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Kyle Hilliard

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REVIEW OF SEX DETERMINATION IN FISH AND REPTILES AND  
ESTIMATION OF SEX RATIO HERITABILITY IN THE COMMON  
SNAPPING TURTLE

by

Kyle Scott Hilliard  
Bachelor of Science, Pennsylvania State University, 2016

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

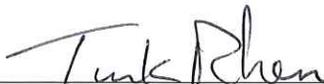
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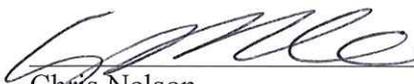
This thesis, submitted by Kyle Hilliard, in partial fulfillment of the requirements for the degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

  
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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

  
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## ABSTRACT

Sex-determining mechanisms in fish and reptiles are incredibly diverse. We first review sex-determination in these groups, including approaches that can be used to identify sex-determining genes and the large body of research showing genetic variation for sex ratio in species with temperature-dependent sex determination (TSD). We then show that sex ratio variation among clutches is due to additive genetic effects in one TSD species, the common snapping turtle. This finding contradicts previous claims that non-genetic maternal factors such as yolk steroid hormones are driving sex determination in this species. Heritable variation for sex determination in the snapping turtle may allow populations to evolve in response to global warming and avoid population decline due to highly skewed sex ratios.

# CHAPTER 1

## REVIEW OF SEX DETERMINATION IN FISH AND REPTILES

### **Abstract**

Sex-determining mechanisms are incredibly diverse and plastic across fish and reptiles, with the two primary mechanisms being genotypic (GSD) and temperature-dependent sex determination (TSD). Although the molecular mechanisms involved in sex determination are largely unknown in these groups, many candidate sex-determining genes have been identified. A variety of methods can and have been used to understand sex determination including controlled breeding studies, genome-wide association studies (GWAS), and reciprocal crosses between different genetic stocks. A large body of literature demonstrates variation in sex determination among species, populations and families in both fish and reptiles. Genetic variation for sex determination in TSD species would allow populations to evolve in response to warming climates.

### **Introduction**

Sex determination in amniotic vertebrates can be broken into two primary modes: genotypic (GSD) and environmental (ESD). In GSD species, an individual's sex is determined by its genotype at conception. In species with ESD, external factors determine whether an individual develops as a male or female after fertilization. Temperature is the only one of these external factors known to affect sex determination in vertebrates (Bull, 1983) and is referred to as temperature-dependent sex determination (TSD). In reptiles, TSD is thought to be the ancestral form of sex determination with GSD being more recently derived, while in fish TSD is very likely

the derived condition (Ospina-Alvarez and Piferrer, 2008; Lang and Andrews, 1994; Ewert et al., 2004, Pokorna and Kratochvil, 2016; Van Doorn, 2013).

Although sex-determining mechanisms in amniotes are quite diverse, the process of gonadal differentiation is conserved (Zarkower, 2001). During development, amniotes have bipotential gonads that can either develop into testes or ovaries during a defined critical period. Other characteristics of the developing embryo are also identical, whether it will become a male or a female. In amniotes, for instance, the bipotential embryo has both Wolffian and Müllerian ducts, which are precursors to the male and female reproductive tracts, respectively. After the bipotential gonad has differentiated into a testis or an ovary, it releases hormones that induce development of the sex-typical phenotype throughout the rest of the body (Morrish and Sinclair, 2002; Rhen et al., 2009). In placental mammals, the Y chromosome carries a master sex-determining gene called Sry (Koopman et al., 1991). If Sry is present, it induces expression of Sox9 and a Sox9/Fgf9 positive feedback loop. These factors in turn inhibit beta-catenin signaling and ovary development (Kashimada & Koopman, 2010; Sinclair et al., 1990). This promotes development of Sertoli cells and testes. Sertoli cells produce AMH (anti-Müllerian hormone), which causes the Müllerian ducts to regress. Fetal Leydig cells differentiate and secrete testosterone, which promotes development of the Wolffian ducts into the epididymis, vas deferens, and seminal vesicles (Zarkower, 2001). Androgens also induce development of male external genitalia and male-typical phenotype through the rest of the body.

If Sry is not present or fails to act at the appropriate time, a Wnt4/beta-catenin positive feedback loop induces transcription of ovary-determining genes such as FoxL2, Rspo1, and Wnt4 and interferes with Sox9 expression (Biaison-Lauber & Chaboissier, 2015). Thecal and granulosa cells then develop. The differentiating ovaries do not secrete AMH so the Müllerian ducts develop

into the fallopian tubes, uterus, the uterine cervix, and the upper portion of the vagina. Likewise, the ovaries do not produce testosterone so the Wolffian ducts regress. In the absence of testosterone, female external genitalia develop. Estrogens produced by the ovaries later in life cause development of female secondary sexual characteristics.

In contrast to mammals, the molecular mechanisms involved in sex determination are largely unknown among fish and reptiles with TSD. However, master regulatory genes analogous to the Sry gene in mammals as well as polygenic models have been proposed across fish species that exhibit GSD. Currently, five master sex-determining genes have been identified in fish: dmY, gsdf, amhy, amhr2, and sdY (Martinez et al., 2014; Matsuda et al., 2002; Myosho et al., 2012; Kamiya et al., 2012; Yano et al., 2012; Hattori et al. 2012). The dmY gene in Medaka (*Oryzias latipes*) is a transcription factor expressed in the testis and is involved in germ cell proliferation and development of Sertoli cells (Martinez et al., 2014, Matsuda et al., 2002). The other sex-determining genes, gsdf, amhy and amhr2, are all involved in cell signaling and control cell proliferation (Heule et al., 2014). The master sex-determining gene is gsdf in *Oryzias curvinotus* (Myoshoetal., 2012), while sdY is sufficient to induce testicular differentiation in salmonids (Yano et al., 2012). The observation that amhy in *Odonthestes hatcheri* (Hattori et al., 2012) and amhr2 in *Takifugu rubripes* (Kamiya et al., 2012) have evolved to become master sex-determining genes is particularly interesting because teleost fish have lost Müllerian ducts during evolution (Pfennig et al., 2015; Adolphi et al., 2019).

### **Approaches for studying sex determination**

A variety of methods can be used to understand sex-determining mechanisms and identify specific sex-determining genes. Controlled breeding studies can be used to determine the

underlying causes of variation in sex ratios, whether the main mode of sex determination is GSD or TSD. One approach is the paternal half-sib, maternal full-sib breeding design in which multiple females are mated to each male (Falconer and Mackay, 1996). In this breeding design, variation among offspring produced by different sires is equal to  $\frac{1}{4}$  of the additive genetic variation, allowing a direct estimate of narrow-sense heritability. Variation among offspring produced by different dams mated to the same sire includes  $\frac{1}{4}$  of the additive genetic variation,  $\frac{1}{4}$  of the dominance variation and all of the non-genetic maternal effects (Falconer and Mackay, 1996). However, dominance variance and maternal effects remain confounded with the paternal half-sib, maternal full-sib design. A more complicated breeding design is needed to partition variance due to dominance versus maternal effects. One example is a fully factorial design in which all possible crosses are made between dams and sires (Lynch and Walsh, 1998). This approach also allows estimation of epistatic variation (gene-gene interactions). A fully factorial design is only feasible for species that produce large numbers of gametes and have external fertilization, like some fish.

Another method to disentangle dominance and maternal effects variance involves reciprocal crosses between populations with distinct phenotypes (Lynch and Walsh, 1998; Roush and Rhen, 2018). If phenotypic differences between the populations are due to maternal effects, the phenotype of reciprocal hybrids will match the phenotype of the maternal population. However, if population differences are due to dominance effects, offspring phenotype will match the phenotype of one population regardless of the parental sex. If differences between the populations are due to additive genetic effects, the phenotype of both reciprocal hybrids will be intermediate between parental populations. Once genetic variation in sex determination is established, then it would be practical to search for specific loci associated with sexual fate via genome-wide association studies (GWAS) or quantitative trait loci (QTL) analyses. Such marker-

based studies could also be used to identify specific loci responsible for genotype-by-temperature interactions in TSD species like the leopard gecko (Rhen et al., 2011) and to discover genetically based differences in pivotal temperatures in other TSD species (Schroeder et al., 2016).

### **Genotypic sex determination**

Using a paternal half-sib, maternal full-sib breeding design, Lozano et al. (2011) were able to estimate additive genetic variation underlying sex determination across six generations in the Nile tilapia (*Oreochromis niloticus*). The additive component was low, but significant, with a narrow-sense heritability estimate of  $0.22 \pm 0.04$  on the underlying liability scale. Until recently, little was known about sex determination in the popular research model the zebrafish. To test whether zebrafish have a GSD mechanism, Liew et al. (2012) analyzed variation in sex ratios among many families. If the sex-determining mechanism were monogenic with a female-determining or a male-determining allele like Sry, sex ratios would be expected to be approximately 0.5. However, the percentage of males across 67 families ranged from 4.8% to 97.3% in that study, making a simple monogenic GSD mechanism unlikely. The authors were also unable to find any major sex-linked markers across the zebrafish genome. Because temperature has not been shown to influence sex ratios (Ospina-Alvarez and Piferrer, 2008), Liew et al. (2012) concluded that the zebrafish most likely has a polygenic sex determination system. More recent work demonstrates a ZZ/ZW sex chromosome system in wild zebrafish and that domesticated zebrafish strains lost the major sex-determining locus (Wilson et al., 2014).

Using a QTL marker-based approach, Martinez et al., (2009) identified the sex-determining region of the genome in turbot and concluded that this species exhibits a ZZ/ZW sex determination system. In addition to a major QTL, this study suggests that minor genetic effects or

environmental factors are involved in sex determination (Martinez et al., 2009). A polygenic sex determination system influenced by temperature is currently proposed in the European sea bass although no GWAS or QTL analyses have been conducted (Piferrer et al., 2005; Vandeputte et al., 2007). Interpreting sex as a threshold trait, Vandeputte et al. (2007) estimated the heritability of sex ratio in the sea bass to be  $0.62 \pm 0.12$ . Similar to classical QTL mapping, an expression quantitative trait loci (eQTL) analysis seeks to identify genetic variants that influence expression of individual genes. Munger et al. (2009) used an eQTL approach to elucidate the transcriptional architecture underlying sex determination in mice and identify putative sex-determining genes besides Sry and the other known players in mammalian sex determination.

