Published in final edited form as:

Funct Ecol. 2017 April ; 31(4): 876–884. doi:10.1111/1365-2435.12801.

Seasonal shifts in sex ratios are mediated by maternal effects and fluctuating incubation temperatures

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Summary

1. Sex-specific maternal effects can be adaptive sources of phenotypic plasticity. Reptiles with temperature-dependent sex determination (TSD) are a powerful system to investigate such maternal effects because offspring phenotype, including sex, can be sensitive to maternal influences such as oestrogens and incubation temperatures.
2. In red-eared slider turtles (*Trachemys scripta*), concentrations of maternally derived oestrogens and incubation temperatures increase across the nesting season; we wanted to determine if sex ratios shift in a seasonally concordant manner, creating the potential for sex-specific maternal effects, and to define the sex ratio reaction norms under fluctuating temperatures across the nesting season.
3. Eggs from early and late season clutches were incubated under a range of thermally fluctuating temperatures, maternally derived oestradiol concentrations were quantified via radioimmunoassay, and hatchling sex was identified. We found that late season eggs had higher maternal oestrogen concentrations and were more likely to produce female hatchlings. The sex ratio reaction norm curves systematically varied with season, such that with even a slight increase in temperature (0.5°C), late season eggs produced up to 49% more females than early season eggs.
4. We found a seasonal shift in sex ratios which creates the potential for sex-specific phenotypic matches across the nesting season driven by maternal effects. We also describe, for the first time, systematic variation in the sex ratio reaction norm curve within a single population in a species with TSD.

Graphical Abstract

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Data Accessibility

Data are appended in the supplementary material.

Data Accessibility:

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.bk5g1> (Carter Bowden & Paitz 2016)

Keywords

maternal effect; season; sex ratio; oestrogen; fluctuating temperature; temperature-dependent sex determination

Introduction

Maternal effects are causal sources of phenotypic plasticity in offspring which stem from maternal phenotype and the environment, and can enhance Darwinian fitness by facilitating context dependent adjustments of offspring phenotype (Mousseau & Fox 1998). When the phenotype that is most advantageous differs with offspring sex (Duckworth, Belloni & Anderson 2015), mechanisms that facilitate sex-specific maternal effects should be favored by selection (Rice 2000, Champman et al. 2003, Fedorka & Mousseau 2004). In egg laying vertebrates, maternally derived steroids transferred to the yolk before the egg is laid are a particularly potent source of maternal influence, acting as a proximate mechanism of adaptive phenotypic modification and serving as a physiological link between mother and offspring (Groothuis et al., 2005, Love & Williams 2008a, Love & Williams 2008b). There has been considerable interest in maternally derived steroids as a mechanism for differential resource allocation to sons versus daughters in avian systems (Badyaev et al. 2005, Uller & Badyaev 2009, Williams 2012, Duckworth et al. 2015). For example, in bluebirds, the concentration of maternally derived testosterone is higher in eggs laid late in the lay sequence, which corresponds to a shift towards the production of males late in the lay sequence and this maternally derived testosterone increases aggressiveness, which benefits males more than females (Duckworth et al. 2015). The ability of a female to influence offspring in a sex-specific manner; however, requires that she can either directly affect the sex of her offspring or that she can match maternal effects to a predictable pattern of sex ratio variation.

Oviparous reptiles provide an excellent system for investigating the evolutionary and ecological significance of sex-specific maternal effects, because offspring phenotype is sensitive to maternal influence and many species demonstrate more labile modes of sex determination. As in many taxa, reptilian phenotype is sensitive to maternal steroid deposition: hatchling dispersal ability (Vercken et al. 2007), survival (Meylan & Clobert 2005), susceptibility to ectoparasites (Uller & Olsson 2003), and post-natal growth (Uller

