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RESEARCH ARTICLE

Measurement and modelling of primary sex ratios for species with temperature-dependent sex determination

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ABSTRACT

For many oviparous animals, incubation temperature influences sex through temperature-dependent sex determination (TSD). Although climate change may skew sex ratios in species with TSD, few available methods predict sex under natural conditions, fewer still are based on mechanistic hypotheses of development, and field tests of existing methods are rare. We propose a new approach that calculates the probability of masculinization (PM) in natural nests. This approach subsumes the mechanistic hypotheses describing the outcome of TSD, by integrating embryonic development with the temperature-dependent reaction norm for sex determination. Further, we modify a commonly used method of sex ratio estimation, the constant temperature equivalent (CTE), to provide quantitative estimates of sex ratios. We test our new approaches using snapping turtles (*Chelydra serpentina*). We experimentally manipulated nests in the field, and found that the PM method is better supported than the modified CTE, explaining 69% of the variation in sex ratios across 27 semi-natural nests. Next, we used the PM method to predict variation in sex ratios across 14 natural nests over 2 years, explaining 67% of the variation. We suggest that the PM approach is effective and broadly applicable to species with TSD, particularly for forecasting how sex ratios may respond to climate change. Interestingly, we also found that the modified CTE explained up to 64% of variation in sex ratios in a Type II TSD species, suggesting that our modifications will be useful for future research. Finally, our data suggest that the Algonquin Park population of snapping turtles possesses resilience to biased sex ratios under climate change.

KEY WORDS: CTE, Fluctuating temperature, Incubation, Natural nests, Nest temperature, Snapping turtle

INTRODUCTION

The temperatures experienced by an embryo during incubation can have many effects on an organism's phenotype (Bobyn and Brooks, 1994; Brooks et al., 1991; DuRant et al., 2010; Shine and Downes, 1999; Shine et al., 1997). Of these, one of the most profound outcomes of incubation temperature is the sexual differentiation in organisms with temperature-dependent sex determination (TSD). During a critical period of embryonic development known as the thermosensitive period (TSP), organisms with TSD undergo gonadal morphogenesis and temperature-mediated physiological signals confer gender (Bull, 1987). Since its discovery (Charnier, 1966), TSD has been found to occur in all crocodilians, most turtles and some fishes (Bergeron et al., 1999; Ospina-Álvarez and Piferrer, 2008; Pieau et al., 1999). There are two categories of TSD, which are defined in terms of the sex ratios produced when incubation temperature is held constant: Type IA, which occurs in most turtles, produces females at high constant incubation temperatures and males at low temperatures; Type IB, which occurs in the tuatara, is the opposite (Cree et al., 1995; Ewert et al., 1994). Type II, in which males are produced at intermediate temperatures, is less common, but occurs in all major reptile clades except Rhynchocephalia (Ewert et al., 1994; Harlow and Taylor, 2000; Lang and Andrews, 1994). TSD is characterized by one or more pivotal temperatures (T_{piv}) that produce a 1:1 sex ratio, as well as the transitional range of temperatures across which both sexes are produced.

Because sex ratio is a key demographic parameter, populations of reptiles with TSD are hypothesized to be influenced by rapid climate change (Janzen, 1994); in fact, green sea turtles from the Great Barrier Reef have already undergone near-complete feminization over the past 20 years (Jensen et al., 2018). Some have argued, however, that taxa with TSD have survived many extreme climatic events over millions of years, and should therefore show some resilience to changes in climate that would skew sex ratios (Brooks, 1995; Silber et al., 2011). Despite the debate surrounding TSD and climate change, there is a paucity of literature describing how sex is determined in natural nests. While many researchers have performed laboratory experiments to determine sex ratios produced at constant temperatures in turtles (e.g. Ewert et al., 1994; Mrosovsky, 1994; Wilhoft et al., 1983; Yntema, 1978, 1976), wild nests are never under constant stationary temperature; therefore, experimental results may poorly reflect real-life situations (Georges et al., 1994; Schwarzkopf and Brooks, 1985; Valenzuela, 2001). Fewer studies have investigated how fluctuating temperatures affect sex ratios, and fewer still have been able to explain sex ratios when nests are *in situ*, with high thermal fluctuation (Bowden et al., 2014; Bull, 1985; Demuth, 2001; Georges, 1992; Georges et al., 1994; Janzen, 1994; Shine et al., 1997).

Two existing approaches to estimating sex ratios in the wild are the mean nest temperature, which translates the mean incubation temperature into a sex ratio, and the constant temperature equivalent (CTE), which takes into account the developmental leverages of different temperatures (Georges, 1989; Georges et al., 2004). The mean nest temperature is generally considered an unreliable proxy when nests experience thermal variance (Bull, 1985; Georges et al., 1994; Schwarzkopf and Brooks, 1985), largely because development rate of an embryo varies non-linearly with temperature (Georges et al., 2005; Gillooly et al., 2002; Girondot and Kaska, 2014). For example, from the perspective of a developing

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embryo, one hour spent at low temperatures results in less anatomical differentiation than one hour spent at high temperatures, such that the mean temperature experienced is not equivalent to the development-weighted mean (Sharpe and DeMichele, 1977). To address this issue, Georges et al. (1994, 2004) developed the constant-temperature equivalent (CTE) statistic, which represents a development-weighted median nest temperature (i.e. the temperature above and below which 50% of development occurs). In other words, the CTE model attempts to improve upon the mean nest temperature statistic by taking anatomical development into account. Once the CTE has been calculated, it can be mapped onto a temperature–sex reaction norm for the population in question to estimate sex ratios. Numerous studies in the laboratory have found the CTE method to be an acceptable predictor of sex in cases where there is thermal variance (Georges et al., 2005; Les et al., 2007; Mitchell et al., 2008). However, the CTE method was not designed to estimate sex ratios in species with two pivotal temperatures (Type II TSD), and performs best when there is homogenous thermal variance and a stationary thermal mean (Georges et al., 1994, 2004). These conditions may not be met in natural nests, especially in seasonal climates, and so the CTE method has been modified to better accommodate heterogeneous thermal variance and a changing thermal mean (Carter et al., 2018; Telemeco et al., 2013). Nevertheless, there is a paucity of literature quantitatively reporting the variation in sex ratio explained by the CTE method in natural nests: Demuth (2001) found that the CTE was able to explain 76% of the variation in sex ratios across a very small sample of 3 natural nests, whereas Carter et al. (2018) found the CTE could explain only 35% of the variation across over 20 years of natural nest data. Therefore, the ability of the CTE to quantitatively predict sex ratios in natural nests is currently unclear.