### **Temperature-dependent sex determination**

Species that exhibit TSD are characterized by a sensitive developmental period (the thermosensitive period or TSP) when temperature can affect the fate of the bipotential gonad. Additionally, different species and populations have distinct pivotal temperatures, which are the incubation temperatures that produce a 50:50 sex ratio. In reptiles, three major TSD patterns have been observed, referred to here as MF, FM, and FMF. The MF pattern has a single pivotal temperature and produces males at low temperatures and females at high temperatures. The FM pattern produces females at low temperatures and males at high temperatures. The FMF pattern has two pivotal temperatures, producing females at both low and high temperatures and males across the intermediate range of temperatures. Because the FMF pattern is found in all groups of TSD reptiles, it is hypothesized to be the ancestral condition, with the MF (found only in turtles) and FM (absent in turtles) patterns being derived (Ewert, Jackson and Nelson; 1994). Although the TSP is relatively short in reptiles (comprising about 8 to 33% of embryogenesis), it can be much

more extended in fish, lasting for weeks or even years in some species (Saito and Tanaka, 2009; Berbejillo et al., 2012).

Although 59 fish species have been reported to display TSD, Ospina-Alvarez and Piferrer (2008) have recently shown that a much smaller number of species truly have TSD. For a species to be recognized as having TSD, there had to be no evidence of heteromorphic sex chromosomes (Valenzuela et al., 2003). Additionally, in laboratory settings, temperatures are often used that species do not experience in nature. Sex-ratio shifts under these circumstances may be due to thermal effects on GSD mechanisms that are not ecologically relevant. Therefore, the authors included a second criterion to assign TSD as the sex-determining mechanism for a species: sex-ratio shifts in response to temperature treatments must occur within a range of temperatures experienced by the species in nature. With these criteria, the authors found that 33 cichlid species of the genus *Apistogramma* as well as seven other species have true TSD, while all other reported cases violate one of the two criteria. Furthermore, only one pattern of sex determination across incubation temperature exists in fish (males at high temperatures and females at low temperatures) instead of three as was previously thought.

#### *Genetic variation in TSD*

Much of the current knowledge of genetic variation in TSD in fish is from one species, the Atlantic silverside (*Menidia menidia*). Conover and Kynard (1981) provided the first evidence of TSD in this species. They observed that offspring produced early in the breeding season experienced low temperatures and were primarily females. Offspring produced late in the breeding season experienced higher temperatures and were mostly males.

In this species, different sex-determining mechanisms are even observed in different populations (Conover and Heins, 1987; Duffy, Hice and Conover, 2015). Conover and Heins (1987) collected recently fertilized eggs of the Atlantic silverside (*Menidia menidia*) from six locations, South Carolina, North Carolina, Virginia, New York, Prince Edward Island and Nova Scotia, and reared them at five constant incubation temperatures. They found that the thermal reaction norm for sex ratio varied greatly among populations from different latitudes. Incubation temperature had a large effect on the South Carolina, North Carolina and Virginia populations, an intermediate effect on the New York and Prince Edward Island populations and no effect on the Nova Scotia population (Conover and Heins, 1987). Although the Nova Scotia is at a lower latitude than Prince Edward Island, the absence of environmental sex determination is correlated with low temperatures and a short growing season in Nova Scotia. Duffy et al. (2015) conducted a similar analysis in this species but collected eggs from 31 locations across the species' entire range. Latitudes of the populations ranged from Amelia Island, Florida on the southern end to Magdalen Islands, Quebec on the northern end. By studying sex determination in this species on a larger scale, the authors found that the level of temperature-dependent sex determination varies in a non-linear pattern with latitude as opposed to the linear pattern initially observed by Conover and Heins (1987). In the more recent analysis, the authors observed a high level of TSD from Florida through New Jersey. Here, a breakpoint occurs where the level of TSD declines steeply until a latitude of 44°N, with populations displaying almost total GSD north of this latitude. Another shift then occurs as the level of TSD rises to intermediate levels in Gulf of St. Lawrence populations. The specific gene (or genes) responsible for the latitudinal cline in GSD versus TSD has not yet been discovered.

Additionally, population sex ratio has been shown to evolve in response to frequency-dependent selection in the laboratory (Conover and Van Voorhees, 1990). In this study, the authors established five laboratory populations of Atlantic silversides (*Menidia menidia*): a South Carolina stock reared at 17°C, a South Carolina stock reared at 28°C, a New York stock reared at 17°C, a New York stock reared at 28°C and a Nova Scotia stock reared at 28°C. The level of TSD in this species varies greatly across these latitudes (Conover and Heins, 1987). Increases in the minority sex occurred in the four populations that began with a skewed sex ratio (all except the Nova Scotia population), and sex ratios stabilized at 0.5 after one to five generations. Sex determination in Nova Scotia populations is controlled almost exclusively by genetic effects (Conover and Heins, 1987), so sex ratios in the Nova Scotia group oscillated around 0.5 for the duration of the experiment. Given heritable variation, these results clearly show that sex determination mechanisms can evolve rapidly to ensure production of even population sex ratios (Conover and Van Voorhees, 1990).

Although GSD and TSD are often treated as mutually exclusive alternatives, some argue that they may be endpoints on a sex determination continuum (Sarre, Georges and Quinn, 2004). Supporting this idea, recent work has shown that TSD and GSD mechanisms can also coexist in a single species of reptile (Holleley et al., 2015). The bearded dragon (*Pogona vitticeps*) has a sex determination system in which females are the heterogametic sex (ZZ/ZW) and males are homogametic (ZZ/ZZ). In males, however, extremely warm incubation temperatures can override genotypic sex determination mechanisms to produce sex-reversed females that have two copies of the Z chromosome. When these sex-reversed females were mated to normal males from a low incubation temperature, they produced offspring whose sex was determined primarily by

temperature with no major genetic influence, demonstrating a transition from a GSD to a TSD mechanism in a single generation (Holleley et al., 2015).

Many studies have shown among-family variation for temperature-dependent sex determination in reptiles (reviewed in Roush and Rhen, 2018), including species such as the snapping turtle (*Chelydra serpentina*) and leopard gecko (*Eublepharis macularius*) (Janzen, 1992; Rhen and Lang, 1998; Rhen et al., 2011). Janzen (1992) did not detect family-by-temperature interactions in the snapping turtle but did detect significant broad sense heritability for sex determination. In this same species, however, Rhen and Lang (1998) did find significant family-by-temperature interactions. Both studies provide evidence that sex ratios in the snapping turtle may have the capacity to evolve in response to climate change. One drawback of these studies is that the analyses were conducted using clutches of eggs collected from the wild. Individual nests contained eggs from single females, but paternity was unknown. Both studies assumed that all offspring within a clutch were full siblings (i.e., sired by the same male). In addition to additive genetic effects, variation among full-sib families also includes dominance, epistatic and non-genetic maternal effects, possibly inflating the estimates of genetic variation for sex ratio.

To avoid this limitation, Rhen et al. (2011) used the ‘animal model’ to analyze the inheritance of sex in a multi-generational pedigree of leopard geckos. The authors directly show additive genetic variation for sex ratio in this species as well as significant genotype-by-environment interactions due to a change in the amount of additive genetic variance across incubation temperatures. In this study, there was additive genetic variation for sex determination at 30°C, but not at 32.5°C. Interestingly, dominance variance for sex determination was significant at 32.5°C in the leopard gecko. Differences in nesting phenology and nest-site choice among females have also been documented in at least one TSD reptile, the painted turtle (Schwanz and

Janzen, 2008; Mitchell, Maciel and Janzen, 2013). These two mechanisms, genetic variation for sex ratio in embryos and nest site selection by the mother, may allow TSD reptiles to respond to warming climates and evolve in a manner similar to a TSD fish (Conover and Van Voorhees, 1990).

Although evidence for genetic variation in sex ratio has been documented in several TSD species, the evolvability of sex ratios in nature is still not certain. One critique of sex ratio heritability estimates is that most are obtained using constant incubation temperatures in the laboratory. Bull, Vogt and Bulmer (1982) and Janzen (1992) point out that variable temperatures in natural nests could limit the capacity for sex ratio evolution because nests may be located in environments with such extreme temperatures that genetic variance for TSD is not expressed. Nests that produce just one sex are indeed observed in nature, but so are nests with both sexes. These authors therefore calculated a value they called “effective heritability” that includes an environmental variance term intended to model variation in nest temperatures in the wild. This additional variance component contributes to total phenotypic variance and is included in the calculation of “effective heritability”. While broad-sense heritability for sex determination at a constant incubation temperature was 0.82, “effective heritability” was estimated at just 0.06 in the map turtle (Bull, Vogt and Bulmer, 1982). Estimates of “effective heritability” were similarly low in the snapping turtle (Janzen, 1992).

However, heritability estimates at constant temperatures appear to slightly underestimate heritability under fluctuating thermal regimes resulting in underestimation of “effective heritability” (McGaugh and Janzen, 2011). More importantly, McGaugh and Janzen (2011) used field estimates of phenotypic variance and nest temperature variance to calculate “effective heritability.” The McGaugh and Janzen (2011) estimate of phenotypic variance of pivotal

temperature in nature was much greater than estimated by Bull, Vogt and Bulmer (1982). This resulted in higher “effective heritability” ranging from 0.193 to 0.315 in various species. Thus, underestimates of variance in pivotal temperatures among clutches may have caused “effective heritability” to be underestimated originally. Studies in more species will be needed to corroborate these results and provide a general picture of phenotypic and genetic variation in TSD in nature.

### *Adaptive significance of TSD*

If TSD reptiles exhibit genetic variation for sex determination, as recent research suggests, populations should be able to adapt to warming temperatures via evolutionary shifts in pivotal temperatures to produce near even sex ratios. Bull and Charnov (1977) presented a model to explain the adaptive benefits of environmental sex determination. Their model states that if some environmental conditions benefit one sex more than the other, selection should favor ESD over GSD. In TSD species, males and females may have different optimal developmental temperatures.

