatchlings. This decision meant that dead embryos remained incubating at 29°C, likely causing their DNA to degrade. The

implications of not being able to construct the genotypes of un-hatched eggs are twofold: firstly, because I was restricted to using only the DNA of live hatchlings, the number of families that I could use in genetic analysis and paternity analysis (with at least eight emerging hatchlings) was reduced significantly down from 37 to 14. Thus, not only was I able to look at fewer hatchlings per clutch (I detected a maximum of three sires by looking at eight offspring per clutch, I may have been able to detect more paternal alleles given more hatchlings), but my subsequent analyses of fitness indicators from clutches sired by one, two, or three males were comparing very small groups (only two families in my comparisons were sired by at least three males), and my results were only marginally significant. Secondly, the clutches used in my analyses were the high-success clutches, and the clutches in which very few eggs hatched were excluded. If there were differences in paternity between the "successful" and "unsuccessful" clutches, then these differences were undetected. For instance, I am unable to assess the possibility that most of the clutches that failed completely were sired by a single male. Increasing the number of families studied, and constructing the multilocus genotypes of un-hatched eggs could improve the power and resolution of future studies.

The genetic compatibility hypothesis posits that females mate multiply to avoid mating with incompatible males, and the results of the current study provide indirect support for this hypothesis; however, since I did not look at the genetic relatedness of the population, I cannot determine if multiple mating is related to inbreeding avoidance.

An unavoidable limitation of this study is that the link between multiple mating and multiple paternity remains unclear. Ideally, observational studies on mating frequency of female map turtles in concert with analyses of multiple paternity would give a much clearer picture of the map turtle mating systems. However, given that map turtles mate at hibernacula, the design of observational studies on the actual mating frequency of female turtles would be challenging. In addition, that females can store viable sperm across mating seasons hinders the ability to make conclusions about the mating system based solely on the frequency of multiple paternity. This study looked at multiple paternity from the female perspective; a comprehensive understanding of the map turtle mating system would require the male perspective to be included: are certain males more successful, and if so, do they display specific characteristics? If few males in the population dominate mating, what is the operational sex ratio and effective population size? Are multiply sired clutches biased towards one father, or are they equally distributed? What is the frequency of infertile males in the population, and are the chances of mating with an infertile male great enough to lead to female promiscuity? Similarly, what is the degree of genetic relatedness and gene flow in this population? Although the QUBS population of map turtles is not small, it is at the northern fringe of the species range, and the genetic diversity and potential for gene flow in this population could be less than that of other, more central populations, and thus may be more susceptible to inbreeding. If female promiscuity is indeed an adaptive strategy to avoid inbreeding depression, is the frequency of multiple mating or multiple paternity related to the genetic structure of the population? Addressing such questions through observational and experimental studies will lead to a greater

understanding of the causes, mechanisms, and effects of mating systems and the importance of multiple mating and multiple paternity in natural populations. As reptiles are proportionally the most threatened vertebrates in Canada, uncovering this aspect of life history has important implications for understanding threats to their breeding behaviour and developing management programs.

Conclusions

There is a growing body of evidence that suggests that multiple paternity is common in the mating systems of reptiles, and that promiscuity may benefit females indirectly through increased viability of offspring. While I acknowledge that improving the limited sample size could have increased the power of the results, this study has shown that the frequency of multiple paternity in map turtles is among the highest observed in turtles and seems to be associated with increased offspring success, although the mechanism by which this occurs is unclear.

Table 1. Frequency of multiple paternity in freshwater and marine species of turtles. Genetic markers include microsatellites (M) or DNA fingerprinting (F).

Species	Marker	No. loci	No. clutches	Frequency of multiple paternity (%)	Source
Chrysemys picta	M	2 or 3	227	30	Pearse *et al.*, 2001
Emys blandingii	M	4	16	56	Refsnider, 2009
Clemmys insculpta	F	n/a	10	50	Galbraith, 1991
Chrysemys picta	M	3	20	0	McTaggart, 2000
Lepidochelys kempi	M	3	26	58	Kichler *et al.* 1999
Caretta caretta	M	4	21	95	Zbinden, 2007
Chelonia mydas	M	5	18	61	Lee and Hays, 2004
Lepidochelys olivacea	M	2	10	20	Hoekert *et al.*, 2002
Chelonia mydas	M	5	22	1	Fitzsimmons, 1998
Natator depressus	M	4	16	69	Theissinger *et al.* 2009