and Olsson 2007). But unlike birds and mammals, where evidence for facultative primary sex ratio skew is relatively rare (Ewen, Cassey & Moller 2004), exposure to exogenous steroids can directly affect sex in reptiles, even those with genetic sex determination (Bull, Gutzke & Crews 1988, Lovern & Wade 2003, Freedberg et al. 2006), thus highlighting the value of utilizing reptiles to investigate sex-specific maternal effects. However, due to the timing of dosing and the supraphysiological doses used to alter sex and other phenotypic traits, our understanding of how natural maternal steroid allocation influences offspring is lacking (Crews, Bull & Wibbels 1991, Crews 1996, Warner et al. 2014). Previous research has found that endogenous (yolk) steroids are also correlated with offspring sex (Bowden, Ewert & Nelson 2000, Lovern & Wade 2003) as in green anole lizards (*Anolis anolis*) where maternally derived yolk testosterone is higher in male-producing eggs (Lovern & Wade 2003). From the wealth of exogenous dosing studies and the limited examples using endogenous steroids, it seems female reptiles may have the capacity to alter the endocrine environment experienced by embryos in a sex-specific manner (Bull et al. 1988, Bowden et al. 2000, Lovern & Wade 2003, Freedberg et al. 2006, Radder 2007).

Reptilian sex and phenotype can also be influenced by incubation temperature, creating a second avenue through which females might match maternal effects to offspring sex. Incubation temperature is a pervasive environmental factor that drives phenotypic change in reptiles (Deeming 2004). Most of these findings come from studies that incubate eggs at different constant incubation temperatures and then compare resulting phenotypes (reviewed in Bowden, Carter & Paitz 2014). In species with temperature-dependent sex determination (TSD), offspring sex is a trait that is consistently and predictably affected by incubation temperature. In most reptile species with TSD, cool incubation temperatures produce males and warm incubation temperatures produce females (Type 1 TSD). There is a thermal threshold where embryonic development switches from male to female, the pivotal temperature (T_{piv}), and a narrow range of constant temperatures (approximately 1°C) that produces mixed sex ratios, termed the transitional range of temperatures (TRT) (Crews et al. 1994, Valenzuela & Lance 2004). These sex ratio reaction norms (which encompass the T_{piv} and TRT) have been characterized by examining sex ratios produced under a range of constant temperature incubations, conditions that do not reflect the thermal stochasticity inherent to natural nests (Bowden et al., 2014). Thermal variability in nests can often result in embryos experiencing temperatures above and below the T_{piv} frequently; therefore, defining sex ratio reaction norms under fluctuating conditions should allow us to better predict how TSD operates under natural thermal conditions. Because sex is determined by environmental temperatures in species with TSD, sex ratios can shift when incubation temperatures change across the course of a nesting season, and the seasonality of the production of sons and daughters has been repeatedly demonstrated in reptiles (Mrosovsky 1994, Shine 1999, Warner & Shine 2005). Seasonal variation in incubation temperatures that causes predictable variation in sex ratios would facilitate sex-specific matching of maternal effects to offspring phenotype. Since much of our understanding of TSD stems from research using constant temperature incubation conditions, we do not know if subtle increases in thermally fluctuating conditions, as would occur with season in the field, contribute to seasonal sex ratio variation. While laboratory studies have been vital to advancing our understanding of how steroids and incubation temperatures influence sex

ratios in species with TSD, it is only through an examination of these effects under more natural conditions that we will be able to discern true ecological and evolutionary implications of these processes.

