Title: Heritable variation in thermosensitive sex determination provides the raw material for adaptation to climate change

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Abstract

Climate change undoubtedly poses a challenge to species with temperature-dependent sex determination (TSD). Some researchers paint a dire picture of demographic collapse due to extremely biased population sex ratios, but that scenario presumes TSD species lack the potential to evolve. Here we address this key assumption by testing whether variation in thermosensitivity is heritable. We use controlled breeding studies to measure the relative contribution of additive genetic effects versus genetic dominance and/or maternal effects to sex ratio variability within and between snapping turtle populations. While egg and hatchling size were strongly impacted by non-genetic maternal effects and/or dominance variance, hatchling sex was highly heritable in various thermal regimes. Furthermore, Texas sires and Minnesota dams produced hybrid offspring with sex ratios halfway between southern and northern populations at warm temperatures, but sex ratios like the northern population at cool temperatures. Together, these findings show genetic variants with additive effects are the primary cause of variation in sex ratio within populations and underlie population differences at warm temperatures. Yet, maternal effects and/or genetic variants that display dominance cause population differences at cool temperatures. With high heritability and genotype-by-temperature interactions, TSD pattern should evolve, allowing snapping turtles to persist in spite of warming global temperatures. At the same time, this study highlights an urgent need to measure genetic variation for TSD in species that are threatened, endangered, or endemic to ecosystems impacted by rapid climate change.

Introduction

Species with temperature-dependent sex determination (TSD) have evolved a key phenotypic (sex) and demographic (sex ratio) trait in direct response to climatic variation. This mode of sex determination is common in ectothermic vertebrates, being found in all crocodilians, most turtles, some squamates, and a few fish[1-7]. Variation in sex ratio is observed at various spatial and temporal scales and is correlated with nest temperatures in all of these taxonomic groups[8-17], indicating that TSD occurs in nature and is not an artifact of exposure to constant temperatures in laboratory experiments. Incubation temperature also influences many other traits in TSD species, including the developmental rate of embryos and post-embryonic growth, metabolism, physiology and behavior[18-20]. Species with TSD are therefore ideal models for understanding the direct impact of global warming on individuals and populations. To be sure, research shows a warming climate over the past few decades has already produced extremely female biased sex ratios in sea turtles from some rookeries, but not others[21].

There is active debate over the long-term impact of global warming and increasingly biased primary sex ratios on sustainability of TSD species. Some argue that populations may be resilient because operational sex ratios in adults are more balanced than primary sex ratios in hatchlings[21-23]. Behavioral changes in nest site selection, nesting phenology, or shifts in species range could also mitigate the effect of global warming on sex ratios[13,14,21,24,25]. On the other hand, theoretical models suggest that extremely biased sex ratios will lead to demographic collapse due to a paucity of individuals of one sex or the other[26]. A major assumption of the latter model is that TSD pattern cannot evolve.

Phenotypic and genotypic variation in thermosensitivity is necessary for evolutionary responses to a changing climate[27]. Phenotypic variation in TSD within and among species is the

norm, but the underlying causes of this variance are uncertain[28]. Some authors hypothesize variation in sex ratio among clutches of eggs produced by different females is due to non-genetic maternal factors like sex steroids deposited in egg yolk prior to oviposition. Others propose that variation in sex ratio is genetically based (i.e., zygotic genotype affects thermosensitivity). However, it is important to point out that these hypotheses are not mutually exclusive: TSD may be a complex trait influenced by a mix of genetic factors, non-genetic maternal factors, and their interaction with temperature[28]. To date, only two studies in reptiles have used experimental designs capable of distinguishing these effects and both show significant genetic variation for TSD but minimal maternal effects on TSD[28-30].

Quantification of both genetic and non-genetic causes of variation in sex determination is essential for understanding, predicting, and mitigating the potential impact of climate change on TSD species. As described by Fisher[31], biased sex ratios cause negative frequency-dependent selection on sex-determining mechanisms and favor alleles that induce development of the rare sex. Indeed, even sex ratios evolved rapidly in a laboratory setting that initially produced biased sex ratios in a TSD fish[32]. To assess the potential for an evolutionary response to global warming, we carry out the first prospective quantitative genetic analysis of variation in thermosensitivity within and between populations of a TSD reptile.

Results and Discussion

We first examined traits (i.e., egg and hatchling size) expected a priori to display strong maternal effects as positive controls to confirm that our paternal half-sib, maternal full-sib breeding design parsed additive genetic variation from maternal effects and dominance variation. We then quantified the underlying causes of variation in TSD. In this breeding design, sire effects directly demonstrate additive genetic variation[51,52]. Maternal effects and/or variation due

94 to genetic dominance are only significant if the variance among dams nested within sires (a.k.a.
95 dam effect) is larger than the variance among sires[51,52]. We used sire variance to calculate
96 narrow-sense heritability (h^2) and the difference between dam variance and sire variance to
97 estimate maternal effects (m^2).

98 As anticipated, maternal effects strongly influenced the size of snapping turtle eggs. Dam
99 identity had significant effects on egg mass, but sire identity had no impact on egg mass (Table
100 1). Similarly, dam identity had strong effects on egg diameter, but the sire effect was
101 insignificant (Table 1). Maternal effects were large for egg mass ($m^2 = 0.64 \pm 0.28$) and egg
102 diameter ($m^2 = 0.52 \pm 0.25$). Conversely, heritability was zero for egg mass ($h^2 = 0.00 \pm 0.57$)
103 and diameter ($h^2 = 0.00 \pm 0.45$). In agreement with these results, the population origin of sires
104 had no impact on egg size: eggs containing Texas x Minnesota (TX x MN) hybrids did not differ
105 from eggs containing MN offspring (Table1). Our breeding design clearly teased apart maternal
106 effects on egg size from potential sire effects. These results are consistent with research showing
107 that maternal body size and physiology are primary determinants of egg size in turtles[33-35].

