Micro-evolutionary potential of temperature dependent sex determination in a wild population of painted turtles, Chrysemys picta

by

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I dedicate this work to:

My Parents, Beverly and Larry Burmeier

and Mickey and Nancy McGaugh

and my fiancé, Lex Flagel
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ABSTRACT

The ways in which organisms determine sex are diverse. Sex determination systems are important because of the effect they have on sex ratios within a population, which affects reproductive success, levels of inbreeding, and population viability. In environmental sex determination (ESD), sex is determined in response to immediate environmental factors after conception. A common form of ESD in non-avian reptiles and some fish is temperature-dependant sex determination (TSD). Individual sex is determined by the temperature during the middle one-third of embryonic development, and the threshold between male and female development is often over a very small (< 2°C) range. While this form of sex determination has been maintained in a species or population over millions of years, TSD species may experience drastic skews in sex ratio in response to large climatic upheavals. Theoretically, response to selection for the rarer sex may produce evolutionary change at two levels in reptiles: 1) maternal nesting behavior with respect to thermal conditions and 2) thermal sensitivity of the threshold between male and female development.

The results from this dissertation reveal that both onset of nesting and nest-site vegetation cover have low heritability and thus low potential to respond to selection. Nesting behavior is dependent on the winter preceding the nesting season, however, and environment-specific analyses suggest that additive genetic variance increases for onset of nesting after warmer winters and increases for vegetation cover over the nest after cold winters. As a result, heritability may be dependent on the winter preceding the nesting season in this system. Estimates of repeatability corroborate these results. There is a
significant, genetic-based tendency for turtles to nest in areas with minimal vegetation cover after colder winters, while there is a significant, genetic basis for earlier first nesting dates after warmer winters. Genetic correlations between warmer and colder winters for vegetation cover are very high and suggest that no potential genotype-by-environment (G × E) interaction exists, while potential G × E between winter environments for first nesting date between winter environments is inconclusive.

Threshold temperatures may respond to selection for sex ratio biases as well. Maternal half-sib analysis facilitated by natural multiple paternity suggests that family effects on hatchling sex in TSD species are not exclusively driven by maternal effects and that there is a detectable genetic variance of the sire. Thus, the sex determination pathway’s sensitivity to temperature (i.e. primary sex ratio) may evolve in response to sex-ratio selection. The effective heritability, which predicts the relative rate of change of threshold temperature, was estimated to be 0.11, while the effective heritability of nesting behavior was estimated to be 0.079.

Since sire genetic variance can substantially influence the sex of offspring when the clutch is incubated at temperatures that produce both sexes, mating with multiple males may have a homogenizing effect on sex ratio variance within and between nests. No significant reduction in sex ratio variance for multiple paternity clutches was observed in the study presented here, but theoretically TSD may provide an ideal situation for bet-hedging. Clutches with multiple sires had higher hatching success rate and lower variance for hatching relative to clutches with a single sire. The incidence of multiply sired clutches
increased with plastron length (a proxy for age), although there was no significant interaction between fitness, plastron length, and multiple paternity.

In conclusion, these studies suggest that temperature dependent sex determination may respond to selection from sex ratio biases either through threshold temperature or nesting behavior, but both are likely to respond to selection slowly. Further, multiple paternity does not seem to homogenize sex ratios between clutches for the population examined here; however, additional studies are needed to exclude possible confounding factors.
CHAPTER 1.

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized in five chapters. The first chapter provides a general introduction to environmental and temperature dependent sex determination (ESD and TSD, respectively), the potential for micro-evolution of TSD, evolutionary genetics in wild populations, polyandry, and the aims of this dissertation. The second through fourth chapters are studies addressing the specific objectives delineated in the introduction.

The second chapter, entitled “Inheritance of nesting behaviour in the field in a turtle with temperature-dependent sex determination,” examines the micro-evolutionary potential of spatial and temporal nesting behaviour in a wild population of painted turtles. This is a collaborative project with Rachel Bowden, Assistant Professor of Ecological Physiology in the School of Biological Sciences at Illinois State University; Lisa Schwanz, Postdoctoral Researcher at the Cary Institute of Ecosystem Studies; Julie Gonzalez, Biology and Biotechnology Instructor at Des Moines Area Community College; and Fredric Janzen, Professor at Iowa State University. Bowden and Janzen authored the NSF grant IBN-0212935 responsible for funding this work and provided the initial impetus for the study as well as intellectual guidance. Schwanz organized much of the phenotypic data and provided important discussion and comments regarding the manuscript. Gonzalez extracted approximately half of the DNA for the project. McGaugh generated data for reconstructing pedigrees, collected some field data, conducted the analysis, and wrote the manuscript.
The third chapter, entitled “Quantitative genetic variation for thermal sensitivity of offspring sex in the wild in a turtle with temperature-dependent sex determination,” investigates the potential for temperature sensitivity of parts of the sex determination reaction norm to evolve in a field population of turtles with TSD. Again, this project required the contributions of multiple authors. Chih-Horng Kuo, a postdoctoral research associate in Department of Ecology and Evolutionary Biology at University of Arizona, helped extract DNA and genotype individuals for this analysis. Bowden and Janzen contributed similar roles as in Chapter 2. McGaugh generated much of the genotype data, collected data on clutch sex-ratios, conducted the analysis, and wrote the manuscript.

The fourth chapter, “Multiple paternity enhances maternal fitness and increases with female size in the painted turtle,” examines the association between multiple paternity, survival, and age and the relationship of multiple paternity and between-clutch sex ratio. Janzen is a co-author on this work as it utilized data from the long-term database maintained by the Janzen lab and data generated with funding from multiple grants to F. J. Janzen. McGaugh generated data for the multiple paternity analyses, conducted the analysis, and wrote the manuscript.

The final chapter outlines the general conclusions of this dissertation and highlights potential future research avenues.

**Introduction**

Many developmental programs, such as body plans or eye evolution, are an illustration that the natural world is filled with endless forms (Carroll et al. 2005). Sex determination systems are no exception (Bull 1983; Janzen and Paukstis 1991) and the
phylogenetic labiality of these systems shows that they are largely plastic among taxonomic groups (Janzen and Krenz 2004; Organ and Janes 2008). Yet, the sex determination method of a particular organism affects aspects of its biology beyond sex. For instance, the sex determination system may affect sex ratios within a population (Williams 1979), which in turn may affect reproductive success, levels of inbreeding, and population viability. In the same vein, different sex determination systems have different implications for the evolution of certain traits. For instance, ZW systems (female heterogamety) are more conducive to the evolution of sexual selection because the chance of loss of a male ‘showy’ or female preference allele is reduced as compared to XY systems (Reeve and Pfenning 2003). Much can be learned about broad areas of biology such as evolution and development, frequency dependent selection, genome organization, and evolution in wild populations from the study of sex determination (Bull 1983).

Generally, the proximate causes of sex determination in animals are well studied. We know, generally, how sex is determined in a variety of organisms. In genotypic sex determination (GSD) sex is permanently fixed at conception by genetic factors. The mammalian XY system (male-heterogamety) and bird and snake ZW system (female-heterogamety) are

![Thermal regimes of temperature-dependent sex determination in reptiles. TSD IA (MF) and TSD II (FMF) are found in turtles. P = pivotal temperatures.](image)
among the most well-known forms of GSD. But GSD is quite diverse. For example, in
arrhenotokous insect species males develop from unfertilized eggs and are haploid (Cook
1993); in several Diptera species sex determination is associated with a mobile genetic
element which initiates chromosomal differentiation (Kraemer and Schmidt 1993; Disney
2008). In vertebrates, such as the guppy and Japanese frog, alternate forms of
heterogamety exist within the same species (Volff and Schartl 2001; Ogata et al. 2003). And
comparative genomics and cytogenetics have revealed transitions between chromosomal
mechanisms at a more ancient level (Grutzner et al. 2004; Ezaz et al. 2006). In
environmental sex determination (ESD), sex is determined in response to immediate
environmental factors after fertilization (Bull 1983). First described in *Bonellia virdis*, where
the sex is determined to be male if the larva stage settles on adult females (Leutert 1975),
environmental sex determination has been found in relation to pH in fish, nutritional status
in nematodes, and temperature in fish and all non-avian reptile groups except snakes (Bull

One form of ESD, temperature-dependant sex determination (TSD), where individual
sex is determined by the temperature during the middle one-third of embryonic
development (Janzen and Paukstis 1991), was first discovered in lizard *Agama agama* in
1966 (Charnier 1966). In 1971-1972, TSD was shown in two turtles *Emys orbicularis* and
*Testudo graeca* (Pieau 1971; 1972). TSD contains multiple thermal regimes (Fig. 1; Janzen
and Paukstis 1991). TSD Ia (MF) produces males at lower temperatures and females at
higher temperatures. TSD Ib (FM) produces females at low temperatures and males at high
temperatures. Lastly, TSD II (FMF) produces females at extremes of the thermal limits for
survival and males are produced in between those extremes. Each pivotal temperature spans only 1-2°C (Janzen and Paukstis 1991). Further, possible genetic constraints in some temperature-sensitive species result in fully unisex clutches never being exhibited (turtle species *Kinosternon* and *Terrapene*, Ewert et al. 2004) or in systems where one of the sexes is never produced independently at a certain temperature (the lizard *Bassiana duperreyi*; Shine et al. 2002), pointing to the multi-faceted nature of temperature regulation (Ewert et al. 2004).

In turtles, TSD is basal (Janzen and Krenz 2004; Organ and Janes 2008), and genotypic controls (GSD) have arisen in at least five phylogenetically independent groups, with one of these groups (the Bataguridae; Carr and Bickham 1981; Olmo and Signorino 2005) containing both XY and ZW systems (Janzen and Krenz 2004; Organ and Janes 2008). In squamates, an incomplete phylogenetic history and knowledge about sex determining mechanisms complicates evolutionary inferences about sex determination (reviewed in Warner 2009), but TSD appears to be basal for all sauropsids (Janzen and Krenz 2004). Thus, TSD and genotypic sex determination vacillate throughout the reptilian phylogenetic history (Janzen and Krenz 2004; Organ and Janes 2008; Warner 2009) although both are ancient mechanisms (> 270 mya; Ezaz et al. 2006; Organ and Janes 2008).

The ultimate causes, “why” there are such diverse sex determination systems, are less understood. Darwin, Düsing, and Fisher noted that selection for the rarer sex should produce fluctuating selection to maintain a 1:1 sex ratio (Uller et al. 2007). Experimentally, selection for the rarer sex occurs and produces balanced sex ratios (Conover and Van Voorhees 1990; Basolo 1994; Carvalho et al. 1998; Blows et al. 1999). Yet, sex ratio selection
is thought to potentially account for change in sex determination mechanism (Volf and Schartl 2001; Ogata et al. 2003).

TSD may be an evolutionary stable strategy and may even be adaptive (Charnov and Bull 1977; Conover and Hiens 1987; Warner and Shine 2008), but is not necessarily at a selective disadvantage to GSD (Morjan 2002). TSD may especially be adaptive or neutral in many reptile populations since populations of these long-lived species contain overlapping generations (Bull and Bulmer 1989). Temporary environmental fluctuations around a stable mean may produce yearly sex ratio bias, but this will be buffered by the cumulative effect of multiple cohorts (Girondot and Pieau 1996; Bull and Bulmer 1989).

The enigma, however, remains regarding how primary sex ratio adapts to climate fluctuations around an instable mean (Janzen 1994). Phylogenetic character state reconstruction indicates that TSD persisted across many severe and abrupt climatic shifts (Rage 1998; Janzen and Krenz 2004; Organ and Janes 2008). In times of large climatic shifts or any large sex ratio bias, selection for the rarer sex may produce evolutionary change at two levels: 1) maternal nesting behaviour with respect to thermal conditions and 2) thermal sensitivity of the threshold temperature, or that temperature at which an individual becomes female (in the case of Ia), of the sex determination pathway (Bulmer and Bull, 1982; Bull et al. 1982a). Understanding the way in which sex ratio responds to selection may provide insight on how the diversity of TSD patterns evolved. For instance, FM and MF patterns are hypothesized to have arisen from shifts in the FMF pattern along a temperature continuum (Deeming and Ferguson 1988; Pieau 1996). Understanding the
evolvability of the thresholds to move from male to female may inform this hypothesis (Bull et al. 1982a; Janzen 1992; Rhen and Lang 1998; Dodd et al. 2006).

Maternal nest-site choice has been expected to respond to selection faster than the thermal sensitivity of the sex determination pathway, as nest microclimate may be sufficiently extreme to mask genetic variation for threshold temperature (Bull et al. 1982a; Janzen 1992). Further, geographic variation in nest site microclimate with respect to overstory vegetation cover has been documented, indicating that local adaptation is possible (Cagle 1950; Vogt and Flores-Villela 1992; Ernst et al. 1994; Tucker 1997; Morjan 2003b) and significant repeatability is seen in this trait (Janzen and Morjan 2001; Kamel and Mrosovsky 2005). Much phenotypic variation exists for nest placement, as females can also alter the incubation temperature experienced by embryos by digging deeper or shallower nests, laying at different times during the season (Georges et al. 1992; Schwanz and Janzen 2008), or choosing areas with different slopes or albedo (Wilhoft et al. 1983; Schwarzkopf and Brooks 1987; Hays et al. 2001; Doody et al. 2006).

To produce an evolutionary response to selection against sex ratio bias, nesting behaviour must have a heritable basis (Bulmer and Bull 1982; Bull et al. 1988). The quantitative genetics of this trait have not been extensively examined. Repeatability, a measure of self-similarity, is the upper bound for heritability (Lessells and Boag 1987; Dohm 2002) and has been estimated for nest-site choice with respect to thermal parameters in the lab and in the field. Estimates of repeatability range from low to high and so are relatively inconclusive in providing a thorough understanding of the potential for
evolutionary change in nesting behaviour (Bull et al. 1988; Janzen and Morjan 2001; Kamel and Mrosovsky 2005). Timing of nesting, or phenology, has also been examined recently for repeatability, but this trait was revealed to be highly dependent on winter environmental conditions prior to the nesting season (Schwanz and Janzen 2008). This dissertation provides the first estimate of the heritability for temporal and spatial nest-site choice in the field in a species with TSD by investigating a natural population of painted turtles, *Chrysemys picta*, on the Mississippi River in Illinois, USA.

Estimating heritability in the wild has been aided substantially by the adoption of mixed model methodology. By fitting the “animal” as a random effect in a linear mixed model, the “animal model” estimates the contribution of genetic and environmental variance components to the phenotypic variance of a trait through restricted maximum likelihood (Kruuk 2004). This approach has special utility for estimating variance components in natural populations because it can remove the variance from fixed effects such as sex or treatment, utilize incomplete cross-generational pedigrees, and model individual-specific environmental and additive genetic effects from repeated measures of the same individuals (Kruuk 2004).