Despite its intuitive appeal, empirical support of the Bull-Charnov model has been difficult to demonstrate. One hurdle is that the effects of incubation temperature and offspring sex are confounded in TSD species. Both sexes must be produced across a broad range of incubation temperatures in order to determine the sex-specific effects of incubation temperature on offspring fitness. In a clever experiment, Rhen and Lang (1995) decoupled the effects of temperature and sex by using hormonal manipulations of eggs to produce females at male-producing temperatures and vice versa. After controlling for potential sex effects, they found that incubation temperature had a strong effect on hatchling growth, with normal male-producing temperatures causing faster hatchling growth. These findings indicate that incubation temperature contributes significantly to observed sex differences in growth rate and adult body size of snapping turtles in nature. A

combination of thermal programming of post-hatching growth rate and selection for sexual size dimorphism may explain the adaptive significance of TSD in this species. These results are consistent with the Bull-Charnov model.

A more recent test of the Bull-Charnov hypothesis comes from Warner and Shine (2008). In that study, the authors also used hormone manipulations to separate the normally confounded effects of temperature and sex in the Jacky dragon (*Amphibolurus muricatus*). This species matures within one year of hatching and has a short lifespan of three or four years, which allowed lifetime reproductive success to be measured in males and females from a range of incubation temperatures. The authors found that males produced at normal male-producing temperatures sired more offspring than sex-reversed males incubated at female-producing temperatures. Similarly, reproductive success was greatest for females that were incubated at female-producing temperatures. The finding that the fitness of each sex was maximized at the incubation temperatures that normally produce that sex in nature provides strong support for the Bull-Charnov model.

## **Conclusion**

In summary, a range of factors influence sex determination in fish and reptiles. In some species, a single locus determines sex. Sex determination in other species appears to be polygenic. Finally, genotype and temperature interact to determine sex in many species. Classical and quantitative genetic approaches have allowed researchers to uncover genetic variation in sex determination in both GSD and TSD species. In conjunction with molecular genetic approaches, researchers have also been able to identify specific sex-determining genes in some species. Future studies can apply the same approaches in non-model organisms. Such work has implications for

aquaculture of fish. In many fish species, the sexes grow and mature at different rates, making sex determination an important economic trait. In addition, rising global temperatures pose a major threat to TSD species. TSD is prevalent in reptiles, including species that are already endangered for other reasons. Identifying additional TSD species and quantifying the level of genetic variation for TSD is increasingly important to understand the potential for evolutionary responses. Without sufficient levels of heritable variation for sex ratio, TSD species may suffer severe population declines or even extinction.

## CHAPTER 2

### ESTIMATION OF SEX RATIO HERITABILITY IN THE COMMON SNAPPING TURTLE

#### **Abstract**

Temperature-dependent sex determination (TSD) is observed in many lizards, turtles and crocodilians. Among-family sex ratio differences are well-documented in reptiles with TSD, however, the underlying causes of phenotypic variance in TSD are unclear. To determine the relative contribution of additive genetic effects versus genetic dominance and/or non-genetic maternal effects to sex ratio variability, we used a paternal half-sib, maternal full-sib breeding design in a captive population of common snapping turtles. We directly show additive genetic effects and genotype-by-temperature interactions for TSD. Variances among sires and among multiple dams mated to a single sire are approximately equal, indicating that dominance variation and non-genetic maternal effects have negligible roles in causing inter-family sex-ratio differences. In comparison, the same analysis conducted for various egg and hatchling size traits demonstrate substantial variance due to non-genetic maternal effects. With the large estimates of narrow-sense heritability and the presence of genotype-by-temperature interactions underlying sex determination, TSD pattern in common snapping turtle populations should be able to evolve, allowing populations to persist in spite of warming global temperatures.

#### **Introduction**

During the early stages of sexual development, amniotes have a bipotential gonad that has the capacity to develop into a testis or an ovary until a specific developmental stage. Sex determination refers to the mechanism that determines the developmental fate of the bipotential gonads as testes or ovaries. Sex-determining mechanisms in animals are diverse, including

genotypic sex determination with heteromorphic and homomorphic sex chromosomes, polygenic sex determination, haplo-diploidy and environmental sex determination (Bachtrog et al., 2014). The two primary modes of sex determination seen in amniotic vertebrates are genotypic (GSD) and environmental (ESD). GSD is genetic in nature (often involving distinct sex chromosomes), with sex being determined at conception by inheritance of sex-determining genes. ESD is dependent on external factors to determine whether an individual develops as a male or female. Although other extrinsic factors such as photoperiod and social environment influence sex in some animals, temperature is the only environmental cue known to influence sex determination in amniotic vertebrates (reviewed in Bull, 1983; Rhen and Schroeder, 2010). This is referred to as temperature-dependent sex determination (TSD). These two main modes of sex determination evolved independently among amniotes (Bull, 1980; Matsubara et al., 2006). For example, birds and mammals have a GSD mechanism while many turtles and all crocodylians display TSD. In reptiles, TSD is suggested to be the ancestral form of sex determination with GSD being more recently derived (Lang and Andrews, 1994; Ewert et al., 2004, Pokorna and Kratochvil, 2016; Van Doorn, 2014). Due to the extraordinary evolutionary plasticity of sex-determining mechanisms, GSD and TSD have even been found to coexist in a single species, with transitions between the two mechanisms occurring (Holleley et al., 2015).

Once the fate of the bipotential gonad has been determined, the gonads differentiate into morphologically distinct testes or ovaries. This process of gonad differentiation is highly conserved across amniotes (Morrish and Sinclair, 2002; Schartl, 2004; Zarkower, 2001). Expression of ovary-determining genes causes the outer cortex of the bipotential gonad to develop into an ovary, whereas expression of testis-determining genes induces development of the inner medulla into a testis (Roush and Rhen, 2018). Following gonad differentiation, testes and ovaries

produce sex-typical hormones that induce development of sex-specific phenotype throughout the rest of the body (Morrish and Sinclair, 2002, Rhen et al., 2009).

Ronald Fisher (1930) originally proposed a model that explains why 1:1 sex ratios are so common in nature. In GSD species, an equal sex ratio is often due to Mendelian inheritance of a master sex-determining gene. In ESD species, however, sex ratio is maintained by frequency-dependent selection in which the rarer sex obtains a fitness advantage over the more common sex. Selection should then favor alleles that overproduce the rarer sex, leading to an approximately even sex ratio. This could be one avenue by which sex ratios can evolve in the face of changing climates. Bull and Charnov (1977) postulated that ESD could be adaptive in situations where the environment has a differential effect on male and female fitness. For example, if warmer temperatures are more beneficial for female fitness than male fitness, then eggs incubated at the warmer temperatures should develop as females. Conversely, eggs incubated at cooler temperatures should develop as males. Recent work in species with ESD has provided empirical support to the Bull-Charnov model (Conover and Heins, 1987; Rhen and Lang, 1995; Warner and Shine, 2008).

The common snapping turtle has several advantages as a model species for TSD. It is an abundant species with a large range, inhabiting much of North America east of the Rocky Mountains. The species is easy to breed and raise in captivity and females lay large clutches of 20-95 eggs. Furthermore, many aspects of its sexual development are well studied. The snapping turtle has a short TSP of about 5 days out of a 65-day long incubation period in a Minnesota population (Rhen et al., 2015). In comparison, the TSP comprises 20-35% of embryonic development in most other TSD species (Rhen et al., 2015). The pivotal temperature that produces a 1:1 sex ratio varies among populations and has been shown to change along a latitudinal cline

(Ewert et al., 2005). Turtles with TSD display two primary patterns of sex determination across incubation temperatures. In the first, males develop at cool temperatures and females at warm ones with a single pivotal temperature. There are two pivotal temperatures in the second pattern. Females develop at cool temperatures, males at intermediate temperatures, and females again develop at warm temperatures (Ewert et al., 1994). Snapping turtles fall into this second pattern. In our captive population of turtles from Minnesota, some females develop at low incubation temperatures, males are produced at intermediate temperatures, while high temperatures produce exclusively females (Rhen and Lang, 1998; Ewert et al., 2005).

A large body of research shows intra-specific variation in temperature sensitivity in the snapping turtle. Ewert et al. (2005) showed that pivotal temperatures vary significantly among populations across a latitudinal cline, suggesting that this aspect of sex determination is adaptive. Differences in pivotal temperatures have also been found at a much smaller scale. Schroeder et al. (2016) found a pivotal temperature difference of 0.5°C between snapping turtle populations from northern and southern Minnesota. Previous work has also shown that sex ratio in this species varies among clutches within populations (Janzen, 1992; Rhen and Lang, 1998).

Although variation in temperature-sensitivity in snapping turtles is well established, the cause of this variation is not. The two main hypotheses regarding variation in sex ratio are that it is due to genetically-based differences in thermosensitivity among embryos, or that the variation is due to non-genetic maternal effects, primarily yolk steroids deposited into eggs by the mother (reviewed in Roush and Rhen, 2018). Variation in embryo thermosensitivity or maternal steroids would need to be heritable in order for TSD to evolve. Because prior studies were conducted with eggs collected from the wild, parentage could not easily be determined, and only broad-sense heritability was estimated. In these cases, variance due to additive genetic effects is confounded

with variance due to dominance effects and non-genetic maternal effects. One approach to disentangle these effects is to use a full-sib, half-sib breeding design in which a single male is mated to multiple females (Falconer and Mackay, 1996; Lynch and Walsh, 1998). With this design, phenotypic variation among offspring produced by different sires is equal to  $\frac{1}{4}$  of the additive genetic variance. Multiplying this value by 4 and dividing by total phenotypic variance provides a direct estimate of narrow-sense heritability. Variation among offspring produced by different dams mated to the same sire is equal to  $\frac{1}{4}$  of the additive genetic variance plus  $\frac{1}{4}$  of the dominance variance plus all non-genetic maternal effects. When the sire and dam variance components are equal, this indicates that dominance and maternal effects are negligible, and that all phenotypic variation is due to additive genetic effects. If variance among dams is greater than sire variance, then dominance and/or maternal effects are significant. Although this breeding design cannot be used to tease apart dominance and maternal effects, variance among dams can be used to place upper and lower bounds on maternal effects and dominance variance. The maximum potential value for maternal effects can be estimated by assuming dominance variance is zero.

Rhen et al. (2011) were able to conclusively show additive genetic variance, dominance variance, and genotype-environment interactions for sex ratio in a captive population of leopard geckos by using the animal model (Kruuk, 2004). Here, we use a full-sib, half-sib breeding design to partition additive genetic variance from dominance variance and/or non-genetic maternal effects in a captive breeding colony of snapping turtles. Specifically, we were interested in estimating narrow-sense heritability and determining the contribution of non-genetic maternal effects to sex determination. We also tested for genotype-by-environment interactions in sex determination. We did these same analyses for egg mass, egg diameter, hatchling mass, hatchling carapace length and hatchling plastron length. We hypothesized that sex determination would be highly heritable,

whereas the egg size and hatchling size should primarily be influenced by non-genetic maternal effects (i.e., allocation of maternal resources to the eggs).