108 We also detected maternal effects on the size of hatchling snapping turtles. The main
109 effect of dam was significant for hatchling mass, carapace length, and plastron length, but the
110 main effect of sire was not significant for any of these traits (Table 1). At the same time,
111 temperature during embryogenesis had a major effect on all measures of hatchling size (Table 1).
112 Hatchlings from 22°C and 22.5°C did not differ from each other but were significantly smaller in
113 mass, carapace length, and plastron length than hatchlings from 26°C, 27°C and 28°C (Figure 1).
114 In addition, there were significant dam-by-temperature interactions for hatchling mass and
115 carapace length, but not for plastron length (Table 1). Sire-by-temperature interactions were
116 significant for hatchling mass and plastron length, but not for carapace length (Table 1). Sire-by-

117 temperature interactions indicate that zygotic genes with additive effects influence embryo
118 growth in at least one thermal environment.

119 To gain insight into the underlying cause of sire-by-temperature and dam-by-temperature
120 interactions for hatchling size, we estimated narrow-sense heritability and maternal effects for
121 each trait at each temperature. Heritability estimates were large at cool temperatures and
122 decreased significantly with increasing temperature for all measures of hatchling size (Figure
123 2A), though estimates at individual temperatures were not greater than zero because of large
124 standard errors (Table 2). The opposite pattern was observed for maternal effects, which were
125 small at cool temperatures and increased significantly with increasing temperature for carapace
126 and plastron length, but not for hatchling mass (Figure 2B). Maternal effects were significant or
127 nearly so for all measures of hatchling size at 27^{o}C and 28^{o}C (Table 2).

128 The negative correlation between heritability and maternal effects on hatchling size
129 (Figure 2A vs. Figure 2B) is likely an indirect consequence of thermal effects on incubation
130 duration (Table 1). Snapping turtle embryos developed faster and hatched sooner as temperature
131 increased (Figure 3). Thus, hatchlings from warmer temperatures were younger than hatchlings
132 from cooler temperatures with regard to time since oviposition (i.e., total age). In a wide range of
133 species, maternal effects on offspring size are significant earlier in development and diminish as
134 maternally derived resources are depleted and offspring age[36-37]. Indeed, incubation temperature
135 influences the amount of residual yolk (a maternally derived resource) in snapping turtles 4 days
136 after pipping their eggshell: hatchlings have more residual yolk with increasing temperature and
137 younger total age[38]. Yolk is also metabolized after turtles emerge from their eggs and is nearly
138 exhausted by 30 days after pipping[38], again showing maternally derived resources are used up as
139 turtles age.

In contrast, genetic effects on body size increase as offspring age in various species[36-37]. Prior research in snapping turtles shows that initial egg mass is strongly correlated with hatchling mass at 5-10 days of age, but has no impact on post-hatching growth when turtles start feeding and fueling their own growth[39]. Growth rate, however, is strongly affected by clutch identity (i.e., genetic effects) for at least the first 6 months of life[39]. Maternal effects on offspring size in snapping turtles are robust at early ages, but fade as offspring age and genotype starts to have a greater influence on growth.

In addition, sire origin had a significant effect on all measures of hatchling size (Table1). With our breeding design, differences in the size of TX x MN hybrids and MN offspring are due to additive effects of alleles contributed by the TX and MN sires mated to MN dams. TX sires generally produced larger offspring than MN sires (Figure 1), but this effect varied with trait and temperature. The sire origin-by-temperature interaction was significant for hatchling mass, but not for carapace or plastron length (Table1). The difference in mass between TX x MN hybrids and MN offspring was pronounced at cooler incubation temperatures and disappeared at warmer temperatures (Figure 1A), which parallels the pattern of higher heritability for hatchling size at cooler temperatures. In contrast, differences in carapace and plastron length were more consistent across temperatures (Figure 1B,C).

Sire origin also had a strong impact on incubation duration (Table 1). On average, MN offspring pipped 7.5 days sooner than TX x MN hybrids across all temperatures (Figure 3), which is consistent with a prior report that turtles from northern populations hatch sooner than southern turtles when incubated at the same temperatures[40]. Countergradient variation in developmental rate with latitude strongly suggests genetic adaptation to regional thermal

environments[41,42]. In other words, northern alleles speed embryonic developmental rate to counteract the negative impact of cooler soil temperatures at higher latitudes[40].

While we focus on the impact of embryonic genotype and maternally derived egg components in this paper, we would be remiss if we did not mention that maternal nest site selection is also important. Overstory vegetation, soil albedo, soil type, and nest depth all affect nest temperatures[9,16,17,40,44-48]. Ewert et al.[40], for example, reported a latitudinal cline in shading of snapping turtle nests in nature: nests at the lowest latitudes are usually shaded whereas nests at the highest latitudes are usually exposed. Shading minimizes exposure to temperatures above the critical thermal maximum for embryos in the south, whereas solar exposure in the north provides the heat necessary for turtles to develop, emerge from the nest, and reach water for overwintering before temperatures become too cold. Selection for emergence and migration to water in the fall is strong because survival of snapping turtles that overwinter in the nest is very low at northern latitudes[49]. Laboratory studies in a mid latitude population further support the idea that overwinter survival of hatchlings is higher in aquatic environments than terrestrial environments[50]. Thus, maternal behavior (selection of warmer nest sites) and zygotic genotype (faster developmental rate) both mitigate the impact of cooler temperatures at higher latitudes.

To eliminate the potential effect of nest site selection on offspring phenotype, we collected eggs within 24 hours of oviposition and incubated eggs in the same incubators. This allowed us to focus on the impact of zygotic genotype and maternally derived components of the egg itself. Maternal effects determined egg size, while maternal effects, additive genetic effects, and incubation temperature all contributed to variation in hatchling size. In addition, controlled crosses revealed differences in developmental rate and size between TX x MN hybrids and MN offspring. These results confirm our ability to disentangle additive genetic effects from maternal

185 effects and dominance variation. They also show that we can detect additive genetic
186 differentiation between populations near the southern and northern ends of the species range. We
187 can therefore use a paternal half-sib, maternal full-sib breeding design to quantify the underlying
188 causes of variation in TSD within and between populations.