This methodological advance also allows finer examination of the effect of climatic changes on evolutionary potential (Charmantier and Garant 2005; Gienapp et al. 2004; Brommer et al. 2008; Charmantier et al. 2008). Several studies have undertaken the quantification of heritability as a function of the environment in wild populations, and the general findings have suggested that heritability in natural populations is not static
(Charmantier and Garant 2005; Brommer et al. 2008). Several potential explanations for the change in heritability among environments include a change in additive genetic variation ($V_A$) or environmental variance ($V_E$) or that the trait actually has a different underlying genetic architecture in different environments (Charmantier and Garant 2005). Each of these explanations has different implications for the evolutionary potential of a trait to respond to selection.

Average first nesting date and average vegetation cover over a painted turtle nest are correlated with September–April temperatures prior to the nesting season, and individual reaction norms vary significantly (Schwanz and Janzen 2008). Environment-specific heritability and genotype-by-environment interactions, which are required to understand the true micro-evolutionary response of a trait, can be estimated by treating specific environments as separate traits or by more advance random regression or character process models (Via et al. 1995; Nussey et al. 2007; Gienapp et al. 2008).

Regardless of the method used, an environment-specific estimate of heritability will provide a more accurate view of the trait's evolutionary potential. In this dissertation, winter environments prior to the nesting season were treated as separate traits, and the environment-specific heritability showed potential differences after warm or cold winters. This information is directly applicable to understanding specific expectations of the evolution of nesting behaviour in response to climate change.

In the second chapter, the heritability of temperature sensitivity in the sex determination pathway was examined. Several points suggest that threshold temperatures can respond to selection. First, pivotal temperatures, the temperatures that produce a 1:1
population or clutch sex ratio, increase with latitude (Bull et al. 1982b; Ewert et al. 1994; Ewert et al. 2004; Ewert et al. 2005), suggesting that zygotic sensitivity to temperature might respond measurably to selection. Second, a gradual increase or decrease of 1°C over 1000 years altered threshold temperature relatively more than nest-site choice in a simulated population of turtles with TSD (Morjan 2003a). Lastly, incubation at a constant temperature in the transitional range between male and female reveals substantial among-clutch variation for sex ratio in the lab, indicating that temperature sensitivity of the sex determination pathway may have an additive genetic component to enable micro-evolution (Bull et al. 1982a; Janzen 1992; Ewert et al. 1994; Rhen and Lang 1998). As an example, some clutches of the alligator snapping turtle (*Macrochelys temminkii*) resemble a temperature-insensitive pattern similar to GSD, and some are highly affected by temperature (Ewert et al. 1994).

Lab estimates of genetic variance for temperature sensitivity of the sex determination pathway did not parse the among-clutch variation in sex ratio into additive genetic effects and maternal effects, although maternal hormones are known to influence sex-ratio in TSD species (Bowden et al. 2000; Elf 2004). To remove maternal effects from heritability estimates, animal breeding experiments typically utilize breeding designs not often possible in natural systems (Lynch and Walsh 1998). However, the contributions of each sire in a nest with multiple paternity can be teased away from the maternal contribution (King et al. 2001). The half-sib analysis allowed by this natural design can provide an estimate of heritability that is free from maternal effects (King et al. 2001). The estimate of heritability of threshold temperature was calculated by this thesis to be 0.26
and is independent of maternal effects. Such an estimate will provide a more accurate understanding of the potential response to selection of threshold temperature (Janzen 1992). This dissertation investigated the genetic variation for threshold temperature by examining sire genetic variance in this way.

In the third chapter, we found evidence for indirect genetic benefits of polyandry. Since the sensitivity of threshold temperature is heritable (Bull et al. 1982a; Janzen 1992), the genetics of the sire have the potential to bias a nest to one extreme (all females) or another (all males). Mating with multiple males may increase the genetic variance for threshold temperature. Thus, polyandry may reduce sex ratio variance within and across nests and this may be an indirect benefit. Theoretical conditions favouring this reduction in variance, called “bet-hedging,” are common in many reptile populations. Namely, small female population size and fluctuating conditions make picking the “correct” male difficult. Specifically in TSD species, yearly climatic fluctuations, which drastically bias cohort sex ratio (Janzen 1994a), may provide the unpredictable environment as required by theory.

Indirect benefits of polyandry may also manifest as increased offspring survival or decreased variance in offspring survival. Post-copulatory sperm biasing (i.e. increased offspring survival) and bet-hedging (i.e. decreased variance in offspring survival) are defined by different mathematical expectations. A higher variance in fitness must be observed in monandrous females relative to polyandrous females to accept bet-hedging (Hopper et al. 2003). When polyandrous females have an overall higher fitness than monandrous females, post-copulatory sperm biasing is accepted (Yasui 1998; Madsen et al. 2005).
Turtles provide an excellent system for examining the indirect benefits of multiple paternity because no direct benefits are gained from mating, forced copulations are unlikely, and females can store sperm for at least three years in the wild (Pearse et al. 2001). Thus, females can minimize the frequency of mating. Turtles are also long-lived, and size can be used as a proxy for age, so changes in polyandry across age classes can be examined.

The subject of all of these studies, the painted turtle, *Chrysemys picta*, is quickly becoming the “model” system for Testudines. For instance, the painted turtle genome and expressed sequence tag (EST) sequencing are currently being undertaken at Washington University under the grant entitled “Evolution of the human proteome: Completing the Chordate Nodes” by John Gerhart, Marianne Bronner-Fraser, Scott Edwards and Peter Holland. *Chrysemys picta* is holding this role for several reasons. By spanning from Canada to Mexico, it is one of the most geographically widespread turtles in North America (Starkey et al. 2003). Several long-term study populations exist in latitudinally disparate zones (Morjan 2003b). Much is known about its reproductive biology (Pearse et al. 2001; 2002), nesting phenology (Schwanz and Janzen 2008), and nesting ecology (Janzen 1994b; Weisrock and Janzen 1999). It also has some medically useful features such as showing little reproductive decline with age (Congdon et al. 2003) and being able to super-cool and survive freezing (Storey 2006).
Objectives

This research involves three projects designed to evaluate the potential for micro-evolution of temperature-dependent sex determination and the indirect benefits of polyandry. In all papers, the western painted turtle is used as a study organism. The specific objectives are to:

(1) evaluate the heritability of nesting behaviour across different environments.

(2) evaluate the heritability of threshold temperature of the sex determination pathway, independent of maternal effects such as sex hormones and nesting behavior.

(3) evaluate whether increasing sire genetic variance for sex ratio can provide justification for polyandry.
CHAPTER 2. INHERITANCE AND PLASTICITY OF NEST-SITE CHOICE IN THE FIELD IN A TURTLE WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

A paper to be submitted to Proceedings of the Royal Society B

Suzanne E. McGaugh, Lisa E. Schwanz, Rachel M. Bowden, Julie E. Gonzalez, and Fredric J. Janzen

Abstract

Nesting behaviour is critical for reproductive success in oviparous organisms with no parental care. And in organisms where sex is determined by incubation temperature, nesting behaviour may be a prime target of selection in response to unbalanced sex ratios suggesting that components of nest-behaviour should be heritable. We estimated the field heritability of two components of nesting behaviour in a population of painted turtles (Chrysemys picta) with temperature-dependent sex determination by applying the ‘animal model’ to a pedigree reconstructed from genotype data. We obtained an estimate of heritability using repeated records across all environments and then evaluated environment-specific heritability by grouping records with similar temperatures for the winter preceding the nesting season, a variable known to be associated with our two traits of interest: date of nesting and vegetation cover over the nest. The heritability of date of nesting was significantly greater than zero when evaluating data across environments and records from only warm winters. Heritability for nest vegetation cover over the nest, a trait highly correlated with sex ratio of the nest, was not significantly different from zero when data across environments were evaluated, but significant heritability was found for
vegetation cover after cold winters. The potential genotype-by-environmental (G × E) interaction between HOT and COLD environments for vegetation cover is deemed to be low as the genetic correlation was essentially +1 across environments. Thus, generally females are exhibiting relatively similar phenotypes after hot and cold winters. Overall our analysis revealed the potential for evolutionary change of nesting behaviour to be dependent on the thermal conditions of the preceding winter, a season whose characteristics are predicted to be especially subject to climate change.

Keywords: Chrysemys, phenology, animal model, climate change, G × E

1. INTRODUCTION

Nesting behaviour is a major factor in determining maternal fitness in oviparous species (Weisrock & Janzen 1999; Reguera & Gomendio 2002). Poor nest-site choice can result in increased predation (Sargent & Gebler 1980; Hatchwell et al. 1996; Downes & Shine 1999; Kolbe & Janzen 2001), reduced hatching success (Cagle et al. 1993; Wilson 1998; Warner & Andrews 2002), and reduced offspring fitness (Shine & Brown 2002; Patterson & Blouin-Demers 2008). Thus, for species with no maternal care after oviposition, finding a suitable nest site is especially critical (Kolbe & Janzen 2001; Blouin-Demers et al. 2004; Hughes & Brooks 2006).

Still, nesting behaviour likely represents a compromise that minimizes the cost of finding an appropriate oviposition site while balancing the factors affecting maternal and offspring fitness (Thompson 1988; Tucker et al. 1999; Spencer 2002; Spencer & Thompson 2003). As a result, selection may act on multiple components of nesting behaviour, yet the
genetic architecture of this complex trait has received little attention (but see Singer et al. 1988; Fox et al. 2004).

Maternal nest-site choice is central in theoretical explanations of the evolution, adaptive value, and maintenance of temperature-dependent sex determination (TSD; Bulmer & Bull 1982; Bull et al. 1988; Roosenburg 1996; Reinhold 1998; Roosenburg & Niewiarowski 1998; but see Valenzuela & Janzen 2001; Morjan & Janzen 2003). With TSD, the thermal environment of the nest during incubation determines sex, rather than a genotypic cue at conception (Janzen & Paukstis 1991). This form of sex determination is widely distributed among reptile lineages and has been maintained throughout rapid climatic upheavals such as the Cretaceous-Tertiary boundary (Rage 1998; Janzen & Krenz 2004; Organ & Janes 2008). The relative response to selection in restoring equilibrium sex ratios during these periods (Fisher 1930) are unknown for specific traits in TSD species (Bulmer & Bull 1982). Nest-site choice in TSD species has been hypothesized to have a more dominant role in the evolutionary response to sex ratio bias than thermal sensitivity of the sex determination pathway (Bulmer & Bull 1982; Bull et al., 1988; Doody et al. 2006), as nesting in extreme microclimates may override variation for thermal sensitivity of the actual sex determination pathway (Bull et al. 1982). Previous research has documented that geographic variation in nest site microclimate occurs, indicating that local adaptation is possible (Ewert et al. 2005; Doody et al. 2006). Further, much phenotypic variation exists for nest placement, as females can also alter the incubation temperature experienced by embryos by digging deeper or shallower nests or laying at different times during the season (Georges 1992; Morjan 2003b; Doody et al. 2006; Schwanz & Janzen 2008).
An important corollary to the hypothesis that nesting behaviour plays a role in the response to selection against sex ratio bias is that nest-site choice has a heritable basis (Bulmer & Bull 1982; Bull et al. 1988), but few studies have tested this possibility. In the lab, nest-site choice in the leopard gecko (*Eublepharis macularius*) exhibited a repeatability of 0.20 (i.e. upper bound of heritability; Bull et al. 1988). In the field, overstory vegetation cover provides a stable cue for nesting turtles that is predictive of nest sex ratio (Janzen 1994b). Measures of repeatability of this trait in the painted turtle (*Chrysemys picta*) and hawksbill turtle (*Eretmochelys imbricata*) are different but not negligible (repeatability = 0.18 - 0.20 and 0.7, respectively; Janzen & Morjan 2001; Kamel & Mrosovsky 2005). Moreover, adjusting nesting phenology could permit a female to regulate the sex ratio of a clutch, although this trait is not known to be repeatable (repeatability = 0.03, Schwanz & Janzen 2008). While, these studies provide some insight into the inheritance of nesting behaviour in TSD species, estimates of the heritability for temporal or spatial nest-site choice in the field are lacking.

Estimating the additive genetic variation underlying nesting behaviour in the field over repeated measures for a single female is complicated by individual plasticity and changes in additive genetic and environmental variance across different years (Charmantier & Garant 2005; Nussey et al. 2007; Brommer et al. 2008). Indeed, date of first nesting in a population of painted turtles is correlated with September–April temperatures prior to the nesting season, and individual reaction norms vary significantly (Schwanz & Janzen 2008). Traits of this nature, with among-individual variability in response to an environmental variable, are excellent systems for investigating environment-specific heritability and
genotype-by-environment interactions, which are essential to assess the micro-evolutionary response to climate change (Via et al. 1995; Nussey et al. 2007; Gienapp et al. 2008; Uller 2008).

We estimated field heritability of nest-site choice with respect to Julian date and vegetation cover for a wild population of painted turtles by applying the animal model. The animal model estimates the contribution of genetic and environmental variance components to the phenotypic variance of a trait by fitting the “animal” as a random effect in a linear mixed model (Kruuk 2004). This approach is especially well suited for estimating variance components in natural populations because it can utilize incomplete (i.e. unbalanced) datasets, information across generations without a breeding design, and can incorporate repeated measures from the same individuals (Kruuk 2004). In the first analysis, we used repeated measures across all environments, and accommodated year-to-year variation by including year as a random effect. Next, we treated data from nesting seasons following the four hottest and coldest winters for which we had data as separate “environment-trait”s for date and vegetation cover in order to more finely examine environment-specific heritability. In this analysis, we assessed whether heritability differed across environments and whether there was any support for genotype-by-environment interactions (i.e. G × E; Via et al. 1995; Nussey et al. 2007).

2. MATERIALS AND METHODS
(a) Field data collection

*Chrysemys picta* ranges from southern Canada to northern Mexico (Ernst *et al*. 1994; Starkey *et al*. 2005). Data were collected from a well-characterized population of painted turtles at the Thomson Causeway Recreation Area along the Mississippi River near Thomson, IL (Janzen 1994a, b; Schwanz & Janzen 2008). We focused on the southeast portion of the island where the nesting beach is a level grassy area, soil moisture is relatively uniform (Janzen 1994b), and variable levels of overstory vegetation cover are present (Morjan 2003a). In this population, clutch sex ratios have been evaluated over the past 20 years (Janzen 1994b, unpublished data). Females in this population mature in 5-7 years (Morjan 2003a) and oviposit 1-3 clutches from late May to early July.

From 1995-2008, the nesting grounds were monitored from 0600 hr to 2000 hr during the May-July nesting season. Turtles in this population typically emerge from the water, nest and return to the water within 2 hr, and nearly all nesting events were observed. Nesting turtles were marked uniquely and after 1996 a blood sample was collected from the postcranial sinus using a 28ga insulin syringe. The sample was preserved in lysis buffer and stored in liquid nitrogen or at -20ºC.