Further complicating sex ratio predictions is the dynamic nature of the thermosensitive period. Specifically, a relatively warm incubation regime may act to increase the range of anatomical stages where sex is influenced by temperature, whereas a relatively cool incubation regime may decrease this range (Yntema, 1979). In snapping turtles, Yntema (1979) showed that eggs incubated at hot (30°C) temperatures experience a longer TSP (Yntema stages 14–19), while eggs incubated at cool temperatures (20°C) experience a shorter TSP (Yntema stages 14–16). Yet, many authors have approximated the TSP in turtles as occurring in the range of 33–66% development (Norris and Lopez, 2010; Stubbs et al., 2014; Yntema and Mrosovsky, 1982). There has been little effort to delineate the TSP under natural conditions, despite its importance for accurate prediction of sex ratios (Girondot et al., 2018).

In this study, we present a new approach for predicting sex ratios, one that can easily accommodate different assumptions regarding TSP duration, and we compare our approach with a mean nest temperature method and a CTE approach. Importantly, we also modify the mean nest temperature and CTE statistic to be weighted by daily embryonic development, in order to provide a fair comparison across all methods. Our new approach for predicting the sex ratios of oviparous reptiles with TSD does not represent a mechanistic model, but it is rooted in mechanistic theory. Briefly, TSD is thought to occur when genetic, epigenetic and hormonal cascades push embryos towards one sex or the other through sexual differentiation of the gonads, in response to male or female-producing temperatures (Ge et al., 2018; Shoemaker and Crews, 2009). Many authors have postulated that temperature appears to exert a dosage effect on sex determination, given that initial changes in sex can be reversed if embryos are subject to drastic temperature changes, and the magnitude of the effect on sex seems to rely on the potency of a given temperature (Bull et al., 1990; Crews et al., 1991; Wibbels et al., 1991, 1998). If we assume that temperature indeed exerts a dosage effect on sex determination, we expect male sex ratio and exposure to temperatures that mediate the masculinizing cascade to exhibit a degree of proportionality. Specifically, we predict that the male sex ratio produced in a clutch is proportional to the development-weighted probability of the clutch masculinizing over the duration of the thermosensitive period. Our approach is intended to be universally applicable to species with both types of TSD and to accurately predict sex ratios, even with non-stationary thermal means and heterogeneous thermal variation during incubation. We hereafter refer to this approach as the probability of masculinization (PM).

We first determined the temperature–sex ratio reaction norm at constant incubation temperatures for a population of snapping turtles from Algonquin Park. We then manipulated nests to determine which method of sex ratio prediction (mean nest, CTE or PM) best explains variation in sex ratios, all while allowing for the possibility of a short or a long thermosensitive period. Lastly, we use the best model from the second experiment to predict sex ratios in real, unmanipulated nests across two years, to determine if the explanatory power of the model holds true in natural situations.

MATERIALS AND METHODS

Study population and history

The present study is part of a long-term research programme on a population of snapping turtles [*Chelydra serpentina* (Linnaeus 1758)] in Algonquin Park. The study was initiated in 1972, and is centred around a dam on Lake Sasajewun, at the Algonquin Wildlife Research Station (AWRS; Algonquin Provincial Park, ON, Canada, 45°35′ N, 78°31′ W). All animal work was done in accordance with the University of Toronto Animal Care Committee protocols.

Estimating the temperature–sex reaction norm

In June 2016 and 2017, we collected snapping turtle eggs of known maternity on the Lake Sasajewun Dam, within 12 h of being laid. We kept the eggs at the AWRS field laboratory at <20°C to slow development, then brought the eggs back to the University of Toronto within 6 days of being laid. In 2016, we collected subsamples of 9 clutches totalling 234 eggs, which were randomly divided with respect to clutch, then evenly allocated to six treatment groups at 23, 25, 26, 27, 28 and 29±0.5°C. The eggs treated at 23°C were incubated in a commercial refrigerator outfitted with a thermostat (Learn to Brew LLC, Moore, OK, USA) and a fan for circulation; the other incubators were Reptibator incubators (ZooMed, San Luis Obispo, CA, USA) with retrofitted circulation fans. In 2017, we also incubated a subsample of 12 eggs from three different nests at 20.5°C in an IN55 ECHOtherm Chilling Incubator (Torrey Pines Scientific, Inc., Carlsbad, CA, USA). Temperatures within each incubator were monitored hourly using DS1921H iButtons (Maxim Integrated, San Jose, CA, USA) iButtons, which have an accuracy of ±1°C. We incubated embryos at constant temperatures until they reached an advanced stage (Yntema stage 23–25), at which point they were euthanized and sexed.

