

Field-measured heritability of the threshold for sex determination in a turtle with temperature-dependent sex determination

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ABSTRACT

Problem: For temperature-dependent sex determination to respond to selection, there should be genetic variance underlying the threshold that switches development from a male-producing program to a female-producing program. Genetic variance for this threshold in reptiles has never been estimated under field conditions.

Methods: We estimated variance components of the thermal sensitivity of the sex determination threshold under field conditions for the painted turtle, *Chrysemys picta*, a species that has temperature-dependent sex determination. Multiple paternity within clutches was identified by genotyping females and their offspring. We endeavoured to statistically account for common nest and maternal effects and estimated the genetic variance underlying the sex determination threshold under field conditions by isolating the contribution of sires within a single clutch.

Results: With 51 clutches containing 393 offspring, we estimated significant heritability for the sex determination threshold ($h^2 = 0.351$, 95% CI = [0.164, 0.832]). Using a more restrictive dataset, which included only those clutches where each sire was represented by at least two offspring (34 nests, 273 hatchlings), heritability was not significantly different from zero ($h^2 = 0.173$, 95% CI = [0.000, 0.628]). Paternal siring success did not influence hatchling sex; thus, we have no evidence to support differential sex allocation across different sires within the same nest.

Conclusion: We used a natural ‘breeding design’ under field conditions to show that the threshold of temperature-dependent sex determination may have a heritable genetic basis.

Keywords: animal model, *Chrysemys picta*, quantitative genetics, reptiles, temperature-dependent sex determination.

INTRODUCTION

An accurate estimation of the additive genetic variance, or the variance responsible for parent–offspring resemblance, is essential for understanding the potential evolutionary response to selection for a trait (Falconer and MacKay, 1996; Lynch and Walsh, 1998). Phenotypes, however, are influenced by a host of environmental effects and non-additive genetic effects, which are difficult or impossible to control in a free-ranging population (Kruuk, 2004; Kruuk and Hadfield, 2007), rendering accurate estimates of additive genetic variance a challenge to obtain. Controlled breeding designs in the laboratory can remove unwanted sources of phenotypic variance (e.g. Fox *et al.*, 2004; Delph *et al.*, 2005), but may result in measures of additive genetic variance that are unrealistic for predicting response to selection in the wild (Holloway *et al.*, 1990; Hoffman, 2000; Conner *et al.*, 2003; Geber and Griffen, 2003). Regardless, this approach is not practical for long-lived organisms (e.g. Wilson *et al.*, 2005; Brommer *et al.*, 2008; DiBattista *et al.*, 2009). Left unaccounted for, though, maternal and common environmental effects may substantially inflate measures of heritability (Wilson *et al.*, 2005; Kruuk and Hadfield, 2007). For instance, in cross-fostered collared flycatcher families, heritability of tarsus length was overestimated by about 2.6-fold when early common environmental effects were ignored (Kruuk and Hadfield, 2007).

Researchers have explored methods that experimentally [cross fostering in birds (reviewed in Merilä and Sheldon, 2001)] and statistically [the ‘animal model’ (Kruuk, 2004)] separate genetic and environmental components while maintaining organisms in natural field conditions. These methods have provided striking insights regarding additive genetic (Kruuk *et al.*, 2002) and maternal genetic effects (McAdam *et al.*, 2002; Wilson *et al.*, 2005), as well as the genetics underlying plasticity (Nussey *et al.*, 2007; Brommer *et al.*, 2008), ageing (Wilson *et al.*, 2007), and fitness (Kruuk *et al.*, 2000), but often require large, multi-generational pedigrees (but see DiBattista *et al.*, 2009) and tremendous sample sizes (e.g. Kruuk *et al.*, 2002) to achieve statistical accuracy. In species with low early-life survival and delayed maturation, tagging and tracking individuals for even a single generation can be logistically challenging.

A maternal half-sib design is naturally created when multiple sires mate with a single female, and the effect of each sire on the phenotype of his offspring can potentially be statistically partitioned from maternal genetic, maternal environmental, and common environmental effects (Olsson *et al.*, 1996; Weigensberg *et al.*, 1998; King *et al.*, 2001). Thus, heritability may be estimated from the sire’s contribution to the phenotype based on the assumptions that neither dominance nor epistasis affect the trait and that maternal effects are random with respect to sire (King *et al.*, 2001). This approach may be particularly well-suited to facilitate field-based measures of heritability where sample sizes may be limited (e.g. several hundred individuals). However, few empirical examples of maternal half-sib analysis under field conditions are available (but see Berenbaum *et al.*, 1986).