189 True to form, incubation temperature had a strong effect on sex determination in
190 offspring from our breeding colony (Figure 4). In contrast to other traits, both sire and dam
191 identity influenced sex determination (Table 1). The sire-by-temperature interaction was also
192 significant, but the dam-by-temperature interaction was not quite significant (Table 1). Variation
193 among sires directly demonstrates additive genetic effects on sex determination, while the sire-
194 by-temperature interaction suggests these effects vary with incubation temperature. Genotype-
195 by-environment interactions can result from changes in rank of family means in different
196 environments (i.e., crossing reaction norms) and/or changes in scale across environments (i.e.,
197 differences in the magnitude or the nature of allelic effects)[51]. In leopard geckos, sire-by-
198 temperature interactions were due to crossing reaction norms for sex ratio and a change in
199 heritability across temperatures[30]. Heritability was significant at 30^{o}C but not at 32.5^{o}C. The
200 decline in additive variance at 32.5^{o}C was accompanied by a significant increase in dominance
201 variance, suggesting the nature of allelic effects change with temperature. In contrast, maternal
202 effects did not influence sex determination at any temperature in leopard geckos. To understand
203 the underlying cause of sire-by-temperature interactions in the snapping turtle, we estimated
204 heritability and maternal effects on sex determination at each temperature and examined reaction
205 norms for paternal half-sibs.

206 We calculated heritability and maternal effects on sex determination on the underlying
207 liability scale using the logit link (i.e., the threshold model)[52]. Heritability of sex was high at all

five temperatures (Table 2; Figure 2A). There were differences in heritability among hatchling traits ($F_{3,12} = 21.0$, $p < 0.0001$), a significant temperature effect ($F_{1,12} = 48.3$, $p < 0.0001$), and a significant trait-by-temperature interaction ($F_{3,12} = 10.6$, $p = 0.001$). This interaction reflects consistently high heritability for sex determination across all temperatures, but decreasing heritability for body size with increasing temperature (Figure 2A). Thus, sire-by-temperature interactions for sex determination are the result of crossing reaction norms (Figure 5) rather than temperature-dependent changes in heritability as we observed for body size.

In contrast to heritability, maternal effects on sex determination were small (Figure 2B). Estimates of maternal effects were in fact negative at four of five temperatures (Table 2) because variance among dams was smaller than variance among sires. When we analyzed variation in maternal effects, there were differences among traits ($F_{3,12} = 20.7$, $p < 0.0001$) and a significant temperature effect ($F_{1,12} = 33.3$, $p < 0.0001$), but the trait-by-temperature interaction was insignificant ($F_{3,12} = 1.3$, $p = 0.33$). On average, maternal effects were smaller for sex determination and plastron length than for hatchling mass and carapace length (Figure 2B).

In accord with the high heritability for sex described above, sire origin influenced sex determination (Table 1). On average, TX x MN hybrids were more likely to develop ovaries than MN offspring (Figure 4). Although the sire origin-by-temperature interaction was not quite significant, there were differences between TX x MN hybrids and MN offspring at 27^{o}C and 28^{o}C but not other temperatures. Hybrids exhibited sex ratios halfway between northern and southern populations at 27^{o}C and 28^{o}C, while sex ratios of hybrids were like the MN population at 22^{o}C and 22.5^{o}C (Figure 4). Together, these results show that variation in TSD pattern is highly heritable and that genetic variants with additive effects cause population differences in sex ratio at warm temperatures. They also show maternal effects and/or dominance variation

cause population differences in sex ratio at cool temperatures. More complicated breeding designs will be required to quantify the relative contribution of maternal effects versus genetic dominance to population differences in sex ratios at cool temperatures[51].

While incubation of eggs at constant temperatures is a convenient way to estimate pivotal temperatures and characterize TSD patterns, temperatures in natural nests normally fluctuate. We therefore carried out temperature shift experiments using eggs from crosses between MN sires and MN dams. We incubated eggs at a male-producing temperature (26.5^{o}C) until embryos reached stage 17.5, which is the most thermosensitive stage during the sex-determining period in snapping turtles[53]. Eggs were then shifted to a female-producing temperature (31^{o}C) for 2.5 days and shifted back to 26.5^{o}C for the remainder of embryogenesis. This brief exposure to 31^{o}C produces mixed sex ratios and reveals variation in thermosensitivity in MN turtles[54]. Our controlled breeding studies reveal significant variation among sires ($LR\chi^2 = 4.0$, $p = 0.046$), but not among dams ($LR\chi^2 = 1.5$, $p = 0.22$) or between years ($LR\chi^2 = 1.65$, $p = 0.20$). Accordingly, heritability for sex determination was high ($h^2 = 0.87 \pm 0.49$) and maternal effects were low ($m^2 = -0.11 \pm 0.19$). These estimates are surprisingly similar to heritability and maternal effects on sex at constant incubation temperatures (Table 2). This again demonstrates additive genetic variation for TSD: i.e., some MN genotypes are sensitive to a 2.5-day exposure to 31^{o}C and develop ovaries while other MN genotypes are not as sensitive and develop testes.

We have previously shown population differences in sensitivity to temperature shifts. A 6-day exposure to 31^{o}C is sufficient to produce 100% females in MN embryos while 12 days at 30^{o}C only produced 25% females in TX embryos[53]. Embryos from MN are more sensitive to high temperature feminization than TX embryos even when exposure duration is normalized by total incubation duration (7.6% of embryogenesis in MN versus >11% of embryogenesis in TX).

This difference in thermosensitivity likely has an additive genetic basis because population differences at constant warm temperatures are heritable.

Both the magnitude and the duration of thermal exposure have quantitative effects on sex determination in TSD species[53,55,56]. Temperatures further from the pivotal temperature have more potent feminizing or masculinizing effects than temperatures closer to the pivotal temperature. Longer exposures to female or male temperatures have more potent feminizing or masculizing effects than shorter exposures to the same temperatures. Sex determination is likewise affected by the cumulative time embryos spend developing above the pivotal temperature in fluctuating thermal regimes[26,57-60]. These observations suggest temperature regulates production of a biochemical factor (or factors) in a quantitative way and that this signal is translated into a binary response (ovary vs. testis) via a threshold-like mechanism[20].

That biochemical factor (or factors) is conceptually equivalent to liability in the threshold model for binary traits[51,52]. Here, we demonstrate additive genetic variation for liability as measured by 1) sex ratios at constant temperatures and 2) the duration of thermal exposure required to induce ovarian development in temperature shifts. Identification of genes that determine liability will provide insight into the mechanism that transduces temperature into a biological signal for sex determination. One such gene is cold-inducible RNA binding protein, which accounts for ~25% of heritable variation for sex in the temperature shift paradigm[54]. Other genes that influence temperature sensitivity remain to be identified.