(b) Molecular markers

Genotypes of five microsatellite loci (GmuD79, GmuD21, GmuD62, GmuD70, GmuD28; King & Julian 2004) were obtained from 340 nesting females using standard molecular techniques (supplementary material). All homozygotes and any ambiguous alleles were rerun for confirmation, resulting in greater than half of the dataset being evaluated at least twice to confirm the genotype. Each of the amplified loci contained a four base-pair repeat
motif and was hypervariable (number of alleles: GmuD79: 30, GmuD21: 26, GmuD62: 25, GmuD70: 64, GmuD28: 19). An exclusion analysis performed with GenAlEx v6.0 (Peakall & Smouse 2006) determined that these five loci provided exclusionary probability of greater than 0.9999 when neither parent is known (Jamieson & Taylor 1997).

Analysis using GenePop v4.0 (Raymond & Rousset 1995) with the default parameters indicated that GmuD28 and GmuD70 significantly deviated from Hardy-Weinberg equilibrium and linkage among loci was evident (tables S1, S2). We interpreted these heterozygote deficiencies (as GmuD62 was not linked to GmuD21) and high error rates in Gmu28 and GmuD70 (supplementary material) to reflect the presence of null alleles and specified that these loci had null alleles when reconstructing genealogies. Reconstruction of relationships omitting GmuD28 resulted in more relationships being identified; therefore, we took the conservative approach and left this locus in the analysis to provide higher exclusion power.

(c) Pedigree reconstruction

All pedigree links were inferred mainly from genotypic data in this study for two reasons. First, chelonian reproductive biology (e.g. sperm storage across years and multiple paternity within clutches; Pearse et al. 2001, 2002) makes pedigree reconstruction solely from field observations difficult. Second, high mortality from hatchling to reproductive maturity (estimated annual juvenile survivorship is 21-51%; Ernst et al. 1994) renders uniquely marking individuals at the neonate stage time- and cost-inefficient. For these reasons, relationships cannot be derived solely from field observations.
The parent-offspring (PO) and sibling relationships were determined as the most likely relationship between a pair of individuals as deduced from genotype data by maximum-likelihood with ML-RELATE (Kalinowski et al. 2006; Wagner et al. 2006). We refined PO pairs initially output by ML-RELATE by creating an enriched dataset that contained only female pairs with five years (the earliest they can mature in this population) or more between each of their first recorded nesting events. These field observations also provided unambiguous assignment of the parent and offspring as the individual with the first nesting date five years earlier than the other was inferred to be the parent of the parent-offspring pair. Requiring this time between first recorded nesting events may have also removed full-sib (FS) or half-sib (HS) pairs that were misclassified as PO. Any offspring with multiple individuals classified as being their parent were removed from the final pedigree (N = 30), as these likely represent false positives. For moms with multiple offspring, the assigned relationships of putative siblings were confirmed to be FS or HS. If these relationships were not concordant, the offspring was removed (N = 1). In all, 54 parent-offspring pairs were identified and 10 of these moms had multiple offspring (4 FS links and 17 HS links). We focused on PO pairs because exclusion power for parent-offspring designations were high and reconstruction of more distance relationships require additional loci.

(d) Traits of interest

We evaluated both onset of nesting and vegetation cover over a nest as crucial measures of nesting behaviour. Onset of nesting was measured by recording Julian date for the first nesting event of the season for each female, from 1995-2008. Our total dataset for onset of
nesting contained 1965 first nesting events of the season from 631 females (mean nesting events per female = 3.11, range = 1-12). We focus on all females, even if no pedigree information was available, because additional records contribute to the estimation of non-genetic effects in the model.

Vegetation cover over a nest was determined using a spherical densiometer (Janzen 1994b; Weisrock & Janzen 1999). The percent of south and west cover was summed to obtain a single vegetation cover measurement for each nest. This measure is used here, as opposed to total vegetation cover from all cardinal directions, because it is more strongly correlated with nest sex ratio (Janzen 1994b). Our total dataset for vegetation cover over the nest contained 2212 (1676 first nests of the season, 536 second nests of the season) nesting events from 631 females (mean nesting events per female = 3.51, range = 1-18). Vegetation cover was collected from 1995–2008, excluding 2004 and 2005 because vegetation cover was measured differently in those years.

Julian nest date and vegetation cover deviated from a normal distribution (all Shapiro-Wilks $p < 0.050$). However the deviations were minimal (Fig. S1, S2 supplementary materials) so both traits were used in statistical genetic analyses as the animal model is fairly robust to slight deviations from normality (Kruuk 2004). For both traits, the only genetic data included were pedigree links mentioned in the above section.

Average first nesting date of the season and average overstory vegetation cover of the nest in this population of painted turtles are associated with the temperature of the winter preceding the nesting season (Fig. 1), and the relative influence of winter temperatures on nesting date is variable across females (Schwanz & Janzen 2008). Given
that this population has significant individual-by-environment interactions for date (I X E; Schwanz & Janzen 2008), we explored whether heritability changed across winter environments by binning winter environments and treating each binned environment as a separate trait. To measure winter environment, heating degree-days for September through April preceding the nesting season were used (HDD; for HDD calculation see Schwanz & Janzen 2008). Specifically, measurements from the four coldest (COLD), four hottest (HOT), and four mid-temperature (MED) years of data collection were lumped as separate traits so that the new traits consisted of date and vegetation cover measures for each of the three individual environments. This binning strategy allowed the finest scale sub-setting of the environment that preserved enough power to estimate heritability and maintained a consistent number of winters in each bin. These six traits are hereafter referred to as “environment-traits” and Table 1 lists the number of individuals and records used to estimate heritability for these environment-traits. This approach allowed us to assay for evidence of G × E by examining the genetic correlation between environments (Via et al. 1995).

(e) Heritability calculations

Several versions of the animal model were run in ASREML v2.0 (Gilmore 2006; Kruuk 2004). In all models except where noted, random effects included year, ‘animal’, which is referenced back to the pedigree, and a permanent environmental effect that accounts for non-genetic within-individual variation accompanying multiple measures per individual (pe; Lynch & Walsh 1998). Clutch order in the season for each nest was not significant and did not increase the log-likelihood, so it was not included in the model for vegetation cover. In
all ASREML runs, starting values for variance and covariance were calculated from the original dataset and refined by using output values from runs as new starting values. Residual plots were examined for normality in ASREML.

Three variations of the animal model were used to evaluate the quantitative genetics of nesting date and vegetation cover: 1) Two univariate animal models (one for date and one for vegetation cover) utilized a pedigree reconstructed from molecular marker data and field observations to estimate overall heritability of date and vegetation cover from all of the data points available (Kruuk 2004). In this model, no fixed effects were specified but random effects included year of nesting, animal, and permanent environmental effects. 2) The measurements for phenotypic traits of date and vegetation cover were binned by similar environments, as described above, and treated as separate traits. These environment-trait traits were each separately used as a response variable in a univariate animal model with the same random effects as in the first model. 3) If two traits have a genetic correlation that is not significantly different from one, the genetic architecture of the two traits is inferred to be very similar (Charmantier & Garant 2005). Thus, we may be able to infer the presence of G × E for date or vegetation cover, if the genetic correlation between the environment-trait traits is significantly different from one. To estimate genetic correlations, two multivariate animal models with the “COLD” trait and the “HOT” trait as response variables were run (one for date and one for vegetation cover; Via et al. 1995; Nussey et al. 2007). These two models contained the same random effects as the first model and allowed for estimation of genetic correlations, providing an indication of G × E between the COLD and HOT environments. Due to our low sample sizes, for the
analysis of vegetation cover, all factors, except correlations, had to be constrained to be positive and the residual correlation was constrained to be zero in order for the model to converge and produce residuals that were normally distributed. Year was also treated as a fixed effect in this case because our sample sizes were not sufficient to estimate year as a random effect. Attempts to fit more sophisticated models such as the random regression animal model or character process models to estimate G × E (reviewed in Jaffrézic & Pletcher 2000; Nussey et al. 2007) were unsuccessful because of lack of power.

Because our estimates of heritability are based purely on pedigrees reconstructed from genotypes, we also calculated repeatability for each of the six environment-traits and for the total datasets of vegetation cover and date (Lessells & Boag 1987). Year was included as a factor in the ANOVAs used for repeatability estimation. Repeatability is simply a measure of the degree of self-similarity for nesting behaviour, and this measure is independent of any pedigree designations (Lessells & Boag 1987; Dolm 2002). Concordance between environment-specific heritability and environment-specific repeatability in direction and magnitude would bolster our conclusions from the animal model analyses.

3. RESULTS

For the univariate analysis of the heritability of date and nest vegetation cover, including all records, estimates were $\hat{h}^2_{date} = 0.0613$ (95% CI = 0.0270, 0.0956) and $\hat{h}^2_{veg} = 0.0038$ (95% CI = -0.2042, 0.2118), respectively. The estimate for date was similar to the estimate of repeatability ($date = 0.0961, p < 0.001$). But much of the repeatability for vegetation cover ($vegetation cover = 0.1326, p < 0.001$) was due to permanent environmental effects ($\sigma_{pe}^2 / total \sigma^2$; date: $0.0 \pm 0.0$; vegetation cover: $0.1361 \pm 0.1079$). Permanent
Heritability of first nesting date and vegetation cover may be environment-specific (Table 1). Heritability of nesting date was significantly different from zero following hot winters ($h^2 = 0.1152; 95\% \text{ CI} = 0.0145, 0.2159$), whereas heritability of vegetation cover over the nest was significantly different from zero after cold winters ($h^2 = 0.2629; 95\% \text{ CI}=0.1494, 0.3764$). The confidence intervals for these estimates overlapped with the overall analysis and other environment-specific analyses, though (Table 1). Permanent environmental effects were constrained to be positive if these were estimated as zero or negative regardless of the starting values. The environment-specific heritability measures were corroborated by the repeatability estimates that exhibit the same trend (Table 1).

The genetic correlation for first nesting date between cold and hot winters was not significantly different from zero or one (Table 2; $r_G = 0.4691, 95\% \text{ CI}=-0.4450, 1.3832$). The genetic correlation for vegetation cover over the nest, however, was very close to one (Table 2; $r_G = 0.9964 95\% \text{ CI}=0.6446, 1.3482$). This estimate provides essentially no support for $G \times E$ for this trait, indicating that different genotypes are maintaining vegetation cover phenotypes in relative similarity to one another in separate environments.

4. DISCUSSION

Nesting behaviour is a key component of individual fitness in oviparous organisms, yet little is known about its inheritance in free-ranging animals. Our study sought to quantify the additive genetic variance underlying aspects of nest-site choice in a natural population of turtles with temperature-dependent sex determination. By applying a
reconstructed pedigree to the animal model, we estimated heritability for female preference for Julian date of nesting (i.e. date), and south + west overstory vegetation cover. Our results revealed that both onset of nesting and nest-site vegetation cover have low heritability, and heritability may be environment specific in this system.

Our assessments using all records across environments detected levels of heritability for first nesting date and vegetation cover that are similar in magnitude to traits associated with oviposition behaviour in other systems (e.g. brood mass weight in dung beetles, Hunt & Simmons 2002; oviposition behaviour in crickets, Réale & Roff 2002; oviposition preference in seed beetles, Fox et al. 2004). Upon closer inspection, however, environment-specific analyses revealed that the potential for evolutionary change of nesting behaviour might be dependent on the temperature of the winter before the nesting season. There is a significant, genetic-based tendency for turtles to nest in areas with minimal vegetation cover after colder winters (Fig. 1), while there is a significant, genetic basis for earlier first nesting dates after warmer winters. Furthermore, examination of the additive genetic variance across environments indicates that any potential changes in heritability across environments are due not to increased environmental variance, but to changes in additive genetic variance. Still, the estimates of heritability in all cases are low indicating that potential responses to selection for these traits will be slow or small in magnitude. The very high estimate for the genetic correlation for vegetation cover over nests between cold and hot winters indicates that the genetic architecture of the trait is similar across environments and that different genotypes are maintaining phenotypes with relative similarity across environments (i.e. no G × E). Potential G × E is suggested by the low genetic correlation for
nesting date between cold and hot winters, and future years of data may render this estimate more conclusive.

Our study contributes to a broader body of work on the response of nesting date to climatic conditions. Some evidence of G × E of nesting date across different climatic conditions has been found in the collared flycatcher, a Dutch population of great tits, and the common gull (Brommer et al. 2005; Nussey et al. 2005; Brommer et al. 2008). Yet, no individual variation in laying date response to temperature was found in a United Kingdom population of great tits or in the common guillemot (Reed et al. 2006; Charmantier et al. 2008). Two factors hypothesized to influence G × E in these systems include 1) stabilizing selection on the correspondence of time of highest food provisioning to the season’s highest food abundance (Nussey et al. 2005; Charmantier et al. 2008) and 2) maintaining population-level breeding synchrony. In our system, however, timing of resource abundance and reproductive synchrony most likely do not apply, as females do not provision offspring after oviposition, and hatchlings overwinter in the nest without feeding and emerge the following spring (Weisrock & Janzen 1999). Earlier nesting turtles, however, do have a higher probability of laying subsequent clutches in the season than late nesters (Schwanz & Janzen 2008; Tucker et al. 2008), so advancing nesting date may confer a fitness advantage at least equivalent to that for the bird populations.

Overall, our study suggests that past theoretical work predicting the relative roles of nest-site choice and thermal sensitivity of the sex determination pathway in the response of TSD to sex ratio biases may have insufficiently appreciated the complexity of inheritance in this system (Bulmer & Bull 1982; Bull et al. 1982, Bull et al. 1988; Morjan 2003a). The likely
environment-specific heritability found in this study suggests that nest-site choice and thermal sensitivity of sex determination may each respond more efficiently to sex ratio bias in different situations. For instance, under predicted climate warming, warmer nests may overproduce females (e.g. Janzen 1994a; Rage 1998; Morjan 2003a; Doody et al. 2006). Our data suggest that heritability of vegetation cover over the nest may decline after warm winters, leaving less fodder for a direct response to selection. Substantial heritability in nesting date in warmer years may allow a greater evolutionary response to selection in nesting date. However, simply advancing nesting date is unlikely to correct sex ratio bias (Schwanz & Janzen 2008), thus it is unlikely that selection on offspring sex would manifest as selection on nesting date. In this situation, sex ratio biases may be more effectively countered via selection on the thermal sensitivity of sex determination rather than on nest-site choice. Still, it is unknown how selection acting on plasticity itself and how constraints caused by the potential correlations between slope and intercepts of reaction norms will affect selection in response to sex ratio bias.

Our study must be interpreted with several considerations. First, if unaccounted factors contribute to phenotypic variance, such as maternal or common environment effects, the model will yield inaccurate additive genetic variance estimates. Since our pedigree analysis consisted mainly of parent-offspring relationships, teasing apart the relative contributions of imprinting and maternal genetic effects remains difficult (Kruuk 2004; Kruuk & Hadfield 2007). Imprinting in this population, though, was previously dismissed as an explanation for repeatable nesting behaviour (Janzen & Morjan 2001; Morjan 2003a). We also expect any common environmental effects to be negligible, as the
probability of any two individuals sharing common nest environments (i.e. the frequency of siblings and half siblings in the dataset) is small. Further, due to sperm storage and multiple paternity (Pearse et al. 2001; 2002), even these molecularly identified siblings may not have experienced the same nest environment since they may have been laid in different nests within or even between years. Second, although, our study employed a relatively low number of microsatellite loci and two of these loci exhibited problems with null alleles, the number of alleles per locus was high, exclusion analysis indicated that the loci used were sufficient to accurately assign parents to their offspring, and the pedigree reconstruction methods accounted for null alleles.