In the present study, we estimated genetic variance for the thermal sensitivity of the sex determination pathway in a turtle with temperature-dependent sex determination (Janzen and Paukstis, 1991) using entirely field-incubated nests. Broad-sense heritability under laboratory conditions has been previously estimated for this trait in turtles (Bull *et al.*, 1982; Janzen, 1992; Rhen and Lang, 1998; McGaugh and Janzen, 2011); however, maternal and common environmental effects could be profound (Cagle *et al.*, 1993; Janzen and Warner, 2009; Schwanz *et al.*, 2010) such that broad-sense heritability obtained from full-sib families may overestimate the potential for an evolutionary response to selection (Kruuk and Hadfield, 2007). We utilized a well-studied wild population of painted turtles, *Chrysemys picta* (Schwanz *et al.*, 2009, 2010), that has multiple sires in at least 30% of clutches (Pearse *et al.*, 2002), to investigate the genetic variance of this trait

using the natural maternal half-sib design afforded by multiple paternity. In the painted turtle, the maternal half-sib approach is particularly useful to study genetic variance under field conditions because eggs hatch in late summer and offspring overwinter in the nest (Weisrock and Janzen, 1999; Colbert *et al.*, 2010); thus, eggs complete development in natural nests from which hatchlings are easily retrieved for phenotyping (Warner *et al.*, 2010). Furthermore, traditional pedigrees are unattainable because turtles store sperm across years (Pearse *et al.*, 2001), mating is generally unobservable (Ernst and Lovich, 2009), and permanent marking of neonates is challenging.

Hatchling sex is notable because for many reptiles, including most turtles, this trait is determined permanently by the temperature that embryos experience during incubation [temperature-dependent sex determination, TSD (Janzen and Paukstis, 1991)]. Microevolution of TSD may theoretically proceed via selection on maternal nest-site choice (Bulmer and Bull, 1982), which has low genetic variance in the field (McGaugh *et al.*, 2010), or selection on the thermal sensitivity of the sex determination pathway (Bulmer and Bull, 1982; Bull *et al.*, 1982). The thermal sensitivity of sex determination governs the threshold for a male developmental program to switch to a female developmental program (Bull *et al.*, 1982) (hereafter called the sex determination threshold). In the laboratory, this trait exhibits moderate narrow-sense heritability in leopard geckos (Rhen *et al.*, 2011) and high broad-sense heritability in several turtles (Bull *et al.*, 1982; Janzen, 1992; McGaugh and Janzen, 2011), and among-family effects on sex ratio has been documented extensively (Rhen and Lang, 1998; Dodd *et al.*, 2006; Janes and Wayne, 2006). Still, non-genetic factors such as maternal hormones (Bowden *et al.*, 2000; Elf, 2004) and common nest effects in the field (Janzen, 1994b; Schwanz *et al.*, 2010) can substantially influence offspring sex ratio in species with TSD. An accurate estimate of additive genetic variance of the sex determination threshold is important for more clearly gauging the microevolution of TSD in response to sex-ratio biases expected under climate change (Janzen, 1994a; Morjan, 2003a; Mitchell and Janzen, 2010).

In our study, we identified the sires of hatchlings within clutches using molecular markers and statistically parsed the genetic variance for the sex determination threshold from the variance attributable to the nests (Bull *et al.*, 1982; Lynch and Walsh, 1998). Our study provides the first estimates of genetic variance for the sex determination threshold in reptiles from field-incubated nests [for an example in a fish with TSD, see Conover and Heins (1987)].

METHODS

Natural history of the painted turtle

The painted turtle, *Chrysemys picta*, is one of the most widely distributed freshwater turtles in the world, with a range that extends from Mexico to Canada (Starkey *et al.*, 2003; Ernst and Lovich, 2009). Adult survivorship is high [e.g. for turtles > 10 years of age, survivorship is 95% (Mitchell, 1988)], and individuals exhibit indeterminate growth (Congdon *et al.*, 2003). Hatchling survival is low, in that only ~8% of all oviposited eggs hatch and complete successful migration from the nest to water (Wilbur, 1975). In our study population, average cohort sex ratio over the past couple of decades is male-biased (Schwanz *et al.*, 2010; *contra* Freedberg *et al.*, 2001; Freedberg and Bowne, 2006).