Our research group has demonstrated in the red-eared slider turtle (*Trachemys scripta*), a species with TSD, that levels of maternally derived yolk oestrogens (both oestradiol and oestrone sulfate) show a seasonal increase that is concurrent with the seasonal increases in soil temperatures (Fig. 1) (Paitz & Bowden 2009, Paitz & Bowden 2013). Further, both of these maternally derived oestrogens increase the production of females when delivered exogenously (Crews et al. 1991, Paitz & Bowden 2010, Paitz & Bowden 2013). *T. scripta* provides an excellent system to investigate whether maternally derived oestrogens (not exogenous doses) correspond to offspring sex naturally. Experimental manipulations of oestrogens were first shown to affect sex determination in the red-eared slider in 1991 as part of a line of research aimed at deciphering how temperature cues are transduced into a biochemical signal that influences gonadal fate (Crews 1991). Since that time, numerous studies have replicated those results (Wibbels et al. 1991, Crews et al. 1994, Wibbels & Crews 1995, Wibbels et al. 1998, Bergeron et al. 1999) and expanded our understanding of how oestrogens trigger ovarian differentiation by pharmacologically blocking the production of oestrogens (*aromatase* inhibitor, Fadrazole) (Wibbels, Bull & Crews 1994) or by blocking the ability of oestrogens to bind their respective receptors (oestrogen receptor antagonist, Tamoxifen) (Dorizzi et al. 1991). Studies have also characterized the molecular cascade underlying how the differentiating gonad responds to oestrogens (Crews et al. 2001, Murdock et al. 2006, Ramsey & Crews 2007a, Ramsey & Crews 2007b, Barske et al. 2010) and identified how temperature influences oestrogen production in the gonad by altering methylation of regulatory genes (Matsumoto & Crews 2012). Because of the breadth and depth of this foundational work, studies that examine endogenous maternally derived oestrogens are now possible, and are needed to advance our understanding of how natural variation in steroids may affect sex determination.

Since incubation temperatures and maternally derived oestrogens increase over the nesting season, and artificial manipulations of each factor can individually affect sex determination, we hypothesized the existence of a natural seasonal shift in sex ratios in hatchlings of the red-eared slider turtle. Specifically, we had three goals: 1) to determine if late season eggs, which contain higher levels of oestrogens, are more likely to produce female hatchlings than early season eggs, 2) to define the sex ratio reaction norm (i.e., the TRT and T_{piv}) under a range of fluctuating incubation temperatures, and 3) to determine if there are seasonal changes in the sex ratio reaction norm, given that there is a seasonal pattern in maternal oestradiol deposition. Addressing these three goals will allow us to determine whether offspring sex ratios shift across the nesting season in a manner concordant with seasonal maternal oestradiol deposition and incubation temperatures, facilitating the ability of females to influence offspring development in a sex-specific manner.

Methods

Egg Collection and Incubation

To determine if hatchling sex ratios change across the nesting season, we collected clutches of red-eared slider eggs both early and late in the 2014 and 2015 nesting seasons. Nesting takes place over ~5 weeks at our field site and occurs in two fairly distinct temporal peaks (hereafter early and late season), generally corresponding to females' first and second clutches (Paitz & Bowden 2009) (Fig. 1). Importantly, clutches laid in these two seasons predictably differ in the concentrations of maternally derived oestradiol, with late season clutches containing significantly higher concentrations than early season clutches (Paitz & Bowden 2009, Paitz & Bowden 2013). Thus, the terms early and late season refer to both oviposition phenology and yolk oestradiol concentrations. We sampled these two nesting peaks by collecting early season clutches (which contain low oestradiol) between May 27–June 6 and late season clutches (which contain high oestradiol) between June 15–21, from Banner Marsh State Fish and Wildlife Area (Glasford, IL; 40.4619°N 89.9236°W). In addition to matching our sample collection to the two nesting peaks, we also verified that our clutches were either early or late season by quantifying yolk oestradiol (see *Oestradiol Quantification* below). In 2014, we collected 8 early and 13 late season clutches, and in 2015 we collected 9 early and 11 late season clutches. Clutches were obtained by excavating freshly laid nests (<6 hrs post-lay), by collecting gravid females on nesting forays, or by trapping gravid females in baited hoop net traps. Gravid females were returned to the lab where oviposition was induced following an oxytocin injection (Carter et al. 2016) (IDNR permits NH15.2084; IACUC protocol 08-2014).