## **Methods**

### *Captive breeding colony*

Adult turtles were captured from Minnesota and Texas and used in a full-sib, half-sib breeding design. Each male was housed with 3-4 females. This experimental design is used to partition phenotypic variance into additive variance (among sires) and dominance variance/non-genetic maternal effects (among dams nested within sires) (Falconer and Mackay, 1996). With this breeding design, among-sire variation is equal to  $\frac{1}{4}$  of the additive genetic variance (Falconer and Mackay, 1996). Variation among offspring produced by different dams mated to the same sire is equal to  $\frac{1}{4}$  of the additive genetic variance plus  $\frac{1}{4}$  of the dominance variance plus all non-genetic maternal effects (Falconer and Mackay, 1996). Adult turtles from Minnesota were the only animals in the colony in 2013 and 2014. Eggs produced by the breeding colony were used for other experiments in 2015 and are not included in this study. Adult turtles from Texas were captured and introduced to the colony in the late summer of 2015 and turtles were reassigned to new tanks after eggs were laid. Thus, eggs in 2016 could have been sired by a prior mate (Minnesota males) or a new mate (Texas males). We did not use offspring from 2016 because of the potential for multiple paternity within clutches.

### *Egg collection and incubation*

Eggs were collected from females housed in the Biology Department at the University of North Dakota. Approximately equal numbers of eggs from each clutch were incubated in thermal

regimes that produce mixed sex ratios. Eggs were labeled with a unique clutch and egg number, randomly assigned to the various temperatures, placed in plastic boxes, and covered in moist vermiculite (1-part water: 1-part vermiculite by mass). In 2013 and 2014, eggs were incubated at 26.5°C until embryos reached Yntema stage 17 (Yntema, 1968). Eggs were then shifted to 31°C for 2.5 days and then shifted back to 26.5°C for the remainder of incubation. In 2017 and 2018, eggs were incubated at constant temperatures of 22°C (in 2018 only), 22.5°C (in 2017 only), 26°C, 27°C, and 28°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 was measured shortly after oviposition. Hatchlings were weighed and carapace length and plastron length were measured 30 days after hatching. Hatchlings were euthanized according to approved animal care protocol IACUC #1809-6C and sex was determined by inspection of the gonads and genital ducts (i.e., presence or absence of oviducts) under a dissecting microscope. Adrenal-kidney-gonad complexes were dissected, placed in RNAlater, and stored at -20 C for long-term storage.

#### *DNA extraction and quantification*

Whole blood was drawn from 67 adult individuals that comprise the breeding colony and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit. Offspring numbers for each study year were 2013 (n = 1062), 2014 (n = 1035), 2017 (n = 1358), 2018 (n = 1422). Four mg of tissue was cut from adrenal-kidney-gonad complexes of individual hatchlings across the four study years. Scissors and forceps were rinsed in sterile 1X PBS in between samples. The

Beckman-Coulter DNAdvance protocol was modified slightly for use with the Beckman Coulter Biomek FXp Liquid Handler. In brief, 4 ul of 10 mg/ml of RNase A was added to each sample after tissue lysis and incubated for 30 minutes at 37° C prior to the running the standard protocol. The concentration and purity of DNA was measured using a Beckman Coulter DTX 880 Fluorescence Plate Reader and the Quant-iT Picogreen dsDNA assay kit. All samples were then normalized to equal concentrations of 5 ng/ul and stored at -80 C until used for genotyping.

### *Sequencing*

Specimens were sequenced at 7 highly polymorphic microsatellite loci by amplicon sequencing following the procedure of Darby et al. (2016). First-round PCR was conducted in separate reactions for each locus. For a given individual, 4.8 microliters of PCR product from each locus was pooled together and purified using a Zymo DNA Clean and Concentrator Kit in a final elution volume of 36 microliters. Two microliters of this pooled PCR product was used as template for a second-round PCR using barcoding primers. Unique combinations of 16 forward and 24 reverse barcodes allowed a maximum of 384 individuals to be pooled into a single sequencing run. These primers allowed DNA sequences from each individual to be identified. Following the second-round PCR, five microliters from each sample was pooled together and purified again using the Zymo DNA Clean and Concentrator Kit in a final elution volume of 30 microliters, which was submitted for 300 bp paired-end sequencing on an Illumina MiSeq sequencer.

### *Bioinformatics and genotyping*

Demultiplexed paired-end sequencing reads were trimmed of adapter sequences and de-replicated with USEARCH (Edgar, 2010). Read frequency histograms were used to manually genotype individuals according to traditional microsatellite allele-calling principles (Guichoux et al., 2011). This process is analogous to using electropherograms to genotype individuals when using capillary electrophoresis. Additionally, parental genotypes from amplicon sequencing were verified against genotypes from capillary electrophoresis on an Applied Biosystems 3130 Genetic Analyzer.

### *Parentage analysis*

Although we controlled breeding among turtles, there were some instances where parentage was uncertain. During the nesting season, gravid females were placed in an outdoor aviary to allow nesting in soil. The aviary was checked every 2 hours to directly observe females in the act of nesting to match each clutch of eggs with its dam. Occasionally two or more females nested simultaneously in less than 2 hours and were not observed nesting. In these cases, DNA-based genotyping was the only way to tell which nest belonged to which female. Finally, on very rare occasions male turtles escaped from their own tank into the tank of another breeding group where they could have mated. It has been shown that females can store sperm from previous matings and multiple paternity can happen within single clutches of eggs (Galbraith et al., 1993). Paternity and maternity were therefore assigned for each offspring using exclusion probability in the CERVUS program (Kalinowski et al., 2007).

### *Quantitative genetic analysis*

In this study, we were interested in quantifying the relative contribution of additive genetic effects and non-genetic maternal effects to six phenotypic traits: egg mass, egg diameter, hatchling mass, hatchling carapace length, hatchling plastron length and hatchling sex. We fitted egg and hatchling trait models using ASREML-R (Butler et al., 2017) and sex models using JMP version 13. Egg mass, egg diameter, hatchling mass, hatchling carapace length and hatchling plastron length were treated as continuous variables. Sex was first analyzed as a binary dependent variable with a logistic model and then as a continuous trait with female = 0 and male = 1.

Year, incubation temperature, sire and dam (nested within sire) were treated as categorical factors. Incubation temperature was treated as a fixed effect while 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. To test for the significance of fixed effects (i.e. incubation temperature), we conducted a Type III ANOVA followed by post-hoc Tukey HSD tests. We tested random effects by conducting likelihood ratio tests that compare a reduced model (with the effect of interest removed) to the ‘full’ model containing all relevant parameters.

To determine the cause of significant sire-by-temperature and dam-by-temperature (nested within sire) interactions in the egg and hatchling trait models, we tested for variation among sires and among dams (nested within sire) at individual temperatures. Likelihood ratio tests were used to determine the significance of sire effects and dam (nested within sire) effects at individual temperatures. We used variance components to estimate narrow-sense heritability (based on variance among sires) and the maximum maternal effects contribution (based on variance among dams minus variance among sires) for the egg and hatchling traits. Without a fully factorial breeding design, maternal effects and dominance variance are confounded and cannot be directly

estimated. However, the maximal potential value for maternal effects can be estimated by assuming dominance variance is zero. We used this strategy to place upper bounds on maternal effects (based on variance among dams minus variance among sires) for each trait. Because egg mass and diameter measurements were taken immediately after oviposition before eggs were exposed to any temperature treatment, narrow-sense heritability ( $h^2$ ) and maximum maternal effects ( $m_{2Max}$ ) were estimated without considering incubation temperature. For measures of hatchling size,  $h^2$  and  $m_{2Max}$  were estimated within single incubation temperatures.

Binary traits such as sex can be analyzed in several ways. Three common approaches include the logistic model, the threshold model and a model where sex is treated as a continuous variable with females assigned a phenotypic value of 0 and males a value of 1 (Bull, Vogt and Bulmer, 1982; Falconer and Mackay, 1996; Rhen et al., 2011). The logistic model uses the logit link function to transform the response variable (sex) into the log of ratios of probabilities of becoming female or male. Using the logit link, the implicit residual variance is  $\pi^2/3 \sim 3.899$ . Our analysis of sex using the logistic model is not yet complete, so although we have provided a description of the model here, the results are not presented below. The threshold model utilizes the concept of ‘liability’. In brief, sex determination is assumed to be influenced by an underlying continuous trait that has a normal distribution with genetic and environmental factors contributing to the trait (Bull, Vogt and Bulmer, 1982; Falconer and Mackay, 1996). When individuals have liability below a certain threshold, they develop as one sex. If their liability is above the threshold, they develop as the other sex. The continuous model treats sex as a continuous variable with females assigned a phenotypic value of 0 and males a value of 1 (Falconer and Mackay, 1996). The same assumptions of the threshold model apply for the continuous model with the addition that measurement error increases as the population sex ratio deviates from 0.5 (Falconer and

Mackay, 1996; Rhen et al., 2011). Here, we used Equation (18.4) in Falconer and Mackay (1996) to transform heritability from the 0–1 scale to the underlying liability scale. This transformation removes the variance due to measurement error and allows a direct comparison with heritability estimates from the threshold model (Falconer and Mackay, 1996; Rhen et al., 2011). In addition to calculating heritability using the threshold and continuous models, we also estimated heritability on the observed scale.

## **Results**

### *Analysis of egg mass and egg diameter*

REML estimates of variance among sires, variance among dams nested within sires, and variance between years for egg mass and egg diameter are shown in Tables 1 and 2, respectively. Dam (nested within sire) and year had strong effects on egg mass, but sire had no impact on egg mass (Table 3). Similarly, dam (nested within sire) and year had strong effects on egg diameter, but the sire effect was insignificant (Table 4). Consistent with these findings, estimates of narrow-sense heritability were zero for both traits (Table 5 and Table 6). Estimates of non-genetic maternal effects were large for both traits (Table 5 and Table 6). Comparison of variance among dams (nested within sire) to variance among sires for egg mass and egg diameter revealed that non-genetic maternal effects and/or dominance variance contribute significantly to phenotypic variation in both traits (Table 7 and Table 8).