Polyandry unquestionably occurs across reproductive seasons, but female painted turtles likely do not mate between nesting events in a single season (Pearse *et al.*, 2001, 2002; Ernst and Lovich, 2009). Obligate female cooperation in mating renders forced copulation unlikely in *C. picta* (Berry and Shine, 1980; Ernst and Lovich, 2009), and pre- and post-copulatory mate choice is poorly

understood (Ernst and Lovich, 2009; but see Niblick *et al.*, 1994; Poschadel *et al.*, 2006). No provisioning of direct resources to females by males has been documented [e.g. nuptial gift (Pearse and Avise, 2001; Uller and Olsson, 2008)], and it is unclear if turtles derive indirect benefits (e.g. genetic) from multiple mating (Pearse and Avise, 2001; Lee and Hays, 2004; Uller and Olsson, 2008). Post-oviposition parental care is absent (Ernst and Lovich, 2009). Sperm can be stored in this species and may be viable for at least 3 years in the wild (Pearse *et al.*, 2001). Follicles are ovulated as a group (Moll, 1979) and are uniform in size (Tucker and Janzen, 1998).

Field data collection

This investigation focused on a long-studied, high-density population of *C. picta* from the Thomson Causeway Recreation Area (TCRA; Illinois, USA) on the Mississippi River (e.g. Janzen, 1994a, 1994b; Valenzuela and Janzen, 2001; Schwanz *et al.*, 2010). Females in this population mature at a plastron length of 101 mm (the smallest recorded nesting female), with the average nesting female being 154 mm and the largest being 187 mm (F.J. Janzen, unpublished data). Nesting occurs from late May to early July, and females typically lay one to three clutches per year. Clutch sizes in this population average 10.5 eggs [observed range 3–21; mode = 10 (Schwanz *et al.*, 2009)] and nest depths are no greater than 11.5 cm (Morjan, 2003b; F.J. Janzen, unpublished data). The annual mean recorded number of nesting females is 168 [range 114–251 (F.J. Janzen, unpublished data)].

From mid-May until early July of 1997–2007, the southeast beach of the island in the TCRA was monitored from dawn to dusk on an hourly basis for nesting females. Females were captured immediately upon completion of nesting and identified or marked by notches along the margin of the carapace (Cagle, 1939). For paternity analysis, approximately 0.5 ml of blood was drawn from the cranial sinus or caudal vein, stored in lysis buffer, and frozen. In mid-September, all hatchlings were excavated from nests and transported to Iowa State University. Hatchlings were sacrificed with an overdose of anaesthetic injected into the pericardial cavity (protocols approved annually by IACUC at Iowa State University to F.J.J.). Because we sampled a large number of clutches, only a subset of each clutch was sacrificed to avoid adversely affecting the population (see Schwanz *et al.*, 2010). The sex of each hatchling was ascertained by visual inspection of the internal gonadal structures under a dissecting microscope by F.J.J. (Janzen, 1994b). Liver samples were removed from each sacrificed hatchling, stored in ethanol, and frozen. Potential sires were not sampled in this study.

Molecular analyses

DNA was extracted from field-collected blood samples from mothers and laboratory-collected liver samples from hatchlings with the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) or the DNeasy kit (Qiagen Inc., Valencia, CA). Samples were amplified at three or four loci using the methods described in McGaugh *et al.* (2010). Polymerase chain reactions (PCRs) were not multiplexed because allele sizes overlapped. The PCRs were diluted to one-fiftieth the concentration of pure product and electrophoresed on an ABI3100 using dye set 'D' with a ROX internal size standard at the Iowa State DNA Facility. Peaks were reviewed in Genotyper v.2.0 software (Applied Biosystems Inc., Foster City, CA), compared with negative controls, and scored manually. To confirm allele sizes and homozygotes, we repeated the genotyping procedure for more than one-third of the individuals.