In 2014, eggs from both early and late season clutches were allocated to one of three incubation treatments in a split clutch design, while in 2015, eggs from both early and late season clutches were allocated to one of four incubation treatments in a split clutch design. Table 1 lists all temperature treatments with both the average temperature $\pm$ fluctuation amplitude and the corresponding constant temperature equivalent (CTE; Georges, Limpus & Stoutjesdijk 1994). The CTE better estimates sex ratios from thermally fluctuating conditions than the average alone, by taking into account that development occurs faster at warmer temperatures, and estimates the temperature where half of development occurs above and half below (Georges et al. 1994). Our incubation treatments were chosen based on thermal data from the field (Fig. 1) and previous incubation experiments in the lab (e.g. Carter et al. 2016), with the goal of defining the full range of conditions that produce mixed sex ratios. The temperature in the fluctuating incubation treatments sinusoidally increased and decreased on a 24-hour cycle, mimicking the daily temperature fluctuations experienced by embryos incubating in the field (Fig. 1, Table 1). The total collection of fluctuating incubation conditions facilitated a test of the minimum and maximum temperatures at which maternal effects may be capable of affecting sex determination. In 2015 only, we included a constant temperature incubation condition to more directly examine the impact of thermal fluctuations *per se* on sex ratios by matching the constant temperature to the CTE of the 28.5 ± 3 °C (Table 1). Differences in sex ratios between the 28.5 ± 3 °C and constant temperature treatment would therefore be attributable to interactions between seasonal maternal effects and thermal fluctuations.

Developing eggs were incubated in boxes of moist vermiculite (~150 kPa) and water was added regularly to maintain similar hydric conditions. Boxes were also rotated within the incubators (IPP 400, Memmert GmbH+ Co. KG, Schwabach, Germany; Fisher Isotemp Model 307C, Fisher Scientific, Pittsburg, PA) and thermal loggers (iButton, Dallas-Maxim, Dallas, TX) monitored incubator conditions throughout the experiment. Hatchlings were maintained in individual containers with water beginning at pipping, and housed at room temperature (IACUC-08-2014). At six weeks post-hatch, turtles were euthanized and sex was determined via macroscopic examination of the gonads (Carter et al. 2016).

Oestradiol Quantification

Within 24 hours of oviposition, one egg per clutch was frozen for later yolk oestradiol quantification using a modified version of Wingfield and Farner's radioimmunoassay (RIA) (Wingfield & Farner 1975). In turtles, yolk is added to all eggs within a given clutch simultaneously and all eggs for a clutch are later laid in a single nesting bout, resulting in minimal variation in steroid concentrations among eggs within a clutch (Bowden et al., 2000; Conley et al., 1997; Janzen et al., 1998). Thus, concentrations in a single egg can be used as an estimate of the oestradiol concentration at oviposition for all remaining eggs in the clutch. We elected to sacrifice a single egg for steroid concentration determination rather than conduct yolk biopsies on each egg as prior work has found that biopsies result in prohibitively high mortality (Bowden, Smithee, & Paitz 2009), and that steroids are not ubiquitously distributed within the yolk, but rather are present in layers which can introduce significant sampling variation when using this technique (Bowden et al. 2001).

Yolk samples were prepared by homogenizing the entire yolk and then diluting 50 mg of yolk in 100 µl of distilled water, adding 2000 cpm of tritiated oestradiol (PerkinElmer Life and Analytical Sciences, Boston, MA), homogenised with two glass beads, and refrigerated overnight at 4 °C. The assay also contained four standard samples with 250 pg of oestradiol (Sigma-Aldrich, Inc., St. Louis, MO) and two blank samples made from distilled water. Steroids were extracted using 6 mL of petroleum ether and diethyl ether in a 30:70 ratio, in two rounds of 3 mL, and reconstituted in 90% ethanol and stored at -20 °C for three days. Samples were then centrifuged at ~2000 rpm for 5 min to consolidate neutral lipids after which they were decanted and dried under nitrogen gas. We used 500 µl of 10% ethyl acetate in isooctane to resuspend the steroids and fractionated in chromatography columns using the 0, 10, 20, and 40% fractions of ethyl acetate in isooctane. The 40% fraction was collected, dried under nitrogen gas, resuspended in 550 µl of PBSg, and refrigerated overnight. Samples were aliquoted such that 100 µl were used to measure recoveries, and two 200 µl duplicates were run through a competitive binding RIA using an E₂ specific antibody (Biogenesis Inc., Kingston, NH). Concentrations of oestradiol were calculated based on a standard curve that ranged from 1.95 – 500 pg. The cpm was averaged across duplicates and then corrected for individual sample recovery and yolk sample mass (average recovery 2014 = 48%, 2015= 35%). All samples were randomised within a single assay per year with intra-assay variations of 1% and 8% in 2014 and 2015 respectively, calculated as the coefficient of variation using the standards.