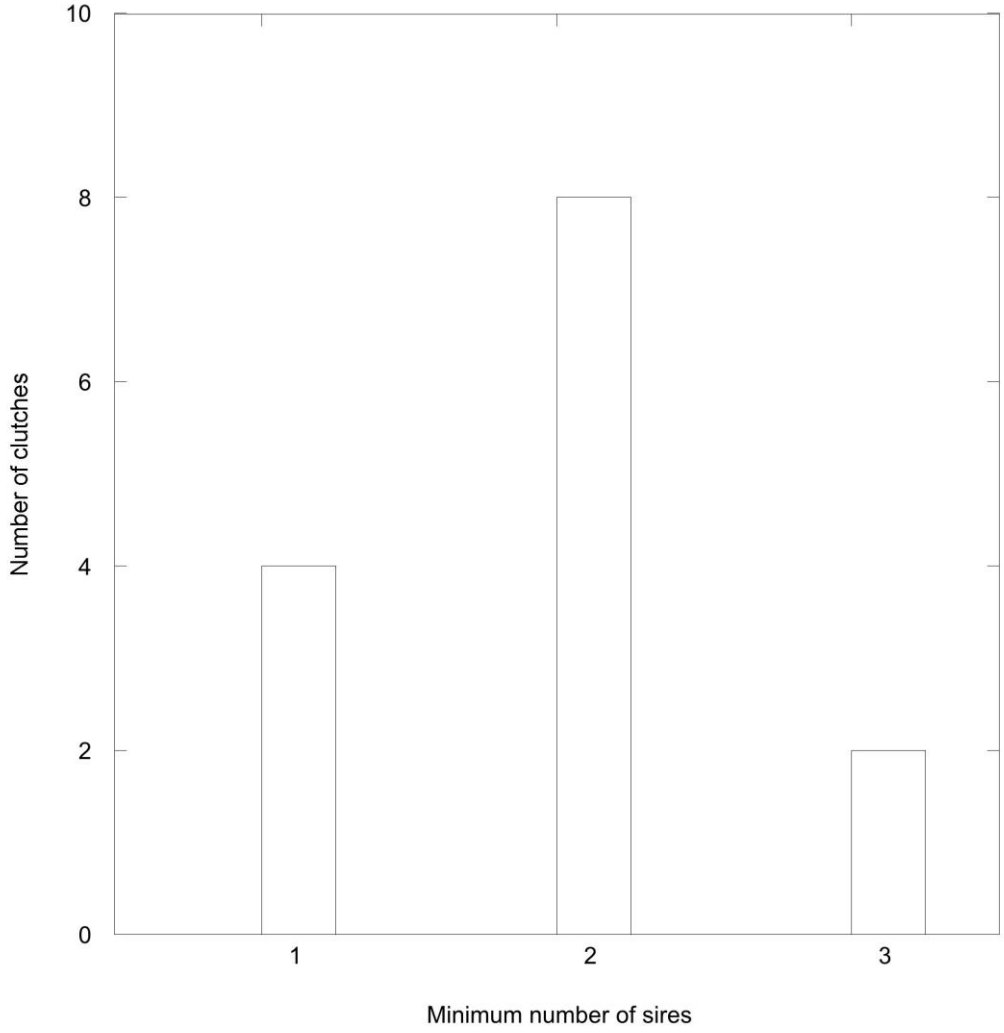

Figure 1. Percentage of clutches sired by a minimum of one, two, or three males (*N* = 14 clutches)

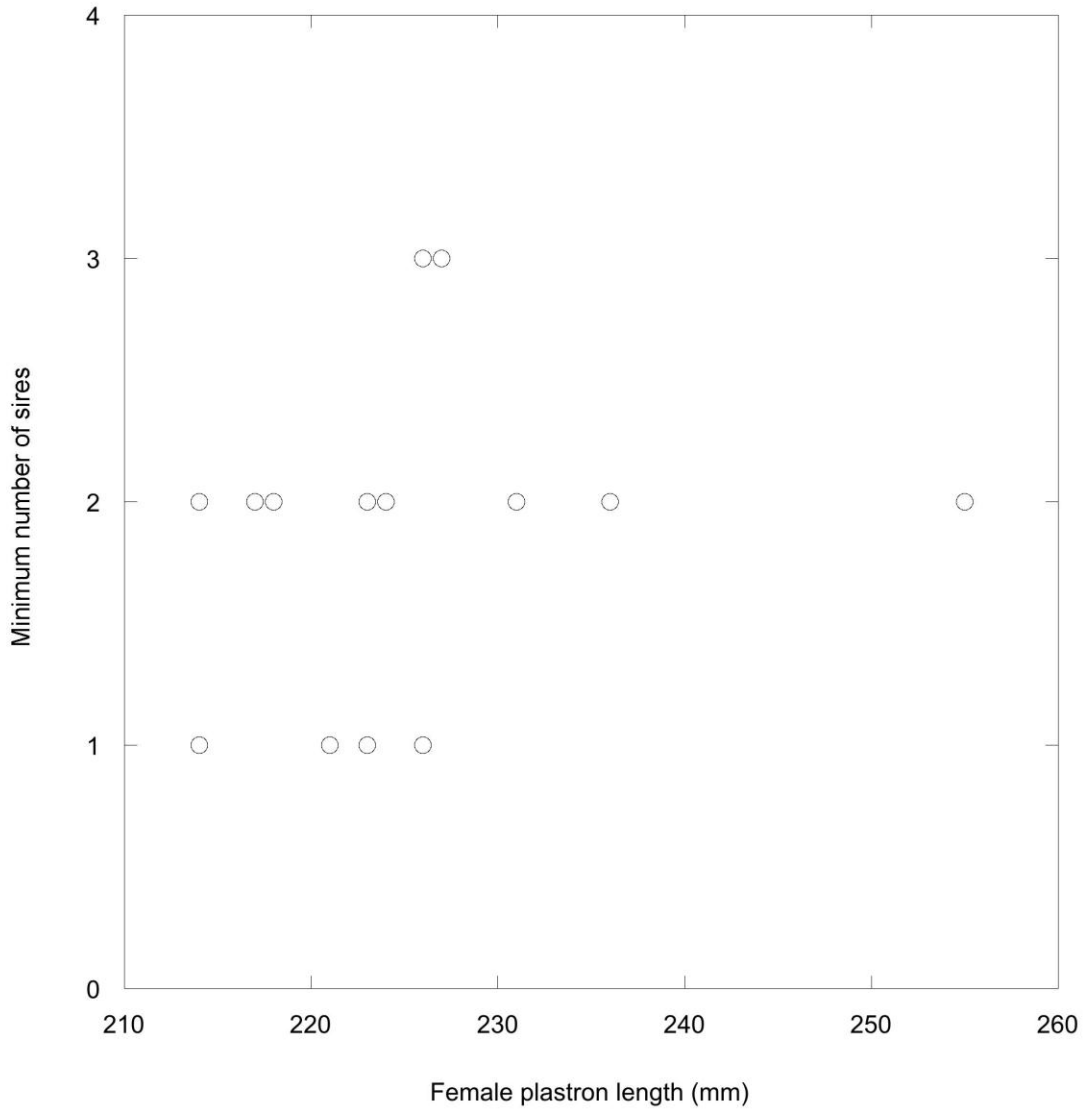

Figure 2. Minimum number of sires as a function of female plastron length (N = 14)

