

Measurement and Modelling of Primary Sex Ratios for Species with Temperature- Dependent Sex Determination

by
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Abstract

For many oviparous animals, incubation temperature influences primary sex ratios through temperature-dependent sex determination (TSD). Currently, few reliable methods are available to estimate sex ratios under natural incubation conditions using a temperature proxy. This study proposes a new, semi-mechanistic approach for estimating sex ratios under TSD in natural nests, based on a nest's probability of masculinization (PM). I test the PM approach against existing approaches using two experiments in snapping turtles (*Chelydra serpentina*) from Algonquin Park. I show that the PM method is overwhelmingly supported as the best method for estimating primary sex ratios, and is effective under a wide range of thermal conditions. My findings also suggest that the PM approach is universally applicable to all types of TSD. Finally, my data suggest that the Algonquin Park population of snapping turtles possesses resilience to climate-driven biased sex ratios.

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Table of Contents

Acknowledgements	iii
Table of Contents.....	v
List of Tables	vi
List of Figures.....	vii
1 Introduction.....	1
1.1 Temperature-dependent sex determination	2
1.2 TSD in a changing climate.....	4
1.3 Approaches to estimating sex ratios under TSD.....	5
1.4 The probability of masculinization method.....	7
2 Methods and Materials.....	9
2.1 Study population and history	9
2.2 Estimating the temperature-sex reaction norm.....	9
2.3 Model selection and the thermosensitive period.....	12
2.4 Explaining sex ratio variation in natural nests.....	20
2.5 Animal care protocol	20
3 Results.....	21
3.1 The temperature-sex reaction norm.....	21
3.2 Model selection and thermosensitive period.....	22
3.3 Explaining sex ratio variation in natural nests.....	25
4 Discussion	29
4.1 Summary of findings	29
4.2 Criticisms of the PM method and other sources of variation in sex ratio.....	30
4.3 Evaluation of the modified CTE.....	32
4.4 Summary of the modified mean nest temperature approach	33
4.5 The thermosensitive period.....	33
4.6 Applications of the PM method.....	34
4.7 Additional conclusions	35
References.....	37

List of Tables

Table 1. Table of Yntema's (1968) stages of development for <i>Chelydra serpentina</i> 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).....	16
Table 2. Model rankings of six candidate models predicting variation in sex ratio in semi-natural snapping turtle nests across two putative thermosensitive periods (TSP).....	23
Table 3. 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.....	25
Table 4. Summary information for the PM (Short TSP) model applied to natural nest data (model $r^2 = 0.671$).	28

List of Figures

- Figure 1.** Patterns of TSD at constant incubation temperatures. In Type IA TSD (left), males are produced at low temperatures, and females are produced at high temperatures. In Type II (right) TSD, males are produced at intermediate temperatures, and females are produced at high and low temperatures. Arrows denote the pivotal temperature (T_{piv}) at which the nests' sex ratio is 50%; note that Type II TSD has two pivotal temperatures. Grey blocks denote the transitional range of temperatures across which mixed-sex nests are produced..... 3
- Figure 2.** Macroscopy of female (A) and male (B) late-stage snapping turtle embryo gonads and accessory organs. Females have long, thin gonads (i) and continuous oviducts (ii). Males have short, stocky gonads (iii) and lack oviducts. Mesonephros (m) are also noted. 11
- Figure 3.** Diagram illustrating the layout of semi-natural nests. Each semi-natural nest is composed of 12 eggs on a 3×4 horizontal plane. I divided 27 semi-natural nests into three replicate groups (each with a shaded [blue], natural [orange], and warm [red] treatment) composed of 9 semi-natural nests each. Pictured is a single replicate group with three semi-natural nests at depths of 10cm, 15cm, and 20cm (9 nests total). Ultimately, this replicate group was replicated 3 times, making 27 semi-natural nests..... 13
- Figure 4.** 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 20°C (Table 1). By extension, an embryo taken from any given incubation environment, once staged, can be assigned an equivalent age at 20°C. 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). 15

Figure 5. The 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.	21
Figure 6. The linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in semi-natural nests. Point size reflects sample size (weight).	24
Figure 7. The linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in natural nests from 2016-2017. Point size reflects sample size (n).	27

1 Introduction

The embryo is a critical stage of life-history. During development, the evolutionary blueprints for the body's form and function are effectuated, and organisms begin travelling along their corporeal trajectories. The embryonic stage of an organism's life, however, is also one of the the most vulnerable to environmental perturbations; critical processes can easily be disrupted, causing changes in adult phenotype, deformity, or mortality (Lourdais et al., 2004; Feretti et al., 2006; Ji et al, 2006). Of contemporary interest to biologists, especially in the context of global climate change, is how the thermal environment of embryos can affect developmental outcomes.