Importantly, the repeatability estimates, which are independent of the pedigree, exhibited the same magnitude and pattern of difference across environments. Thus, our conclusions regarding the potential differences in heritability between nesting seasons following different winter environments are strengthened and it is likely that future predictive models of the micro-evolution of TSD in response to sex ratio bias may be more informative if the complexity of G × E were more thoroughly investigated.

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Figure 1. Relationship between mean nesting trait values in each nesting season of the painted turtle, *Chrysemys picta*, in Illinois, USA and the heating degree days (HDD) from September to April preceding the nesting season (see Schwanz & Janzen 2008). (a) average percent south + west vegetation cover of first nest laid by females in different years in response to HDD ($y = -0.0075x + 134.52$, $R^2 = 0.289$). (b) average Julian nest date of the first nest laid by females in different years in response to HDD ($y = 0.0063x + 124.94$, $R^2 = 0.3641$).
Table 1. Repeatability compared to heritability calculated by a univariate animal model for onset of nesting and South + West vegetation cover over a nest for female painted turtles, *Chrysemys picta*, from Illinois, USA. Reconstruction of parent-offspring pairs, full-sib, and half-sib links by maximum likelihood analysis of genotypes were used as a pedigree. Each row represents a separate univariate analysis. All records available were fit by the animal model for the ‘Total’ dataset and other measures represent environment-specific analyses. The number of individuals (N) for which records were available is in subscript to the number of records. The subscript number in parentheses in the ‘mean’ column is the variance. Asterisks denote cases where the 95% confidence intervals do not span zero (heritability) or ANOVA indicated significance (repeatability). For vegetation cover after medium winters, additive genetic variance had to be constrained to be positive and so this analysis was essentially uninformative (denoted by ‘NA’).

<table>
<thead>
<tr>
<th></th>
<th>Records</th>
<th>Mean</th>
<th>VA</th>
<th>Repeatability</th>
<th>Heritability</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total data set</td>
<td>1965(631)</td>
<td>161.55(86.04)</td>
<td>0.064 ± 0.018</td>
<td>0.0961*</td>
<td>0.0613 ± 0.0175</td>
<td>(0.0270, 0.0956)*</td>
</tr>
<tr>
<td>Cold Winters Only</td>
<td>395(277)</td>
<td>166.67(51.62)</td>
<td>0.053 ± 0.059</td>
<td>0.0669</td>
<td>0.0455 ± 0.0523</td>
<td>(-0.0570, 0.1481)</td>
</tr>
<tr>
<td>Med Winters Only</td>
<td>531(373)</td>
<td>158.95(95.57)</td>
<td>0.066 ± 0.068</td>
<td>0.0890</td>
<td>0.0644 ± 0.0671</td>
<td>(-0.0671, 0.1959)</td>
</tr>
<tr>
<td>Hot Winters Only</td>
<td>693(405)</td>
<td>160.72(83.07)</td>
<td>0.116 ± 0.053</td>
<td>0.1552*</td>
<td>0.1152 ± 0.0514</td>
<td>(0.0145, 0.2159)*</td>
</tr>
</tbody>
</table>
### Table 1 (continued)

**South + West vegetation cover of the nest**

<table>
<thead>
<tr>
<th>Records</th>
<th>Mean</th>
<th>$V_A$</th>
<th>Repeatability</th>
<th>Heritability</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total data set</td>
<td>2212 (N=631)</td>
<td>89.46 (1834.375)</td>
<td>0.004 ± 0.106</td>
<td>0.1326*</td>
<td>0.0038 ± 0.1061</td>
</tr>
<tr>
<td>Cold Winters Only</td>
<td>531 (N=298)</td>
<td>83.60 (1799.968)</td>
<td>0.261 ± 0.064</td>
<td>0.2506*</td>
<td>0.2629 ± 0.0579</td>
</tr>
<tr>
<td>Med Winters Only</td>
<td>792 (N=444)</td>
<td>89.28 (1807.729)</td>
<td>NA</td>
<td>0.0545</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Hot Winters Only</td>
<td>889 (N=421)</td>
<td>93.12 (1848.79)</td>
<td>0.051 ± 0.182</td>
<td>0.1667*</td>
<td>0.0505 ± 0.1815</td>
</tr>
</tbody>
</table>
Table 2. Multivariate animal model for onset of nesting and South + West vegetation cover over a nest for female painted turtles, *Chrysemys picta*, from Illinois, USA. Reconstruction of parent-offspring pairs, full-sib, and half-sib links by maximum likelihood analysis of genotypes were used as a pedigree. Asterisks denoted cases where the 95 % confidence intervals do not span zero.

<table>
<thead>
<tr>
<th></th>
<th>Heritability Date</th>
<th>95%CI</th>
<th>Heritability Shade</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold winters only</td>
<td>0.0469 ± 0.0522</td>
<td>(-0.05541, 0.1545)</td>
<td>0.1683 ± 0.0564*</td>
<td>(0.0578, 0.2788)*</td>
</tr>
<tr>
<td>Hot winters only</td>
<td>0.1319 ± 0.0515*</td>
<td>(0.03096, 0.2328)*</td>
<td>0.0047 ± 0.0002*</td>
<td>(0.0043, 0.0051)*</td>
</tr>
<tr>
<td>Hot-Cold genetic correlation</td>
<td>0.4691 ± 0.4664</td>
<td>(-0.4450, 1.3832)</td>
<td>0.9964 ± 0.1795*</td>
<td>(0.6446, 1.3482)*</td>
</tr>
</tbody>
</table>
**ELECTRONIC SUPPLEMENTARY MATERIALS**


*Inheritance and plasticity of nest-site choice in the field in a turtle with temperature-dependent sex determination*

Table S1. Locus information for the painted turtle, *Chrysemys picta*, at Thomson Causeway Recreation Area, Thomson, IL, USA. Error rates for the loci were measured by counting the offspring genotype that did not match the maternal genotype. The error rates represent study-wide error rate (e.g. mislabeling/identifying turtles in the field, genotyping error, mutation) and null alleles. We interpreted the high error rates of GmuD28 and GmuD70, in combination with the significant heterozygote deficiency to be indicative of null alleles. $H_e$ = frequency of expected heterozygotes, $H_o$ = frequency of observed heterozygotes, HWE= p-value for test of Hardy Weinberg Equilibrium, Hetero Def = p-value for test of heterozygote deficiency. All tests were implemented in GenePopv4.0 with default parameters. Number of alleles, $H_e$, $H_o$, HWE, and Hetero Def were calculated from the 340 females genotyped for this study.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alleles</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>HWE</th>
<th>Hetero Def</th>
<th>Error Rate (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GmuD21</td>
<td>17</td>
<td>0.859</td>
<td>0.909</td>
<td>0.569</td>
<td>0.9707</td>
<td>0.36$_{N=2458}$</td>
</tr>
<tr>
<td>GmuD62</td>
<td>22</td>
<td>0.881</td>
<td>0.868</td>
<td>0.3496</td>
<td>&lt; 0.001*</td>
<td>1.79$_{N=2384}$</td>
</tr>
<tr>
<td>GmuD79</td>
<td>25</td>
<td>0.920</td>
<td>0.918</td>
<td>0.0508</td>
<td>0.0250*</td>
<td>2.48$_{N=2440}$</td>
</tr>
<tr>
<td>GmuD28</td>
<td>18</td>
<td>0.857</td>
<td>0.444</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
<td>35.55$_{N=470}$</td>
</tr>
<tr>
<td>GmuD70</td>
<td>57</td>
<td>0.943</td>
<td>0.894</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.0259*</td>
<td>11.81$_{N=608}$</td>
</tr>
</tbody>
</table>
Table S2. Results from linkage disequilibrium tests of loci from the painted turtle, *Chrysemys picta*. Test was implemented in GenePopv4.0 with default parameters.

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus2</th>
<th>ChiSq</th>
<th>Df</th>
<th>Bonferroni corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21 &amp; D62</td>
<td>7.519</td>
<td>2</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>D21 &amp; D79</td>
<td>4.933</td>
<td>2</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>D62 &amp; D79</td>
<td>6.595</td>
<td>2</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>D21 &amp; D28</td>
<td>Infinity</td>
<td>2</td>
<td>Highly sign.</td>
<td></td>
</tr>
<tr>
<td>D62 &amp; D28</td>
<td>9.639</td>
<td>2</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>D79 &amp; D28</td>
<td>7.721</td>
<td>2</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>D21 &amp; D70</td>
<td>9.916</td>
<td>2</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>D62 &amp; D70</td>
<td>10.861</td>
<td>2</td>
<td>0.04</td>
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</tr>
<tr>
<td>D79 &amp; D70</td>
<td>7.908</td>
<td>2</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>D28 &amp; D70</td>
<td>7.615</td>
<td>2</td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>
Figure S1. Left: distribution of Julian date of nesting for the entire dataset. The red line represents a normal distribution. Right: Q-Q plot.

Figure S2. Left: distribution of S+W vegetation cover of a nest for the entire dataset. The red line represents a normal distribution. Right: Q-Q plot.
Supplementary information for materials and methods

Molecular marker data

DNA was extracted from field collected blood samples obtained from 340 females with the Roche High Pure PCR Template Preparation Kit or the Qiagen DNeasy kit and stored at -20°C. The final 12.5 ul PCR reaction included 0.1uM dNTP, 1U taq, 1XBuffer, 1.6mM MgCl₂, 0.4uM forward primer (fluorescently labeled), 0.4uM reverse primer.

Six microsatellite loci (GmuD79, GmuD21, GmuD62, GmuD88, GmuD70, GmuD28; King & Julian 2004) were fluorescently tagged with HEX or FAM. All PCRs experienced a 94°C initial denaturation for 2 min, followed by 34 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30s, and one final extension of 10 min at 72°C. Samples were amplified in either an Eppendorf Mastercycle Gradient or a Techne TC-412. PCRs were not multiplexed because allele sizes overlapped. PCRs were diluted to be one-fiftieth the concentration of pure product and genotyped on an Applied Biosystems Prism 3100 Genetic Analyzer by the Iowa State DNA facility using dye set ‘D’ with a ROX internal size standard. Peaks were reviewed visually in Genotyper software v. 2.0, (PE Biosystems, USA), compared to negative controls, and scored manually. All homozygotes and any ambiguous alleles were rerun for confirmation, resulting in greater than half of the dataset being rerun at least once to confirm the genotype. Each of the amplified loci contained a four base-pair repeat motif and was hypervariable (Table S1). GmuD88 often amplified four major alleles, therefore this locus was excluded from the analysis. An exclusion analysis performed with GenAlEx v6.0
determined that the five remaining loci provided exclusionary probability of greater than 0.99999 when neither parent is known (Jamieson & Taylor 1997; Peakall & Smouse 2006).

GmuD79 and GmuD62 exhibited a small frequency of alternate alleles that were shifted 2bp over from the 4bp repeat motif. In total, 8.3% of the data contained these "alternate motifs" for GmuD79 and 3.5% for GmuD62. Representative normal individuals and individuals containing the alternative motifs were amplified using nonfluorescent primers for GmuD79 and GmuD62, cloned using pGEM-T Easy Vector System I (Promega, USA) and One Shot Mach I Competent Cells (Invitrogen, USA), and sequenced using BigDye v3.1 and an Applied Biosystems 3730xl DNA Analyzer at the Iowa State DNA facility. Sequencing revealed that alternate motifs were caused in both GmuD62 and GmuD79 by a 2bp indel in the 3’ flanking region associated with an additional microsatellite (repeat motif ‘TG’ GmuD62 and ‘TA’ in GmuD79; GenBank Accession GmuD79 normal: EU872151, GmuD79 alternate=EU872152, GmuD62 normal: EU872154, GmuD62 alternate: EU872153). These alternate motifs were treated as alleles of each of these loci. The alternate motifs are not a product of polymerase error because 1) they were repeatable across multiple microsatellite amplification attempts in the same subset of individuals and 2) hatchlings taken directly from nests of alternative-allele individuals also exhibited the alternative alleles (unpublished data, McGaugh).

The entire dataset contained 2.1% missing genotypes (GmuD21: 0%, GmuD62: 0.58%, GmuD79: 0.87%, GmuD28: 2.89% and GmuD70: 3.47%). Error rates were measured by comparing maternal genotypes to genotypes of offspring collected from nests of known females for a companion study and are given in table S1. Null allele rates were quantified with ML-RELATE.
CHAPTER 3. QUANTITATIVE GENETIC VARIATION FOR THERMAL SENSITIVITY OF OFFSPRING SEX IN THE WILD IN A TURTLE WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

A paper to be submitted to Evolution, shorter contributions

Suzanne McGaugh, Rachel M. Bowden, Chih-Horng Kuo, and Fredric J. Janzen

Fluctuating selection maintains equilibrium sex ratios in polygenic sex determination systems. In organisms with temperature-dependent sex determination, where sex is determined by incubation temperature, thermal sensitivity of the sex determination pathway could be a target of this selection. Previously detected, among-family variance indicates that this trait may be heritable, but the genetic component of variance has never been separated from non-genetic maternal effects. By utilizing natural multiple paternity, we performed a maternal half-sib analysis using 39 field-incubated mixed-sex nests of the painted turtle, *Chrysemys picta*. We calculated heritability of threshold temperature that was free from the inflation of maternal effects as 0.26 (95% CI 0, 1). In addition, the effective heritability of threshold temperature in the field was estimated using a relative variance term calculated from field data. This calculation of effective heritability was 0.106, which is an order of magnitude higher than past approximations. Thus, in responding to sex ratio bias in the field, the potential role for the temperature sensitivity of the sex determination pathway has been underappreciated.

**KEYWORDS:** *Chrysemys*, TSD, multiple paternity, heritability, threshold
INTRODUCTION

Sex determination systems are diverse, but many appear to follow the Fisherian prediction of equal investment in males and females (Fisher 1930; Bull and Charnov, 1988). Polygenic systems may adapt more efficiently than chromosomal sex determination systems to deviations from the Fisherian equilibrium, as primary sex ratios are modulated by several to many loci instead of the segregation of chromosomes (Bull and Charnov, 1988; Basolo 1994; Vandeputte et al. 2007). Response to a sex ratio bias by a polygenic system such as environmental sex determination (ESD), where sex is determined after fertilization in response to an environmental cue, may result in a more efficient reestablishment of an evolutionarily stable sex ratio (Bull and Charnov 1988; Janzen 1992).