**Table 1.** Variance component estimates for egg mass in snapping turtles.

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.0000000948	0.17190671	0.000000551
Dam (nested within sire)	0.846425	0.23849055	3.549092
Year	0.06086276	0.08695343	0.6999467
Residual	0.5231307	0.01469404	35.60154

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 2.** Variance component estimates for egg diameter in snapping turtles.

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.000000341	0.10758756	0.00000317
Dam (nested within sire)	0.5158621	0.14695181	3.510417
Year	0.1233114	0.17519689	0.703845
Residual	0.4814178	0.01352239	35.60153

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 3.** Likelihood ratio tests of random effects on egg mass in snapping turtles.

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	1719.06	1	<0.0001
Year	91.31	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an *LR<sub>x2</sub>* -value greater than the observed value; *Df*, degrees of freedom.

**Table 4.** Likelihood ratio tests of random effects on egg diameter in snapping turtles.

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	1211.20	1	<0.0001
Year	202.00	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an *LR<sub>x2</sub>* -value greater than the observed value; *Df*, degrees of freedom.

**Table 5.** Estimates of narrow-sense heritability (*h<sub>2</sub>*) and maximum maternal effects (*m<sub>2Max</sub>*) of egg mass  $\pm$  standard error.

<i>Parameter</i>	<i>Estimate</i>
<i>h<sub>2</sub></i>	0.00 $\pm$ 0.48
<i>m<sub>2Max</sub></i>	0.59 $\pm$ 0.24

**Table 6.** Estimates of narrow-sense heritability (*h<sub>2</sub>*) and maximum maternal effects (*m<sub>2Max</sub>*) of egg diameter  $\pm$  standard error.

<i>Parameter</i>	<i>Estimate</i>
<i>h<sub>2</sub></i>	0.00 $\pm$ 0.38
<i>m<sub>2Max</sub></i>	0.46 $\pm$ 0.20

**Table 7.** Comparison of variance among dams (nested within sire) to variance among sires to test for non-genetic maternal effects and/or dominance variance for egg mass.

<i>Effect</i>	<i>Df</i>	<i>Variance</i>	<i>F</i>	<i>p</i>
Dam (nested within sire)	21	0.846425	8926336.10	<0.0001
Sire	12	0.00000009482334		

Abbreviations: *p*, probability of obtaining an F-value greater than the observed value; *Df*, degrees of freedom; *F*, F-ratio of dam (nested within sire) variance divided by sire variance.

**Table 8.** Comparison of variance among dams (nested within sire) to variance among sires to test for non-genetic maternal effects and/or dominance variance for egg diameter.

<i>Effect</i>	<i>Df</i>	<i>Variance</i>	<i>F</i>	<i>p</i>
Dam (nested within sire)	21	0.5158621	1513883.77	<0.0001
Sire	12	0.0000003407541		

Abbreviations: p, probability of obtaining an F-value greater than the observed value; Df, degrees of freedom; F, F-ratio of dam (nested within sire) variance divided by sire variance.

*Analysis of hatchling mass*

REML estimates of variance among sires, variance due to the sire by temperature interaction, variance among dams nested within sires, variance due to the dam by temperature interaction (nested within sire), and variance between years for hatchling mass are shown in Table 9. Incubation temperature had a strong effect on hatchling mass in the full model that included all interactions (Table 10). A post-hoc Tukey HSD test showed that hatchling masses at 22°C and 22.5°C were not different from each other. However, hatchlings from 22°C and 22.5°C were significantly lighter than hatchlings from 26°C, 27°C and 28°C (Figure 1). Year had a strong effect on hatchling mass (Table 10). There was also significant variation among dams (nested within sire), a significant sire by temperature interaction, and a significant dam by temperature interaction (nested within sire) (Table 10). Sire had no influence on hatchling mass (Table 10).

Given the sire-by-temperature interaction and the strong dam-by-temperature interaction (nested within sire), we tested for variation among sires and variation among dams (nested within sire) at each incubation temperature. The sire effect was insignificant at all incubation temperatures but was almost significant at 22°C (Tables 11-15). This likely caused the significant sire-by-temperature interaction (Table 10). Narrow-sense heritability estimates are not significantly different than zero at any temperature (Table 16). There was significant variation

among dams (nested within sire) at each incubation temperature (Tables 11-15). Maximal maternal effects estimates are significantly greater than zero at all temperatures (Table 16).

**Table 9.** Variance component estimates for hatchling mass using the full model

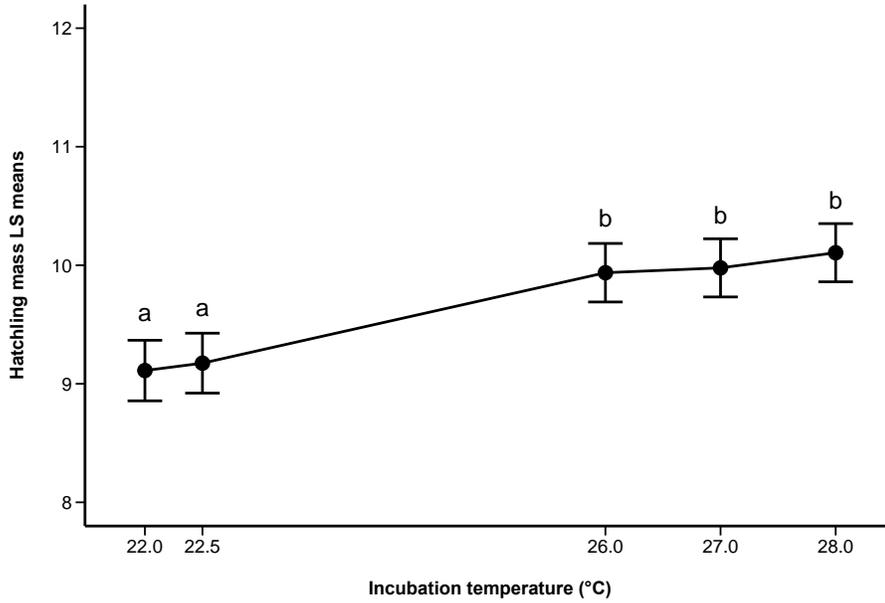
<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.06516561	0.10171873	0.6406452
Sire x temperature	0.07335905	0.02994627	2.4496890
Dam (nested within sire)	0.34903706	0.11107732	3.1422891
Dam x temperature (nested within sire)	0.07152781	0.02153801	3.3210038
Year	0.07114914	0.10229598	0.6955224
Residual	0.47510101	0.01690082	28.1111250

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 10.** Analysis of fixed and random effects on hatchling mass using the full model

<i>Effect</i>	<i>Df</i>	<i>LR<sub>x2</sub></i>	<i>p</i>
Incubation temperature	4	79.12	<0.0001
Sire	1	0	1.0
Sire x temperature	1	9.24	0.0024
Dam (nested within sire)	1	68.05	<0.0001
Dam x temperature (nested within sire)	1	25.68	<0.0001
Year	1	54.88	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value; *SS*, sums of squares; *MS*, mean squares; *F*, F-ratio.



**Figure 1.** The effect of incubation temperature on hatchling mass

**Table 11.** Variance component estimates for hatchling mass at 22.0C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.5563802	0.41149916	1.352081	0.0882
Dam (nested within sire)	0.5257005	0.22045897	2.384573	0.0085
Residual	0.3312297	0.03521914	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 12.** Variance component estimates for hatchling mass at 22.5C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.07562408	0.1345829	0.5619145	0.2871
Dam (nested within sire)	0.37605509	0.1723786	2.1815652	0.0146
Residual	1.10109649	0.1088462	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect

**Table 13.** Variance component estimates for hatchling mass at 26°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.1046573	0.1341424	0.7801954	0.2176
Dam (nested within sire)	0.4099313	0.1454321	2.8187116	0.0024
Year	0.2624866	0.3776188	0.6951100	0.2435
Residual	0.4831685	0.0362520	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 14.** Variance component estimates for hatchling mass at 27°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.05230412	0.09877333	0.5295369	0.2982
Dam (nested within sire)	0.40896096	0.12745866	3.2085771	0.0007
Year	0.05197004	0.07660904	0.6783799	0.2488
Residual	0.31957830	0.02242219	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 15.** Variance component estimates for hatchling mass at 28°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.00000036	0.07624595	0.0000048	0.5000
Dam (nested within sire)	0.4170965	0.12816341	3.254412	0.0006
Year	0.03979660	0.05944841	0.6694308	0.2516
Residual	0.3557414	0.02396346	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 16.** Estimates of narrow-sense heritability ( $h_2$ ) and maximum maternal effects ( $m_{2Max}$ ) of hatchling mass at individual incubation temperatures  $\pm$  standard error

<i>Incubation temperature</i>	$h_2$	$m_{2Max}$
22°C	1.00 $\pm$ 0.81	0.37 $\pm$ 0.17
22.5°C	0.19 $\pm$ 0.34	0.24 $\pm$ 0.10
26°C	0.33 $\pm$ 0.42	0.33 $\pm$ 0.14
27°C	0.25 $\pm$ 0.46	0.49 $\pm$ 0.13
28°C	0.00 $\pm$ 0.38	0.51 $\pm$ 0.12

### *Analysis of hatchling carapace length*

REML estimates of variance among sires, variance due to the sire by temperature interaction, variance among dams nested within sires, variance due to the dam by temperature interaction (nested within sire), and variance between years for hatchling carapace length are shown in Table 17. Incubation temperature had a strong effect on carapace length (Table 18). A post-hoc Tukey HSD test showed that carapace lengths at 22°C and 22.5°C were not different from each other. However, hatchlings from 22°C and 22.5°C were significantly smaller than hatchlings from 26°C, 27°C and 28°C (Figure 2). Year had a strong effect on carapace length (Table 18). There was also significant variation among dams (nested within sires) and a significant dam-by-temperature interaction (nested within sire) for carapace length (Table 18). The main effect of sire was insignificant, but the sire-by-temperature interaction was almost significant (Table 18).