During development, the temperatures experienced by embryos can exert profound effects on individuals, both during and beyond the embryonic life-history stage. Factors affecting embryo fitness, such as hatching success and growth rate, are directly influenced by temperature (Deeming & Ferguson, 1991; Lourens et al. 2005); cascading effects of the embryonic thermal regime can later influence adult morphology, behavior, and sex (Janzen & Paukstis, 1991; Shine, Elphick, & Harlow, 1997, Downes & Shine, 1999; DuRant et al., 2010). And unlike placental mammals, whose embryonic relationship to external temperatures is buffered by their maternal environment, oviparous animals are directly exposed to external conditions, and are thus more vulnerable to the effects of variable thermal regimes. Furthermore, larger eggs generally experience longer incubation times, increasing their exposure to environmental fluctuations (Deeming & Ferguson, 1991). This is especially true for oviparous amniotes, when contrasted with rapidly-developing amphibians and fishes (Iverson & Ewert, 1991; Gillooly et al., 2002). This study is concerned with the effects of the embryonic thermal environment on the phenotype of oviparous amniotes: specifically, their sex determination.

1.1 Temperature-dependent sex determination

Of the processes governed by temperature during development, one that has garnered much contemporary interest in the scientific community is temperature-dependent sex determination (TSD). Many reptiles exhibit TSD, in which sex is determined during embryonic development and is influenced by temperature. Since the discovery of TSD in 1966 by Madeleine Charnier in the Rainbow Agama (*Agama agama*), TSD has been found to occur in all crocodilians, most turtles, some lizards, and recently, fishes (Bergeron et al., 1999; Pieau et al., 1999; Ospina-Alvarez et al., 2008).

During a critical period of embryonic development known as the thermosensitive period (TSP), these organisms undergo gonadal morphogenesis, conferring gender through temperature-mediated physiological changes (Bull, 1987). The TSD sex-determining cascade is thought to begin when temperature-sensitive molecules, whose identities are currently unknown, respond to temperature at the onset of the thermosensitive period, and influence the release of sex-specific genes and hormones (Shoemaker and Crews 2009). Recently, the histone modifier KDM6B has been shown to influence the male-producing pathway, showing that epigenetic mechanisms also factor into sex determination under TSD (Ge et al. 2018). In the male pathway, genes downstream of the temperature-sensing molecule(s) are upregulated in response to male-producing temperatures; the male-determining factors *Sox9* and *Dmrt1* have been shown to respond to temperature through complex regulatory mechanisms, and both are necessary and sufficient to induce maleness in reptile embryos (Sekido and Lovell-Badge 2008; Ge et al. 2017). Likewise, in the female pathway, male-determining factors are downregulated in response to female-producing temperatures, and other female-associated factors such as *Foxl2* (Rhen et al. 2007) become expressed, though much less is currently known about how females gonads are produced

(Shoemaker and Crews 2009). Interestingly, the male-associated and female-associated factors involved in TSD sex-determining pathways are deeply evolutionarily conserved, and also play roles in genetic sex determination in vertebrates (Sreenivasulu et al. 2002; Loffler et al. 2003).

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 (Fig. 1) (Ewert et al. 1994; Cree et al. 1995). 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; Lang and Andrews 1994; Harlow and Taylor 2000). 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 (Fig. 1).