Each microsatellite locus [GmuD21, GmuD62, GmuD79, GmuD70 (King and Julian, 2004)] contained a microsatellite four base-pair repeat motif, was hypervariable, and was fluorescently 5' modified with HEX or FAM [number of alleles: GmuD21 = 16, GmuD62 = 27, GmuD79 = 28 (King and Julian, 2004)]. When paternity assignments by hand (*sensu* Pearse *et al.*, 2001) were inconclusive, one additional tetranucleotide motif locus [GmuD70, number of alleles = 43 (King and Julian, 2004)] was added. An exclusion analysis based on allele probabilities, performed with GENALEX v.6.0 (Peakall and Smouse, 2006), determined that the four microsatellite loci (GmuD21, GmuD62, GmuD79, GmuD70) provided a paternity exclusion probability of 0.999 when the mother was known (Jamieson and Taylor, 1997). Using the first three loci, the exclusion probability was 0.996.

Genotyping error was measured by comparing the maternal genotypes to the offspring's genotype. Hatchling mismatches to the mother were tallied. Proportion of error obtained for each locus was as follows: GmuD21 = 0.012, GmuD62 = 0.015, GmuD79 = 0.046, and GmuD70 = 0.025. These proportions represent study-wide error (e.g. mislabelling/identifying turtles in the field, genotyping error, mutation) and null alleles. The program ML-RELATE (Kalinowski *et al.*, 2006) was used to test for null alleles. The frequency of null alleles was estimated as: GmuD21 = 0, GmuD62 = 0.029, GmuD79 = 0, and GmuD70 = 0.102. Analysis of a sample of 435 genotyped females from a companion study (McGaugh *et al.*, 2010) using GENEPOP v.1.2 default parameters (Raymond and Rousset, 1995) indicated that all loci were in Hardy-Weinberg equilibrium except GmuD70, which contained an excess of homozygotes. We interpreted this result to reflect the presence of null alleles for this latter locus.

Paternity assessment

We sampled six or more individuals per clutch for 100 clutches collected between 1997 and 2007 (excluding the years 2004 and 2005). In total, 730 hatchlings and 99 mothers were included in the analysis (average of 7.3 individuals from each clutch; range 6–15). On average, 69.6% of each clutch was genotyped (range 37.5–100%). These 100 mixed-sex, field-incubated clutches were assayed for multiple paternity using the program Colony v.1.2 (Wang, 2004). Colony determined 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 (Wang, 2004). Colony does not require user-provided constraints on the level paternity skew (Wang, 2004).

A detection bias could exist, whereby those offspring that did not hatch or were not sampled were sired by different males than the embryos that were genotyped. This possibility could not be directly evaluated because dead embryos were degraded and not saved, and we sampled only a subset of each clutch to avoid adversely affecting the population. The detection of multiple paternity did not heavily depend on the percent of the clutch that was genotyped (see Table S1 at: evolutionary-ecology.com/data/2612suppl.pdf). Regardless, a failure to detect multiple sires because of sampling error would likely not affect our heritability estimates, as clutches with single sires detected were not included.

Statistical analysis

We used a logistic regression to determine whether the success of a sire affected the sex of his offspring relative to the sex ratio of the entire nest. Fifty-one nests with multiple sires detected (115 sires, 51 dams, and 393 hatchlings) were included. No sires or dams were

duplicated across nests. The response variable was untransformed offspring sex, which was treated as a factor in the logistic regression. The effects in the model were the nest identity (character variable), and logit-transformed proportion of the clutch that was sired by a particular male (Warton and Hui, 2011). Hatchling sex was measured as 1 = male, 0 = female. Four inter-sex individuals were excluded from the logistic regression, although results from an ordinal regression that included these individuals were consistent. We also used a generalized linear mixed model with binomial error term with all of the factors fitted as random effects and compared the deviance information criterion (DIC; smaller value indicates the favoured model) for a model that did not contain the random effect ‘percent of clutch sired’ relative to a model that did.

We used a generalized linear model to test for an interaction of maternal size and paternity status of a clutch on clutch sex ratio. Maternal carapace length was not normally distributed (Shapiro-Wilks test, $W = 0.9726$, $P = 0.037$) and was log-transformed to achieve normality ($W = 0.983$, $P = 0.241$). Sex ratio was measured as proportion male and was logit transformed with 1 minus the largest proportion added to both the numerator and denominator (Warton and Hui, 2011). Transformed sex ratio was used, as opposed to a GLM with binomial error (*sensu* Wilson and Hardy, 2002), so as to include inter-sex individuals. A GLM with binomial error, excluding inter-sex individuals, is included in the supplementary materials (see Online Appendix at: evolutionary-ecology.com/data/2612suppl.pdf). All analyses were performed in R 2.11.0 (R Development Core Team, 2010) or JMP 8.0.2.