Given the nearly significant sire by temperature interaction and the strong dam by temperature interaction (nested within sire), we tested for variation among sires and variation among dams (nested within sire) at each incubation temperature. The sire effect was insignificant at all incubation temperatures but was almost significant at 22°C (Tables 19-23). This likely caused the weak sire-by-temperature interaction. Narrow-sense heritability was not significantly different from zero at any temperature (Table 24). However, there was significant variation among dams (nested within sire) at each of the temperatures (Tables 19-23). Maximal maternal effects estimates are significantly greater than zero at all temperatures and generally increase from lower to warmer incubation temperatures (Table 24).

**Table 17.** Variance component estimates for hatchling carapace length using the full model

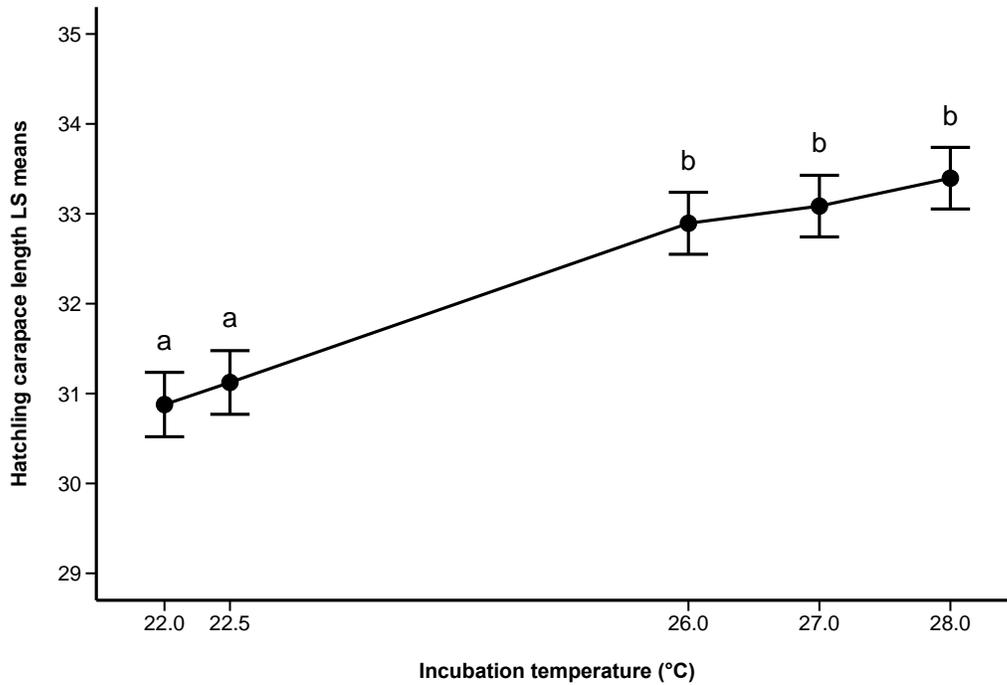
<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.32333355	0.31268101	1.0340684
Sire x temperature	0.08515581	0.05281927	1.6122110
Dam (nested within sire)	0.97515159	0.31779724	3.0684709
Dam x temperature (nested within sire)	0.16837526	0.05370011	3.1354731
Year	0.09415225	0.13776737	0.6834147
Residual	1.34150680	0.04773113	28.1054872

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 18.** Analysis of fixed and random effects on hatchling carapace length using the full model

<i>Effect</i>	<i>Df</i>	<i>LR<sub>x2</sub></i>	<i>p</i>
Incubation temperature	4	279.75	<0.0001
Sire	1	0	1.0
Sire x temperature	1	3.01	0.0828
Dam (nested within sire)	1	68.83	<0.0001
Dam x temperature (nested within sire)	1	20.71	<0.0001
Year	1	25.05	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value; *SS*, sums of squares; *MS*, mean squares; *F*, F-ratio.



**Figure 2.** The effect of incubation temperature on hatchling carapace length

**Table 19.** Variance component estimates for hatchling carapace length at 22°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	1.0882094	0.67023797	1.623616	0.0522
Dam (nested within sire)	0.4884951	0.24055509	2.030699	0.0211
Residual	0.8874005	0.09436042	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 20.** Variance component estimates for hatchling carapace length at 22.5°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.2474174	0.3519895	0.7029113	0.2410
Dam (nested within sire)	0.9489209	0.4114261	2.3064188	0.0105
Residual	2.1176877	0.2093748	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 21.** Variance component estimates for hatchling carapace length at 26°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.5997033	0.4764778	1.258618	0.1041
Dam (nested within sire)	1.0752386	0.3971625	2.707301	0.0034
Year	0.3527461	0.5196996	0.678750	0.2486
Residual	1.4679837	0.1103297	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 22.** Variance component estimates for hatchling carapace length at 27°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.2698293	0.32025105	0.8425555	0.1997
Dam (nested within sire)	1.1784653	0.39086615	3.0150098	0.0013
Year	0.1920618	0.28439202	0.6753417	0.2497
Residual	1.3532618	0.09535311	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 23.** Variance component estimates for hatchling carapace length at 28°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.2841502	0.2728784	1.041307	0.1489
Dam (nested within sire)	0.8562537	0.2769406	3.091831	0.0010
Year	0.0000001005589	0.000000006767874	14.858272	<0.0001
Residual	0.9937349	0.06688092	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 24.** Estimates of narrow-sense heritability ( $h^2$ ) and maximum maternal effects ( $m_{2Max}$ ) of hatchling carapace length at individual incubation temperatures  $\pm$  standard error

<i>Incubation temperature</i>	<i><math>h^2</math></i>	<i><math>m_{2Max}</math></i>
22°C	1.00 $\pm$ 0.68	0.20 $\pm$ 0.11
22.5°C	0.30 $\pm$ 0.41	0.29 $\pm$ 0.12
26°C	0.69 $\pm$ 0.49	0.31 $\pm$ 0.11
27°C	0.36 $\pm$ 0.41	0.39 $\pm$ 0.11
28°C	0.53 $\pm$ 0.47	0.40 $\pm$ 0.11

*Analysis of hatchling plastron length*

REML estimates of variance among sires, variance due to the sire by temperature interaction, variance among dams nested within sires, variance due to the dam by temperature interaction (nested within sire), and variance between years for hatchling plastron length are shown in Table 25. Incubation temperature had a strong effect on plastron length (Table 26). A post-hoc Tukey HSD test revealed that plastron lengths at 22°C and 22.5°C are not different from each other, but are smaller than plastron lengths at 26°C, 27°C and 28°C (Figure 3). Dam (nested within sire) and the sire-by-temperature interaction had significant effects on hatchling plastron length, while the dam-by-temperature interaction (nested within sire), sire, and year effects were insignificant (Table 26).

Variation among dams (nested within temperature) was highly significant at each of the incubation temperatures except for 22°C (Tables 27-31). Variation among sires was insignificant at all incubation temperatures except for 22°C (Tables 27-31), which likely caused the significant sire-by-temperature interaction (Table 26). Narrow-sense heritability for plastron length was greater than zero at 22°C, but was not different from zero at the other temperatures (Table 32). Maximal maternal effects on plastron length are significantly greater than zero at all temperatures except 22°C (Table 32).

**Table 25.** Variance component estimates for hatchling plastron length using the full model

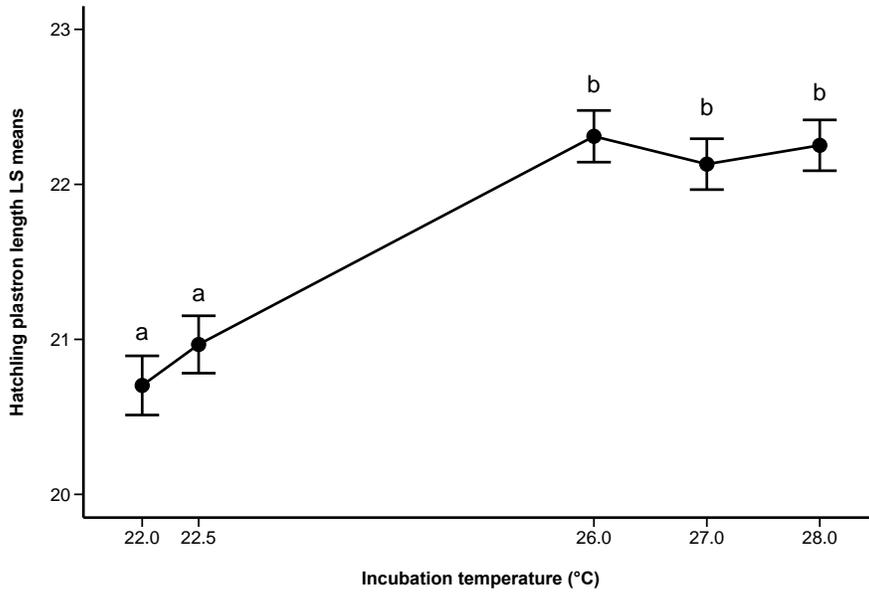
<i>Effect</i>	<i>Variance</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.0000000532	0.07621341	0.000000698
Sire x temperature	0.151565	0.04878399	3.106859
Dam (nested within sire)	0.2685465	0.08628291	3.112395
Dam x temperature (nested within sire)	0.02220995	0.02255900	0.9845274
Year	0.00536533	0.01164114	0.4608938
Residual	1.262362	0.04461308	28.29578

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 26.** Analysis of fixed and random effects on hatchling plastron length using the full model

<i>Effect</i>	<i>Df</i>	<i>LRx2</i>	<i>p</i>
Incubation temperature	4	119.17	<0.0001
Sire	1	0	1.0
Sire x temperature	1	22.54	<0.0001
Dam (nested within sire)	1	53.81	<0.0001
Dam x temperature (nested within sire)	1	1.38	0.2401
Year	1	0.81	0.3681

Abbreviations: *LRx2*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LRx2* -value greater than the observed value; *SS*, sums of squares; *MS*, mean squares; *F*, F-ratio.