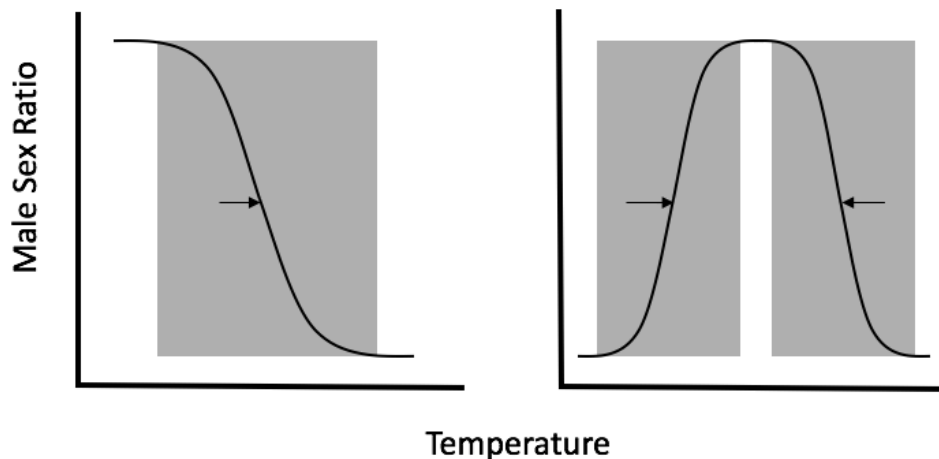


Figure 1. Patterns of TSD at constant incubation temperatures. In Type IA TSD (left), males are produced at low temperatures, and females are produced at high temperatures. In Type II (right) TSD, males are produced at intermediate temperatures, and females are produced at high and low temperatures. Arrows denote the pivotal temperature (T_{piv}) at which the nests' sex ratio is 50%; note that Type II TSD has two pivotal temperatures. Grey blocks denote the transitional range of temperatures across which mixed-sex nests are produced.

1.2 TSD in a changing climate

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 (*Chelonia mydas*) from the Great Barrier Reef have already undergone near-complete feminization over the past 20 years (Jensen et al. 2018). Warming over the past 25 years has increased production of females in loggerhead sea turtles (*Caretta caretta*) from North Carolina (Reneker and Kamel 2016), and, interestingly, a population of red-eared sliders (*Trachemys scripta*) in Illinois has become increasingly male-biased due to climate-induced phenological shifts (Tucker et al. 2008). Oviparous reptiles with TSD have thus been likened to the “canaries in the coal mine” for the biological impacts of rapid climate change (Mitchell and Janzen, 2010). 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. Although 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, Hotelling, & Franks, 1983; Yntema, 1978; Yntema, 1976), wild nests are never under constant stationary temperature; therefore, experimental results may poorly reflect real-life situations (Schwarzkopf and Brooks 1985; Georges et al. 1994; 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 (Bull 1985; Georges 1992; Georges et al. 1994; Janzen 1994; Shine et al. 1997; Demuth 2001).

1.3 Approaches to estimating sex ratios under TSD

Currently, there is no non-invasive method available for determining offspring sex in species with TSD. As juveniles do not possess dimorphic characters, most methods of precisely determining sexual phenotype are invasive, requiring observation of gonads through dissection (Yntema 1981), histology (Maffucci et al. 2013), or laparoscopy (Wood et al. 1983). Blood assays can also be used to determine juvenile sex (Owens et al. 1978; Allen et al. 2015; Jensen et al. 2018). However, all of these methods are labor-intensive, and require either sacrifice or invasive sampling of individuals. Therefore, there is great need for non-invasive proxy estimations of sex ratios in species with TSD, especially in species that are already imperiled. Furthermore, reliable proxies for estimating sex ratios can also be leveraged to predict sex ratios under future climate change scenarios.

Naturally, temperature is the most common proxy used to estimate sex ratios in species with TSD. The most basic temperature statistic used is the mean nest temperature, which translates the mean incubation temperature into a sex ratio (Bull 1980). A more advanced temperature statistic is the constant temperature equivalent (CTE), which takes into account the developmental leverages of different temperatures (Georges et al. 2004; Georges 1989). The mean nest temperature is generally considered an unreliable proxy when nests experience thermal variance (Bull 1985; Schwarzkopf and Brooks 1985; Georges et al. 1994), largely because development rate of an embryo varies non-linearly with temperature (Gillooly et al., 2002). For example, from the perspective of a developing embryo, one hour spent at low temperatures results in less anatomical differentiation than one hour at a high temperature, 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, Paitz, & Bowden, 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 heterogenous thermal variance and a changing thermal mean (Telemeco et al. 2013; Carter et al. 2018). 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 20 natural nests. Therefore, the ability of the CTE to quantitatively predict sex ratios in natural nests is 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 during which 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 from 33-66% of development (Norris & Lopez, 2010; Stubbs et al., 2014; Yntema & 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).