Heritability of sex determination threshold

Variance components and standard errors for the sex determination threshold were estimated using a generalized linear mixed model [GLMM (Lynch and Walsh, 1998; Kruuk, 2004; Wilson *et al.*, 2010)] in the R package MCMCglmm (Hadfield, 2010). Maternal carapace length, year, and nest of origin explained significant amounts of deviance for offspring sex in exploratory GLM analyses (see Table S2 at: evolutionary-ecology.com/data/2612suppl.pdf). Thus, the model to estimate genetic variance of the sex determination threshold contained maternal carapace length (log-transformed and standardized to a mean of zero and unit standard deviation), year, nest of origin, and sire as random effects (Kruuk and Hadfield, 2007; Wilson *et al.*, 2010). In binomial threshold models that estimate the variance components of an underlying continuous variable, the residual variance must be fixed to a value (here we use 1; see MCMCglmm package documentation), and the calculation of heritability requires that the residual variance be set to the variance of the link function [logit in this case, which is $\pi^2/3$ (section D in Goldstein *et al.*, 2002; Browne *et al.*, 2005)]. Thus, heritability was determined by dividing twice the sire component by the sum of the total variance components plus $\pi^2/3$ (section D in Goldstein *et al.*, 2002; Browne *et al.*, 2005).

We estimated variance components for the sex determination threshold ($n = 393$, mean = 0.494, s.d. = 0.498) with a univariate sire model with binomial error structure (Goldstein *et al.*, 2002; Browne *et al.*, 2005). We ran the model for 300,000 iterations, with the first 30,000 discarded as burn-in and then sampled every 1000 iterations thereafter. We used two datasets: (1) all clutches including those with such high-paternity skew that some sires were represented by only one offspring per clutch (115 sires, 51 dams, and 389 hatchlings, excluding four inter-sex individuals) and (2) only those clutches where each sire was represented by at least two offspring per clutch (73 sires, 34 dams, 273 hatchlings, excluding four inter-sex individuals), which we refer to as the low-paternity skew dataset. We assessed

significance of heritability by comparing the DIC values (smaller value indicates the favoured model) for a model that did not contain the random effect 'sire' relative to a model that did and by examining the confidence intervals provided by MCMCglmm.

RESULTS

We detected multiple sires for 51 of the 100 clutches. In 39 clutches, we detected two sires. In 11 clutches, we detected three sires, and we detected four sires in one clutch. In our dataset, there were two cases where a sire's genotype was identical in two different nests; thus, a very small number of paternal half-sib links may be present in the dataset. In both cases, Colony confidently predicted the male genotype for only two loci; thus, we lacked complete confidence in stating that these genotypes represented the same male.

Maternal size positively predicted if multiple sires were detected for a clutch ($P = 0.004$). This effect was independent of clutch size ($P = 0.386$; see Table S1 at: evolutionary-ecology.com/data/2612suppl.pdf) and was marginally independent of the number of hatchlings genotyped ($P = 0.076$). Each male sired an average of 3.4 offspring (s.d. = 2.3, range = 1–12, median = 3, mode = 2).

Maternal size and paternity status of a clutch interacted to influence hatchling sex (Fig. 1, Table 1; see also Table S3 at: evolutionary-ecology.com/data/2612suppl.pdf). Despite this opportunity for differential sex allocation, the primary sire did not influence offspring sex (d.f. = 1, 337, $P = 0.219$; Table 2; DIC for GLMM including percent of clutch sired = 508.200, DIC for GLMM excluding percent of clutch sired = 506.413).