Additional sex ratio data were available from a previous unpublished experiment, and were included in our estimation of the temperature–sex reaction norm. Heather Passmore and R.J.B. (unpublished results) collected 663 eggs from the Algonquin Park population of 18 snapping turtles in June of 1991. After removal from the nest, they were marked with a graphite pencil to identify the clutch of origin, and kept at <20°C in order to slow development. The eggs were transported to University of Guelph before they

reached Yntema stage 6. Any infertile or damaged eggs were removed prior to incubation. The remaining 586 eggs were then allocated evenly among four incubators (Koolatron Corp., Brantford, ON, Canada) set at 21.5, 22.0, 26.5 and 27.5°C. Within each incubator, eggs were separated into boxes of ∼40 eggs in moist vermiculite. To estimate water loss, individual boxes were weighed to the nearest 0.1 g at the beginning of incubation, and then subsequently weighed throughout; if the weight decreased, distilled water was added in order to maintain moisture levels. Thermal variance in each box was monitored using temperature-sensitive probes (Grant Instruments Ltd, Royston, UK) and kept within 0.5°C of the target temperatures. Turtles were euthanized just after hatching, and sexed.

In all cases, sex was determined using macroscopic examination of the gonads, based on the morphology of the gonad and presence of oviducts. Macroscopic gonadal examination is commonly used, and accurate when verified with histological examination (Cotter and Sheil, 2014; Spencer and Janzen, 2014; St. Juliana et al., 2004; Yntema, 1960). If the embryo had short (i.e. half the length of the mesonephros or less), rounded and thick gonads, while lacking oviducts, it was identified as a male. If the embryo had long (i.e. greater than half the length of the mesonephros), pointed and thin gonads, with thick, continuous oviducts, it was identified as female. Occasionally (in 5/192 cases; <3%), we found individuals with a mix of these characters: for example, a long, continuous oviduct on one side of the body or a feminine-appearing gonad with patchy oviducts. We treated these individuals first as 'intersex', marking down which characters they possessed. As intersex turtle embryos develop into males post-hatching, and the ovary retains male potential throughout development (Pieau et al., 1998), we later treated them as males.

We took the average incubation temperature as the constant temperature for each treatment. We then used the *nls* package in the R environment (2013) to find the least-squares parameter estimate for a double-logistic equation, a bell-shaped function described by Eqn 1:

$$\text{Sex ratio} = \frac{1}{1 + e^{((1/S_1)\times(T_{P1}-t))}} \times \frac{1}{1 + e^{((1/S_2)\times(T_{P2}-t))}}, \quad (1)$$

where parameters T_{P1} and T_{P2} are the lower and upper pivotal temperatures, S_1 and S_2 are curvature parameters for either half of the function, and t is the independent temperature variable. Each treatment produced one sex ratio that was based on n embryos, and therefore the sex ratio was weighted by n in the model.

Model selection and the thermosensitive period

We conducted an experiment to compare which method of sex ratio prediction best explains variation in sex ratios in semi-natural nests; this experiment also tested which window of the thermosensitive period, long versus short (Yntema, 1979), was most appropriate for estimating sex ratios. A key aim of our design was to experimentally increase the mean and variance in temperature across 27 semi-natural nests, thereby producing a large range of nest sex ratios with which to compare different methods of predicting sex ratios. We collected 324 eggs from 11 female snapping turtles nesting on the Sasajewun Dam (AWRS); all clutches were laid during the night of 10 June or the morning of 11 June in 2001. The eggs were brought back to the lab and kept at ∼18°C to slow development. The eggs were randomized with respect to clutch, creating 27 semi-natural nests of 12 eggs each, and then reburied on the dam. Within each nest, eggs were buried on a horizontal plane in a 3×4 egg grid, to minimize variation in temperature between eggs (Fig. 1). To vary the mean nest temperature, nests were placed into three temperature treatment groups: 9 nests were covered with elevated wooden slats to reduce direct sunlight, 9 nests were placed in an area of the dam with no shade, and 9 were placed beneath a 30 cm×30 cm clear plastic polypropylene tarp that was elevated 30 cm off the ground, which emulated a greenhouse and produced a warming effect. Within each temperature treatment, three nests were buried at a depth of 10 cm, three were buried at a depth of 15 cm, and three were buried at a depth of 20 cm, in order to vary temperature fluctuations between nests (Fig. 1). This design minimized the variation between temperatures for eggs within a nest, but maximized variation in temperature across nests. Each semi-natural nest had an iButton DS1921H temperature logger (Maxim Integrated, San Jose, California, USA) programmed to record hourly temperatures. Eggs were allowed to incubate without interference, until approximately 1 week before hatching, when nests were excavated and placed in a 25°C incubator. All hatchlings were sexed macroscopically (Yntema, 1976).

After the experiment was completed, we predicted when embryos in each nest had entered the thermosensitive period. To do so, we leveraged the incubation temperature profile specific to each semi-natural nest, in conjunction with a thermal performance curve for embryonic development specific to our population of *C. serpentina* (Rollinson et al., 2018). The thermal performance curve is expressed in equivalent development units (Webb et al., 1986, 1983), which allows estimation of current Yntema stage based on the temperatures to which the embryo was previously exposed (Fig. S1, Table S1). Given that onset of the TSP in *C. serpentina* occurs at Yntema stage 14 (Yntema, 1979), we calculated the amount of development, in equivalent development units, that occurred at each hourly time interval, and these units were summed to estimate Yntema stage at each time interval, allowing us to estimate when Yntema stage 14 was reached (Rollinson et al., 2018). A similar method of development summation was first developed by Georges et al. (2005), and has been found to be more accurate at delineating the TSP than an estimate using only the middle-third period of development (Girondot et al., 2018). For the period of time between egg laying and egg reburial, we used the average field laboratory temperature of 18°C to estimate developmental increments. Using the method above, we estimated the timing of both a short (Yntema stage 14–16) and long (Yntema stage 14–19) thermosensitive period for each of the 27 semi-natural nests, ultimately allowing us to focus on the thermal profile experienced only during the thermosensitive period, thereby ignoring temperatures that are putatively irrelevant to sex determination.