1.4 The probability of masculinization method

In this thesis, I develop and evaluate a new tool for the temperature-based prediction of sex ratios in a species with Type II TSD, called the probability of masculinization (PM) method. I compare my approach with the two most commonly used methods in the field: a mean nest temperature method, and a CTE approach. Importantly, I 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. The PM approach for predicting the sex ratios of oviparous reptiles with TSD does not represent a mechanistic model, but it is rooted in mechanistic theory. 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 effect on sex seems to rely on the potency of a given temperature (Bull et al. 1990; Wibbels et al. 1998,1991; Crews et al. 1991). If we assume that temperature indeed exerts a dosage effect on sex determination, we expect male sex ratio and exposure to temperatures which mediate the masculinizing cascade to exhibit a degree of proportionality. Specifically, I 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. My approach is intended to be universally applicable to species with both types of TSD, and accurately predict sex ratios even with non-stationary thermal means and heterogeneous thermal variation during incubation.

I first determine the temperature-sex ratio reaction norm at constant incubation temperatures for a population of snapping turtles from Algonquin Park. I then manipulate nests to determine which method of sex ratio prediction (mean nest, CTE, or PM) better explains variation in sex ratios, all while allowing for the possibility of a short or a long thermosensitive period. Lastly, I 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.

2 Methods and Materials

2.1 Study population and history

The present study is part of a long-term research program on snapping turtles. The study was spearheaded by Dr. Ronald J. Brooks and Dr. Martyn Obbard (University of Guelph) in 1972, and is centred around a manmade dam running across Lake Sasajewun, at the Algonquin Wildlife Research Station (AWRS; Algonquin Provincial Park, ON, Canada, 45°35' N, 78°31' W). The gravel-covered slopes of the dam are popular with Snapping Turtles, who travel up to 8.0 km to nest there during the June nesting season (Obbard and Brooks, 1980). As snapping turtles generally exhibit nest site fidelity (Obbard and Brooks, 1980; Congdon, 1987), the long-term study has been able to identify multiple clutches from the same mother through the decades.

At nearly a half-century long, the long-term study has accumulated a wealth of knowledge on the population at large, and specifically on sex ratios and incubation temperatures experienced by nests, presenting a rare opportunity to put to use decades of collected data. In addition to this, the extreme Northern location and pristine environment experienced by the Algonquin snapping turtles make them a desirable study population for the effects of climate change in fringe populations.

2.2 Estimating the temperature-sex reaction norm

In June 2016 and 2017, I collected snapping turtle eggs from clutches with known maternity on the Lake Sasajewun Dam within 12hrs of being laid. I 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 six

days of being laid. In 2016, I collected 9 clutches totaling 405 eggs, which I randomly divided into six treatment groups at 23, 25, 26, 27, 28, and $29 \pm 0.5^{\circ}\text{C}$. The 23°C treatment was incubated in a commercial refrigerator outfitted with a thermostat (Learn to Brew LLC, Moore, OK) and a fan for circulation; the other incubators were Reptibator incubators (ZooMed, San Luis Obispo, CA) with retrofitted circulation fans. In 2017, I 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). Within an incubator, all eggs were placed at the same level (*i.e.*, were the same distance from the heating or cooling element). Eggs were buried in moist vermiculite, and the top-half of each egg was left exposed, in order to check for eggs that failed to develop. Water was sprayed onto the eggs and substrate every other day to qualitatively maintain moisture levels as needed, such that vermiculite was moist but not saturated with water. Embryos were developed until they reached late-term (Yntema stages 23-25) before sexing.