**Figure 3.** The effect of incubation temperature on hatching plastron length

**Table 27.** Variance component estimates for hatching plastron length at 22°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.34158436	0.20198076	1.6911728	0.0454
Dam (nested within sire)	0.03907798	0.07617322	0.5130147	0.3040
Residual	1.19839627	0.12721566	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 28.** Variance component estimates for hatching plastron length at 22.5°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.1986210	0.2349196	0.8454851	0.1989
Dam (nested within sire)	0.4356813	0.2219063	1.9633573	0.0248
Residual	1.5334880	0.1513996	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 29.** Variance component estimates for hatchling plastron length at 26°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.1659546	0.1574826	1.0537965	0.1460
Dam (nested within sire)	0.2594676	0.1255266	2.0670336	0.0194
Year	0.1767703	0.2683838	0.6586474	0.2550
Residual	1.4178461	0.1058077	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 30.** Variance component estimates for hatchling plastron length at 27°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.02102603	0.09106648	0.2308866	0.4087
Dam (nested within sire)	0.3707662	0.1359389	2.7274468	0.0032
Year	0.0000001181529	0.000000008274722	14.2787763	<0.0001
Residual	1.167601	0.08177177	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 31.** Variance component estimates for hatchling plastron length at 28°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>	<i>p</i>
Sire	0.04450678	0.07803328	0.5703564	0.2842
Dam (nested within sire)	0.27214	0.1035825	2.6272775	0.0043
Year	0.0000001108955	0.000000007437468	14.858272	<0.0001
Residual	1.095882	0.07349793	NA	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 32.** Estimates of narrow-sense heritability ( $h^2$ ) and maximum maternal effects ( $m_{2Max}$ ) of hatchling plastron length at individual incubation temperatures  $\pm$  standard error

<i>Incubation temperature</i>	$h^2$	$m_{2Max}$
22°C	0.87 $\pm$ 0.44	0.02 $\pm$ 0.05
22.5°C	0.37 $\pm$ 0.42	0.20 $\pm$ 0.10
26°C	0.33 $\pm$ 0.30	0.13 $\pm$ 0.06
27°C	0.05 $\pm$ 0.23	0.24 $\pm$ 0.08
28°C	0.13 $\pm$ 0.22	0.19 $\pm$ 0.07

### Analysis of sex ratio

REML estimates of variance among sires, variance due to the sire by temperature interaction, variance among dams nested within sires, variance due to the dam by temperature interaction (nested within sire), and variance between years for hatchling sex (treated as a continuous variable) are shown in Table 33. Incubation temperature had a strong effect on hatchling sex (Table 34). Sire, the sire-by-temperature interaction, dam (nested within sire) and year had significant effects on hatchling sex, while the dam-by-temperature interaction (nested within sire) had an insignificant effect (Table 34).

Variation among dams (nested within temperature) was significant at 26°C, 27°C and 28°C and insignificant at the other temperatures (Tables 35-40). Variation among sires was significant at all incubation temperatures (Tables 35-40). Sex ratio reaction norms for individual sires are plotted in Figure 4. Crossing reaction norms indicate that paternal half-sib families do not maintain their “rank” across incubation temperatures (Figure 4). We used three different methods to calculate narrow-sense heritability for sex determination (Table 41). Estimates of  $h^2$  were 1.00 at four temperatures and  $\geq 0.78$  at two temperatures (Table 41).

**Table 33.** Variance component estimates for hatchling sex using the full model

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Z ratio</i>
Sire	0.0081211	0.0064298	1.263041
Sire x temperature	0.0168935	0.006046	2.794161
Dam (nested within sire)	0.0067849	0.0034385	1.973215
Dam x temperature (nested within sire)	0.008437	0.0033486	2.519560
Year	0.004376	0.004721	0.926922
Residual	0.1012319	0.0036225	NA

Abbreviations: *SE*, standard error; *Z ratio*, ratio of the variance component to its associated standard error for a given random effect.

**Table 34.** Analysis of fixed and random effects on hatchling sex using the full model

<i>Effect</i>	<i>Df</i>	<i>LR<sub>x2</sub></i>	<i>p</i>
Incubation temperature	4	36.32	<0.0001
Sire	22	146.38	<0.0001
Sire x temperature	47	206.67	<0.0001
Dam (nested within sire)	46	81.75	0.0009
Dam x temperature (nested within sire)	78	64.23	0.8688
Year	3	19.35	0.0002

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value; SS, sums of squares; MS, mean squares; F, F-ratio.

**Table 35.** The effect of sire and dam (nested within sire) on hatchling sex at 22°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	21.87	10	0.0158
Dam (nested within sire)	13.07	15	0.5970

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table 36.** The effect of sire and dam (nested within sire) on hatchling sex at 22.5°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	31.98	10	0.0004
Dam (nested within sire)	16.68	16	0.4068

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table 37.** The effect of sire and dam (nested within sire) on hatchling sex at 26°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	77.50	12	<0.0001
Dam (nested within sire)	53.67	24	0.0005
Year	0.13	1	0.7202

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table 38.** The effect of sire and dam (nested within sire) on hatchling sex at the 26-31-26°C temperature shift treatment

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	114.66	13	<0.0001
Dam (nested within sire)	31.13	20	0.0534
Year	0.13	1	0.7160

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table 39.** The effect of sire and dam (nested within sire) on hatchling sex at 27°C

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	180.23	12	<0.0001
Dam (nested within sire)	84.20	25	<0.0001
Year	21.70	1	<0.0001

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table 40.** The effect of sire and dam (nested within sire) on hatchling sex at 28°C

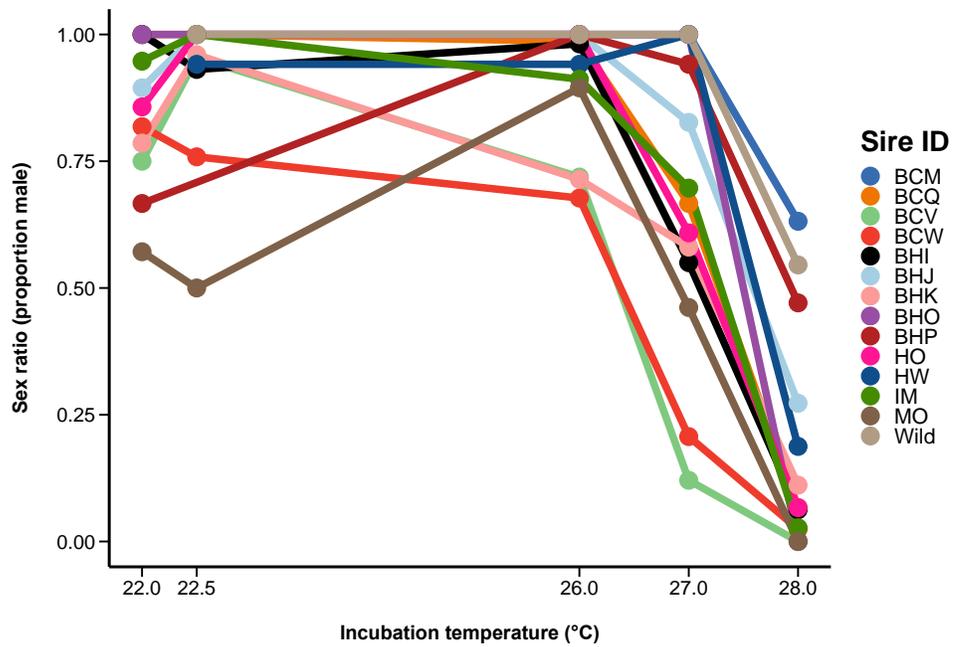
<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	113.41	12	<0.0001
Dam (nested within sire)	50.87	24	0.0011
Year	2.90	1	0.0883

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table 41.** Estimates of narrow-sense heritability (*h<sub>2</sub>*) of hatchling sex at individual incubation temperatures with their 95% confidence intervals. Estimates are shown using the observed scale, and two liability methods as described in the text.

<i>Incubation temperature</i>	<i>Continuous</i>	<i>Liability (Bull)</i>	<i>Liability (F &amp; M)</i>
22°C	0.34, CI = [0.11, 1.00]	0.78, CI = [0.29, 1.00]	0.80, CI = [0.27, 1.00]
22.5°C	0.61, CI = [0.20, 1.00]	1.00, CI = [0.71, 1.00]	1.00, CI = [0.76, 1.00]
26°C	0.29, CI = [0.08, 1.00]	0.80, CI = [0.34, 1.00]	0.86, CI = [0.22, 1.00]
26-31-26°C	0.50, CI = [0.21, 1.00]	1.00, CI = [0.50, 1.00]	1.00, CI = [0.50, 1.00]
27°C	1.00, CI = [0.52, 1.00]	1.00, CI = [0.80, 1.00]	1.00, CI = [0.83, 1.00]
28°C	0.90, CI = [0.36, 1.00]	1.00, CI = [0.86, 1.00]	1.00, CI = [0.92, 1.00]

Abbreviations: *CI*, 95 % confidence interval of the narrow-sense heritability estimate.



**Figure 4.** Sex ratio reaction norms as a function of incubation temperature

**Table 42.** Summary of results for each trait analyzed. Results show the likelihood ratio chi-square value and the associated p-value for the effect that each independent variable has on each trait.

Trait	Incubation Temperature	Sire	Sire x temperature	Dam (nested within sire)	Dam x temperature (nested within sire)	Year
Egg mass		0, p = 1.0		1719.06, p = <0.0001		91.31, p = <0.0001
Egg diameter		0, p = 1.0		1211.20, p = <0.0001		202.00, p = <0.0001
Hatchling mass	79.12, p = <0.0001	0, p = 1.0	9.24, p = 0.0024	68.05, p = <0.0001	25.68, p = <0.0001	54.88, p = <0.0001
Carapace length	279.75, p = <0.0001	0, p = 1.0	3.01, p = 0.0828	68.83, p = <0.0001	20.71, p = <0.0001	25.05, p = <0.0001
Plastron length	119.17, p = <0.0001	0, p = 1.0	22.54, p = <0.0001	53.81, p = <0.0001	1.38, p = 0.2401	0.81, p = 0.3681
Hatchling sex	36.32, p = <0.0001	146.38, p = <0.0001	206.67, p = <0.0001	81.75, p = 0.0009	64.23, p = 0.8688	19.35, p = 0.0002

## **Discussion**

Variation in TSD within and among populations is well documented in the common snapping turtle. However, the underlying cause of this variation is still uncertain. Understanding whether phenotypic variation has a genetic basis is crucial for predicting the response of a trait to selection. In this study, we used a paternal half-sib/maternal full-sib breeding design to partition phenotypic variation into additive genetic variance and dominance variance/non-genetic maternal effects (Falconer and Mackay, 1996; Lynch and Walsh, 1998). We found that sexual phenotype is highly heritable and also detected significant sire-by-temperature interactions. Analysis of phenotypic variation in egg mass, egg diameter, hatchling mass, hatchling carapace and plastron length for the same offspring revealed that these traits were influenced by non-genetic maternal effects. The finding that maternal effects contribute greatly to variation in egg and hatchling size, while maternal effects are essentially non-existent for hatchling sex in the same turtles, refutes the hypothesis that non-genetic maternal factors play a role in sex determination in the common snapping turtle. These results are important because genetic variation in sex determination would allow evolution of TSD pattern in response to climate change, allowing populations of snapping turtles to remain viable for longer periods of time.