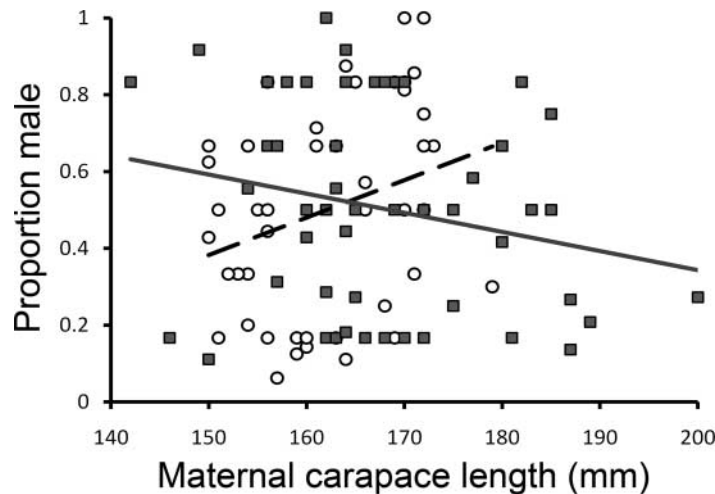


Fig. 1. The interaction between paternity status of a clutch (multiply sired or singly sired) and maternal carapace length on the sex ratio of a clutch is significant (Table 1) for our dataset of painted turtles, *Chrysemys picta*. Within singly sired clutches (open circles and black dashed trend line), maternal carapace length is positively correlated with the degree of male bias of the clutch ($P = 0.032$, $r = 0.307$, d.f. = 47, $t = 2.215$). Within multiply sired clutches (solid squares and grey solid trend line), the relationship is inverted, but is not statistically significant ($P = 0.131$, $r = -0.217$, d.f. = 48, $t = -1.5372$). Turtles have indeterminate growth, and size may be used as a crude proxy for age (Ernst and Lovich, 2009).

Table 1. Linear model used to test for an interaction of maternal size and paternity status of a clutch on clutch sex ratio in the painted turtle, *Chrysemys picta*

	d.f.	SS	MS	<i>F</i>	<i>P</i>
A. Response: Sex ratio					
Maternal CL	1	0	0	<0.001	0.998
Num. sires	3	1.923	0.641	0.495	0.687
Maternal CL*Num. sires	2	7.814	3.907	3.019	0.054
Residuals	92	119.085	1.294		
B. Response: Sex ratio					
Maternal CL	1	0	0	<0.001	0.998
Num. sires	1	0.081	0.081	0.064	0.801
Maternal CL*Num. sires	1	8.890	8.890	7.047	0.009
Residuals	95	119.851	1.261		

Note: Sex ratio was measured as proportion male and was logit transformed with 1 – the largest proportion added to both the numerator and denominator (Warton and Hui, 2011). Maternal carapace length (CL) was log transformed. In part A, paternity was treated as an ordinal effect (1, 2, 3, 4) to account for the number of detected sires contributing to each clutch. In part B, paternity was treated as a binomial variable (multiple sires detected or not).

Table 2. Generalized linear model with binomial error variance used to analyse 51 field-incubated nests with multiple paternity from the painted turtle, *Chrysemys picta*

Response: Hatchling sex	d.f.	Deviance	Residual d.f.	Residual deviance	<i>P</i>
NULL			388	539.20	
Maternal CL	1	4.663	387	534.54	0.031
Year	7	13.291	380	521.25	0.065
Nest	42	94.503	338	426.75	<0.001
Percent of clutch sired	1	1.510	337	425.24	0.219

Note: ‘Proportion clutch sired’ represents the logit-transformed (Warton and Hui, 2011) proportion of a clutch that each male sired. Hatchling sex was delineated as male = 1, female = 0. Nest and year were treated as categorical variables. An ordinal regression, which included four inter-sex individuals, also showed no effect of paternal success on hatchling sex. CL = carapace length.

Heritability of the sex determination threshold

Heritability of the sex determination threshold, calculated using all 51 clutches, was $h^2 = 0.351$ (95% CI = [0.164, 0.832]; DIC with sire component = 491.961, DIC without sire component = 506.369). The sum of the nest and maternal size components of variance, which is the proportion of maternal genetic, maternal environment, and common environment effects that comprised the total variance, was 0.017 (95% CI = [0.001, 0.217]).

Heritability of the sex determination threshold was lower and not significantly different from zero when calculated using the smaller dataset with two or more individuals per sire ($h^2 = 0.173$, 95% CI = [0.000, 0.628]; DIC with sire component = 345.729, DIC without sire component = 351.056). In this case, the sum of the nest and maternal size components of variance was over four-fold higher, yet still small, at 0.074 (95% CI = [0.000, 0.301]).

DISCUSSION

Fluctuating selection is expected to maintain primary sex ratios at an evolutionary equilibrium (Fisher, 1930; Bull and Charnov, 1988; Conover *et al.*, 1992; Basolo, 1994; Blows *et al.*, 1999). In organisms with temperature-dependent sex determination (TSD), thermal sensitivity of the sex-determining pathway may be a target of this selection, but the underlying genetic variance of this trait in the wild is largely unknown. Our study is the first to document the genetic variance under field conditions in a reptile with TSD. We found weak genetic variance for the sex determination threshold, but we also potentially documented relatively weak maternal and nest effects.