Additional sex ratio data were available from a previous experiment, and these data were also used to help estimate the shape of the temperature-sex reaction norm. Passmore & Brooks (unpubl.) collected 663 eggs from the Algonquin Park population of 18 snapping turtles in June of 1991. After being removed from the nest, they were marked with a graphite pencil to identify the clutch of origin, and kept at $<20^{\circ}\text{C}$ 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) set at 21.5°C , 22.0°C , 26.5°C , and 27.5°C . Within each incubator, eggs were separated into boxes of approximately 40 eggs, in moist vermiculite. To estimate water loss, individual boxes were weighed to the nearest 0.1g 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., Taft Cambridge England) and kept within 0.5°C of the target temperatures. Turtles were euthanized just after hatching and sexed macroscopically, as described below.

I examined gonads of late stage embryos macroscopically to determine sex. Macroscopic gonadal examination is commonly used, and accurate when compared with histological examination (Yntema 1960; St. Juliana et al. 2004; Cotter and Sheil 2014; Spencer and Janzen 2014). To do so, I assessed sex based on two characters: the appearance of the oviducts, and the appearance of the gonads. Females have long, thin gonads, and continuous, thick oviducts; males have short, stocky gonads, and lack oviducts, or possess degenerate oviducts (Fig. 2) (S. Holt, pers. comm.; Yntema, 1960).

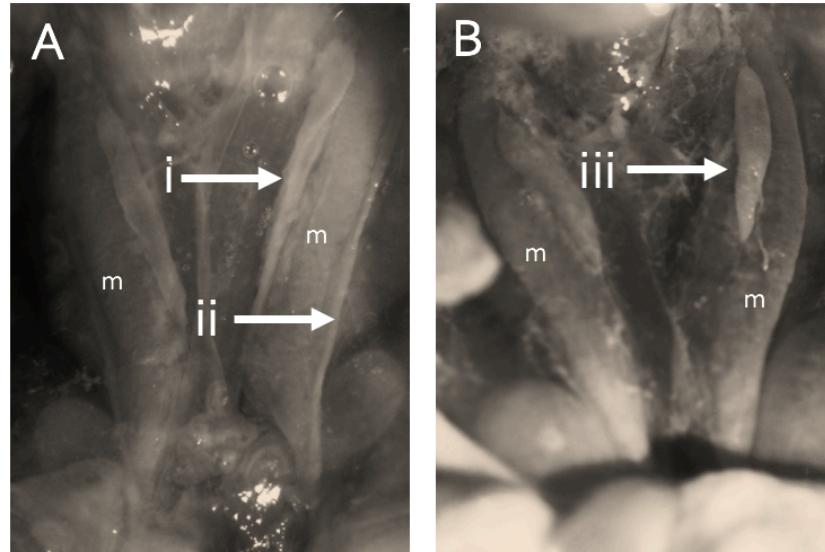


Figure 2. Macroscopy of female (A) and male (B) late-stage snapping turtle embryo gonads and accessory organs. Females have long, thin gonads (i) and continuous oviducts (ii). Males have short, stocky gonads (iii) and lack oviducts. Mesonephros (m) are also noted.

Occasionally (in 5/192 cases; <3%), I found individuals with a mix of these characters: for example, a long, continuous oviduct on one side of the body or a long, feminine-appearing gonad with patchy oviducts. I 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), I later treated them as males.

I took the average incubation temperature as the constant temperature for each treatment. I then used the *nls* package in the R environment (R Development Core Team, 2016) to find the least-squares parameter estimate for a double-logistic equation, a bell-shaped function used to model the temperature-sex reaction norm (Godfrey et al. 2003). The double-logistic function is described by the following equation:

$$Sex\ Ratio = \frac{1}{1 + e^{\left(\frac{1}{S1} \times (P1 - t)\right)}} \times \frac{1}{1 + e^{\left(\frac{1}{S2} \times (P2 - t)\right)}}$$

Where parameters *P1* and *P2* are the lower and upper pivotal temperatures, *S1* and *S2* are curvature parameters for either side of the function, and *t* is the independent temperature variable. Each treatment produced one sex ratio that was based on *n* embryos, and therefore each data point was weighted by *n*.