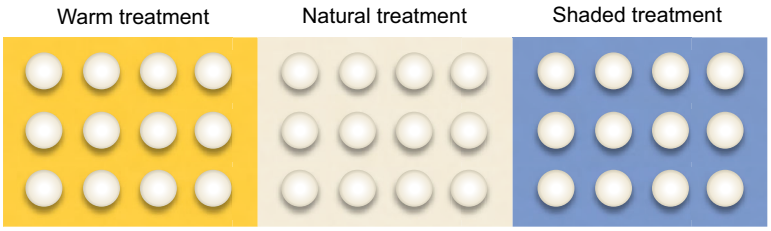

Fig. 1. Layout of semi-natural nests. Each semi-natural nest is composed of 12 eggs on a 3×4 horizontal plane. We divided 27 semi-natural nests into three replicate groups composed of 9 semi-natural nests each. Pictured is one layer of a single replicate group; each had three layers buried directly below one another, at depths of 10 cm, 15 cm and 20 cm. Each replicate group had a warm (yellow) treatment, a natural (beige) treatment and a shaded (blue) treatment. Ultimately, this replicate group was itself replicated 3 times, totalling 27 semi-natural nests. Total sample size=324 eggs.

Using the thermal profile experienced during the estimated TSP, we compared the explanatory power of three methods in predicting sex ratio in semi-natural nests: a modified CTE (Georges et al., 2005), a development-weighted mean nest temperature and PM. To calculate the modified CTE, we determined the amount of anatomical development that occurred at each hourly interval within the thermosensitive period, as described immediately above. Within each day of the TSP, the temperatures experienced and their respective developmental increments were sorted numerically by temperature, and the temperature above and below which 50% of development occurs was taken as the daily CTE. We then found the average CTE value for the entirety of the CTE, with each daily CTE value being weighted by the development occurring in that day. A similar approach was taken by Doody et al. (2004), although their approach averaged daily CTEs to calculate a final CTE value. The CTE was then translated into a sex ratio using the temperature–sex reaction norm specific to this population. In order to make a fair comparison of the mean nest temperature statistic with the other models, we calculated the daily mean nest temperature for each nest during the TSP, and weighted each day by the amount of development that occurred in that day. The resultant development-weighted mean was translated into a sex ratio, as in our calculation of the CTE.

For the PM method, we calculated the amount of development each nest experienced between each hour-long interval at a given temperature during the TSP, and used the temperature–sex reaction norm to determine the probability of masculinization. To weigh each hour's probability of masculinization by the amount of development that had occurred in each interval, we multiplied the two values together. We then summed the development-weighted probabilities of masculinization across the entire thermosensitive period, and divided by the total amount of development that occurred in the thermosensitive period, following Eqn 2:

$$\mathrm{PM} = \sum_{i=0}^{n}(D_i \times \mathrm{PM}_i)/\sum_{i=0}^{n}(D_i), \qquad (2)$$

where i=0 is the first time interval (temperature log) during TSP and i=n is last time interval before the end of the TSP, D_i is the amount of development that occurred during the interval, taken from the thermal performance curve for this population, and PM_i is the probability of masculinization at the temperature experienced during the time interval, taken from the temperature–sex reaction norm for this population. The denominator $\sum_{i=0}^{n} D_i$ should be equivalent to 2 equivalent development stages for the short TSP window (Yntema stages 14–16; equivalent development ages 6–8) and 5 equivalent development stages for the long TSP window (Yntema stages 14–19; equivalent development ages 6–11).

We used an information-theoretic approach to compare the mean nest temperature, the CTE and the PM as a predictor of the sex ratios, relying on the Akaike information criterion adjusted for small sample sizes (AICc) (Akaike, 1973; Burnham and Anderson, 2002). Further, we tested whether a short TSP or a long TSP better predicted sex ratios. This resulted in six candidate models, which took the form: (1) $\mathrm{SR}=\beta_0+\beta_1\mathrm{MNT}_{\mathrm{short}}+\varepsilon$; (2) $\mathrm{SR}=\beta_0+\beta_1\mathrm{MNT}_{\mathrm{long}}+\varepsilon$; (3) $\mathrm{SR}=\beta_0+\beta_1\mathrm{CTE}_{\mathrm{short}}+\varepsilon$; (4) $\mathrm{SR}=\beta_0+\beta_1\mathrm{CTE}_{\mathrm{long}}+\varepsilon$; (5) $\mathrm{SR}=\beta_0+\beta_1\mathrm{PM}_{\mathrm{short}}+\varepsilon$; (6) $\mathrm{SR}=\beta_0+\beta_1\mathrm{PM}_{\mathrm{long}}+\varepsilon$, where SR is the logit-transformed sex ratio, MNT represents the development-weighted mean nest temperature sex ratio predictions, CTE represents the modified constant temperature equivalent sex ratio predictions and PM represents the development-weighted probability of masculinization, β_0 and β_1 are parameters to be estimated and ε is error. Short and long refer to the length of the thermosensitive period (equivalent development ages 6–8 and ages 6–11, respectively). All models were fitted using maximum likelihood estimation.

Explaining variation in sex ratio in natural nests

On 11–18 June 2016 and 17–24 June 2017, we surveyed for nesting turtles on the Sasajewun Dam (AWRS). We removed eggs from natural nests shortly after laying, numbering them as we removed them from the nest. Eggs were measured and weighed, then returned to their original nest cavity in approximately the order they were retrieved. We placed DS1921H iButtons (Maxim Integrated, San Jose, California, USA) in the centre of each nest to record hourly nest temperatures, and enclosed the nests in mesh cages to protect them from predators. In early September (2016) and mid-October (2017), we returned to retrieve a subsample of presumed late-stage eggs from the nests. The embryos were brought back to the University of Toronto to be incubated until hatching, at which point they were euthanized by immediate decapitation and pithing. The embryos were subsequently sexed using macroscopic gonadal examination.