Statistics

All statistics were performed in SAS (v 9.3, Cary, NC, USA) and significance was determined using an alpha of 0.05. We used mixed model ANOVA (Proc Mixed) to test for differences in endogenous oestradiol levels between first and second clutches in 2014 and 2015 separately since these samples were run in separate assays. To determine if sex ratios significantly differed between first and second clutches across our incubation treatments, we used a generalised linear mixed model (Proc Glimmix). The model specified a binomial distribution (dist = binomial) and a logit link function (link = logit). Incubation treatment and clutch as a binary variable (first or second) were included as main effects. In 2014, all the treatments produced males, and in 2015 the $29.0 \pm 3^\circ\text{C}$ incubation treatment produced all females in both early and late season groups. Since these groups had no variance, leaving them in prevented model convergence. We therefore only included incubation treatments that produced mixed sex ratios and subsequently allowed a test of our hypothesis. Female nested within season was included as a random effect to take clutch-of-origin and interdependence of siblings into account. To directly test the effect of thermal fluctuations on sex ratios by season, we used two Fisher's Exact tests that compared sex ratios in the $28.5 \pm 3^\circ\text{C}$ (CTE 29.2°C) to the 29.2°C constant temperature treatment by season. We used a generalised linear mixed model to calculate predicated probabilities of sex ratios and 95% confidence intervals across our incubation temperatures to generate reaction norm curves for each season.

Results

Oestradiol levels significantly differed between early and late season clutches (2014: $F_{1,19} = 50.1$, $p < 0.0001$; early season clutch average 0.55 ng/g , late season clutch average 4.7 ng/ml ; 2015: $F_{1,19} = 30.05$, $p < 0.0001$, early season clutch average 0.5 ng/g , late season clutch average 8.6 ng/g , Fig. 2).

Among incubation treatments that produced mixed sex ratios, late season clutches were significantly more female biased than early season clutches ($F_{1,52} = 4.72$, $p = 0.03$, Fig. 3). Specifically, within the $28.0 \pm 3^\circ\text{C}$ (CTE 28.7°C) treatment, late season clutches produced 26% more females than early season clutches. Late season clutches produced 15% and 12% more females than early season clutches in the $28.5 \pm 3^\circ\text{C}$ (CTE 29.2°C) and 29.2°C constant treatments respectively. There was a significant effect of incubation temperature on hatchling sex ratios ($F_{2,52} = 6.24$, $p = 0.004$) where treatments with higher CTEs produced more females (Fig. 3). There was not a significant season*incubation temperature interaction. In 2014, all incubation treatments produced males, and in 2015, the warmest incubation treatment produced all females (Fig. 3), providing lower and upper bounds to the transitional range of temperatures that produce mixed sex ratios under fluctuating conditions. The sex ratios in the $28.5 \pm 3^\circ\text{C}$ (CTE 29.2°C) and 29.2°C constant treatments, did not significantly differ within a season (Fisher's Exact Test early season: $p = 0.49$, late season: $p = 0.63$).

Discussion

We wanted to determine if sex ratios show a seasonal shift concomitant with the shift in maternal estrogens, to define the sex ratio reaction norm under thermally fluctuating incubation conditions, and to decipher if there are seasonal changes in the sex ratio reaction norm. Embryos from late season eggs, which contained more maternally derived oestrogens, were more likely to develop as females than embryos from early season eggs across all temperatures that produced mixed sex ratios. Additionally, we characterized the sex ratio reaction norm in a reptile with TSD under thermally fluctuating conditions, demonstrating that the shift from 100% male to 100% female occurs across a narrow thermal range ($\sim 1.5^{\circ}\text{C}$), which subsequently results in seasonal differences in the T_{piv} (Fig. 4). To our knowledge, we are the first to describe a seasonal shift in the sex ratio reaction norm within a single population for any species with TSD. Overall our findings demonstrate that female turtle hatchlings are more likely to develop late in the nesting season in an environment marked by high maternally derived oestrogens and warmer incubation temperatures, and this creates the potential for sex-specific maternal effects.