Our measurement of the genetic variance for the sex determination threshold provides a more realistic measure compared with previous studies in two important ways. First, the heritability of the sex determination threshold, when evaluated in systems where sex determination has a substantial environmental component, has been estimated mostly in the broad-sense (Bull *et al.*, 1982; Janzen, 1992; Rhen and Lang, 1998; Janes and Wayne, 2006; but see Conover and Heins, 1987; Vandeputte *et al.*, 2007; Rhen *et al.*, 2011). Broad-sense heritability includes all among-family variance and may inflate our assessment of the potential response to selection for a trait (Lynch and Walsh, 1998). The DIC values of the models indicate that the sire component of variance is likely important; thus, underlying genetic variation (and not purely maternal effects) may be a substantial component of the sex determination threshold. Second, heritability for this threshold has previously been estimated only in the laboratory. Such studies typically use constant-temperature incubation conditions (but see McGaugh and Janzen, 2011), neglecting daily temperature fluctuations that appear to be important for embryonic sex determination (Les *et al.*, 2007; McGaugh and Janzen, 2011). Furthermore, laboratory-measured heritability may be an overestimate relative to field-measured heritability, because the laboratory is a novel environment (Holloway *et al.*, 1990) and environmental variance may be lower than what is experienced in the field (Hoffman, 2000; Geber and Griffen, 2003; but see St. Juliana and Janzen, 2007). A maternal half-sib design allowed us an unprecedented opportunity to understand whether genetic variation for the sex determination threshold plays any role under natural field conditions. Our maternal half-sib design is prone to yield different values for genetic variance depending on how phenotypically (or, obviously, genetically) distinct the sires are within a single clutch. Excluding the clutches with the greatest paternity skew may inadvertently exclude clutches with the greatest genetic or quality difference between sires. Excluding clutches with the greatest paternity skew also reduces the sample size and power. Our estimates of heritability differ based on whether we utilized all clutches or only those with low-paternity skew ($h^2 = 0.351$ and $h^2 = 0.173$, respectively). However, both values are lower than most broad-sense laboratory heritability estimates for this trait, which range as high as 0.82 [95% CI = 0.31–1 (Bull *et al.*, 1982)], and, for both datasets, the model that includes the effect ‘sire’ has a favoured DIC value over a model that excludes the term ‘sire’.

In both datasets we utilized, maternal effects (embodied by nest micro-environment and maternal carapace length in the model) accounted for little variance in sex determination threshold. Nesting biology substantially impacts the sex ratio of natural nests (Janzen, 1994b; Schwanz and Janzen, 2008; Schwanz *et al.*, 2010). Importantly, we used nests that contained mixed sex ratios, which experienced less extreme environments than the totality of nests in the population (McGaugh and Janzen, 2011); thus, by focusing only on mixed-sex nests, we excluded some of the maternal effects that contribute to total cohort sex ratio. Our model seems to indicate that, at least for the nests included in this study, nest-site choice and within-nest

maternal effects (e.g. hormone allocation and maternal body condition) may have had little impact on nest sex ratio. Similarly, little variance in the sex determination threshold in the leopard gecko (*Eublepharis macularius*) was attributable to maternal identity or body condition (Rhen *et al.*, 2011). These quantitative genetic findings are in accordance with evidence that maternal hormone allocation to embryos may have only weak effects on nest sex ratio (Janzen *et al.*, 1998; St. Juliana *et al.*, 2004; Radder *et al.*, 2007; Warner *et al.*, 2007, 2008; but see Bowden *et al.*, 2000).

Our measures of genetic variance for the sex determination threshold rely on two assumptions. First, we assume that sex allocation, if it exists in this system, is random with respect to sire. We detected no relationship between a male's within-clutch reproductive success and the sex of his offspring [Table 2 (*contra* Calsbeek and Sinervo, 2004)], thus our data support the validity of this assumption. Second, we assume that no dominance or epistasis affects the sex determination threshold. Dominance variance at a male-biased temperature accounted for ~22% of the phenotypic variance in *E. macularius* (Rhen *et al.*, 2011), and additive genetic variance accordingly decreased to nearly zero, suggesting a mechanism for TSD of environment-dependent increase in dominance at male-biased temperatures. Since we detected additive genetic variance in our data, the nests in our study may not have been in environments that induced expression of dominance, or the genetic architecture for the sex determination threshold is different between *C. picta* and *E. macularius*.