Until now, the underlying cause of variation in TSD in the snapping turtle was uncertain because eggs were collected from nests in the wild and parentage was not known, which precluded direct estimation of additive genetic variance. Prior estimates of broad-sense heritability (0.34 to 0.76) in the snapping turtle[61] are lower than our estimates of narrow-sense

heritability. It is likely that multiple paternity within clutches from the wild[62] decrease phenotypic similarity among clutch mates and explain lower broad-sense heritability.

Our findings of substantial heritability and minimal maternal effects challenge assertions that maternally derived steroids in egg yolk are the primary cause of variation in sex ratio among clutches. Roush and Rhen[28] reviewed a total of 21 studies that measured yolk steroids in TSD reptiles. Only three report a significant correlation between yolk steroids at oviposition and offspring sex ratios[63-65]. Just one study tested for a correlation between steroid levels and sex ratio at the clutch level, while the other two compared eggs laid early versus late in the nesting season. Clutches of painted turtle eggs with higher estradiol/testosterone ratios produced more female-biased sex ratios[63]. The coefficient of determination in that study shows yolk steroids explain a relatively small proportion of the variation in sex ratio among clutches ($r^2 = 0.16$). Painted turtle and red-eared slider turtle eggs laid later in the nesting season had higher estrogen levels and were more likely to produce females than eggs laid early in the season[64,65]. In another study of painted turtles, McGaugh et al.[29] genotyped dams and offspring to infer paternity and directly estimate narrow-sense heritability. Because eggs were left in natural nests, they experienced fluctuating temperatures instead of constant temperatures. Nevertheless, heritability for sex was significant ($h^2 = 0.35$) and accounted for twice as much variation as yolk steroids in the earlier study ($r^2 = 0.16$). This reinforces the key point that genetic and maternal effects are not mutually exclusive. Although narrow-sense heritability was significant in snapping turtles, painted turtles, and leopard geckos, differences among species warn against broad generalizations about the mechanistic basis for clutch effects in other species. It is imperative to quantify heritability, dominance, and maternal effects on a species by species basis.

Genetic variation for sex ratio is essential for the evolution of TSD[32,61,66-68]. Fisherian selection and evolution of even sex ratios has been shown in the Atlantic silverside, a widespread TSD fish. Conover and Van Voorhees[32] established laboratory populations from individuals collected in South Carolina, New York, and Nova Scotia. Experimental populations were reared at constant low or high temperatures that produce biased sex ratios in the source populations. Four laboratory populations began with skewed sex ratios in the first generation. The minority sex increased in frequency until even sex ratios were established in just one to five generations, showing that sex determination is highly heritable. In light of our finding that sex in the snapping turtle is also heritable across a range of constant temperatures and a temperature shift study, it is likely that TSD pattern will evolve in snapping turtles and prevent demographic collapse.

At the same time, our work highlights an urgent need to measure the underlying causes of variation for TSD in endangered and threatened species in order to develop more effective conservation plans in the face of climate change. Different decisions would need to be made for species that exhibit genetic variation for TSD than for species with little or no genetic variation. Additional questions arise. For instance, would it be better to maintain genetic variation for TSD or selectively breed genotypes with a specific pattern of thermosensitivity to ensure long-term sustainability in new thermal environments? Abundant species like snapping and painted turtles serve as tractable models to identify TSD genes that can be studied in threatened and endangered species (i.e., non-destructive tests for genetic associations between DNA variants and sex). Widespread species are also ecological and evolutionary models for understanding how patterns of spatial and temporal variation in temperature (i.e., climate) shape TSD patterns. Snapping turtles display a cline in TSD pattern that is counterintuitive: the upper pivotal temperature increases with increasing latitude[40]. Yet, northern populations are more sensitive to shorter

pulses of high temperature[53]. In contrast, painted turtles do not exhibit clines in TSD pattern despite being widely distributed across climatic regions[69]. We clearly need to develop a better understanding of the complex interplay among nesting ecology, maternally derived egg components, zygotic genotype, and climate in TSD species.

Methods

Captive breeding colony

All procedures were approved by the Institutional Animal Care and Use Committee at UND. We have two rooms set up in identical fashion to house and breed snapping turtles in the animal quarters of Starcher Hall on the UND campus. There are seven "300-gallon" stock tanks in each room (14 breeding tanks). Each tank is filled with roughly 900 liters of water and houses 1 male and 2-4 females. Tanks are of similar width and depth to streams inhabited by snapping turtles in nature, so animals have sufficient room to swim freely. Clean, temperature controlled water flows in one end of each tank. Dirty water flows out the other end of the tank and passes through an automated filtration system (each room has an identical filtration system).

Dirty water from seven turtle tanks flows into a ProfiDrum Eco 45/40 Rotary Drum Filter, which removes solid waste larger than 70 microns. Filtered water flows into a Sweetwater Low-Space Bioreactor with denitrifying bacteria to degrade nitrogenous wastes. Water is then pumped through an Emperor Aquatics SMART High Output UV Sterilizer and an Aqua Logic Multi-Temp Chiller to control water temperature. Filtered, sterilized, temperature-controlled water is fed back into tanks. Water is re-circulated through the system at a rate of two complete water changes per tank per hour. De-chlorinated tap water (~900 liters/day) is fed into the system to provide input of fresh water to the system. Excess dirty water flows into a floor drain.

Environmental conditions are designed to mimic seasonal changes in water temperature and photoperiod in MN. Temperatures range from 4°C during the winter when turtles hibernate to 24°C during the summer when turtles are active. Temperatures are adjusted monthly to match average temperatures in small lakes in MN. Rooms have full spectrum lighting under automatic control to mimic seasonal changes in photoperiod. Photophase and scotophase are adjusted monthly to match average day lengths in MN. Light period ranges from 8.5 hours during January to 16 hours during July. Turtles are fed a diet of whole frozen smelt twice per week and fresh fruits with a vitamin supplement once per week when water is above 12°C from April to September. Turtles do not feed at temperatures below 12°C and hibernate from October to March. Full spectrum lighting consists of eight F32T8 Vita Brite ® 48 inch fluorescent bulbs in each room. These conditions ensure normal reproduction. Fertility of eggs in the breeding colony is the same as eggs collected from natural nests in the wild.