Studies of sex-ratio evolution in polygenic systems with ESD also provide insight on how a trait directly tied to fitness can harbor genetic variation (Bull and Charnov 1988; Uller et al. 2007). In polygenic systems, excluding those with local mate competition, genetic variation for sex is often high (Vanputte et al. 2007, sea bass, $h^2 = 0.62 \pm 0.12$; Janzen 1992, snapping turtle, $h^2 = 0.56 \text{ CI} 0.26-1.0$; Bull et al. 1982, map turtle, $h^2 = 0.82$, CI 0.31-1). This level of genetic variation could result from populations that are often at a Fisherian equilibrium, where no selection on the primary sex ratio occurs (Bull and Charnov 1988; Uller et al. 2007). Thus, while a population is at equilibrium, substantial additive genetic variation would be allowed to accumulate for primary sex ratio (Bull and Charnov 1988). Alternatively, selection for a balanced sex ratio is a classic example of frequency dependent
selection, which is thought to be an important mechanism in maintaining genetic variation (Fisher 1930).

The form of ESD exhibited in reptiles and some fish is temperature-dependent sex determination (TSD), where sex is determined after fertilization by the temperature experienced during sexual differentiation (Conover and Kynard 1981; Bull 1983). In many species of turtles, for instance, females are produced from eggs incubated at warm temperatures, males are produced from eggs in cool incubation temperatures, and a narrow transitional range (~2°C, transitional range temperature: TRT) produces both sexes in a single clutch (Janzen and Paukstis 1991). The temperature at which or above an individual becomes female is the threshold temperature, whereas the temperature producing a 1:1 population or clutch sex ratio is termed the pivotal temperature (Morjan 2003). Primary sex ratio varies considerably across years in TSD species, but in the absence of large environmental extremes, cohort and population sex ratios are expected to be 1:1 (Bull and Charnov 1988; Janzen 1994a). TSD has been maintained through large climatic upheavals such as the Cretaceous -Tertiary boundary (Rage 1998; Janzen and Krenz, 2004; Organ and Janes 2008). Therefore, this form of sex determination has adapted to large sex ratio biases in the past. Genetic variation that could lead to adaptation towards a 1:1 sex-ratio may be present at two levels: 1) threshold temperature and 2) maternal nest-site choice with respect to thermal conditions (Bulmer and Bull 1982). Consideration of these two avenues of adaptation to shifting climates will inform hypotheses of how TSD species coped with past changes and how they might correct deviations from Fisherian equilibrium produced by current and future climate change (Janzen 1994a; Morjan 2003).
Three lines of evidence support an important role for the temperature sensitivity of the sex determination pathway for sex ratio evolution in reptiles. First, pivotal temperatures change with latitude (Ewert et al. 1994; Morjan 2002; Ewert et al. 2004; Ewert et al. 2005), suggesting that zygotic sensitivity to temperature might respond measurably to selection. Second, in a simulation study, a gradual increase or decrease of 1°C over 1000 years altered threshold temperature relatively more than nest-site choice in a population of turtles with TSD (Morjan 2003). Lastly, incubation at a constant temperature in the TRT reveals substantial among-clutch variation for sex ratio in the lab, indicating that temperature sensitivity of the sex determination pathway may have an additive genetic component to support micro-evolution (Bull et al. 1982, Janzen 1992; Rhen and Lang 1998; Dodd et al. 2006; Janes and Wayne 2006; Janzen, 2008).

Although maternal hormones are known to influence sex-ratio in TSD species (Bowden et al. 2000; Elf 2004), lab estimates of genetic variance for temperature sensitivity of the sex determination pathway did not parse the among-clutch variation in sex ratio into additive genetic effects and maternal effects (e.g. Bull et al. 1982; Janzen 1992; Rhen and Lang 1998). Animal breeding experiments typically utilize a strict paternal half-sib design, whereby a single sire is mated to multiple females to tease apart maternal and additive genetic effects (Lynch and Walsh 1998), but in natural populations such a controlled environment is often not available. However, natural multiple paternity (Pearse et al. 2001, 2002; Uller and Olsson 2008) enables a maternal half-sib analysis to be performed within a single reproductive bout (King et al. 2001). With this design, phenotypic differences between the half-sib families can be attributed to the sire if the assumptions are made that
no dominance or epistasis affect the trait and that maternal effects are random with respect to sire (King et al. 2001). Thus half-sib analyses afforded by natural multiple paternity may provide an estimate of heritability that is free from inflation by maternal effects (King et al. 2001). Such an estimate is essential for more accurately determining the potential response to selection of the sex determination pathway’s sensitivity to temperature (Janzen 1994a; Morjan 2003).

To examine the potential for threshold temperature to respond to selection, we obtained estimates of sire genetic variance through maternal half-sib analysis. Through genotyping 176 field-incubated mixed-sex clutches, we identified 39 nests of painted turtles, *Chrysemys picta*, where multiple sires had produced separate, full-sib clutches within a single nest (Pearse et al. 2001). Genetic variance for sex primary ratio was parsed from maternal variance in these clutches and heritability, which was free from maternal effects, and was calculated on the observed scale and transformed to the underlying scale (Bull et al. 1982; Lynch and Walsh 1998).

**MATERIALS AND METHODS**

**FIELD DATA COLLECTION**

Data were collected from a long-studied population of the common, widespread freshwater painted turtle (Ernst et al. 1994; Starkey et al. 2005) at the Thomson Causeway Recreation Area on the Mississippi River near Thomson, IL (Janzen 1994a,b; Pearse et al. 2001, 2002; Schwanz and Janzen, 2008).

From 15 May to 1 July of 1997-2007, nesting females were identified or marked, and less than 0.5 cc of blood was drawn from the cranial sinus or caudal vein, stored in lysis
buffer, and frozen. Each nest was measured to three stationary landmarks to enable the unambiguous relocation of the nest to excavate hatchlings in the fall. Within-nest variation in temperature and moisture is low compared to larger species, as clutches in this population are relatively small (average number of eggs = 10.4) and nest depths are no larger than 11.5 cm (unpublished data).

Two measures that are highly correlated with nest sex ratio were recorded. First, the percent of south and west vegetation cover of a nest was measured with a spherical densiometer and summed (Janzen 1994b; Weisrock and Janzen 1999). Second, for a subset of nests, mean July air temperatures were calculated from hourly measurements recorded with temperature data loggers (iButton model D2191L for years 2002-2007 or HOBO XT temperature logger for years 1997-2001) that were placed in the nest (Weisrock and Janzen 1999). Cubic spline in R 2.7.0 (The R Foundation for Statistical Computing 2008) was used to interpolate measurements recorded at intervals greater than every hour (e.g. every 72 min in years 1998-2001).

In late September each year, nests were excavated and hatchlings were transported to the lab at Iowa State University. Here, a subset of hatchlings was sacrificed, and sex was determined by direct observation of gonads under a dissecting microscope by F.J.J. (e.g. Janzen 1994a,b). Liver was taken from each hatchling, stored in ethanol, and frozen.

Paternity analysis

DNA was extracted from field-collected blood samples from females and liver samples from hatchlings with the Roche High Pure PCR Template Preparation Kit or the Qiagen DNeasy kit.
Individuals from 176 field-incubated mixed-sex nests were successfully amplified for three or four microsatellite loci (King and Julian 2004) using standard PCR procedures described elsewhere (McGaugh et al. *in review*). In total, 1096 hatchlings and 142 females (an average of 6.17 individuals from each clutch) were genotyped.

Peaks were scored manually using Genotyper software (v. 2.0, PE Biosystems, USA) and compared to negative controls. More than one-third of the individuals were rerun at least once to confirm ambiguous alleles and homozygotes.

Each locus contained a tetranucleotide repeat motif and was hypervariable (number of alleles: GmuD21: 18, GmuD62: 30, GmuD70: 43; GmuD79: 31). Four loci (GmuD21, GmuD62, GmuD70, GmuD79) provided paternity exclusion based on allele frequencies with a probability of > 0.997 as the mothers were known (GenAlEx v6.0, Jamieson and Taylor 1997; Peakall and Smouse 2006). The frequency of null alleles was estimated as GmuD21: 0.00, GmuD62: 0.0145, GmuD79: 0.0159, and GmuD70: 0.1169 in ML-RELATE (Kalinowski et al. 2006; Wagner et al. 2006).

Any maternal-offspring mismatched genotypes were used to obtain error rates for each locus. Estimated error rates were GmuD21: 0.36% (N=2458), GmuD62: 1.77% (N=2384), GmuD79: 2.48% (N=2440), and GmuD70: 12.15% (N=608). These numbers represent the study-wide error rate (e.g. mislabeling/identifying turtles in the field, genotyping error, mutation) and null alleles. The high error rate of GmuD70 was attributed to null alleles at that locus. Significant deviations from Hardy-Weinberg equilibrium were observed in GmuD70 (McGaugh et al. *in review*).
The 176 mixed-sex field-incubated clutches were assayed for multiple paternity using the program Colony v1.2 (Wang 2004) which incorporated the maternal genotype, null allele rates, genotyping error rates, and allele frequency into a maximum likelihood framework (all parameters given above; Wang 2004).

QUANTITATIVE GENETICS

The heritability of the threshold temperature, or the temperature above which an individual becomes female (Morjan 2003), must be indirectly measured by the observable threshold character of “male” and “female” (Bull et al. 1982). In other words, we assume that there is an underlying normal distribution of threshold values $X$, at which point, male to female conversion is achieved (Bull et al. 1982). Each embryo inherits a value ($x$) for its specific threshold on that normal distribution. Quantitative genetic variances of $x$ in the field were estimated by using 1) the between-dam mean squares, or 2) the between-sire-within-dam mean squares from an ANOVA implemented in R 2.7.0 in equations 1-3 from Bull et al. (1982). With this experimental design, the dam component represents the variance due to the particular nest (year laid, temperature, moisture, hormone allocation, etc.), and the sire component represents only among-sire-within-nest variance. Typically the sire component of variance would be multiplied by four to achieve a heritability estimate (Lynch and Walsh 1998). The analysis presented here calculated the fraction of the phenotypic variance attributable to full-sib families within single nests; therefore, the intraclass correlation coefficient was multiplied by two ($px$; Bull et al. 1982).

Randomizations, which shuffled hatchling sex among sires within a single nest, were performed 999 times using R 2.7.0.
Since the transitional range of temperature (temperature at which both males and females can be produced in a single clutch) is typically narrow in turtles with TSD (< 2ºC; Janzen and Paukstis 1991, but see Ewert et al. 2004), nest-site choice could mask zygotic sensitivity by the female’s placement of a nest outside the TRT (Bulmer and Bull 1982; Bull et al. 1982; Janzen 1992). The concept of “effective” heritability was developed specifically to account for this interdependence in the rate of response to selection of nest-site choice and threshold temperature in the field (Bull et al. 1982). Effective heritability weights estimated heritability of the threshold or nest-site choice by a relative variance term:

\[ h^2_{ex} = h^2_x \frac{\sigma^2_x}{\sigma^2_x + \sigma^2_t} \]

where \( h^2_{ex} \) is effective heritability of the threshold temperature (\( X \)), \( h^2_x \) is the estimated heritability of the threshold temperature, \( \sigma^2_x \) is the phenotypic variance of threshold temperature, and \( \sigma^2_t \) is the phenotypic variance of the total nesting possibilities (\( T \); Bull et al. 1982). This equation is adjusted to calculate the effective heritability of maternal nest site choice (\( h^2_{et} \)) so that the variance in total nesting possibilities is in the numerator and the estimated heritability of nest site choice is in place of the estimated heritability of threshold temperature. The rate of response to selection for maternal nest-site choice is the effective heritability of maternal nest site choice (\( h^2_{et} \)) multiplied by \( \frac{1}{2} \) because it has female-limited expression and, therefore, is only expressed in half of the population (Bull et al. 1982). We used a predictor of nest sex ratio from nest data from our entire database: overstory vegetation cover of the nest (\( N = 416 \) nests, 1996-2003) to calculate the relative variance...
terms for threshold temperature and nest-site choice (Bull et al. 1982; Janzen 1994a; Schwanz and Janzen 2008).

RESULTS AND DISCUSSION

Colony v.1.2 determined that 88 clutches exhibited multiple paternity (50%). Thirty-nine clutches with multiple paternity had more than one offspring sired by each father and were subsequently used to estimate quantitative genetic parameters. The average sex ratio (proportion males) for the 305 hatchlings in these clutches was 0.448. The average number of individuals per nest (N = 39) was 7.82 with an average of 3.59 individuals per sire (N = 85).

Our analysis revealed that well-documented family effects on hatchling sex in TSD species (e.g. Bull et al. 1982, Janzen 1992, Rhen and Lang 1998) are not driven solely by maternal effects (e.g. steroid hormones; Bowden et al. 2000) and have a substantial genetic basis. Separating the components of the sums of squares into the mean squares for dams, the intraclass correlation coefficient \( \rho_x \) (Bull et al. 1982) was \( \rho_x = 0.769 \) and heritability was \( h^2 = 1.538 \) (df = 38, 95% CI 0.521, 1.681). This estimate is inflated by variance associated with nest-incubation micro-environments and other maternal effects.

The mean squares for sires-within-dams resulted in an intraclass correlation coefficient of \( \rho_x = 0.1315 \) and a heritability of \( h^2 = 0.263 \) (df = 46, 95% CI -0.193, 1.060). Wide confidence intervals can be attributed to the low number of hatchlings in some sire-within-dam clutches and is a result of paternity skew (i.e. one male siring a disproportionate number of hatchlings in a clutch; Pearse et al. 2002) and relatively low total clutch sizes of this species. This study, however, was purposefully conducted on a species with small nest cavities to minimize intra-nest temperature fluctuation differences (e.g. Thompson 1988).
Dominance and epistasis cannot be accounted for by this experimental design and, thus, this heritability estimate may be inflated (Lynch and Walsh 1998). Randomizations indicated that the distribution of hatchling sex was random with respect to sires-within-nests ($p = 0.145$).

The sire-derived heritability estimate and prior direct experimental evidence (Conover and Van Voorhees 1990; Conover 1992) indicate that the sex determination pathway’s sensitivity to temperature (i.e. primary sex ratio) may evolve readily in response to sex-ratio selection. Yet, to understand how TSD will respond to a sex ratio bias in the field, the variance of thermal conditions experienced in multi-sex nests should be evaluated in the context of the variance across all nests. The relative variance terms, as calculated by other studies (Bull et al. 1982), relied on “circumstantial evidence” of mean threshold temperatures and estimated the variance in threshold temperature ($\sigma_x^2$) to be 0.09. Since then, models that incorporate temperature fluctuations characteristic of natural nests have explained why mixed sex ratios occur in more nests than would be expected from the very narrow pivotal temperature range of many species (Georges et al. 1994; Georges et al. 2004; Georges et al. 2005), even in the absence of within-nest thermal gradients (Georges 1992). Using temperature data from 176 nests from 1997-2007, we found that the variance in mean July nest temperature for multi-sex clutches was 1.499 while the variance for all nests for mean July nest temperature ($\sigma_t^2$) was 2.258. Thus, the variance in threshold temperature ($\sigma_x^2$) in natural nests is at least 1.499, pointing to the underestimation of $\sigma_x^2$ in past studies (Bull et al. 1982). With these field-calculated variances for threshold temperature and the total distribution of nest-temperatures within a population, the
relative variance term with which to weight the heritability of threshold temperature ($h_x^2$) is 0.399, while the relative variance term to weight the heritability of nesting behavior is ($h_t^2$) is 0.601. Again, female control of sex ratio through nest site choice acts in only half the population, and, therefore, responds to selection only half as fast as zygotic control through threshold temperature (Bulmer and Bull 1982). Thus, the two relative variance parameters in this population are nearly equal.