We applied the simplest model formulation that best explained variation in sex ratio from semi-natural nests in the previous experiment, in order to find new parameter estimates for natural nests from 2016 to 2017. Because multiple models were not being compared, we simply estimated how much variation in sex ratios could be explained among natural nests, and whether the parameter estimates were statistically significant.

RESULTS

The temperature–sex reaction norm

We collected sex ratios for 11 mean constant incubation temperatures: 29.18, 28.29, 27.50, 27.37, 26.50, 25.99, 24.55, 23.33, 21.50, 22.00 and 20.50°C (Fig. 2). Fluctuations in temperature were within 0.50°C for all treatments. The results of the constant incubation experiment are consistent with the pattern of TSD Type II previously described in *C. serpentina*, where females are produced at extreme constant incubation temperatures and males are produced in the middle range of temperatures (Fig. 2; Ewert et al., 1994; Yntema, 1976). We estimated the pivotal temperatures T_{P1} and T_{P2} as 21.9±0.129°C and 27.2±0.137°C (means±s.e.m.), respectively.

Of 246 eggs from 2016 to 2017, 192 survived and were sexed. Twenty eggs failed to develop at all (no embryo was found in the egg), or failed in early development. The remaining 34 embryos perished in early development due to an incubator malfunction.

Model selection and thermosensitive period experiment

Of 27 semi-natural nests with 12 eggs each, one nest had no survivors, while one had only four survivors; both were in the warm treatment group. The nest with no survivors was removed from analyses. Otherwise, the nest survivorship ranged from 7 to 12 hatchlings with a mean of 10.2. As expected, our experimental design resulted in a large range of temperature variation, as the mean nest temperature experienced by semi-natural nests during the short TSP window ranged from 23.4 to 30.5°C, while over the long TSP window the range was 18.4–31.0°C. The mean duration of the short TSP window for all treatments was 7.5 days, while the longer window lasted for a mean of 22.9 days. Sex ratios in the semi-natural nests varied from 0% to 72.7% male.

Model selection revealed that the PM method using both the long and short windows of the TSP had strongest support (w_i=0.49 and 0.43, respectively, Table 1). The long and short PM models explained 68.7% and 68.4% of the variation in sex

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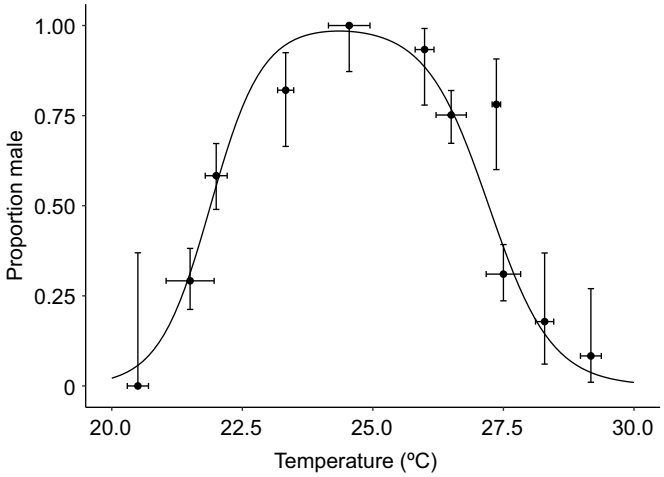

Fig. 2. Temperature–sex reaction norm for Algonquin Park snapping turtles. The pivotal temperatures are 21.89°C and 27.22°C. Error bars are 95% confidence intervals on each estimated sex ratio and standard deviations on each temperature. Total sample size=722 eggs.

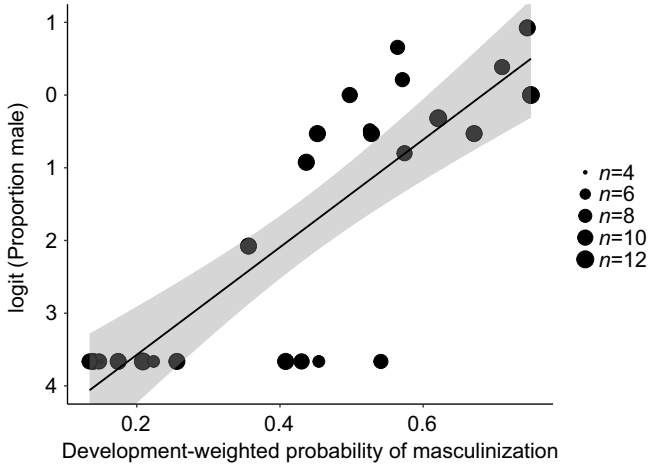

Fig. 3. Linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in semi-natural nests for the long TSP (*P*<0.05). Point size reflects sample size (*n*). Total number of eggs sexed=259.

ratios, respectively (Table 1; Fig. 3). The long and short modified CTE models explained 59.3% and 63.6% of the variation in sex ratios, respectively, and the long and short mean nest models explained 50.5% and 49.3% of the variation in sex ratios, respectively (Table 1). Parameter estimates for the long and short PM method are reported in Table 2.

Each of the three methods of sex ratio estimation (CTE, PM and mean nest) that have been compared so far have been nested within two different TSP lengths, and therefore the model rankings reflect not just the relative performance of methods, but also of the short and long TSP. A fairer comparison of the three methods per se would arise when each method appears only once in the model set. Therefore, we also compared model weights for the PM, modified CTE, and mean nest models using only the long TSP (i.e. three models are compared) and found that the PM had a substantially higher support (w_i=0.97) compared with the modified CTE (w_i=0.03). Similarly, when we compared the three models using only the short TSP, support for the PM method was also much higher (w_i=0.86) than that of the CTE (w_i=0.13).