Adult turtles were captured in MN and TX. Turtles from MN were collected from the full latitudinal range within the state (from the Canadian border in the north to the Iowa border in the south). Turtles from TX were collected on Lake Lewisville U.S. Army Corps of Engineers property, Lewisville, Texas. We permanently labeled turtles with unique combinations of marks on their marginal scutes when they were brought to UND. We housed each sire with 2-4 dams in a paternal half-sib, maternal full-sib breeding design. Details regarding analysis of offspring phenotype for this breeding design are found below in the "Quantitative genetic analysis" section. Adult turtles from MN were the only animals in the colony in 2013 and 2014. A total of 15 males and 31 females produced offspring in 2013 and 2014. Eggs produced in 2015 were used for other experiments and are not included in this study. Adult turtles from TX were captured and brought to UND in the early summer of 2015. TX turtles were held in quarantine to

monitor their health before being introduced to the colony. Turtles were assigned to new breeding groups in late summer of 2015. Roughly equal numbers of MN males (n = 8) and TX males (n = 6) were mated to MN females (n = 34). We did not use eggs from 2016 for this study because we wanted to be sure new mates sired offspring rather old mates (via sperm storage). We used eggs from the new breeding groups in 2017 and 2018 and verified parentage of offspring with molecular markers.

Egg collection and incubation

In early June, we palpated females to assess their reproductive status. Gravid females were transferred to an outdoor aviary with an artificial pond and natural nesting substrate. Aviaries were monitored at 2-hour intervals from 10 pm to 10 am on a daily basis to observe females in the act of nesting. Females were individually identifiable by the unique combination of marks on their marginal scutes. Eggs were collected shortly after nesting was completed. A post-it note with nesting date and dam ID was placed with each clutch. Females were returned to their indoor stock tanks the day after nesting.

Eggs were labeled with a unique clutch and egg number, placed in plastic boxes, and covered in moist vermiculite (1-part water: 1-part vermiculite by mass). Approximately even numbers of eggs from each clutch were assigned to each temperature treatment and further split between replicate incubators to avoid pseudoreplication. In 2013 and 2014, eggs were incubated at 26.5^{o}C until embryos reached stage 17.5 of embryogensis[70]. Eggs were then shifted to 31^{o}C for 2.5 days and then shifted back to 26.5^{o}C for the remainder of incubation. In 2017 and 2018, eggs were incubated at constant temperatures of 22^{o}C (2018 only), 22.5^{o}C (2017 only), 26^{o}C, 27^{o}C, and 28^{o}C until turtles hatched.

Measurement of phenotypic traits and tissue collection

Egg and hatchling morphometric data were collected in 2017 and 2018. Eggs were weighed and egg diameter measured shortly after oviposition. Hatchlings were weighed and carapace length and plastron length measured 30 days after turtles pipped their eggshell. Hatchlings were euthanized via rapid decapitation and sex was determined by inspection of the gonads and genital ducts under a dissecting microscope. Adrenal-kidney-gonad complexes were dissected, placed in RNAlater, and stored at -20^{o}C.

DNA extraction and quantification

Whole blood was drawn from adults in the breeding colony and DNA extracted using the Qiagen DNeasy Blood and Tissue Kit. Total egg numbers for each study year were 2013 (n = 1062), 2014 (n = 1035), 2017 (n = 1358), 2018 (n = 1422). Adrenal-kidney-gonad complexes were removed from long-term storage to cut around 4 mg of tissue from the posterior portion of one kidney for DNA extraction. Scissors and forceps were rinsed in sterile 1X PBS between samples. We used the Beckman Coulter Biomek FX^{P} liquid handling robot and DNAdvance kit to extract DNA from kidney tissues. We made one slight modification to the protocol by adding 4 ul of 10 mg/ml of RNase A to each sample after tissue lysis. Samples were incubated with RNase A for 30 minutes at 37^{o}C prior to the running the standard protocol. DNA concentration was measured using a Beckman Coulter DTX 880 Fluorescence Plate Reader and the Quant-iT Picogreen dsDNA assay kit. Samples were then normalized to 5 ng DNA/ul and stored in 96-well plates at -80^{o}C until used for genotyping.

Genotyping by sequencing

DNA from each turtle was sequenced at 7 highly polymorphic microsatellite loci by amplicon sequencing following the procedure of Darby et al.[71]. We wrote custom scripts to

414 automate all steps in the protocol for high throughput processing of samples on the Biomek FX^P
415 liquid handling robot. First-round PCR was conducted in separate reactions for each locus for
416 each individual. We pooled 4.8 ul of PCR product from each locus (7 PCR products) for each
417 individual. Pooled DNA was purified using ZR-96 Zymo DNA Clean and Concentrator Kit and
418 eluted in 36 ul of elution buffer. We then used 2 ul of the pooled PCR product as template for
419 second-round PCR with barcoded primers. Unique combinations of 16 forward and 24 reverse
420 barcodes allowed pooling of up to 384 individuals in a single sequencing run. Following the
421 second-round PCR to uniquely barcode individuals, 5 ul of PCR product from each individual
422 was pooled together (up to 384 individuals). Multiplexed DNA was purified with the Zymo
423 DNA Clean and Concentrator Kit and eluted in 30 ul of elution buffer. Libraries were submitted
424 for 300 bp paired-end sequencing on an Illumina MiSeq sequencer at the UND Genomics Core.
425 *Bioinformatics and genotyping*
426 We demultiplexed reads, trimmed adapters, and de-replicated reads with USEARCH[72].
427 Read frequency histograms were used to manually genotype individuals according to traditional
428 microsatellite allele-calling principles[73]. This process is analogous to genotyping individuals
429 using capillary electrophoresis. Parental genotypes from amplicon sequencing were verified
430 against genotypes from traditional capillary electrophoresis on an Applied Biosystems 3130
431 Genetic Analyzer.
432 *Parentage analysis*
433 Although we controlled breeding among turtles, there were a few cases where parentage
434 was uncertain. During the nesting season, gravid females were placed in an outdoor aviary. The
435 aviary was checked every 2 hours to observe females in the act of nesting and to directly match
436 females with their eggs. Occasionally two or more females nested simultaneously in less than 2

hours and were not observed nesting. In these cases, we palpated females in the aviary to determine which females had nested (i.e., females without eggs) and narrow the list of potential dams. We then used DNA-based genotyping of putative parents and offspring to determine which nest belonged to which female. Finally, on very rare occasions male turtles briefly escaped from their own tank into the tank of another breeding group where they could have mated. Female snapping turtles can store sperm and multiple paternity can occur within a single clutch of eggs[62]. Paternity and maternity were therefore assigned for each offspring using exclusion probability in the CERVUS program[74]. Given the small number of potential parents and highly polymorphic microsatellite loci, we were able to identify parentage with 100% certainty.