2.3 Model selection and the thermosensitive period

In order to compare which method of sex ratio prediction best explains variation in sex ratios in natural nests, as well as which window of the thermosensitive period (long vs short, following

Yntema, 1979) is most appropriate for estimating sex ratios, I used data from a 2001 experiment conducted by Sarah Holt. A key aim of this 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. Sarah Holt collected 324 eggs from 11 female snapping turtles nesting on the Sasajewun Dam (AWRS); all clutches were laid within 6h of 01:00 hrs on June 11th, 2001. The eggs were brought back to the lab and kept at approximately 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. 3).

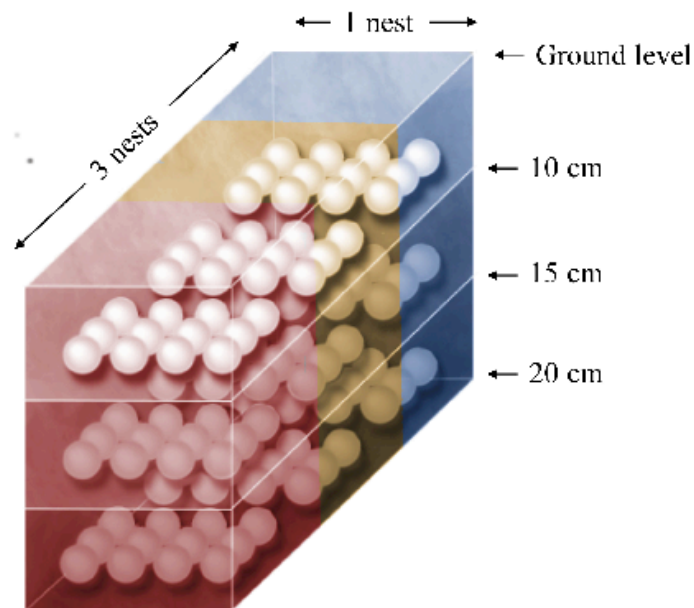


Figure 3. Diagram illustrating the layout of semi-natural nests. Each semi-natural nest is composed of 12 eggs on a 3×4 horizontal plane. I divided 27 semi-natural nests into three replicate groups (each with a shaded [blue], natural [orange], and warm [red] treatment) composed of 9 semi-natural nests each. Pictured is a single replicate group with three semi-natural nests at depths of 10cm, 15cm, and 20cm (9 nests total). Ultimately, this replicate group was replicated 3 times, making 27 semi-natural nests.

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 an 30cm x 30cm clear plastic polypropylene tarp that was elevated 30cm off the ground, which emulated a greenhouse and produced a warming effect. Within each temperature treatment, three nests were buried at a depth of 10cm, three were buried at a depth of 15cm, and three were buried at a depth of 20cm, in order to vary temperature fluctuations between nests. This design minimized the variation between temperatures for eggs within a nest, but maximized variation in temperature across nests. Each semi-natural nest had a temperature logger programmed to record hourly temperatures. Eggs were allowed to incubate without interference, until approximately one week before hatching, when nests were excavated and placed in a 25°C incubator. All hatchlings were sexed macroscopically (Yntema 1976).

Using the nest temperature data from this experiment, I predicted when embryos in each nest had entered the thermosensitive period. To do so, I leveraged the incubation temperature profile specific to each semi-natural nest, in conjunction with a thermal performance curve for embryonic development specific to the focal population of *C. serpentina* (Fig. 4; Rollinson et al., 2018). The thermal performance curve is expressed in Equivalent Development units (Webb, Choquenot, & Whitehead, 1986; Webb et al., 1983), which allows estimation of current Yntema stage based on the temperatures to which the embryo was previously exposed (Table 1).