Seasonal maternal effects mediated a shift in sex ratios with late season clutches having up to 26% (average 17%) more females than early season clutches (Fig. 3). If maternal oestrogen deposition directly affects sex determination, contributing to the observed seasonal sex ratio pattern, by default, maternal oestrogens are allocated to offspring in a sex-specific manner. However, since we did not directly manipulate maternal oestrogen concentrations in eggs, rather we relied on naturally occurring systematic variation, we cannot unequivocally attribute our seasonal sex ratio shift to any particular maternal effect(s). Given the decades of research in *T. scripta* demonstrating that oestrogens are the physiological equivalent to temperature and integral to the sex determination pathway in species with TSD, we believe that the described patterns are likely mediated, at least in part, by maternal oestrogens (Crews et al. 1991, Crews et al. 1994, Wibbels et al. 1994, Crews 1996, Bowden et al. 2000, Matsumoto & Crews 2012). Dosing studies have determined that oestradiol (Crews et al. 1991, Crews et al. 1994, Crews 1996), oestriol (Crews 1996), oestrone (Crews 1996), and oestrone sulfate (Paitz & Bowden 2010, Paitz & Bowden 2013) are capable of reversing sex when embryos are incubated at male-producing temperatures. Moreover, exogenous application of aromatizable androgens applied to the outside of the egg result in female sex determination, presumably due to their conversion to oestrogens, whereas nonaromatizable androgens result in the production of males (Wibbels & Crews 1992, Crews et al. 1994, Crews et al. 1995, Wibbels & Crews 1995). However, dosing studies generally lack biological realism (Crews et al. 1991, Crews 1996, Warner et al. 2014) necessitating the use of correlational studies utilizing natural variation in maternal steroids, like the present one. Designing experiments based on natural variation in maternal oestrogens ensures that the entire suite of oestrogens, their metabolites, and aromatizable androgens that may act as oestrogen sources is captured. Other maternal effects that systematically vary with season that may be capable of affecting sex determination are currently unknown and determining the existence of other seasonal maternal effects (if any) may be a valuable area of future research.

The presence of a seasonal sex ratio shift that co-occurs with a seasonal oestrogen deposition pattern creates the potential for sex-specific maternal effects with evolutionary consequences. The seasonality of maternally derived oestrogens may be a mechanism to match maternally derived steroid concentrations to the offspring sex that most greatly benefits from that developmental endocrine environment to maximise fitness. There is evidence in birds demonstrating fitness advantages of matching maternal steroid deposition/composition to offspring sex (Badyaev et al. 2006, Badyaev et al. 2008, Duckworth et al. 2015). Dominant female leghorn chickens (*Gallus gallus domesticus*) allocate more androgens to eggs that produce sons than eggs that produce daughters, which enhances the growth rates and competitiveness of sons early in ontogeny, and ultimately benefits adult quality (Müller et al. 2002). Males have more variable reproductive success than females in this species, and therefore benefit more from being in good condition (Müller et al. 2002). Maternal oestrogens affect phenotype in many vertebrate taxa, including zebra finches (*Taeniopygia guttata*), which demonstrate increased weight gain and decreased reactive oxygen metabolite production early in ontogeny (Tissier, Williams & Criscuolo 2014). Importantly, maternal oestrogens increase seasonally in several species and populations of turtles suggesting that this seasonal match in maternal oestrogen deposition and the production of females may not be isolated to our population (*Chrysemys picta*: Indiana: (Bowden et al. 2000), Illinois: (Bowden, Paitz & Janzen 2011); *T. scripta*: Illinois: (Paitz & Bowden 2009, Paitz & Bowden 2013), *this study*; and *Caretta Caretta*: Georgia: (Davis *Thesis*)). To fully elucidate this hypothesis in our system, longitudinal research tracking individual phenotype and fitness is necessary.