No accurate estimation of the heritability of nest-site choice with respect to nest temperature is available. Using a predictor of sex ratio, we employed south + west vegetation cover of the nest ($N = 1031$) from 1997-2007 to estimate effective heritability for threshold temperature and nest-site choice. This measure is highly associated with nest sex ratio ($F=164.67, df=1, 1029, p < 0.001$). The variance in s + w vegetation cover that produced multi-sex clutches ($\sigma_x^2$) represents the variance in threshold temperatures. This variance was 1149.17 and while the vegetation cover variance for all nests ($\sigma_t^2$) was 1694.59. Hence, the relative variance terms with which to weight estimated heritability of threshold ($h_x^2$) is 0.4041, while the relative variance term to weight estimated heritability of nest-site choice ($h_t^2$) is 0.5959. We used the sire heritability of 0.2630 and the previously estimated heritability of nest site choice ($h_t^2$) with respect to s + w vegetation of 0.2629 (McGaugh et al., in review) to calculate the effective heritability of the threshold of the sex determination pathway and nest-site choice to be $h_{x,s}^2 = 0.106$ and $h_{t,s}^2 = 0.078$, respectively.

The estimates of “effective” heritability presented here are the first to use field-collected data for the components of the relative variance term, and this result indicates that
temperature sensitivity of the sex determination pathway may play a larger role in response to a sex ratio bias in the field than previously thought (contra Bull et al. 1982; Janzen 1992; but supported by Morjan 2003).

The study of the evolvability of threshold temperature (e.g. Bull et al. 1982; Janzen, 1992) may inform the understanding of different TSD patterns within Reptilia. Three forms of TSD are exhibited: Ia) males are produced at cool temperatures and females are produced at warm temperatures, Ib) the reverse of Ia, and II) females are produced at extremes and males are produced at mid-range temperature (Janzen and Paukstis 1991). The potential for threshold temperature to respond to sex-ratio selection is consistent with the hypothesis that TSD Ia and Ib are shifted forms of the two tail reaction norm of TSD II along a > 10ºC gradient (Janzen and Krenz, 2004; Deeming et al. 2004; Ewert et al. 2004; Harlow et al. 2004; Janzen 2008).

Efficient selection on threshold temperature may provide an explanation of how many reptiles might have maintained TSD through climatic shifts. Yet, a potentially sharp contrast exists between adaptation to past climate change and the prospects for adapting to ongoing warming (Janzen 1994a; Rage 1998). Anthropogenic factors have resulted in nearly two-thirds of turtle species becoming threatened or endangered (IUCN 2004). The small, fragmented remnants of populations of most such species may hinder adaptation because, even with underlying potential to respond to selection, drift may overwhelm selective forces (Lynch and Walsh 1998).

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Figure 1. Representation of within-nest sex ratios among clutches with multiple paternity for the painted turtle, *Chrysemys picta*, in Illinois USA. Each nest (N = 39) is represented as two-three bars (black, grey, and white) representing full-sib clutches sired by separate males (N = 85). Each sex ratio (proportion male offspring in a clutch) was centered among the mean sex ratio for all 305 individuals (0.45) to facilitate ease of viewing.
Table 1. ANOVA table for nest sex ratios among clutches with multiple paternity for the painted turtle, *Chrysemys picta*, in Illinois USA. Each nest (N = 39) contains multiple full-sib clutches (N = 85).

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CHAPTER 4. MULTIPLE PATERNITY ENHANCES MATERNAL FITNESS AND INCREASES WITH FEMALE SIZE IN THE PAINTED TURTLE

A paper to be submitted to Molecular Ecology

Suzanne McGaugh and Fredric Janzen

Abstract

In systems without direct benefits, the advantages of polyandry are often undetectable or absent in the wild. Theoretical conditions promoting multiple paternity are limited, but when costs of remating are low, post-copulatory sperm biasing mechanisms (e.g. cryptic female choice, sperm competition) and genetic bet-hedging (e.g. increasing genetic diversity to lower variance in fitness) can be favored. To examine the indirect fitness benefits to polyandrous females, paternity analysis was performed for 34 mixed-sex clutches of painted turtles incubated in the wild using microsatellite loci. Multiple paternity (MP) clutches had significantly higher rates of hatching success than single paternity clutches (MP: 94.9%, non-MP: 86.5%) and lower variance in hatching success, supporting both the post-copulatory sperm biasing and bet-hedging mechanisms. Increased incidence of MP was observed in larger females (average plastron length for MP: 162.33 mm, non-MP: 153.5 mm), and this difference represents, on average, nine years of growth. Thus, the cost-benefit ratio for multiple mating may be age dependent in painted turtles. The painted turtle also exhibits temperature-dependent sex determination (TSD), whereby sex is determined after oviposition due to the thermal environment of the nest. Having multiple sires per clutch decreased the variance in sex-ratio among nests of multiple paternity
females relative to single paternity females, albeit not significantly, indicating that polyandry could potentially serve as a bet-hedging mechanism for sex-ratio in fluctuating climates. This study is one of the first to examine polyandry in relation to female age in a wild population and the first, to our knowledge, to examine the association between polyandry and offspring sex-ratio in a species with TSD.

**Keywords:** bet-hedging, reproductive strategy, polyandry, temperature-dependent sex determination, *Chrysemys picta*

**Introduction**

Mating strategy should differ between males and females, as male reproductive success is limited by copulation opportunities and female reproductive output is limited by the number of ova produced (Bateman 1948). The advantage of multiple mating in males is evident, while the advantages of polyandry to females, independent of direct benefits, are obscure or may be absent (Bateman 1948; Arnqvist & Kirkpatrick 2005; Westneat & Stewart 2003; Uller & Olsson 2008). Thus the maintenance and evolution of polyandry, which is common in many vertebrate populations (Uller & Olsson 2008) remains enigmatic.

The cost that mating entails for the female (e.g. male inflicted physical harm or death, Byrne & Roberts 1999; reduced time and energy for other activities, Watson *et al.* 1998; exposure to disease or parasites, Hurst *et al.* 1993) balanced with indirect benefits may explain the frequency of polyandry when direct benefits are not apparent (Yasui 2001). Indirect genetic benefits in the form of genetic bet-hedging are favoured in some situations
For instance, under a bet-hedging scenario, a double mating strategy has a higher probability of fixation than a single mating strategy in a small population (< 200 females) or one that experiences large environmental fluctuations (Figure 6c in Yasui 2001) when costs of mating are low (e.g. ≤1% reduction in relative fitness). Indirect genetic benefits in the form of “post-copulatory paternity-biasing” mechanisms (e.g. cryptic female choice and genetic incompatibilities, reviewed in Jennions & Petrie 2000) may also favor the evolution and maintenance of polyandry (Yasui 1997; Lorch & Chao 2003). The post-copulatory paternity-biasing hypotheses of “good genes” or “good sperm” generally require sperm competitiveness to be heritable and directly related to male viability and that female cost of mating be low (Yasui 1997). Polyandry as a form of bet-hedging is favored in the presence of post-copulatory sperm biasing (e.g. Lorch & Chao 2003), and empirical evidence also supports that the two mechanisms may co-exist (Sarhan & Kokko 2007).

Bet-hedging and post-copulatory sperm biasing are defined by different mathematical expectations. In between-generation bet-hedging for a single population, all individuals of the non-bet-hedging genotype experience the same environment. In within-generation bet-hedging for a single population, different individuals of the non-bet-hedging genotype experience different environments. Multiple paternity is a form of within-generation bet-hedging since non-bet-hedging individuals in a population mate with different individuals (i.e. environments; Hopper 1999; Sarhan & Kokko 2007). The variance in fitness must be higher for monandrous females relative to polyandrous females to accept bet-hedging as an explanation for polyandry (Hopper et al. 2003). And while multiple mating has been noted
to increase genetic diversity of the offspring (Garant et al. 2004; Calsbeek et al. 2007), empirical tests of the mathematical predictions bet-hedging have been limited (but see milkweed bug, Fox & Rauter 2003; fritillary butterfly, Sarhan & Kokko 2007). Post-copulatory sperm biasing inferred when polyandrous females have an overall higher fitness than monandrous females (Madsen et al. 1992; Yasui 1998; Fisher et al. 2006).

Empirical evidence for indirect genetic benefits is contradictory (for a recent discussion see Uller & Olsson 2008; Madsen 2008). The discordance between studies may reflect that under some conditions, a multiple mating strategy is more beneficial than in others (Fitze et al. 2005; Richard et al. 2005; Eizaguirre et al. 2007). Conditions conducive to indirect genetic benefits explaining the evolution and maintenance of polyandry may be extrinsic to the female such as (1) when there is a high level of genetic similarity or incompatibility of potential mates (Madsen et al. 1992; Zeh & Zeh 1996), (2) when sperm are immature, costly, or from a male of low quality (Olsson & Madsen 1996; Olsson et al. 1996; Olsson & Madsen 1998; Radwan 2003; Akçkay & Roughgarden 2007; Fricke et al. 2008), (3) when precopulatory signals for the best mate are absent (Jennions & Petrie 2000), or (4) when the environment is fluctuating so that the best mate is unpredictable (Yasui 2001). A notable example of extrinsic conditions altering mating strategies is shown in ants: If a colony has multiple queens, no remating is evident, but if it is founded by a single queen multiple paternity occurs (reviewed in Jennions & Petrie 2000). Alternatively, conditions favoring the evolution and maintenance of polyandry may be intrinsic to the female, such as her age or potential for future reproduction (Fisher et al. 2006; Eizaguirre et al. 2007).
Turtles provide an excellent system for examining the indirect benefits of multiple paternity because forced copulations are unlikely, no direct benefits are gained from mating multiply, and females can store sperm for at least three years in the wild (Pearse et al. 2001). Thus, females can minimize the frequency of mating. Turtles are also long-lived, and size can be indicative of age (Congdon et al. 2003; Bowden et al. 2004). Thus, changes in polyandry across age classes can be examined.

Most turtles, like many reptile taxa, have an environmental form of sex determination where thermal cues during incubation direct sex development (Janzen & Paukstis 1991). In several species with temperature-dependent sex determination (TSD), the temperature sensitivity of the sex determination pathway may have a substantial heritable component (Bull et al. 1982; Janzen 1992), meaning that sire genetic variance (McGaugh et al. in prep) has the potential to bias a nest sex ratio towards a higher production of males or females. Thus, mating with two males may not only provide bet-hedging for hatching success, but it also may reduce variance within and across nests for sex ratio because genetic variance for threshold temperature would be increased.

We hypothesized that indirect genetic effects of multiple paternity will confer a fitness advantage and a reduction in the variance in fitness. We also investigated potential for changes in the frequency of polyandry in relation to size (a proxy for age) and examined relationship between multiple paternity and sex ratio across nests in the painted turtle, *Chrysemys picta*.

**Material and methods**

*Life history of the painted turtle*
The painted turtle, *Chrysemys picta*, is an abundant, long-lived turtle that can be found from Canada to Mexico (Starkey *et al.* 2003). In the wild, adult survivorship is high (e.g. for turtles 1-30 years of age, survivorship is 76%, Wilbur 1975; for turtles >10 years of age, survivorship is 95%, Mitchell 1988; see Congdon *et al.* 2003 Figure 5 for further estimates), individuals can live approximately 60 years and exhibit indeterminate growth (Congdon *et al.* 2003).

Females of this species generally reach sexual maturity at 97-128mm (5-13 years; Congdon *et al.* 2003; Morjan 2003) while males are sexually mature at 70-95mm (4-5 years; Congdon *et al.* 2003; Ernst *et al.* 1994), and this sexual size dimorphism is maintained in the adult population. Courtship culminates in a female sinking to the bottom of shallow water (< 60cm), allowing a male to mount her (Ernst *et al.* 1994), so forced copulation is unlikely. Mating takes place during March to mid-June and also in August and September (Ernst *et al.* 1994); sperm production is highest in the fall (Gist *et al.* 1990). The ovarian cycle begins in July or August, ceases when the female becomes dormant in the fall, and ovulation occurs in May after additional follicular growth in the spring. Thus, mating in the fall may result in sperm stored overwinter. Indeed, stored sperm in this species may be viable for at least three years (Pearse *et al.* 2001). Post-laying parental care is absent, and females receive no direct resources from males (e.g. nuptial gift; Uller and Olsson 2008).

This study focuses on a long-studied, high-density population from the Thomson Causeway Recreation Area (Illinois, USA) on the Mississippi River (e.g. Janzen 1994; Weisrock & Janzen 1999; Schwanz & Janzen 2008). Nesting occurs from late May to early July at the Thomson Causeway population (Janzen 1994). Females in this population
typically lay two clutches per year with some laying up to three clutches in a single year. Our sampling was not biased across parities (i.e. no difference in clutch order in the season was exhibited between clutches with multiple paternity and clutches without multiple paternity [38.89% second clutches; 37.5% second clutches, respectively]). The average number of eggs laid per nest is 10.4 (observed range 2-21; information from long-term database maintained by the Janzen lab). Females in this population reach sexual maturity at a plastron length (PL) of 101 mm (the smallest recorded nesting female), while the largest recorded nesting females was 187 mm (mean 154.4). The mean number of individual females recorded nesting in a single season is 168 (range: 114-251). Climatic variation can produce yearly cohorts of all one sex (Janzen 1994).

Field data collection

From mid-May until early July of 1997-2007, nesting grounds were monitored on an hourly basis. Immediately after oviposition ended, eggs were excavated, counted, weighed, and returned to the nest for incubation. Blood was drawn from the female with a 28 gauge insulin syringe, stored in lysis buffer, and frozen. Nests were measured to enable accurate relocation for excavation of the hatchlings in mid September. Hatching success was calculated as a proportion of hatchlings from an intact nest that successfully hatched to total eggs laid. In some cases, such as when eggs were accidently popped by the mom or researchers or when eggs were used for another study (two eggs from one nest with multiple paternity [MP] and two eggs from a nest without multiple paternity [non-MP]), the actual denominator of the proportion was adjusted to reflect the eggs that went through development in the nest.
Molecular analyses

DNA was extracted from field-collected blood samples from moms and lab-collected liver samples from hatchlings with the Roche High Pure PCR Template Preparation Kit or the Qiagen DNeasy kit.