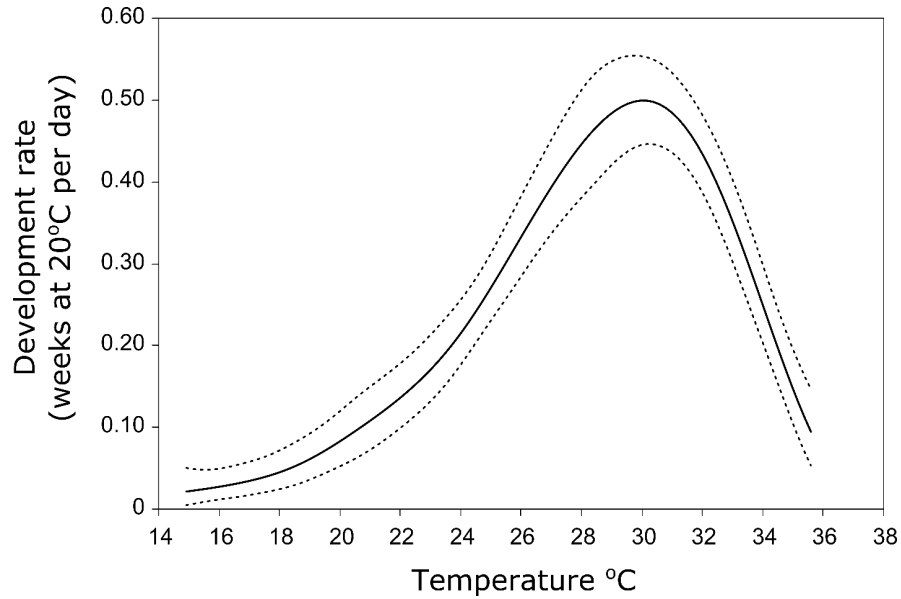


Figure 4. Thermal performance curve for embryonic development rate of snapping turtles from Algonquin Park. Development rate is expressed in Equivalent Development, which leverages the reference series of development stages for *Chelydra serpentina* embryos. The reference series, originally described at a constant incubation temperature of 20°C, maps each distinct developmental stage onto embryonic age (in weeks) at 20°C (Table 1). By extension, an embryo taken from any given incubation environment, once staged, can be assigned an equivalent age at 20°C. 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. Confidence intervals are represented by dotted lines. Modified from Rollinson et al. (2018).

Table 1. 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	

Given that onset of the TSP in *C. serpentina* occurs at Yntema stage 14 (Yntema 1979), I 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, in order to estimate when Yntema stage 14 was reached (Rollinson et al. 2018). A similar method of

development summation was first developed by Georges (2005), and has been found to be more accurate at delineating the TSP than an estimate using only the middle-third of development (Girondot et al. 2018). For the period of time between egg laying and egg reburial, I assumed a temperature of 18°C, the average temperature of the field laboratory the eggs were housed in, to estimate developmental increments. Using the method above, I 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.

Using the thermal profile experienced during the estimated TSP, I 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, I 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. I then found the average CTE value for the entirety of the CTE, with each day being weighted by the development occurring in that day. A similar approach was taken by Doody et al. (2004), though I modified their approach by weighing each day by development. 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, I 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 my calculation of the CTE.

For the PM method, I calculated the amount of development the 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, I multiplied the two values together. I 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 Equation 2:

$$PM = \sum_{i=0}^n (D_i \times PM_i) / \sum_{i=0}^n (D_i)$$

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).

I 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, I tested whether a short TSP or a long TSP better predicted sex ratios. This resulted in six candidate models, which took the form:

1. $SR = \beta_0 + \beta_1 MN_{\text{short}} + \varepsilon$
2. $SR = \beta_0 + \beta_1 MN_{\text{long}} + \varepsilon$
3. $SR = \beta_0 + \beta_1 CTE_{\text{short}} + \varepsilon$
4. $SR = \beta_0 + \beta_1 CTE_{\text{long}} + \varepsilon$
5. $SR = \beta_0 + \beta_1 PM_{\text{short}} + \varepsilon$
6. $SR = \beta_0 + \beta_1 PM_{\text{long}} + \varepsilon$

Where SR is the logit-transformed sex ratio, where MN 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 fit using maximum likelihood estimation.

2.4 Explaining sex ratio variation in natural nests

Between June 11th-18th, 2016 and June 17th-24th, 2017, I surveyed for nesting turtles on the Sasajewun Dam. I removed eggs from natural nests immediately after turtles had finished laying, numbering them as I removed them from the nest. Eggs were measured and weighed, then returned to their original nest cavity in approximately the order they were retrieved. I placed iButtons (DS1921H) in the center 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) I 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.