Our results support a growing appreciation of the potential role that the thermal sensitivity of the sex-determination pathway can play in response to sex-ratio selection relative to maternal nest-site choice (Bulmer and Bull, 1982; Bull *et al.*, 1982; Morjan, 2003a; McGaugh and Janzen, 2011). Nest-site choice was initially thought to be the most likely candidate for a swift evolutionary response to sex-ratio selection, because well-documented geographic clines in nesting behaviour illustrate potential for local adaptation; these behavioural changes appeared large relative to geographic clines in threshold temperature (Bull *et al.*, 1982; Ewert *et al.*, 2005; Doody *et al.*, 2006; Janzen, 2008). However, both nest-site choice and nesting phenology have low genetic variance (McGaugh *et al.*, 2010), and thus are unlikely to serve as substrates for rapid evolutionary response to any sex-ratio bias produced by climate change (Morjan, 2003a; Schwanz and Janzen, 2008; Schwanz *et al.*, 2010; McGaugh and Janzen, 2011).

Our study suggests that there is genetic variance underlying the sex determination threshold and indicates that sex-ratio selection may be effective in producing an evolutionary shift in this key trait. In the field, however, some nests are located in environments so hot or cold that embryonic sex is effectively canalized. In these cases, the response to selection by the sex determination threshold is not directly predicted by the standard breeder's equation (Falconer and MacKay, 1996), because heritability estimates must be weighted to account for this environment-limited expression (Bull *et al.*, 1982; McGaugh and Janzen, 2011). Here we calculated this 'effective' heritability as $h^2 = 0.135$ (0.063, 0.319) for our full dataset and $h^2 = 0.066$ (0, 0.241) for the low-paternity skew dataset (for detailed methodology, see McGaugh and Janzen, 2011). For our study population in Illinois during July (when embryos are undergoing the temperature-sensitive phase of sexual development), climate change is projected to be less intense (<1°C) over the next 30–40 years than in other areas of the United States (Pan *et al.*, 2004; Liang *et al.*, 2006; Portmann *et al.*, 2009). Such a modest change (current mean July nest temperature of 23.9°C increasing to 24.9°C) is expected to decrease the production of males in a typical cohort by ~23.5% (cohort sex ratio = $4.14 - 0.147 \times \text{mean July air temperature } [^{\circ}\text{C}]$) (Schwanz *et al.*, 2010, p. 3021). Even our smaller estimate of effective heritability should be sufficient to return the average cohort sex ratio in our population to nearly equilibrium

within ~75–100 years under a 1°C increase in mean air temperature, if climate change ceases after an initial warming period (Figure 5A in Morjan, 2003a).

Finally, an unexpected and indirect finding of our study is that maternal age (as reflected by carapace length) and paternity status appear to interact in association with the sex ratio of a clutch (Table 2, Fig. 1; see also Table S3 at: evolutionary-ecology.com/data/2612suppl.pdf). Within singly sired clutches, maternal carapace length is positively associated with male sex-ratio bias of a clutch ($P = 0.032$, $r = 0.307$, d.f. = 47). In contrast, within multiply sired clutches, maternal carapace length is negatively associated with male sex-ratio bias of a clutch ($P = 0.131$, $r = -0.217$, d.f. = 48). Since maternal age significantly influences hormone allocation to eggs, nest placement, and survival of offspring during migration from nest to water in this population (Bowden *et al.*, 2004; Harms *et al.*, 2005; Paitz *et al.*, 2007), such an interaction may be both ecologically and evolutionarily relevant and deserves further investigation. Interestingly, increased male production with female age has been documented in other TSD systems as well (Rhen *et al.*, 2011).

In summary, we leveraged a natural feature of our system (i.e. multiple paternity) to gain insight into the evolutionary potential of a key offspring trait in the wild. Obtaining such important quantitative genetic information typically meets resistance generated by the intrinsic biology of long-lived organisms abutting the statistical requirements of experimental design. Here we show how an effective understanding of the natural history of a species can be creatively integrated with modern molecular and statistical approaches to afford valuable progress in predicting micro-evolutionary dynamics. We encourage others working with ‘challenging’ taxa in the field to explore how elements of the biology of their systems can be profitably exploited to gain substantive evolutionary genetic insights.

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