Explaining variation in natural nests

In 2016 and 2017, we sampled a total of 14 natural nests with a range of 10–32 surviving eggs (mean=21.4). We sampled one additional nest in 2017 that was excluded from analyses because it had only three surviving eggs, and did not complete the full thermosensitive period before being removed from the ground, due to extraordinarily cool summer temperatures in 2017. Across the 14 usable nests, male sex ratios varied from 5.6% to 82.60%, representing a wide range of naturally produced sex ratios.

Given that the short PM and long PM models were indistinguishable (Table 1), we applied the short PM method (the simplest model) to the natural nest data from 2016 to 2017, and found that it was able to explain 67.1% of variation in sex ratios, with the development-weighted probability of masculinization exhibiting a significant relationship with the logit-transformed sex ratios (Table 3; Fig. 4).

Table 1. Model rankings of six candidate models predicting variation in sex ratio in semi-natural snapping turtle nests across two putative thermosensitive periods (TSPs)

Rank	Model formulation	*k*	ΔAICc	w_i	logLik	r^2
1	Probability of masculinization (long TSP)	3	0.00	0.49	−36.66	0.687
2	Probability of masculinization (short TSP)	3	0.25	0.43	−37.79	0.684
3	Constant temperature equivalent (short TSP)	3	3.97	0.07	−38.65	0.636
4	Constant temperature equivalent (long TSP)	3	6.83	0.02	−40.08	0.593
5	Mean nest (long TSP)	3	11.96	0.00	−42.64	0.505
6	Mean nest (short TSP)	3	12.57	0.00	−42.95	0.493

DISCUSSION

Organisms with TSD can have their sex ratios skewed towards one sex by a shift as low as 1°C: an alarming fact, considering global mean temperatures are expected to increase by at least 0.3 to 1.7°C in the next 100 years (Paukstis and Janzen, 1990; Trenberth and Josey, 2007). Although concern over climate change and sex ratio bias has been rising since the late 1980s (e.g. Davenport, 1989; Girondot et al., 2004; Hulin et al., 2009; Janzen, 1992, 1994; Mitchell et al., 2010, 2008; Mrosovsky and Provancha, 1992; Ospina-Álvarez and Piferrer, 2008), few methods have been reliably validated in the field, especially in species with Type II TSD. Despite this, the mean nest temperature (Santidrián Tomillo et al., 2014) and constant temperature equivalent (CTE) estimation are popularly used in sex ratio projections under climate change scenarios (Escobedo-Galván et al., 2016; Fuentes et al., 2009, 2010; Hays et al., 2003; Mitchell et al., 2008; Poloczanska et al., 2009; Stubbs et al., 2014; Telemeco et al., 2013; Chu et al., 2008). In addition, no existing method has been purported to predict sex

Table 2. Summary information for coefficient estimates of the probability of masculinization (long and short TSP) model for semi-natural nests with 95% upper and lower confidence intervals

TSP duration		Estimate	95% LCI	95% UCI
Long	Intercept	5.04	−6.00	−4.07
	Slope	7.41	5.41	9.40
Short	Intercept	−5.22	−6.24	−4.21
	Slope	6.38	4.65	8.20

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Table 3. Summary information for the PM (short TSP) model applied to natural nest data (model r^2=0.671)

	Estimate	s.e.m.	*t*	*P*
Intercept	–3.12	0.632	–4.93	<0.001
Slope	5.82	1.17	4.94	<0.001

ratios in natural nests of species with Type II TSD, such as the snapping turtle.

In the present study, we propose a new method of sex ratio estimation based on a clutch's development-weighted probability of masculinization (PM). To test the accuracy of the PM method, we compared it with the development-weighted mean nest temperature and a modified CTE method, and found through model selection that the PM method was supported as the best predictor of sex ratios in semi-natural nests of the snapping turtle – a species with Type II TSD. However, we found that the modified CTE method was also able to explain a respectable amount of variation in sex ratios in semi-natural nests, and that both the PM and CTE methods outperform the use of a mean nest temperature adjusted for daily development, as a predictor for sex ratios. Therefore, the present study presents a new method of estimating sex ratios in the wild, as well as one of the first thorough and quantitative tests of the CTE outside of a laboratory setting. Furthermore, we show that the PM method and modified CTE method are both appropriate for use in environments where seasonal changes in mean nest temperature and its variance are pronounced.

We propose that the PM method provides a stepping stone between use of a basic temperature statistic, such as the mean nest temperature, and a highly detailed physiological mechanistic approach (Delmas et al., 2007). The PM method acts as a dosage model for sexual differentiation, subsuming the complex temperature-mediated genetic, hormonal and epigenetic sex-determining cascade, by multiplying the observed probability of masculinization with the proportion of differentiation occurring at instantaneous time points during the TSP. However, despite the strong model support for the PM method, a third of the variation in sex ratios remains unexplained. Other studies of reptiles with TSD have found that sex is influenced by factors other than temperature, such as seasonal maternal yolk steroid allocation (Bowden et al., 2000), maternal age (McGaugh et al., 2011) or maternal genetics (Warner et al., 2008), as well as environmental factors – such as CO_2 – that are involved in respiration (Etchberger et al., 2002). Therefore, it seems unlikely that any quantitative method based solely on temperature can explain an overwhelming majority of variation in sex ratios among nests. We also note that a limitation of the PM method, in its current form, is that mean parameter estimates are used for the temperature–sex reaction norm and the thermal performance curve. These parameters are measured with error, but measurement error and error propagation was not incorporated into our methodology; similarly, for ethical reasons we sampled a subset of one-third to one-half of all eggs within each natural nest, rather than removing all the embryos, and therefore the sex ratio is estimated with error. Further development of the PM method could therefore include a Bayesian approach that incorporates parameter uncertainty at all levels of the study. Despite this, the PM method was supported as the best model for estimating sex ratios, and given the concept behind its design, we suggest it may be widely applicable to highly seasonal environments and all types of TSD.