Quantitative genetic analysis

We fitted mixed models using ASREML-R[75]. Incubation temperature, sire origin, sire identity, dam identity (nested within sire), and year were independent variables. Temperature, sire origin, and their interaction were treated as fixed effects. Year, sire identity, the sire-by-temperature interaction, dam identity (nested within sire), and the dam-by-temperature interaction (nested within sire) were random effects. Egg mass, egg diameter, hatchling mass, hatchling carapace length and hatchling plastron length were continuous dependent variables. Sex was analyzed as a binary dependent variable with the logit link and residual variance of $\pi^2/3$.

To test the significance of fixed effects, we conducted Type III ANOVA followed by post-hoc Tukey HSD tests. We tested for significance of random effects using likelihood ratio tests that compared reduced models (with the effect of interest removed) to the 'full' model containing all parameters. We used variance components from REML to estimate narrow-sense heritability and non-genetic maternal effects.

With a paternal half-sib, maternal full-sib design, among-sire variation is equal to ¼ of the additive genetic variance[52]. Thus, significant sire effects directly demonstrate additive genetic variation. Narrow-sense heritability is calculated as 4 times the among-sire variance (¼ V_A) divided by total phenotypic variance ($h^2 = V_A/V_T$). Variation among dams (nested within sires) is equal to ¼ of the additive genetic variance plus ¼ of the dominance variance plus all non-genetic maternal effects[52]. Variation due to genetic dominance and/or maternal effects is only significant if the variance among dams nested within sires (a.k.a. dam effect) is larger than the variance among sires. Maternal effects and dominance variance are confounded and cannot be directly estimated from a paternal half-sib, maternal full-sib breeding design. However, we can place an upper bound on maternal effects by assuming dominance variance (V_D) is zero, subtracting the variance among sires (¼ V_A) from variance among dams (¼ V_A + ¼ V_D + V_M), and dividing this value by total phenotypic variance ($m^2 = V_M/V_T$). Because egg mass and diameter measurements were taken immediately after oviposition and before eggs were exposed to any temperature treatment, narrow-sense heritability (h^2) and maternal effects (m^2) were estimated without considering incubation temperature. Heritability and maternal effects on hatchling size and sex were estimated at each incubation temperature.

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Tables and Figures

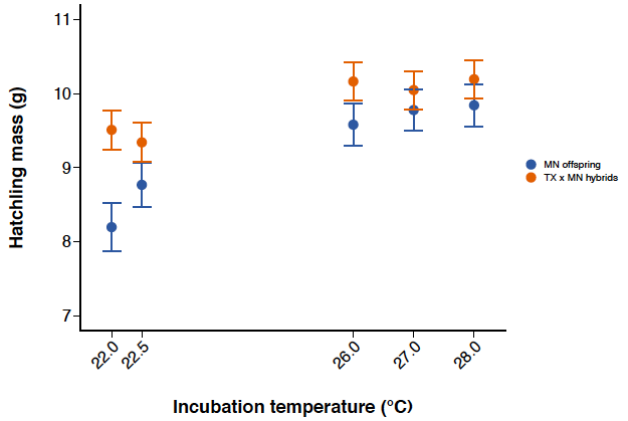
Table 1. Results from mixed model analyses of variation in egg size, hatchling size, incubation duration, and hatchling sex in snapping turtles using ASREML-R. Incubation temperature, sire origin, and their interaction were fixed effects in the model. Year, sire identity, dam identity (nested within sire), the sire-by-temperature interaction, and the dam-by-temperature interaction (nested within sire) were random effects in the model. Egg mass, egg diameter, hatchling mass, hatchling carapace length, hatchling plastron length, and incubation duration were continuous dependent variables. Hatchling sex was a binary dependent variable analyzed with the logit link and residual variance of $\pi^2/3$ (i.e., the threshold model).

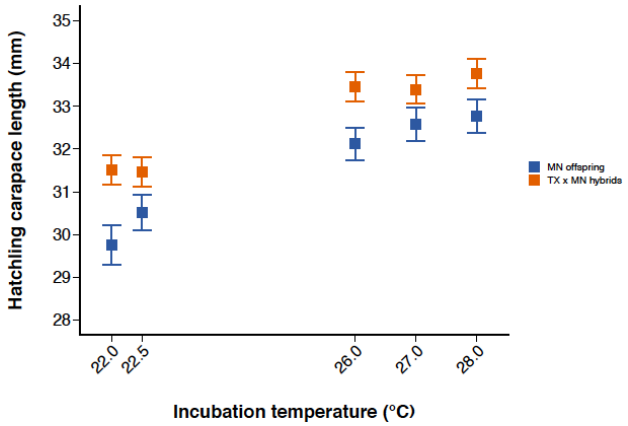
	Egg Mass	Egg Diameter	Hatchling Mass	Hatchling CL	Hatchling PL	Incubation Duration	Sex Determination
Year	$LR\chi^2 = 107$ $p<0.0001$	$LR\chi^2 = 193$ $p<0.0001$	$LR\chi^2 = 49$ $p<0.0001$	$LR\chi^2 = 23$ $p<0.0001$	$LR\chi^2 = 1.4$ $p=0.23$	$LR\chi^2 = 280$ $p<0.0001$	$LR\chi^2 = 8.2$ $p=0.004$
Temp	N/A	N/A	$F_{4,40} = 22$ $p<0.0001$	$F_{4,37} = 65$ $p<0.0001$	$F_{4,42} = 30$ $p<0.0001$	$F_{4,34} = 1586$ $p<0.0001$	$F_{4,46} = 68$ $p<0.0001$
Sire	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 0$ $p=1.0$	$LR\chi^2 = 3.9$ $p=0.05$
Sire x Temp	N/A	N/A	$LR\chi^2 = 10$ $p=0.001$	$LR\chi^2 = 3.5$ $p=0.06$	$LR\chi^2 = 24$ $p<0.0001$	$LR\chi^2 = 1.2$ $p=0.28$	$LR\chi^2 = 8.6$ $p=0.003$
Dam [Sire]	$LR\chi^2 = 1333$ $p<0.0001$	$LR\chi^2 = 944$ $p<0.0001$	$LR\chi^2 = 76$ $p<0.0001$	$LR\chi^2 = 69$ $p<0.0001$	$LR\chi^2 = 53$ $p<0.0001$	$LR\chi^2 = 44$ $p<0.0001$	$LR\chi^2 = 14.6$ $p=0.0001$
Dam x Temp [Sire]	N/A	N/A	$LR\chi^2 = 23$ $p<0.0001$	$LR\chi^2 = 23$ $p<0.0001$	$LR\chi^2 = 1.4$ $P=0.24$	$LR\chi^2 = 50$ $p<0.0001$	$LR\chi^2 = 3.57$ $p=0.06$
Sire Origin	$F_{1,38} = 1.56$ $p=0.22$	$F_{1,38} = 0.6$ $p=0.45$	$F_{1,10} = 5.7$ $p=0.04$	$F_{1,13} = 9.6$ $p=0.01$	$F_{1,52} = 4.9$ $p=0.03$	$F_{1,11} = 20.6$ $p=0.001$	$F_{1,14} = 4.45$ $p=0.05$
Sire Origin x Temp	N/A	N/A	$F_{4,44} = 2.8$ $p=0.04$	$F_{4,43} = 1.0$ $p=0.40$	$F_{4,46} = 0.6$ $p=0.66$	$F_{4,42} = 0.49$ $p=0.74$	$F_{4,45} = 2.51$ $p=0.06$