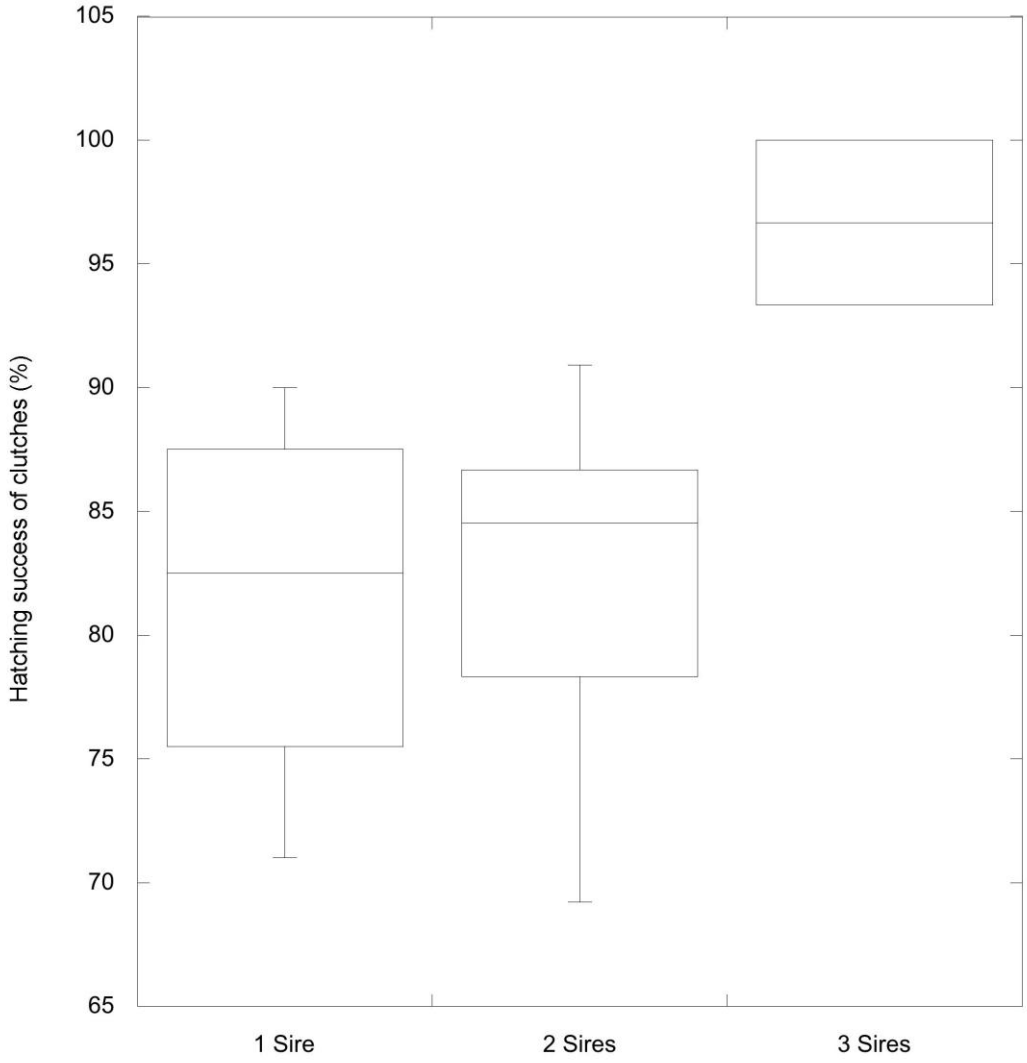

Figure 3. Hatching success of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