Samples were amplified in either an Eppendorf Mastercycle Gradient or a Techne TC-412 using the protocol of 94°C initial denaturation for 2 min, followed by 34 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30s, and one final extension of 10 min at 72 °C. The final concentrations for the 12.5 ul PCR reaction were 1XBuffer, 1.6mM MgCl2, 0.4uM forward primer (fluorescently labeled), 0.4uM reverse primer, 0.1uM dNTP, and 1U taq. PCRs were not multiplexed because allele sizes overlapped. PCRs were diluted to be one-fiftieth the concentration of pure product and genotyped on an ABI3100 using dye set ‘D’ with a ROX internal size standard. Peaks were reviewed visually in Genotyper software (v. 2.0, PE Biosystems, USA), compared to negative controls, and scored manually. Homozygotes and any ambiguous alleles were rerun for confirmation, resulting in greater than one-quarter of the dataset being rerun at least once to confirm proper genotyping.

Each of the loci contained the four base-pair repeat motif, was hypervariable, and fluorescently tagged with HEX or FAM (number of alleles: GmuD21: 15, GmuD62: 26, GmuD79: 23; King & Julian 2004). When multiple paternity assignments by hand (sensu Pearse et al. 2001) were inconclusive, one additional tetranucleotide motif loci (GmuD70, number of alleles = 36; King and Julian, 2004) was added. An exclusion analysis based on allele probabilities was performed with GenAIEx v6.0 (Peakall & Smouse, 2006) and determined that four loci (GmuD21, GmuD62, GmuD79, GmuD70) provided paternity
exclusionary probability of as high as 0.999 when the mom is known (Jamieson & Taylor, 1997).

Genotyping error rates for the loci were measured by comparing the maternal genotypes to offspring’s genotype. Hatchling mismatches to the mom were tallied and in cases where maternal error was evident (e.g. nearly all offspring did not match maternal alleles for all loci) only one error was counted as it was assumed the maternal identification was incorrect. Error rates obtained were GmuD21: 1.2% (N=1946), GmuD62: 1.5% (N=1882), GmuD79: 4.6% (N=1920), and GmuD70: 2.5% (N=552). This level of error represents study-wide error rate (e.g. mislabeling/identifying turtles in the field, genotyping error, mutation) and null alleles. The program Micro-checker v. 2.2.3 (van Oosterhout et al. 2004) tested for null alleles and allelic dropout with 1000 Monte Carlo simulations and a Bonferroni corrected 95% confidence interval. The frequency of null alleles was estimated as D21: 0.00, D62: 0.017, D79: 0.014, and D70: 0.091.

Analysis with a sample of 340 females from another study using GenePop v4.0 (Raymond & Rousset 1995) default parameters indicated that GmuD79 and GmuD70 significantly deviated from Hardy-Weinberg equilibrium. We interpreted this to reflect the presence of null alleles. GmuD79 was not as drastically out of Hardy-Weinberg equilibrium as D70 (table S1).

Paternity assessment

Pearse et al. (2002) detected the highest amount of multiple paternity when greater than six individuals per clutch were genotyped. Thus, our study included only clutches with seven or greater individuals sampled per clutch. In total, 334 hatchlings and 34 moms were
included in the analysis (average of 9.8 individuals from each clutch; range= 7-15). On average 87.7% of each clutch was genotyped (range = 58.3-100%).

Thirty-four multi-sex field-incubated clutches were assayed for multiple paternity using the program Colony v1.2 (Wang 2004). Colony determines full-sib families within each clutch by incorporating the maternal genotype, null allele rates, genotyping error rates, and allele frequencies into a maximum likelihood framework. The null allele rates for each locus and error rates used in the Colony analysis are given above.

**Statistical analysis**

The statistical tests addressed five questions: 1) Do larger clutches have more multiple paternity independent of female size? 2) Do females with larger plastron lengths have more multiple paternity independent of clutch size? 3) Do multiple paternity clutches have higher hatching success independent of female size? 4) Do multiple paternity clutches have lower variance in hatching success (a proxy for fitness) as predicted by bet-hedging? 5) Do multiple paternity clutches have lower variance in sex ratio as predicted by bet-hedging? All analyses were performed in R 2.7.0 (The R Foundation for Statistical Computing 2008) or JMP 7.0.2 (SAS Institute Inc. 2007).

Shapiro-Wilks tests concluded that the number of eggs per clutch (W = 0.9699, p-value = 0.459) and egg mass (W = 0.9444, p-value = 0.2438) were normally distributed, but hatching success (W = 0.8062, p-value < 0.001), sex ratio (W = 0.9042, p-value < 0.006), and plastron length (W = 0.9335, p-value < 0.040) were not normally distributed for this sample. An angular transformation (arcsine-square root) improved normality for proportion of the clutch successfully hatched, and a square root transformation improved normality for sex
ratio, so both were employed in subsequent analyses. No transformation was used for plastron length (PL) because it was not substantially non-normal. For all analyses, multiple paternity was treated as a categorical variable (i.e. MP or non-MP).

Previous analyses that asserted that larger clutches have more multiple paternity neglected to include an important covariate with clutch size, plastron length (Congdon et al. 2003), in the analyses (Pearse et al. 2002). To address whether larger clutches have more multiple paternity independent of female size, successive ANCOVAs were run in R 2.7.0 with eggs laid per clutch as the response variable and PL, MP, and their interaction as effects in the model. Nonsignificant terms were removed from the model, and the model was rerun. Although backward elimination was performed on the ANCOVAs, final model selection was also concordant with corrected Akaike Information Criterion (AICc; Hurvich & Tsai 1989; Johnson & Omland 2004; Whittingham et al. 2006).

To determine if females with larger plastron lengths have more multiple paternity independent of clutch size, a logistic regression was fit using JMP 7.0.2, with MP as the response variable and PL and number of eggs per clutch as effects.

To understand if multiple paternity clutches have higher hatching success independent of female size, successive linear models were run in R 2.7.0 with proportion of the clutch successfully hatched as the response variable and PL, MP, and their interaction as effects in the model. Again, backward elimination simplified the model, and Akaike Information Criterion was used for model selection. The non-parametric Kruskal-Wallis test was run in R 2.7.0 to determine if the difference in hatching success between MP and non-MP clutches was significant.
To test if multiple paternity clutches had lower variance in hatching success (a proxy for fitness) as predicted by bet-hedging, a non-parametric Bartlett test was run in R 2.7.0 to compare variances between hatching success of MP and non-MP clutches.

Lastly, multiple paternity and non-multiple paternity clutches were examined for evidence that polyandry could be a bet-hedging mechanism to reduce sex ratio variance. The equality of the variance in sex ratio among MP and non-MP clutches was assessed using a Bartlett test in R 2.7.0.

**Results**

A total of 18 clutches were identified as having multiple paternity with Colony (52.941%). Our stringent sampling may have detected a higher frequency of multiple paternity than previously reported multiple paternity rates in this population, which range from 10.7-33.3 % depending on how many hatchlings are sampled per nest (Pearse et al. 2002).

Female size, independent of multiple paternity, explains much of the variance in clutch size. The ANCOVA with PL and MP as effects in the model showed that PL, not MP, had a strong relationship with clutch size (Table 1). Model comparison indicated that a model containing PL alone was preferred (AICc= 122.81) over one containing PL and MP and their interaction (AICc= 127.73), or PL and MP as independent effects (AICc= 125.04). Moms of non-MP clutches had a mean plastron length of 153.5, and the plastron length for moms of MP clutches was 162.33 mm (Figure 1). This difference was significant (t = -2.2394, df = 26.698, p-value = 0.03366). Thus, larger moms had a higher incidence of multiple paternity even when clutch size was taken into account.
Females with larger plastron lengths have more multiple paternity independent of clutch size. Logistic regression demonstrated that a model containing plastron length was predictive of multiple paternity (df= 1, $\chi^2 = 4.631$, $p < 0.0314$), whereas one including plastron length and clutch size was not (df= 2, $\chi^2 = 5.0457$, $p < 0.0802$). Clutch size had little predictive power for MP designations (Table 2).

Multiple paternity clutches have higher hatching success independent of female size. A non-parametric Kruskal-Wallis test showed that multiple paternity clutches have higher rates of hatching success ($\chi^2 = 4.4643$, df = 1, p-value = 0.03461). Non-MP clutches exhibited 86.48% average hatching success, whereas MP clutches had 94.88% average hatching success (Figure 1). Model comparison (Table 3) indicated that a model containing MP as the sole effect was preferred (AICc= -4.69) over one containing PL and MP and their interaction (AICc= -1.01) or PL and MP as individual effects (AICc= -2.74). Previous studies indicated an association between egg mass and hatching success of a clutch. The average egg mass of each clutch, did not improve the model (3-way interaction between PL, MP, and average egg mass interaction AICc= 17.20; individual effects of PL, MP, and average egg mass AICc= 2.76) and was always an insignificant factor (p > 0.55 in all cases). These tests suggest that MP, independent of the effects of egg mass or plastron length, is significantly associated with higher hatching success (Table 3; Figure 2).

Multiple paternity clutches have lower variance in fitness (i.e. hatching success) as predicted by bet-hedging. The Bartlett test indicated that the variance of the hatching
success was smaller for MP clutches than for Non-MP (Bartlett's K-squared = 5.5148, df = 1, 
$p = 0.01886; \sigma_{MP}^2 = 0.00475; \sigma_{non-MP}^2 = 0.01601$).

Multiple paternity clutches do not have a lower variance in sex ratio than non-MP clutches ($\sigma_{MP}^2 = 0.056; \sigma_{non-MP}^2 = 0.096$; Bartlett's K-squared = 1.0886, df = 1, p-value = 0.2968). Subsequent analysis accounting for overstory vegetation cover (a known predictor of sex ratio), year, and nest of the season also note no difference in variance in sex ratio. Both the non-MP and MP females produced clutches had sex ratios that were not significantly different from 0.5 (sex-ratio: non-MP: mean= 0.41824, t=-0.2131, df=15, 
p=0.8341; MP: mean=0.48349, t=-1.4598, df=17, p =0.1626).

**Discussion**

Multiple paternity is often documented, but the evolution and maintenance of this mating strategy is enigmatic in species where direct benefits to females are not apparent (Uller & Ollson 2008, Madsen 2008; but see Madsen et al. 1992, Calsbeek & Sinervo 2004; Fitze et al. 2005, DiBattista et al. 2008). Our results indicate that indirect genetic benefits may exist. Specifically, our study showed that multiple paternity is associated with a higher hatching success rate and our results support the hypothesis that females use polyandry as a way to bet-hedge, as the variance for hatching success is lower for MP clutches than non-MP clutches. Further, the incidence of MP increased with plastron length (a proxy for age), although there was no significant interaction between fitness, plastron length, and multiple paternity which would support a fitness trade-off with plastron length. This study is one of the first to examine polyandry in relation to female size in the wild.
Although no significant association between hatching success and multiple paternity was found in a previous study of painted turtles conducted on the same population (Pearse et al. 2002: MP: 90.3%, and non-MP 86.2%; here MP: 94.88% and non-MP: 86.48%), paternity designations were based on more robust analyses here (e.g. tetranucleotide versus dinucleotide repeats, sampling only greater than six individuals per clutch). In addition, the measurement of hatching success here may more accurately reflect survival differences due to multiple paternity because all clutches in this study were incubated at conditions that produce both sexes, thus environmental incubation conditions were more homogenized compared to Pearse et al. (2002). The explanation for increased hatching success may be complex, though.

Genetic bet-hedging, which increases genetic diversity, has not been well-supported as an explanation for polyandry in past studies (Lee & Hays 2004; DiBattista et al. 2008; but see Garant et al. 2004). This may be because conditions that favor bet-hedging (e.g. fluctuating environments and/or small population sizes) are limited (Hopper et al. 2003). However, tests for genetic bet-hedging also do not address the mathematical expectations that the variance in fitness must be higher in monandrous females relative to polyandrous females (Jennions & Petrie 2000; Sarhan & Kokko 2007). Thus, bet-hedging predicts that polyandry should reduce variance in fitness for MP females relative to non-MP females (Hopper 1999; Jennions & Petrie 2000; Yasui 2001; Sarhan & Kokko 2007). A significant decrease in fitness variance for MP clutches is suggested by the data (MP variance in fitness: 0.00475; non-MP variance in fitness: 0.01601). The relative increase in fitness due to
within-generation bet hedging is explained by $W = \mu - \frac{\sigma^2}{m}$ where $W$ is the fitness of a genotype over multiple generations, $\mu$ is arithmetic mean, $\sigma^2$ is the variance in fitness, and $m$ is population size (Gillespie 1974; Hopper et al. 2003). Using our estimates for $\mu$ and $\sigma^2$ and estimating the population size for the non-MP and MP females to be 47.1% and 52.9% of 167.64 (i.e. the rate of non-MP and MP multiplied by the average female population size), respectively, the fitness of within-generation bet-hedging females is 0.94877 versus 0.8646 for non-bet hedging non-MP females if our current estimates are extrapolated to multiple generations (Hopper et al. 2003). Additional hypotheses involving forms of post-copulatory paternity biasing (e.g. good genes or cryptic female choice) cannot be individually evaluated with the data, but generally these mechanisms could provide an explanation for the high arithmetic mean hatching success for MP clutches (Fisher et al. 2006; Sarhan & Kokko 2007).

The relative increase in multiple paternity in larger females observed in this study may illustrate that age is a condition where polyandry is favored by indirect effects. Putative costs of remating may decrease with age because future reproductive potential is reduced or the costs of remating (increased parasite or disease load) may already be incurred (Ezaguirre et al. 2006). However, there is no significant plastron length-fitness-multiple paternity interaction that would be indicative of trade-offs in polyandry as a turtle ages; therefore, data on the costs of mating in turtles is required to determine if this hypothesis is legitimate.
Several points should be addressed to bolster the hypothesis that the size-associated shift in polyandry is related to age and is ecologically relevant. First, younger females may have had fewer opportunities to mate than the older females. Using our long-term field-collected database (1989-present), the average number of nesting years on record between MP and non-MP females is not significantly different between paternity categories (non-MP=2.875; MP=4.72; Kruskal-Wallis $\chi^2 = 1.5945$, df = 1, $p = 0.2067$). On average, females in the MP category may have had a similar number of opportunities to mate than the females in the non-MP category as females usually mate at least once per nesting season (Pearse et al. 2001; 2002). Additional measures of nesting behavior, though, provide a better picture of the differences between the females in the MP and non-MP categories. For instance, the percent of females in each category that were primiparous was qualitatively different (non-MP=37.5%; MP=22%). Second, plastron length may be an imperfect proxy for age (Congdon et al. 2003). Growth rates are variable among females, and overlapping sizes for different age classes are known in this population (Bowden et al. 2004). Still, a 9 mm difference in plastron length, as seen between MP and non-MP females, constitutes approximately 6-12 years of adult growth (average = 9 years) according to the long-term database for this population (limited to individuals 152-156 mm at first record).