Table 2. Heritability and maternal effects estimates for hatchling size and sex at constant incubation temperatures of 22, 22.5, 26, 27, and 28^{o}C. Hatchling mass, carapace length, and plastron length were measured 30 days after turtles pipped their eggshell. Hatchling sex was recorded as a binary dependent variable and analyzed with the threshold model to estimate heritability and maternal effects on the underlying liability scale for sex determination.

	22°C		22.5°C		26°C		27°C		28°C	
	h^2	m^2	h^2	m^2	h^2	m^2	h^2	m^2	h^2	m^2
Hatchling Mass	0.74±0.76	0.30±0.35	0.44±0.44	0.12±0.19	0.26±0.38	0.25±0.19	0.25±0.48	0.43±0.23	0.00±0.39	0.53±0.20
Hatchling CL	0.84±0.65	0.08±0.27	0.39±0.45	0.18±0.20	0.31±0.39	0.26±0.18	0.11±0.33	0.38±0.18	0.13±0.36	0.44±0.18
Hatchling PL	0.84±0.45	-0.20±0.13	0.87±0.54	-0.07±0.20	0.29±0.31	0.07±0.13	0.01±0.22	0.25±0.12	0.02±0.22	0.23±0.12
Hatchling Sex	0.76±0.64	-0.15±0.25	0.87±0.82	-0.14±0.32	0.80±0.79	0.12±0.35	1.05±0.49	-0.10±0.17	0.90±0.54	-0.02±0.22

Figure 1. Measures of hatchling size as a function of incubation temperature and sire origin. Males from MN and TX were mated to females from MN to produce eggs that were incubated at 22, 22.5, 26, 27, or 28°C throughout embryogenesis. Hatchling A) mass, B) carapace length, and C) plastron length were measured 30 days after turtles pipped their eggshell. Least squares means and standard errors are from the mixed model analyses in Table 1.





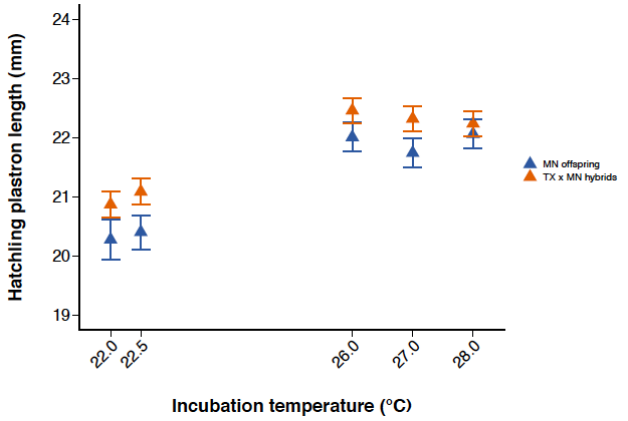
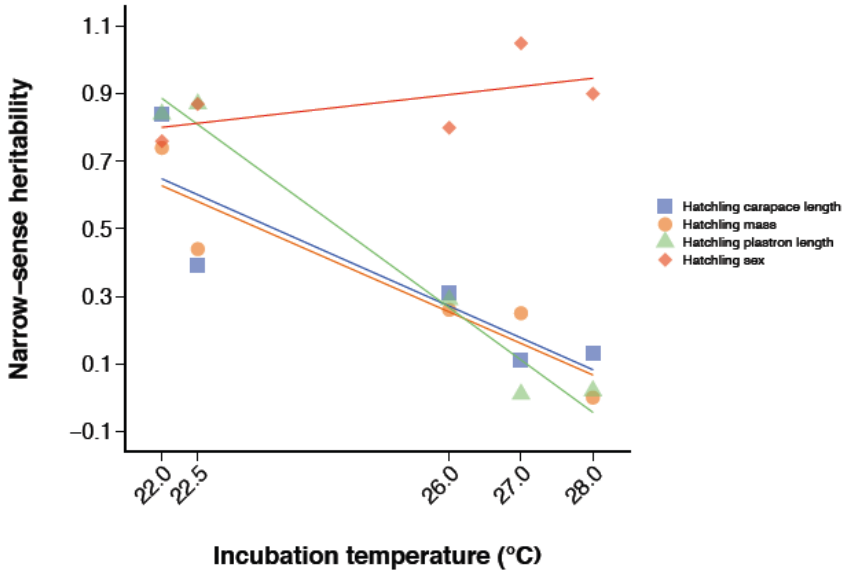


Figure 2. Linear regression of narrow-sense heritability and maternal effects for various traits in hatchling turtles as a function of incubation temperature. Eggs were incubated at constant temperatures of 22, 22.5, 26, 27, or 28°C throughout embryogenesis. Hatchlings were measured 30 days after pipping their eggshell. A) Heritability and B) maternal effects on hatchling mass, carapace length, plastron length, and sex determination were calculated using variance components from mixed model REML analyses carried out separately at each incubation temperature.