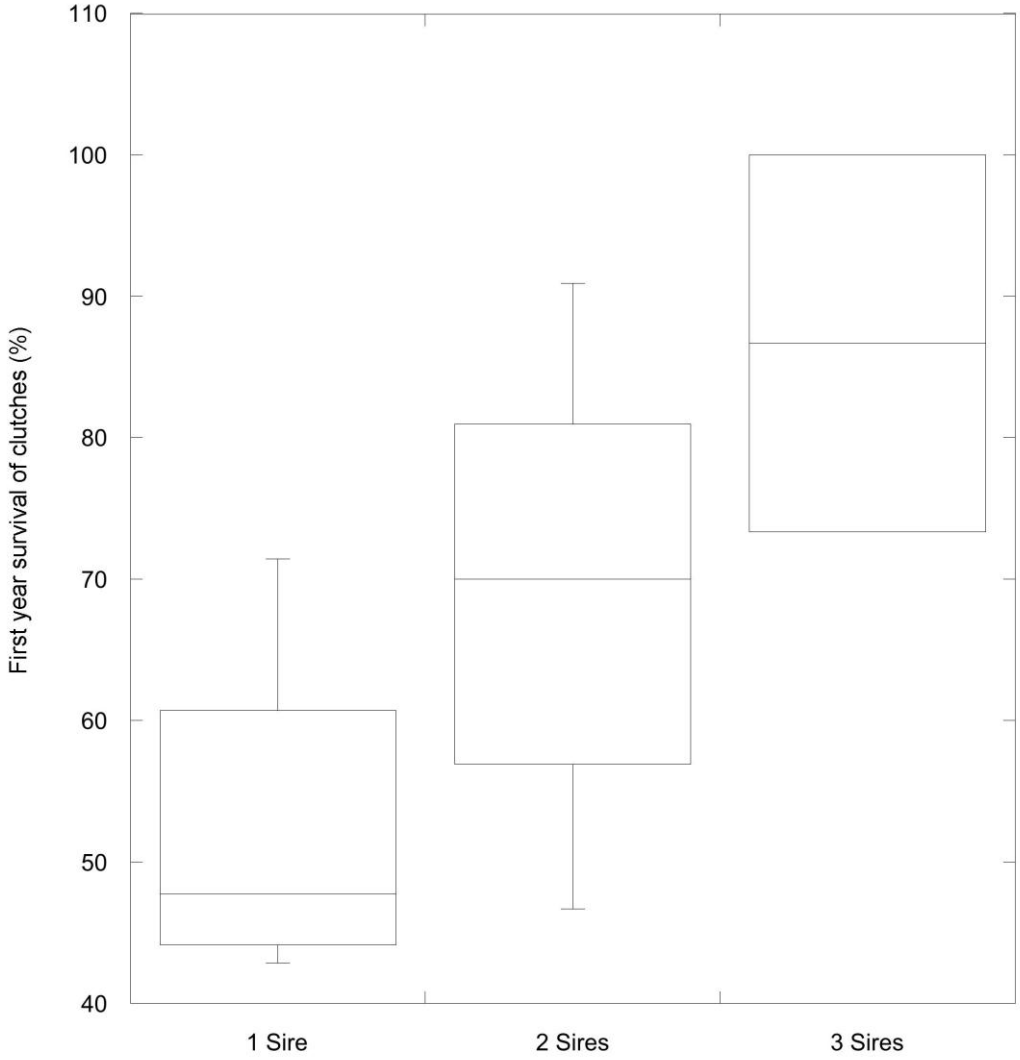

Figure 4. First year survival of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

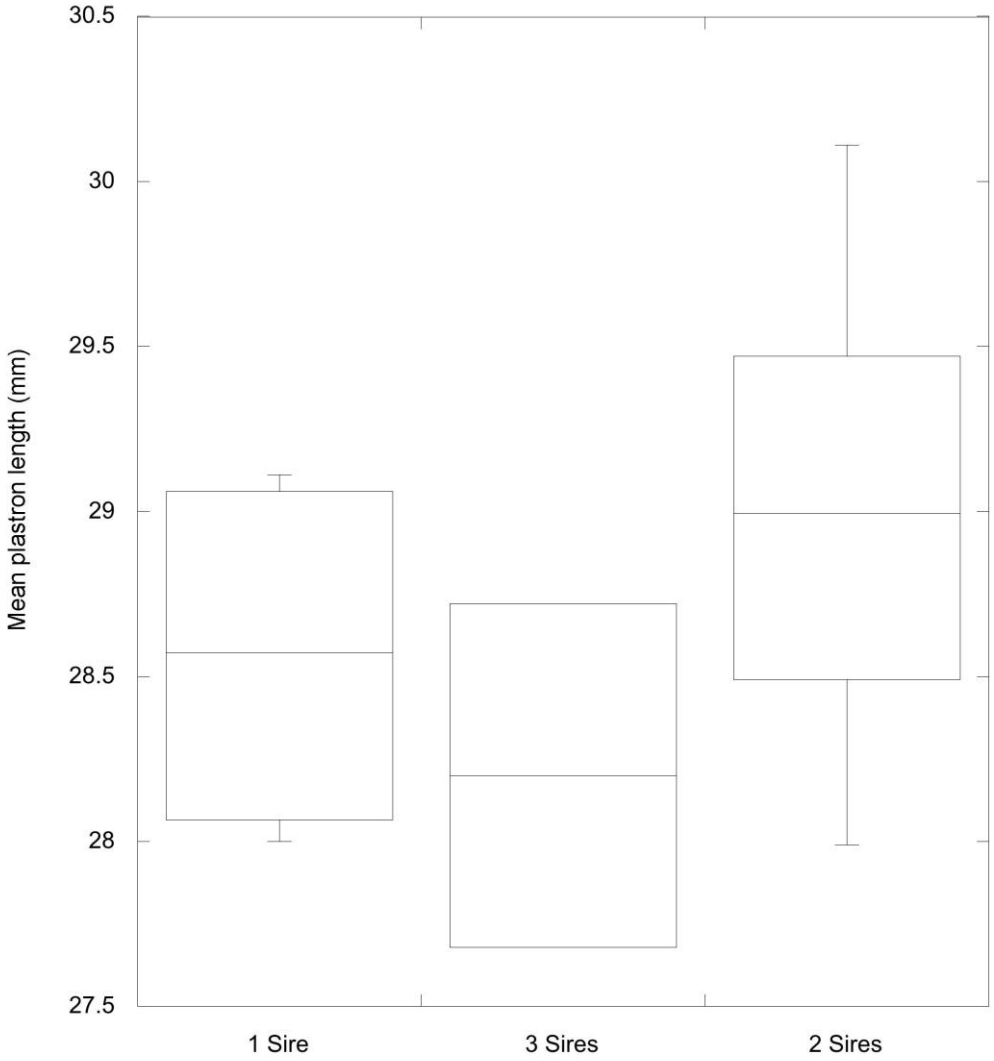

Figure 5. Mean plastron length of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (*N* = 14 clutches)