By defining the reaction norm under thermally fluctuating conditions (CTE's: 28.0–29.5 °C; Fig. 3) our data demonstrate that slight increases in fluctuating temperatures can have significant effects on sex ratios. We demonstrate that the TRT is similarly narrow (~1.5 °C) under thermally fluctuating conditions as it is under constant temperatures (Crews et al. 1994). With these data, we now know that the narrowness of the TRT is not just an artifact of only experiencing constant male or female developmental thermal cues for the entirety of development. Because of the narrowness of the TRT, a 0.5 °C increase in incubation temperature alone, between the early season sex ratios in the 28.7 and 29.2 °C treatments for example, resulted in a 34% increase in the production of females. Interestingly, we found that similar sex ratios were produced under the constant and fluctuating incubation conditions with the same CTE (29.2°C constant temperature versus 28.5 ± 3°C (CTE 29.2°C)), suggesting that the observed seasonal sex ratio pattern is robust to ecologically relevant thermal fluctuations. Defining the range of fluctuating temperatures that produce mixed sex ratios is vital to our understanding of TSD in general, and to our interpretations of the capacity of additional factors, like maternal effects, to affect sex determination.

We were able to describe, for the first time, different sex ratio reaction norm curves that systematically vary within a single population (Fig. 4). Since the TRT was the same for early and late season embryos (28.5–29.6 °C, Figs. 3,4), but the sex ratios at any given temperature varied, the reaction norm curve of late season embryos is more curvilinear than that of the early season. The inflection point on the curves, the T_{piv} , is lower in embryos from late season clutches (28.5 °C) than embryos from early season clutches (28.9 °C; Fig.

4). This 0.4°C seasonal T_{piv} decrease may seem relatively mild, but given that the transition from 100% male to 100% female production happens over a ~1.5°C temperature span (TRT; Fig. 4), it suggests that this seasonal shift can have drastic effects on sex ratios in years where temperatures approximate the TRT. Specifically, an increase of 0.5 °C in incubation temperature in addition to the seasonal maternal effect can cause a 49% increase in the production of females (early season 28.7 °C sex ratios compared to late season 29.2 °C sex ratios; Fig. 3). Slight variations in reaction norm curves have been investigated directly, or indirectly via examination of the T_{piv} at the clutch (Janzen 1992, Bowden et al. 2000, Dodd, Murdock & Wibbels 2006) and population levels (Bull, Vogt & Bulmer 1982, Bull Vogt & McCoy 1982, Ewert, Jackson & Nelson 1994, Ewert, Lang, Nelson 2005, Refsnider et al. 2014, Refsnider & Janzen 2016). Reported differences in the T_{piv} are generally within 1.5°C; however, this variation may be significantly underlain by the seasonal pattern described here. Failure to account for a seasonal shift in sex ratio reaction norms may mask our ability to estimate genetic heritability of sex ratio (via the T_{piv} ; Janzen 1992, Refsnider & Janzen 2016) and to describe latitudinal gradients in the T_{piv} , or lack thereof (Bull, Vogt & Bulmer 1982, Bull Vogt & McCoy 1982, Ewert, Jackson & Nelson 1994, Ewert, Lang, Nelson 2005, Refsnider et al. 2014).

Our data demonstrate that seasonal maternal effects operate to reinforce existing TSD patterns driven by incubation temperatures. Seasonal sex ratio variation is thought to be an integral aspect of the adaptive significance of TSD, such that offspring sex can be matched to incubation temperature and the suite of thermally-sensitive phenotypes produced at that temperature, ultimately maximizing offspring fitness (Charnov & Bull 1977, Conover 1984, McCabe & Dunn 1997). Maternally derived oestrogens may act to increase the likelihood of producing females late in the season when temperatures are warm (i.e. reinforcing a match between sex and incubation temperature). While empirical evidence of sex-specific advantages due to differences in phenotype derived from incubation conditions is lacking in long-lived reptiles like turtles, this idea remains central to our evolutionary understanding of the adaptive significance of TSD in other, short-lived reptiles (Shine 1999, Warner & Shine 2005). Additionally, evidence suggests that there may be detrimental fitness consequences to developing within the TRT, where embryos receive ambiguous sex determination thermal cues (Janzen 1995, Crews 1998). In our population, late season temperatures are more likely to fluctuate within the TRT (Fig. 1) than early season temperatures, which are well below the TRT on average. As such, oestrogens in late season eggs may functionally lower the T_{piv} to avoid development within the TRT (i.e. resulting in late season temperatures being clear female thermal cues for embryos) to alleviate fitness costs of ambiguous thermal cues on sex determination.