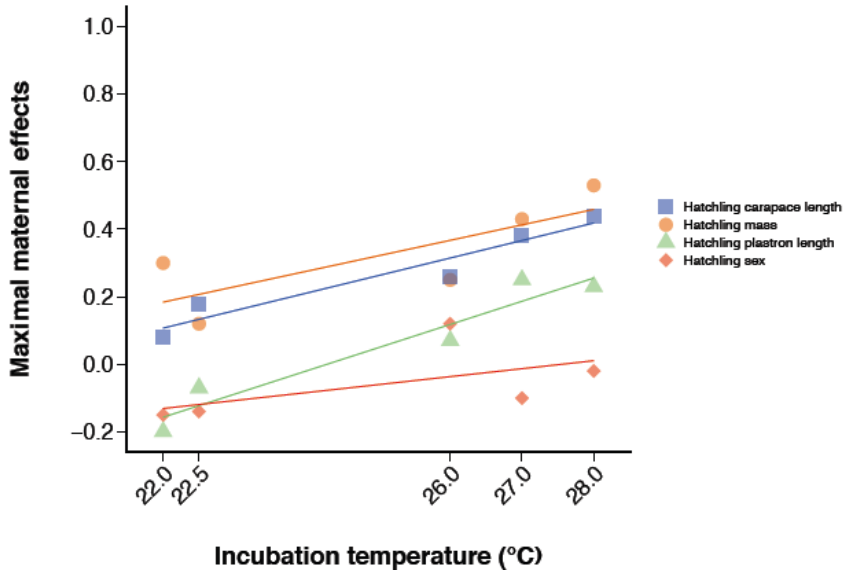


Figure 3. Incubation duration for snapping turtle embryos as a function of incubation temperature and sire origin. Males from MN and TX were mated to females from MN to produce eggs that were incubated at 22, 22.5, 26, 27, or 28°C throughout embryogenesis. Incubation duration was measured as the number of days from oviposition to pipping. Least squares means and standard errors are from the mixed model analyses summarized in Table 1.

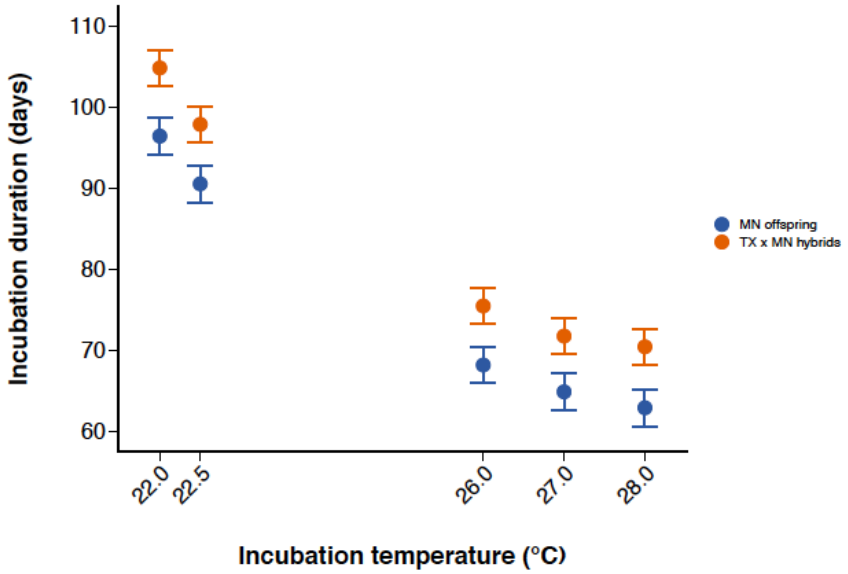


Figure 4. Sex ratio of hatchling snapping turtles as a function of incubation temperature and sire origin. Males from MN and TX were mated to females from MN to produce eggs that were incubated at 22, 22.5, 26, 27, or 28°C throughout embryogenesis. Hatchling sex was diagnosed by examining gonads and genital ducts under a dissecting microscope. Sex ratios for snapping turtles from Claiborne Parish, Louisiana are from a prior study[40]. The Louisiana data are from a population at the same latitude as the source population for TX males.

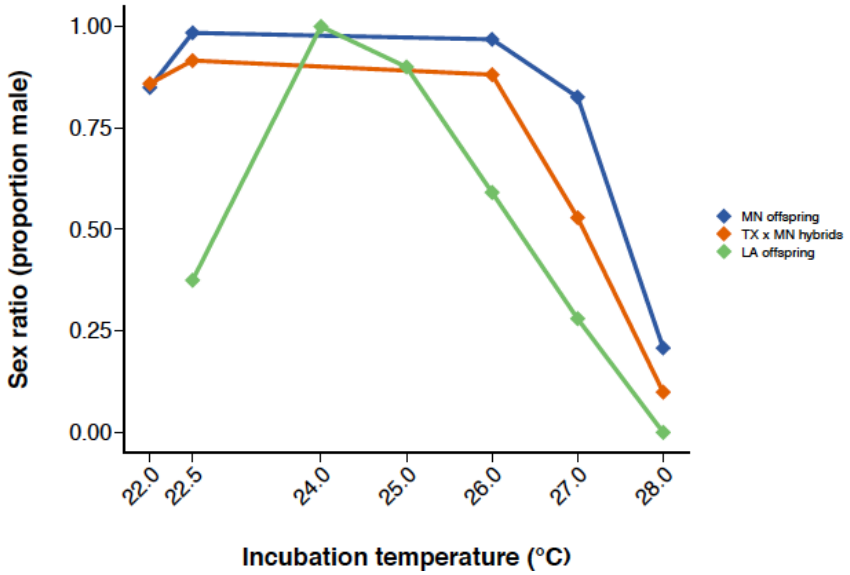


Figure 5. Sex ratio of hatchling snapping turtles as a function of incubation temperature and sire identity. Each line connects sex ratios for offspring sired by a single male (i.e., paternal half sibs). Eggs from each family were divided equally among temperatures and incubated at 22, 22.5, 26, 27, or 28°C throughout embryogenesis. Hatchling sex was diagnosed by examining gonads and genital ducts under a dissecting microscope.