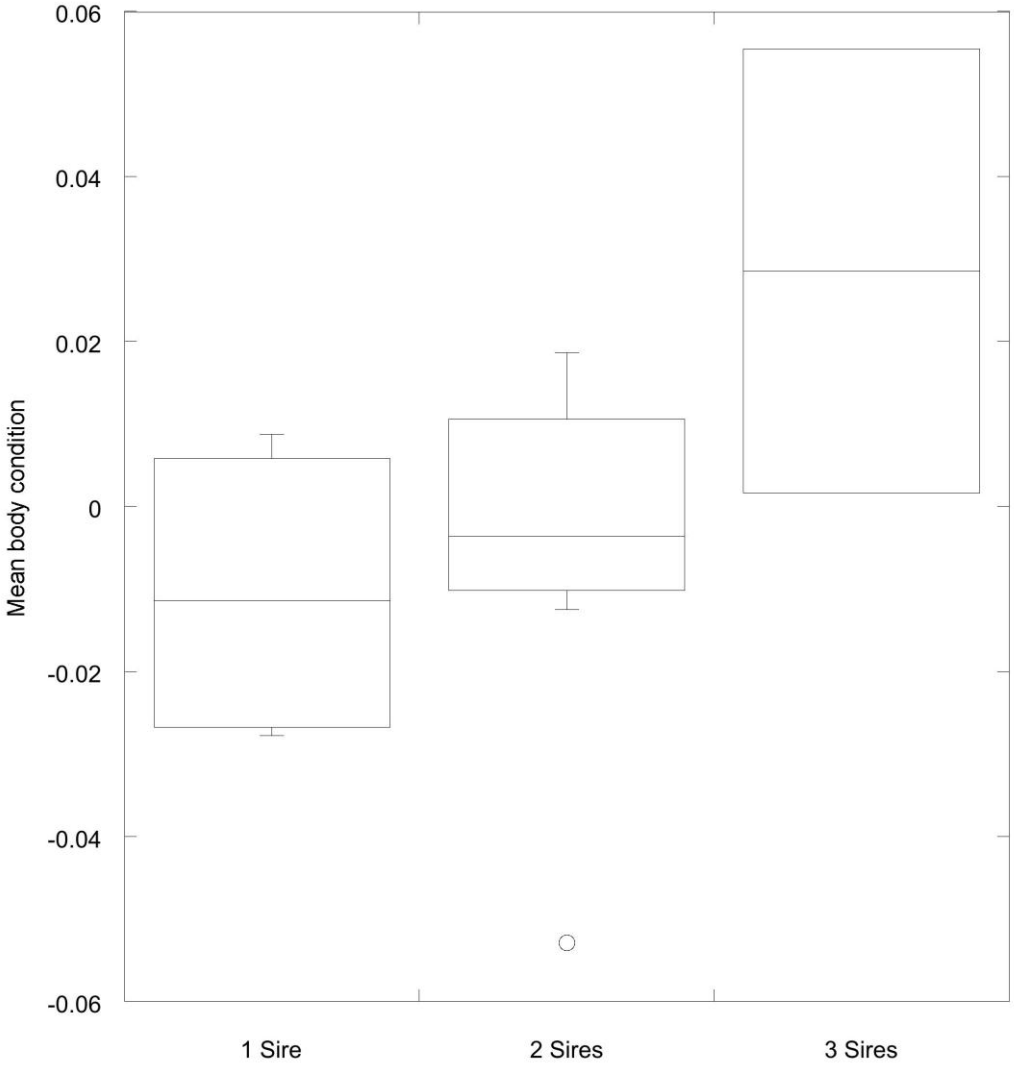

Figure 6. Mean body condition of map turtle hatchlings from clutches sired by a minimum of one, two, or three sires (N = 14)