Lastly, temperature-dependent sex determination may provide a unique influence on bet-hedging due to polyandry. In this population of painted turtles, sire genetic variance can substantially influence the sex of offspring when the clutch is incubated at temperatures (McGaugh et al. unpublished) that produce both sexes. Mating with multiple males may ensure less variance in nest sex ratios than mating with either sire alone. Although we did
not observe a significant reduction in sex ratio variance for multiple paternity clutches, varied incubation conditions across years and across nesting sites confounded our results in this study; controlling for these effects left little power to detect differences in variance. Drastically fluctuating climatic conditions (Janzen 1994), and the number of nesting female painted turtles (mean=168) in this population (and in most reptile populations, in general) is well within the population size that allows polyandry to invade under low-costs (Figure 5c; Yasui 2001). Future studies, especially in shorter lived SD species with greater susceptibility to sex ratio fluctuations, may find a variance-lowering effect of polyandry on sex ratio.

Future modeling and empirical work may be useful to shed light on two hypotheses suggested by this study 1) The impact of multiple-mating costs are decreased with age to an “acceptable” cost-benefit ratio for polyandry and 2) Variance in nest sex ratios may be reduced by polyandry through increasing genetic diversity. Unequivocally, though, we have demonstrated that polyandry results in a maternal fitness benefit in this system.

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Letters, 6, 581-586.


Figure 1. Boxplots for 34 clutches of the painted turtle, *Chrysemys picta*, without multiple paternity (non-MP=16) or with multiple paternity (MP=18) A) Plastron length (mm) is significantly greater (*p* <0.034) for MP females. B) Hatching success, expressed as proportion of eggs in a clutch that successfully hatch, was significantly greater in females with MP (<0.035). Tails of the boxes represent the range of the data. Lines in the center of the boxes represent means.
Figure 2. Proportion of the painted turtle clutches that survived from oviposition to pipping by plastron length in natural incubation conditions in Illinois, USA. Grey boxes and solid line represent clutches with multiple paternity (MP; N=18), white boxes and dashed line represent non-MP clutches (N=16). Equations for trendlines were MP, $y = 0.0016x + 0.6937$; $R^2 = 0.1098$ and non-MP, $y = -0.0003x + 0.9046$; $R^2 = 0.0003$. 
Table 1. Three ANCOVA models testing the effects of plastron length (PL) and multiple paternity (MP) on the number of eggs laid in a clutch. Data from 34 painted turtle, *Chrysemys picta*, nests with greater than six individuals genotyped. Rate of multiple paternity was 52.9%. Smaller corrected Akaike Information Criterion (AICc) values represent favored models. PL has a stronger effect on the number of eggs per clutch than does multiple paternity. This was confirmed with a t-test (t = -2.2394, df = 26.698, p-value = 0.03366). The most parsimonious model (c) is also the model favored by AICc.

A) Response, Eggs per clutch: AICc=127.73

<table>
<thead>
<tr>
<th>Df</th>
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<th>p-value</th>
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<tr>
<td>PL</td>
<td>1</td>
<td>75.160</td>
<td>37.8119</td>
<td>&lt;0.001*</td>
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<tr>
<td>MP</td>
<td>1</td>
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<td>0.620</td>
<td>0.3119</td>
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<tr>
<td>PL*MP</td>
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<td>0.118</td>
<td>0.118</td>
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<tr>
<td>Residuals</td>
<td>30</td>
<td>59.632</td>
<td>1.988</td>
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B) Response, Eggs per clutch : AICc=125.04

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<td>38.9951</td>
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<td>0.620</td>
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<td>Residuals</td>
<td>31</td>
<td>59.750</td>
<td>1.927</td>
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C) Response, Eggs per clutch: AICc=122.81

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<td>39.840</td>
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<tr>
<td>Residuals</td>
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Table 2. Logistic regression testing the effects of plastron length (PL) and eggs per clutch (Eggs) on the incidence of multiple paternity. Data from 34 painted turtle, *Chrysemys picta*, nests with greater than six individuals genotyped. A) The overall model including PL and Eggs is marginally predictive of multiple paternity (df= 2, $\chi^2 = 5.0457$, $p < 0.0802$). B) The overall model including PL is predictive of multiple paternity (df= 1, $\chi^2 = 4.631$, $p < 0.0314$). PL is mainly predictive of clutch multiple paternity while the contribution of the number of eggs per clutch is not significant.

A)  Response, Multiple paternity

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>Std Error</th>
<th>ChiSquare</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>6.0663</td>
<td>3.91</td>
<td>0.0479*</td>
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<tr>
<td>PL</td>
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<td>3.01</td>
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<tr>
<td>Eggs</td>
<td>0.18529</td>
<td>0.29296</td>
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</table>

B)  Response, Multiple paternity

<table>
<thead>
<tr>
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<tbody>
<tr>
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<td>5.2573</td>
<td>3.81</td>
<td>0.0509*</td>
</tr>
<tr>
<td>PL</td>
<td>-0.06589</td>
<td>0.03346</td>
<td>3.88</td>
<td>0.0489*</td>
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</table>
Table 3. ANCOVA using data from 34 painted turtle, *Chrysemys picta*, nests examining the effect on hatching success of A) plastron length (PL), multiple paternity (MP), and average egg mass of the clutch, B) PL and MP, and C) MP alone. At least 6 individuals were genotyped from each clutch. Smaller corrected Akaike Information Criterion (AICc) values represent favored models. The non-parametric Kruskal-Wallis test ($\chi^2 = 4.4643, \ df = 1, \ p = 0.03461$) corroborates the ANCOVA results that a higher proportion of embryos in clutches with multiple paternity (MP) successfully hatched compared to those in clutches without multiple paternity (non-MP). The average proportion of the clutch that hatched is 0.949 for clutches with MP and 0.865 for non-MP clutches. Hatching success was arc-sine and square-root transformed and plastron length was not transformed prior to running the ANCOVA. Additional ANCOVAs which included interaction terms were not more likely. The most parsimonious model (c) is also the model favored by AICc.

A) Response, Proportion of the clutch which successfully hatched: AICc, 2.76

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<td>EggMass</td>
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<td>0.01664</td>
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<tr>
<td>MP</td>
<td>1</td>
<td>0.31813</td>
<td>0.31813</td>
<td>7.3252</td>
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<tr>
<td>Residuals</td>
<td>18</td>
<td>0.78173</td>
<td>0.04343</td>
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B) Response, Proportion of the clutch which successfully hatched: AICc, -2.74

<table>
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<td>0.09845</td>
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<tr>
<td>MP</td>
<td>1</td>
<td>0.13574</td>
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<tr>
<td>Residuals</td>
<td>31</td>
<td>1.39373</td>
<td>0.04496</td>
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</table>
C) Response, Proportion of the clutch which successfully hatched: AICc, -4.69

<table>
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<tbody>
<tr>
<td>MP</td>
<td>1</td>
<td>0.20817</td>
<td>0.20817</td>
<td>4.6921</td>
<td>0.03786*</td>
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<td>32</td>
<td>1.41973</td>
<td>0.04437</td>
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CHAPTER 5.

GENERAL CONCLUSIONS

Temperature dependent-sex determination represents just one pattern in which species determine sex, but its evolution, maintenance, and molecular underpinnings have provided many life-times worth of scientific investigation. This dissertation has presented insights on a small facet of this form of sex determination by examining the evolutionary potential of two traits that may respond to selection against biased sex ratios (Fisher 1930)—maternal nesting behaviour and the sensitivity of the threshold temperature.

The study described in the second chapter revealed that both onset of nesting and nest-site vegetation cover have low heritability, and heritability may be environment specific in a population of painted turtle on the Mississippi River in Illinois. Low levels of heritability for first nesting date and vegetation cover were detected when all records, irrespective of environment, were analyzed. Alternatively, the potential for evolutionary change of nesting behaviour was revealed by environment-specific analysis to be potentially dependent on the temperature of the winter before the nesting season. After cooler winters, turtles have a significant, genetic-based tendency to nest in areas with minimal vegetation cover, while after hotter winters there is a significant, genetic basis for earlier first nesting dates. Repeatability estimates suggest that these conclusions are accurate, as they are of the same magnitude and pattern of difference across environments.

The explanation for these potential shifts in heritability across environments derive from increased additive genetic variance not from increased environmental variance. Thus, some aspect(s) of cooler winter temperatures are constraining additive genetic variance in
nesting date from being expressed but allowing the release of additive genetic variance for south + west overstory vegetation cover preference. Potentially, this could be driven by avoidance of thermal or hydric conditions that are not favourable to development.

The estimate of genetic correlation between cooler and warmer winters for vegetation cover over the nest indicates that the response to winter temperatures by females is consistent relative to one another and the genetic architecture for vegetation cover preference over the nest may be similar across nesting environments after colder and warmer winters (i.e. no G × E). As indicated by the estimate of genetic correlation between colder and warmer winter environments, the genetic architecture of nesting date and response to winter temperatures by different females between cold and hot winters may be different, but wide standard errors make this estimate equivocal.

Broadening this study to include all individuals with blood samples available to reconstruct the pedigree may offer additional insights. Further, methods that do not rely on pedigree reconstruction and take into account all of the genetic relationships would provide additional power when applying advanced methodology in analyzing traits that vary with the environment (Meyer and Hill 1997). Currently, generating a marker-based relatedness matrix for computing heritability is not accurate in most natural populations even though several attempts to legitimize such a method have been made (Ritland 1996; Frentiu et al. 2008). Genome-wide markers are being used to generate a closer estimate of the true relationship matrix in animal breeding (Meuwissen 2007), and applying this method to natural populations would remove one of the greatest constraints to estimating heritability in the wild- the pedigree. With the advent of new sequencing technologies, it is feasible to
envision this study being revisited in the near future with genome-wide markers. Using the power afforded by such a technique may allow successful application of random regression animal models to estimate the additive genetic and permanent environmental components of the reaction norm for each individual (Meyer and Hill 1997).

The third chapter indicated that the sex determination pathway’s sensitivity to temperature potentially has a substantial genetic basis that is independent of maternal effects. To calculate the effective heritability of the threshold temperature of the sex determination pathway and nest-site choice, the estimated sire heritability of threshold temperature of 0.26 and the previously estimated heritability of nest-site choice with respect to $s + w$ vegetation of 0.26 were used. The “effective” heritability of threshold temperature was $h^2_{t,x} = 0.106$ and the effective heritability of nest-site choice was $h^2_{n,c} = 0.079$. This result utilized field records to obtain an ecologically relevant estimate of heritability in the field. This experiment indicates that the temperature sensitivity of the sex determination pathway may play a role in response to a sex ratio bias in the field when, previously, the potential response to selection by this trait was estimated to be negligible (Bull et al. 1982a; Janzen 1992; but see Morjan 2003a).

The major limiting factor in determining the sire contribution for this study was having large enough multiply-sired clutches inside a single nest to assure that the pattern seen was not purely by chance. In fact, our randomization indicated that the distribution of sex phenotypes for the offspring among the half-sib clutches in a single nest were random ($p=0.145$) with respect to sire. With only two individuals representing a single sire in many
instances, it is difficult to discern if patterns observed are truly related to sire genetic variance. Future replications of this study are encouraged to employ the multiple-paternity method here to species with a greater number of eggs per clutch in a laboratory setting. Further, we identified clutches with mixed sexes first (an assay that is terminal to the hatchling) and then genotyped for multiple paternity to reduce genotyping costs. The number of individuals sampled per clutch was limited in order to avoid sacrificing entire cohorts. The number of individuals sampled per clutch could increase in future studies, if sex identification of the hatchlings was performed only after multiple paternity was identified for the clutch. It is also suggested that some lizard species with TSD may be more suitable for a typical sire breeding design.

Overall, these two studies suggest that the relative roles of nest-site choice and thermal sensitivity of the sex determination pathway in the response of TSD to sex ratio biases may have insufficiently appreciated the complexity of inheritance in this system (Bulmer & Bull 1982; Bull et al. 1982a, Bull et al. 1988; Morjan 2003a). It is likely that nest-site choice and thermal sensitivity of sex determination may both respond equally well to sex ratio bias. Alternatively, the most effective response may change in different environments. For example, under climate warming scenarios an overproduction of females would be expected, but the heritability of vegetation cover over the nest is suggested to be reduced after warm winters. Advancing nesting date is unlikely to correct sex ratio bias (Schwanz and Janzen 2008) even though substantial heritability is expected after warm winters for this trait. In this situation, sex ratio biases may be more effectively countered via selection on the thermal sensitivity of sex determination than on nest-site choice.
Finally, since sire genetic variance may influence the sex of offspring when the clutch is incubated at temperatures that produce both sexes, polyandry may homogenize nest sex ratios more than mating with one male. Diverse incubation conditions across years and nesting sites confounded the test for sex ratio variance between polyandrous and monandrous females. Statistically controlling for these varied conditions limited the power to detect reductions in variance in clutches with polyandry. However, the requirements for bet-hedging including low population size and fluctuating, unpredictable conditions (Yasui 2001), are met by most reptile populations and so this is a ripe avenue for potential investigation. Future studies, especially in shorter lived TSD species with greater susceptibility to sex ratio fluctuations, may find a variance-lowering effect of polyandry on sex ratio.

The study of polyandry in this natural population revealed an indirect maternal fitness benefit. Multiple paternity clutches had a higher hatching success rate and females use polyandry as a within-generation bet-hedging mechanism, as the variance for hatching success is lower for clutches with multiple paternity than those without. The incidence of multiple paternity increased with plastron length (a proxy for age), yet no significant interaction between fitness, plastron length, and multiple paternity was found. Thus, a fitness trade-off between plastron length and multiple paternity was not supported in this dataset.

In conclusion, the investigations in the dissertation represent the first attempts in wild populations of turtles to estimate heritability for nesting behaviour and threshold temperature, and the first to hypothesize a link between polyandry and sex ratio variance.
This dissertation provides the first support that the additive genetic variance for traits highly correlated to offspring sex ratio may have an environment-specific component and that the threshold temperature of the sex-determination pathway may have a heritable genetic component that is independent of maternal effects. I also present evidence of indirect fitness benefits for polyandrous females, even though these are not well-documented for reptiles (Uller and Olsson 2008), and provide data that suggests a trend that could indicate a shift in reproductive strategy with age.

LITERATURE CITED


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Girondot, M., & C. Pieau. 1996. On the limits of age-structured models for the maintenance


http://ginux.univpm.it/scienze/chromorep/introduzione.html


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I also thank my committee--Anne Bronikowski, Dennis Lavrov, John Nason, and Jonathan Wendel--for their time, insight, and providing a supportive environment. Their investment in my education has provided a challenging and stimulating atmosphere over the past six years. Additional faculty, including Dorian Garrick and Dean Adams, illuminated much regarding the quantitative genetics and statistical analyses. Tom Peterson and Adam Bogdanove also gave their time and intellectual contributions to earlier stages of these projects.

These studies would not have been possible without the co-authors. Besides the work they completed for these projects, they provided wonderfully thoughtful comments on the final manuscripts.
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