The finding that variation in egg and hatchling size was due to non-genetic maternal effects while variation in sex ratio was due to additive genetic effects shows that the paternal half-sib, maternal full-sib design effectively teased apart maternal effects from additive genetic effects. These results directly contradict prior claims that variation in the concentration of steroid hormones in egg yolk cause variation in sex ratios among clutches. Roush and Rhen (2018) recently examined the literature for papers claiming that maternal effects influence TSD. They found a total of 21 studies measured yolk steroid hormones in TSD reptiles. Of these 21 studies,

only three reported a significant correlation between the concentration of yolk steroid hormones and offspring sex ratios while using appropriate experimental designs (Roush and Rhen, 2018). In one of these studies, Bowden et al. (2000) found that clutches of painted turtle eggs with higher estradiol/testosterone ratios produced more female-biased sex ratios. Bowden et al. (2001) and Carter et al. (2017) reported a seasonal shift in estradiol concentrations between painted turtle eggs laid early and late in the season. This shift in estradiol levels was correlated with a shift towards more female-biased sex ratios in painted turtles (Bowden et al., 2001) and red-eared sliders (Carter et al., 2017). The most conclusive way to demonstrate maternal effects would be to manipulate the levels of hormones deposited in eggs by the mother during oocyte development and note any changes in offspring sex ratios (Roush and Rhen, 2018). Only one study to date has experimentally manipulated circulating hormone levels in females in an attempt to influence deposition of steroids in egg yolk (Janzen, Wilson, Tucker, & Ford, 2002). High rates of female mortality in that study prevented the measurement of egg yolk hormones and offspring sex ratios, leaving the experiment inconclusive. Our quantitative genetic analysis in the snapping turtle clearly demonstrates that natural variation in sex ratio among clutches is not due to maternal factors transmitted to eggs before oviposition (i.e., yolk steroid hormones). One important note is that females can influence sex ratios by selecting microenvironments that produce different nest temperatures (Mitchell, Maciel, & Janzen, 2013; St. Juliana, Bowden, & Janzen, 2004; Warner, Lovern, & Shine, 2008). In this study, we collected and incubated eggs in common environments to eliminate potential effects of maternal nest site selection on incubation temperature.

Similar to our findings, previous work has shown clutch differences in egg and hatchling traits. Rhen and Lang (1995) found that hatchling mortality was affected by clutch identity and the clutch-by-incubation temperature interaction. Furthermore, clutch and the clutch by incubation

temperature interaction had a significant effect on hatchling growth rates (Rhen and Lang, 1995). Clutch identity was a significant source of variation in mortality in other studies of the snapping turtle (Brooks et al., 1991; Bobyn and Brooks, 1994). These two studies also demonstrated a clutch effect on growth in the snapping turtle (Brooks et al., 1991; Bobyn and Brooks, 1994).

Our analysis demonstrates that offspring sex is strongly affected by additive genetic variation, but not maternal effects (e.g., yolk steroids deposited by the mother). In another quantitative genetic study of sex ratio in the snapping turtle, Janzen (1992) reported significant variation in sex ratio among clutches but an insignificant clutch-by-temperature interaction. Because eggs were collected from the wild and parentage was unknown, only broad-sense heritability ( $H_2$ ) was estimated. Broad-sense heritability includes variance due to all genetic factors and non-genetic maternal effects. Therefore, additive genetic variance, dominance variance, and non-genetic maternal effects are confounded in these estimates.  $H_2$  estimates were 0.60, 0.76, and 0.34 at the 27.5°C, 28.0°C and 28.5°C incubation temperatures, respectively. In a later study, McGaugh et al. (2011) genotyped painted turtle dams and their offspring from natural nests to infer paternity. Because eggs were in natural nests in the wild, they experienced fluctuating incubation temperatures instead of constant temperature regimes used in laboratory experiments. By genotyping offspring and determining paternity, the authors were able to directly estimate narrow-sense heritability of sex ratio at 0.35. Rhen et al. (2011) used the “animal model” to analyze a multigenerational pedigree of leopard geckos and found significant genetic variance for sex determination with a  $h_2$  ranging from 0.37 to 0.44 at an incubation temperature of 30°C as well as significant dominance variance at 32.5°C. This difference in additive versus dominance variance caused a statistically significant genotype-by-temperature interaction.

With the breeding design used in the current study of snapping turtles, dominance variation and non-genetic maternal effects remain confounded. However, by assuming dominance variance is zero, we could place an upper limit on the potential contribution of maternal effects to phenotypic variation. This analysis shows that among-family sex ratio variation is caused by additive genetic variation, while variation in egg size and hatchling size is due to maternal effects.

In our analysis of sex determination, we also found significant genotype-by-temperature interactions. This interaction was driven by a change in the magnitude of the sire effect across temperature treatments. Although the effect of sire was highly significant within each incubation temperature, heritability on the observed scale was variable (Table 41). There was also variation in temperature sensitivity, with genotypes changing their “rank” across incubation temperatures (Figure 4). That is, a genotype that produces male-biased sex ratios at one temperature may produce female-biased sex ratios at another temperature when compared to other genotypes. Genetic variation in sex ratio and/or genotype-by-temperature interactions are essential for the evolution of TSD (Conover and Kynard, 1981; Bull, 1983; Conover and Van Voorhees, 1990; Janzen, 1992; Rhen and Lang, 1998).

Fisherian selection on sex determination and evolution of sex ratios in TSD species has been demonstrated over just a few generations. Conover and Van Voorhees (1990) established five laboratory populations of the Atlantic silverside, a fish with TSD, derived from natural populations ranging from South Carolina to Nova Scotia. Four of the populations began with skewed sex ratios in the first generation. In subsequent generations, the minority sex increased in frequency until the sex ratio stabilized at 0.5, with the trajectory by which the sex ratio approached 0.5 differing among the populations. In light of our finding that sex determination in the snapping turtle is highly heritable across a range of constant temperatures and in a temperature-shift study, it

is likely that TSD pattern could evolve in a similar manner to Atlantic silversides. With the life history of the snapping turtle, however, this hypothesis would be difficult to test.

## Supplementary information

Additional likelihood ratio tests and variance component estimates for hatchling mass, hatchling carapace length, hatchling plastron length and hatchling sex

**Table S1.** The effect of sire and dam (nested within sire) on hatchling mass at 22°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	81.35	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S2.** The effect of sire and dam (nested within sire) on hatchling mass at 22.5°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	23.87	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S3.** The effect of sire and dam (nested within sire) on hatchling mass at 26°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	105.70	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S4.** The effect of sire and dam (nested within sire) on hatchling mass at 27°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	213.16	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S5.** The effect of sire and dam (nested within sire) on hatchling mass at 28°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	204.26	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S6.** The effect of sire and dam (nested within sire) on hatchling carapace length at 22°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	25.03	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S7.** The effect of sire and dam (nested within sire) on hatchling carapace length at 22.5°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	32.94	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S8.** The effect of sire and dam (nested within sire) on hatchling carapace length at 26°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	77.53	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S9.** The effect of sire and dam (nested within sire) on hatchling carapace length at 27°C

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	122.51	1	<0.0001

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table S10.** The effect of sire and dam (nested within sire) on hatchling carapace length at 28°C

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	148.41	1	<0.0001

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table S11.** The effect of sire and dam (nested within sire) on hatchling plastron length at 22°C

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	0.35	1	0.5525

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table S12.** The effect of sire and dam (nested within sire) on hatchling plastron length at 22.5°C

<i>Effect</i>	<i>LR<sub>X2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	16.85	1	<0.0001

Abbreviations: *LR<sub>X2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>X2</sub>* -value greater than the observed value.

**Table S13.** The effect of sire and dam (nested within sire) on hatchling plastron length at 26°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	20.42	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S14.** The effect of sire and dam (nested within sire) on hatchling plastron length at 27°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	54.33	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S15.** The effect of sire and dam (nested within sire) on hatchling plastron length at 28°C

<i>Effect</i>	<i>LR<sub>x2</sub></i>	<i>Df</i>	<i>p</i>
Sire	0	1	1.0
Dam (nested within sire)	43.67	1	<0.0001

Abbreviations: *LR<sub>x2</sub>*, likelihood ratio chi-square; *p*, probability of obtaining an F-value or *LR<sub>x2</sub>* -value greater than the observed value.

**Table S16.** Variance component estimates for hatchling sex at 22°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.010381	0.0081782	0.0913534
Dam (nested within sire)	0	0	0
Residual	0.1136357	0.0122485	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

**Table S17.** Variance component estimates for hatchling sex at 22.5°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.0093241	0.0074465	0.1798029
Dam (nested within sire)	0.0026593	0.0038395	0.0512807
Residual	0.0518574	0.0052202	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

**Table S18.** Variance component estimates for hatchling sex at 26°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.0062609	0.006561	0.1059235
Dam (nested within sire)	0.0237954	0.0080668	0.4025739
Year	0	0	0
Residual	0.059108	0.003964	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

**Table S19.** Variance component estimates for hatchling sex at the 26-31-26°C temperature shift treatment

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.0158337	0.0087128	0.1485554
Dam (nested within sire)	0.0034743	0.004186	0.0325966
Year	0	0	0
Residual	0.1065846	0.0075185	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

**Table S20.** Variance component estimates for hatchling sex at 27°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.0707757	0.0381194	0.5247307
Dam (nested within sire)	0.039627	0.0155575	0.2937947
Year	0.0249487	0.0366574	0.1849697
Residual	0.13488	0.0097132	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

**Table S21.** Variance component estimates for hatchling sex at 28°C

<i>Effect</i>	<i>Variance component</i>	<i>SE</i>	<i>Variance ratio</i>
Sire	0.0253313	0.0151507	0.3112385
Dam (nested within sire)	0.0148136	0.0067101	0.1820104
Year	0.0011899	0.0024314	0.0146205
Residual	0.0813887	0.0056875	NA

Abbreviations: *SE*, standard error; *Variance ratio*, The ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance.

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