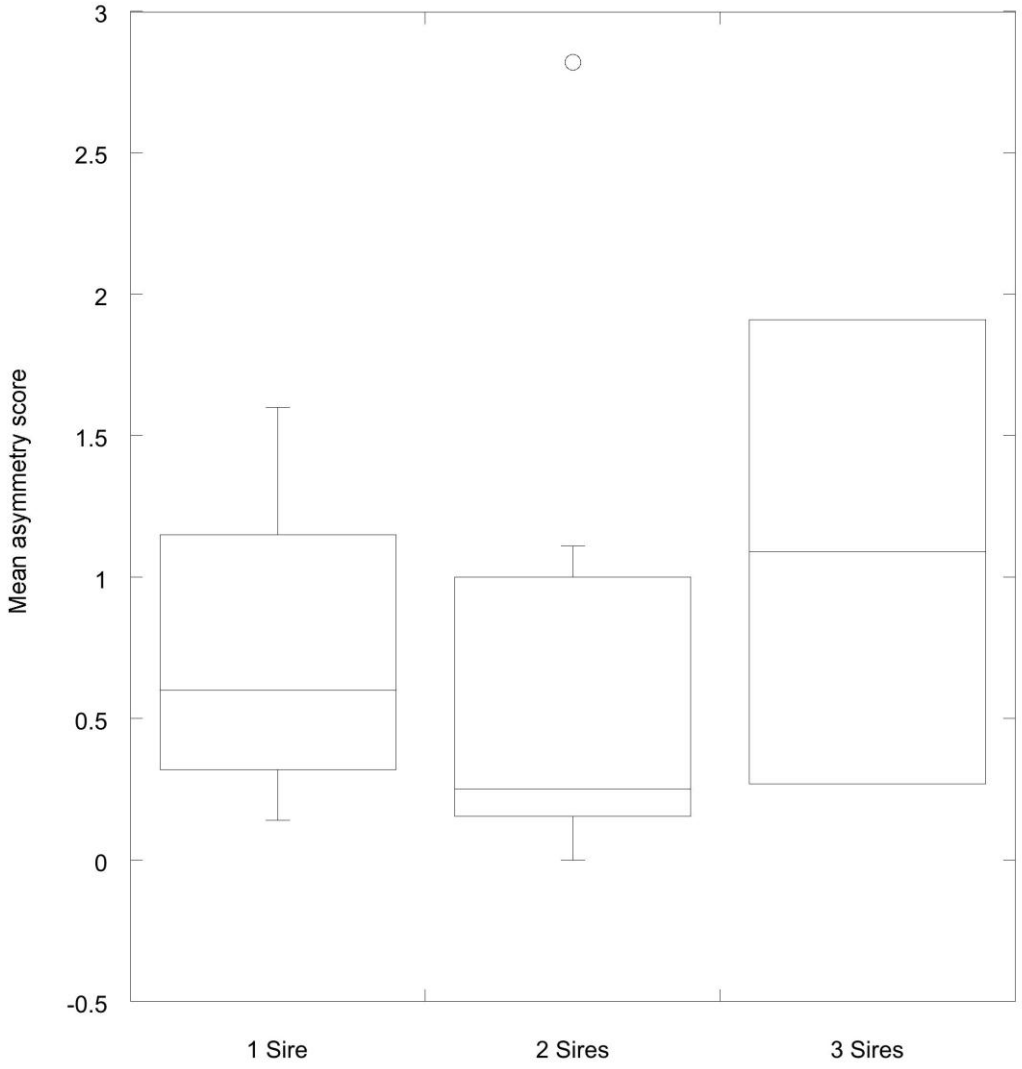

Figure 7. Mean asymmetry score of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

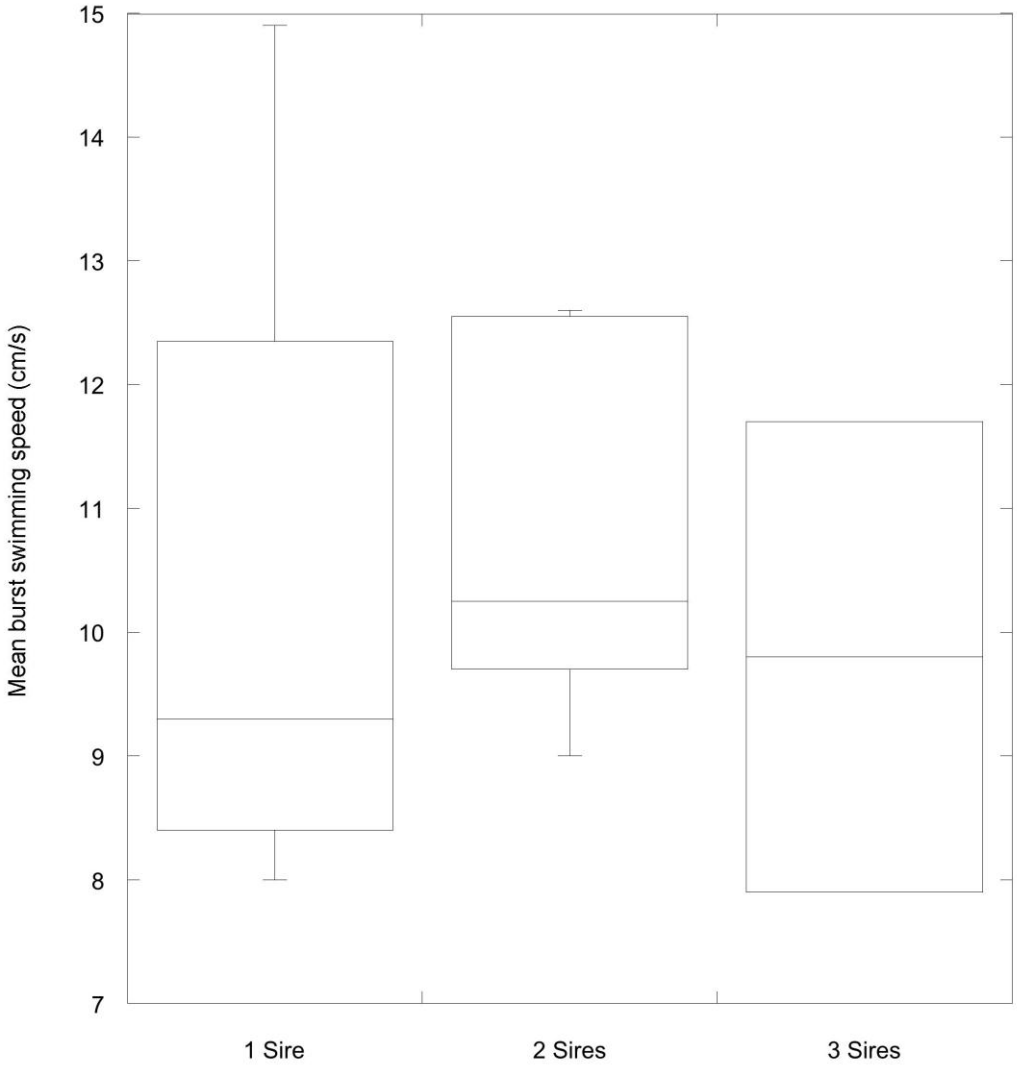

Figure 8. Mean burst swimming speed of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