Conclusions

We demonstrated a seasonal shift in sex ratios that is mediated, in part, by naturally occurring maternal effects, potentially the concurrent seasonal increase in maternally derived oestrogens. The temporal match between the production of females and the seasonal increase in maternal oestrogen deposition suggests that a sex-specific maternal effect may exist, causing female embryos to develop in more oestrogen rich environments than males. For the first time, we demonstrated that the TRT is similarly narrow under fluctuating

temperatures as constant temperatures in a model TSD organism, and as a result, even slight increases in thermally fluctuating conditions, as would occur during the nesting season, can drastically affect sex ratios. Lastly, we found systematic, seasonal variation in the sex ratio reaction norm curves within a single population, a novel contribution to the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank S. Marrochello, L. Treidel, J. Dillard, B. Cohler, H. Nichols, M. Gillard, and P. Hunter for their assistance conducting fieldwork and rearing hatchlings, and the Illinois Department of Natural Resources for access to the field site. AWC would like to thank J. Carter for support, and B. Sadd, J. Casto, S. Juliano, and S. Sakaluk for their comments on the project. We would like to thank two anonymous reviewers for comments on an earlier version of this manuscript. This research was supported by an NSF graduate research fellowship, two Weigel grants from the Beta Lambda Chapter of Phi Sigma, and an ILMA-Lakes Graduate Scholarship to AWC. RTP is supported by an NIH IR15ES023995-01 to RMB and RTP. The authors have no conflict of interest to declare.

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Figure 1.

Daily minimum and maximum soil temperatures averaged from 1993–2014. Soil temperature readings were taken 4 in below the soil surface under sod by the Illinois State Water Survey (2016). Previous research (not shown) demonstrates that these soil temperature readings closely approximate natural *T. scripta* nest temperatures at our field site. Shading reflects the seasonal timing of oviposition in our population, and corresponds to when maternal oestrogens are low and high in early and late season clutches respectively. The timing of hatching is temperature-dependent, and generally occurs between August–October.

Figure 2.

Average oestradiol concentrations of early (white bars) and late season (gray bars) clutches in 2014 and 2015. Late season clutches contain significantly more maternally derived oestradiol than early season clutches in both years (* denotes significant differences). Bars are means $\pm$ 1 standard error; points are individual clutch values.

Figure 3.

Sex ratios of hatchlings were significantly more female biased in late season clutches (gray bars) than in early season clutches (white bars) within treatments that produced mixed sex ratios. Treatments with higher CTEs produced a significantly greater proportion of females. Incubation treatments included: 27.7 ± 2 °C (CTE 28.0 °C) from 2014, 28.0 ± 3 °C (CTE 28.7 °C), 28.5 ± 3 °C (CTE 29.2 °C), a constant 29.2 °C, and 29.0 ± 3 °C (CTE 29.6 °C) from 2015. Numbers within the bars are the number of females/ total number of hatchlings in each treatment.

Figure 4.

Sex ratio reaction norms of early (black line) and late (gray line) season clutches across the TRT. The temperature at which a 50:50 sex ratio is predicted (T_{piv}) is lower in embryos from late season clutches (~ 28.5 °C) than in early season clutches (~ 28.9 °C) (dashed line). Curves were calculated using the predicted probabilities of the proportion of females produced at each fluctuating incubation temperature. Error bars are 95% confidence intervals.

Table 1

Incubation regimes and corresponding constant temperature equivalents (°C).

	Fluctuating Incubation Regime	CTE
2014	26.5 ± 2	26.9
	27.1 ± 2	27.5
	27.7 ± 2	28.0
2015	28.0 ± 3	28.7
	28.5 ± 3	29.2
	29.2 ± 0	29.2
	29.0 ± 3	29.6