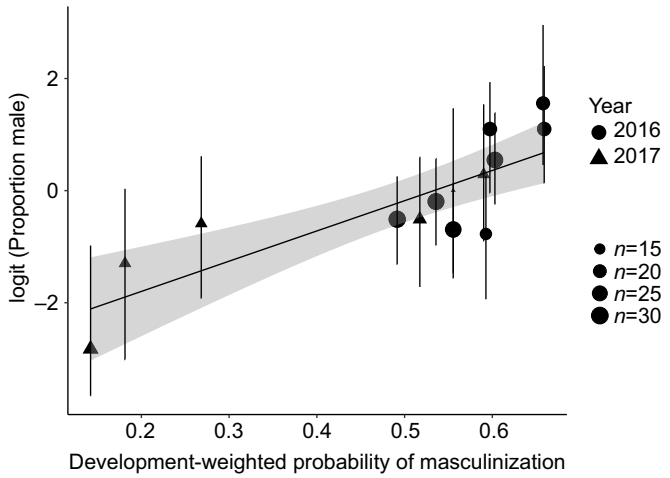

Fig. 4. Linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in natural nests from 2016 to 2017 (*P*<0.05). Point size reflects sample size (*n*). Total number of eggs sexed=299.

An additional criticism of the PM method is that it requires estimation of development rate and the temperature–sex reaction norm before it can be implemented. While this is true, the same is required to estimate the CTE. In fact, use of the PM method could be simplified by assuming that development rate is linear with respect to temperature, as in the classic CTE model, although simplification of thermal performance would presumably come at the cost of prediction accuracy (Rollinson et al., 2018). Nevertheless, development rate could be estimated from a degree–day model, as in Georges et al. (1994, 2005), and provided the relationship between temperature and development can be validated to ensure it is accurate, Eqn 2 could be applied.

In the present study, the modified CTE method also resulted in reasonable quantitative estimates of sex ratios for semi-natural nests of the snapping turtle. We suggest that calculating the CTE on a daily basis, and then weighting each daily CTE value by development to produce a final CTE value, are modifications to the method that overcome difficulties associated with heterogenous thermal variance and a non-stationary thermal mean, that otherwise pose challenges under the classic CTE approach (Georges, 1989; Georges et al., 1994). The modified CTE method used herein also appears to overcome the restriction of the CTE model to Type I TSD (Georges et al., 2004; Warner and Shine, 2011), as predictions were reasonable for our Type II study species. As we found in the PM method, the modified CTE method did not explain all of the variation in sex ratios, which is probably due to the aforementioned reasons for the PM method. Additionally, we question, as others have (Delmas et al., 2007), why the CTE method arbitrarily selects the median development-weighted nest temperature as a constant-temperature equivalent, as there is no mechanistic reason to assume this would yield an estimate equivalent to that of a constant-temperature scenario. It is possible that selecting a value different from the developmental median would yield more accurate results, although this would require further justification. Nevertheless, although the CTE method did not have the strongest model support, we conclude the modified CTE method developed herein provides a reasonable method for estimating sex ratios.

The exact timing and duration of the TSP can be difficult to pinpoint, as the range of anatomical stages it encapsulates varies with incubation temperature (Hewavisenthi and Parmenter, 2002; Yntema, 1979). The lack of certainty surrounding the thermosensitive period has previously been cited as a source of unexplained variation in sex ratio estimates (Girondot et al., 2010).

In our study, we compared the two extreme windows of the thermosensitive period described by Yntema (1979) for snapping turtles. Importantly, we used a nonlinear thermal performance curve for the Algonquin Park population of snapping turtles to estimate the development rate of clutches in the field (Rollinson et al., 2018), an improvement over using time as a proxy for development, and presumably more accurate than assuming development rate increases linearly with temperature (Georges et al., 2004). We could detect no statistical difference between the short TSP and long TSP using the PM method, although the short TSP performed better than the long TSP when using the modified CTE method. Interestingly, Yntema (1979) found that the longer TSP window occurs when incubation temperatures are held constant at 30°C (Yntema, 1979), and given that natural incubation temperatures in Algonquin Park typically average 21°C and rarely exceed 30°C, the longer TSP window may be less biologically relevant for the focal population. More broadly, delineating the thermosensitive period remains a significant problem that warrants further research, and ours is the first study that develops tools to explore how prediction accuracy of empirical models varies under different assumptions of TSP duration.

In conclusion, we emphasize that the PM method is a promising tool, as it is relatively accurate, and can accommodate various assumptions regarding TSP duration. The PM method may be especially useful for facilitating the monitoring or study of primary sex ratios for species at risk, for which non-lethal methods are necessary. Furthermore, if combined with projections of future climate change effects on soil temperature, it may be possible to adapt this method to make primary sex ratio predictions, informing conservation practices such as artificial incubation programmes. Likewise, by using historical soil temperatures to estimate primary sex ratios, researchers can look into the past and infer what a population's naturally occurring sex ratios were before a selected disturbance.

In addition to the insights into TSD and practical applications gleaned from the PM method, we also note some interesting implications of our findings for the Algonquin Park population of snapping turtles. Because of the nature of this population's Type II TSD, and the high thermal variance found in Algonquin Park, natural incubation temperatures frequently fluctuate across ranges of temperatures that are expected to produce mixed clutches. According to the PM model, a probability of masculinization value of only 0.17 is needed to produce males, and our results suggest clutches of 100% males are unlikely to occur in Algonquin Park. As a result, in our study population, mixed clutches are likely to occur as long as expected fluctuations in incubation temperature continue. Therefore, we suggest that this population of snapping turtles, unlike many sea turtle populations (e.g. Jensen et al., 2018), may show future resilience against biased sex ratios as the climate changes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.D.M., N.R., S.M.H., R.J.B.; Methodology: M.D.M., N.R., S.M.H., R.J.B.; Software: M.D.M., N.R.; Validation: M.D.M., N.R., S.M.H.; Formal analysis: M.D.M., N.R., S.M.H.; Investigation: M.D.M., N.R., S.M.H., R.J.B.; Resources: N.R., S.M.H.; Data curation: M.D.M.; Writing - original draft: M.D.M.; Writing - review & editing: M.D.M., N.R., S.M.H., R.J.B.; Visualization: M.D.M., S.M.H.; Supervision: N.R., R.J.B.; Project administration: N.R., R.J.B.; Funding acquisition: N.R.