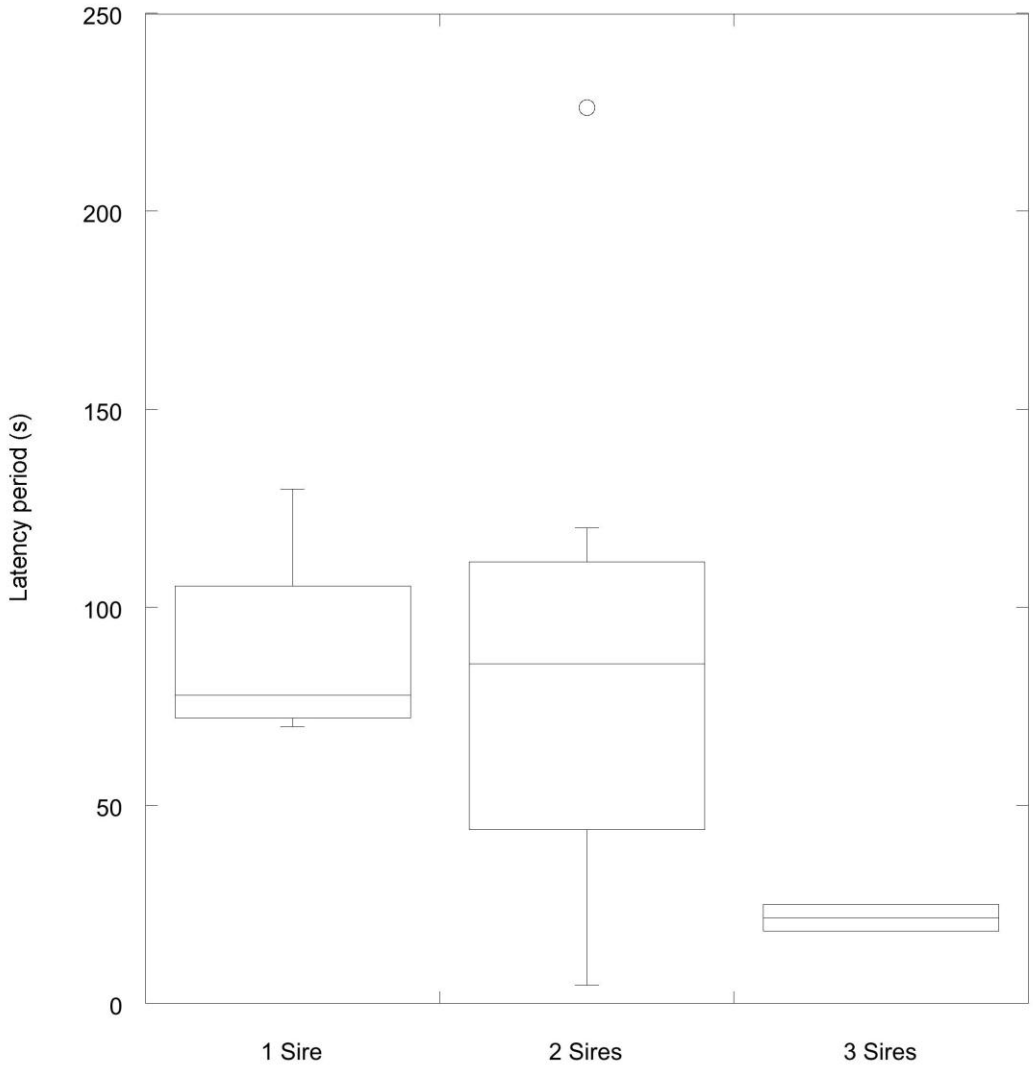

Figure 9. Mean latency period of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

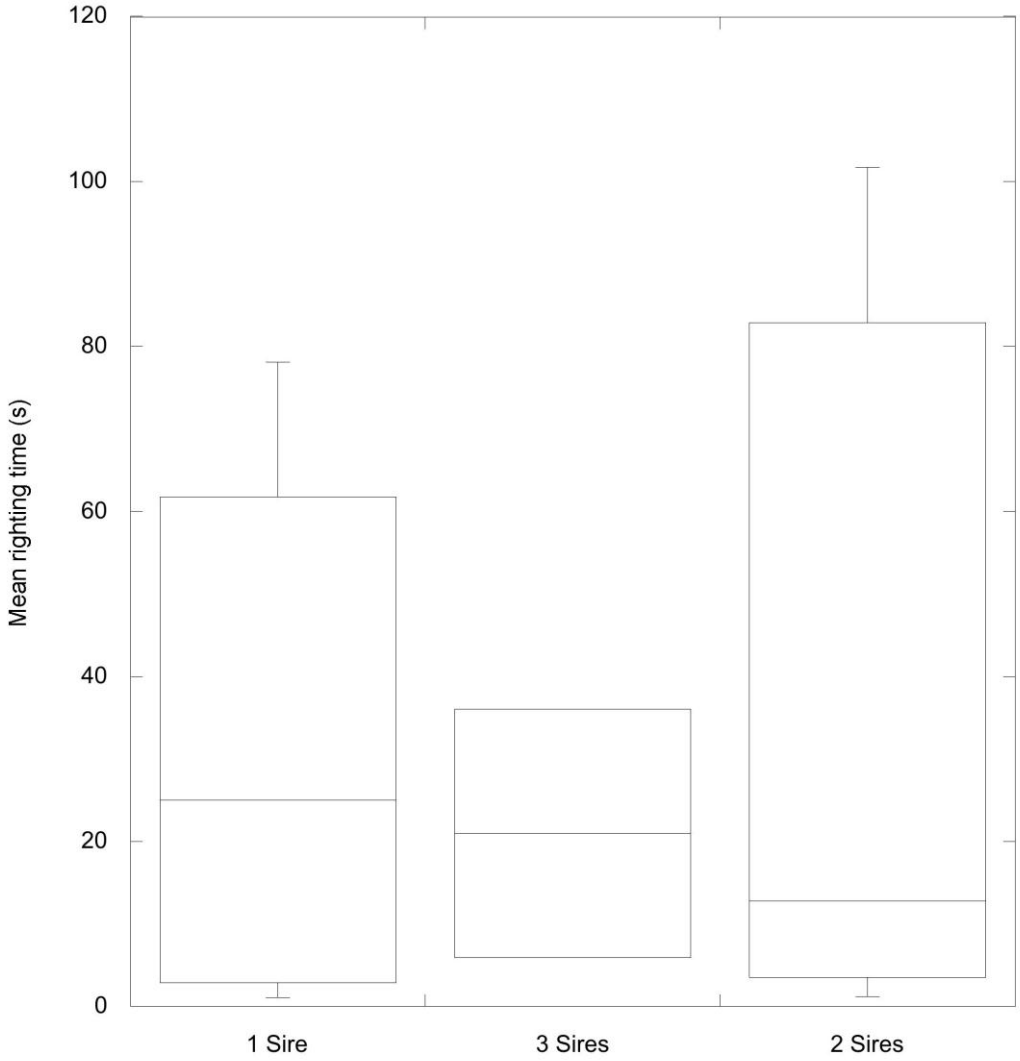

Figure 10. Mean righting time of map turtle hatchlings from clutches sired by a minimum of one, two, or three males (N = 14)

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Appendix

DNA Extraction Protocol – modified from glass fiber protocol for animals (Ivanova *et al.* 2006)

1. For each tube, mix 185ul of Lysis Buffer and 15 ul of Proteinase K (20mg/ml) in a sterile container. Add 200ul of Lysis Mix to each tube.

2. Add a small amount of tissue to each tube (flame sterilize instruments between samples), close lids

3. Incubate at 56°C for a minimum of 6 hours or overnight to allow digestion.

4. Warm water to 56°C for elution.

5. Pulse-centrifuge at 8000 rpm for 15 sec to remove any condensate from the caps

6. Add 400ul Binding Mix to each sample. Make as much as needed

7. Mix by pipetting and transfer the lysate (about 600ul) from the tubes into the spin columns placed on top of the collection tubes.

8. Centrifuge at 8000 rpm for 2 min to bind DNA to the GF membrane

9. **First wash step:** Add 300ul of Protein Wash Buffer (PWB) to column. Make as much PWB as needed each time. Centrifuge at 8000 rpm for 2 min. Pour off the contents of collection tube.

10. **Second wash step:** Add 700ul of Wash Buffer (WB) to column. Centrifuge at 8000 rpm fro 4 min.

11. Replace collection tube with a clean one. Centrifuge at 10000 rpm for 4 min.

12. Replace collection tubes with 1.5 ml tubes. Open the lids and incubate the columns at 56°C or at room temperature for 15-30 min.

13. Add 150ul of ddH_20 (pre-warmed to 56°C) directly onto the membrane of each column and incubate at room temperature for 1 min.

14. Centrifuge at 10000 rpm for 5 min to collect the DNA eluate. You can do it again with a different tube and collect a second eluate.

15. Store temporarily at 4°C or at -20°C for long-term.

Use 1-5ul of the DNA for PCR.

PCR Protocols

I optimized all primers using 19 DNA samples from male map turtles caught in 2005 and 2006 from Lake Opinicon. I carried out all PCR reactions using a 10 µL volume per amplification in 0.2 mL microtubes. Table 2 shows the final optimized concentrations of KCl buffer, $MgCl_2$, forward primer, reverse primer, fluorescent labeled M13 primer, dNTPs, Taq polymerase, and the amount of DNA for a single 10 µL reaction. Table 3 shows the final optimized temperatures and times of the PCR thermocycler protocols.

Table 2. Final concentrations/amounts of reactants for PCR cocktails for all primers used.

Primer	KCl Buffer (mM)	$MgCl_2$ (mM)	FOR (µM)	REV (µM)	M13 (µM)	dNTPs (mM)	Taq (units)	Template DNA (ng)
GmuB08	10	2	0.5	0.5	0.6	0.25	0.5	10-50
GmuA18	10	2	0.5	0.5	0.6	0.25	0.5	10-50
GmuD87	10	2	0.5	0.5	1.2	0.25	0.5	10-50
TerpSH7	10	2	0.5	0.5	0.6	0.25	0.5	10-50
GmuD51	10	3	0.5	0.5	2	0.25	0.5	10-50
GmuD90	10	2	0.5	0.5	0.6	0.25	0.5	10-50

Table 3. PCR Protocols

Primer	Initial amplification (°C x time)	Total # Cycles	Denature (°C x time)	Annealing (°C x time)	Extension (°C x time)	Final extension (°C x time)
GmuB08	94 x 2 min	35	94 x 45 sec	58 x 45 sec	72 x 90 sec	72 x 5 min
GmuA18	94 x 2 min	35	94 x 45 sec	58 x 45 sec	72 x 90 sec	72 x 5 min
GmuD87	94 x 5 min	35	94 x 45 sec	56 x 45 sec	72 x 90 sec	72 x 5 min
TerpSH7	94 x 5 min	35	94 x 30 sec	50 x 45 sec	72 x 45 sec	72 x 5 min
GmuD51	94 x 2 min	40	94 x 45 sec	55 x 45 sec	72 x 90 sec	72 x 5 min
GmuD90	94 x 2 min	35	94 x 45 sec	58 x 45 sec	72 x 90 sec	72 x 5 min