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Table S1. Table of Yntema's (1968) stages of development for *Chelydra serpentina* and their corresponding Equivalent Development ages (weeks at 20°C, ED20), with the onset and end of the TSP noted. Modified from Rollinson et al. (2018

Yntema's Stage	Days of Incubation	ED20	TSP Status
0	0	0	
1	1	0.14	
2	2	0.29	
3	3	0.43	
4	4	0.57	
5	6	0.86	
6	7	1	
7	9	1.29	
8	12	1.71	
9	16	2.29	
10	20	2.86	
11	25	3.57	
12	30	4.29	
13	35	5	
14	42	6	TSP Begins
15	49	7	
16	56	8	End of Short TSP
17	63	9	
18	70	10	
19	77	11	End of Long TSP
20	84	12	
21	91	13	
22	98	14	
23	105	15	
24	119	17	
25	133	19	
26	140	20	

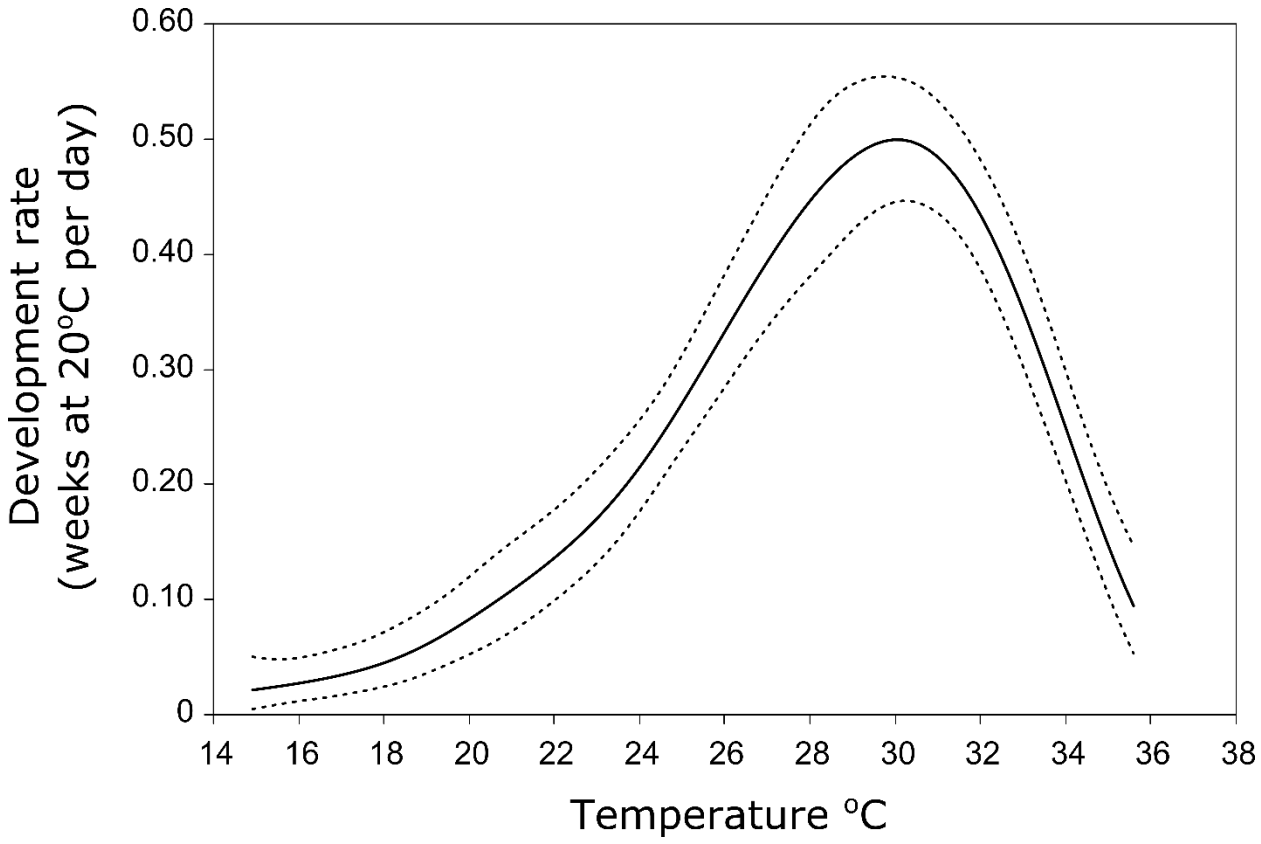

Figure S1. Thermal performance curve for embryonic development rate of snapping turtles from Algonquin Park. Development rate is expressed in Equivalent Development, which leverages the reference series of development stages for *Chelydra serpentina* embryos. The reference series, originally described at a constant incubation temperature of 20°C, maps each distinct developmental stage onto embryonic age (in weeks) at 20ºC (Table 1). By extension, an embryo taken from any given incubation environment, once staged, can be assigned an equivalent age at 20ºC. The performance curve was estimated in the lab across a series of constant temperatures, by observing the amount of development that occurred over a given period of time at a given temperature, then expressing the amount of development that occurred in terms the amount expected at 20°C. Modified from Rollinson et al. (2018).