I 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 2016-2017. Because multiple models were not being compared, I simply estimated how much variation in sex ratios could be explained among natural nests, and whether the parameter estimates were statistically significant.

2.5 Animal care protocol

Animal use was approved by the University of Toronto Local Animal Care Committee (Protocol # 20011948).

3 Results

3.1 The temperature-sex reaction norm

In total, I collected sex ratios for 11 constant incubation temperatures: 29.18, 28.29, 27.50, 27.37, 26.50, 25.99, 24.55, 23.33, 22.00, 21.50, and 20.50°C. Six of these sex ratios were collected in 2016, one was collected in 2017, and the remaining four were taken from Passmore & Brooks (unpubl.). 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 II previously described in *C. serpentina*, in which females are produced at extreme constant incubation temperatures and males are produced in the middle range of temperatures (Fig. 5; M. A. Ewert, Lang, & Nelson, 2005; C. L. Yntema, 1976). I estimated the pivotal temperatures P1 and P2 as $21.9 \pm 0.129^\circ\text{C}$ and $27.2 \pm 0.137^\circ\text{C}$.

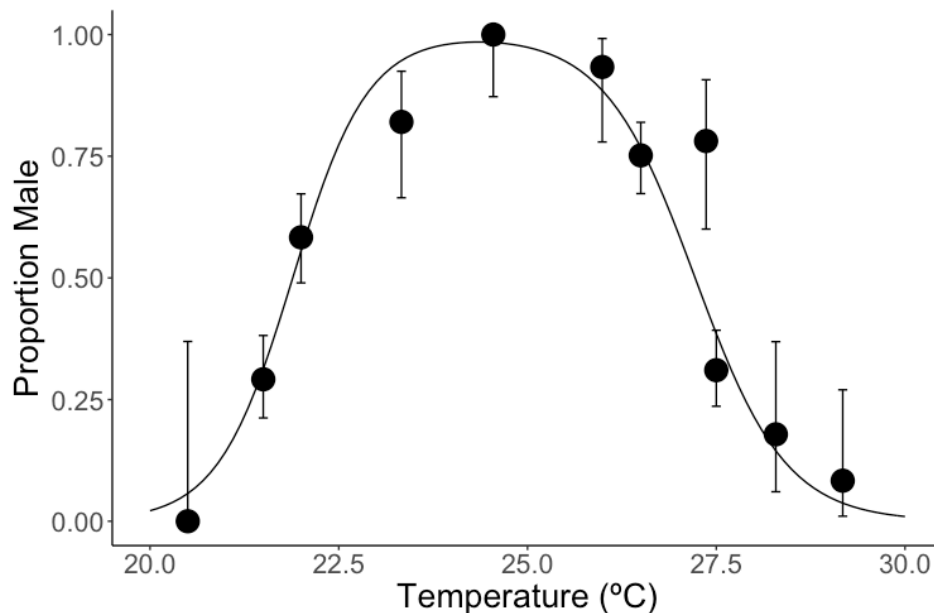


Figure 5. The temperature-sex reaction norm for Algonquin Park snapping turtles. The pivotal temperatures are 21.89°C and 27.22°C. Error bars are 95% confidence intervals on each estimated sex ratio.

Of 234 eggs, 180 survived and were sexed. Twenty eggs failed to develop at all, or failed in early development (no embryo was found in the egg). Further, a group of 34 embryos in a 24°C treatment died due to an incubator malfunction.

3.2 Model selection and thermosensitive period

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 7-12 hatchling with a mean of 10.2. As expected, the experimental design resulted in a large range of temperature variation, as the mean nest temperature experienced by semi-natural natural nests during the short TSP window ranged from 23.4 - 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 the both the long and short windows of the TSP had strongest support ($w_i = 0.49$ and 0.43 , respectively, Table 2). The long and short PM models explained 68.7% and 68.4% of the variation in sex ratios, respectively (Fig. 6, Table 2). 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 2). The parameter estimates for the long and short PM methods in semi-natural nests are shown in Table 3.

Table 2. Model rankings of six candidate models predicting variation in sex ratio in semi-natural snapping turtle nests across two putative thermosensitive periods (TSP).

Rank	Model Formulation	k	$\Delta AICc$	w_i	LogLik	R²
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

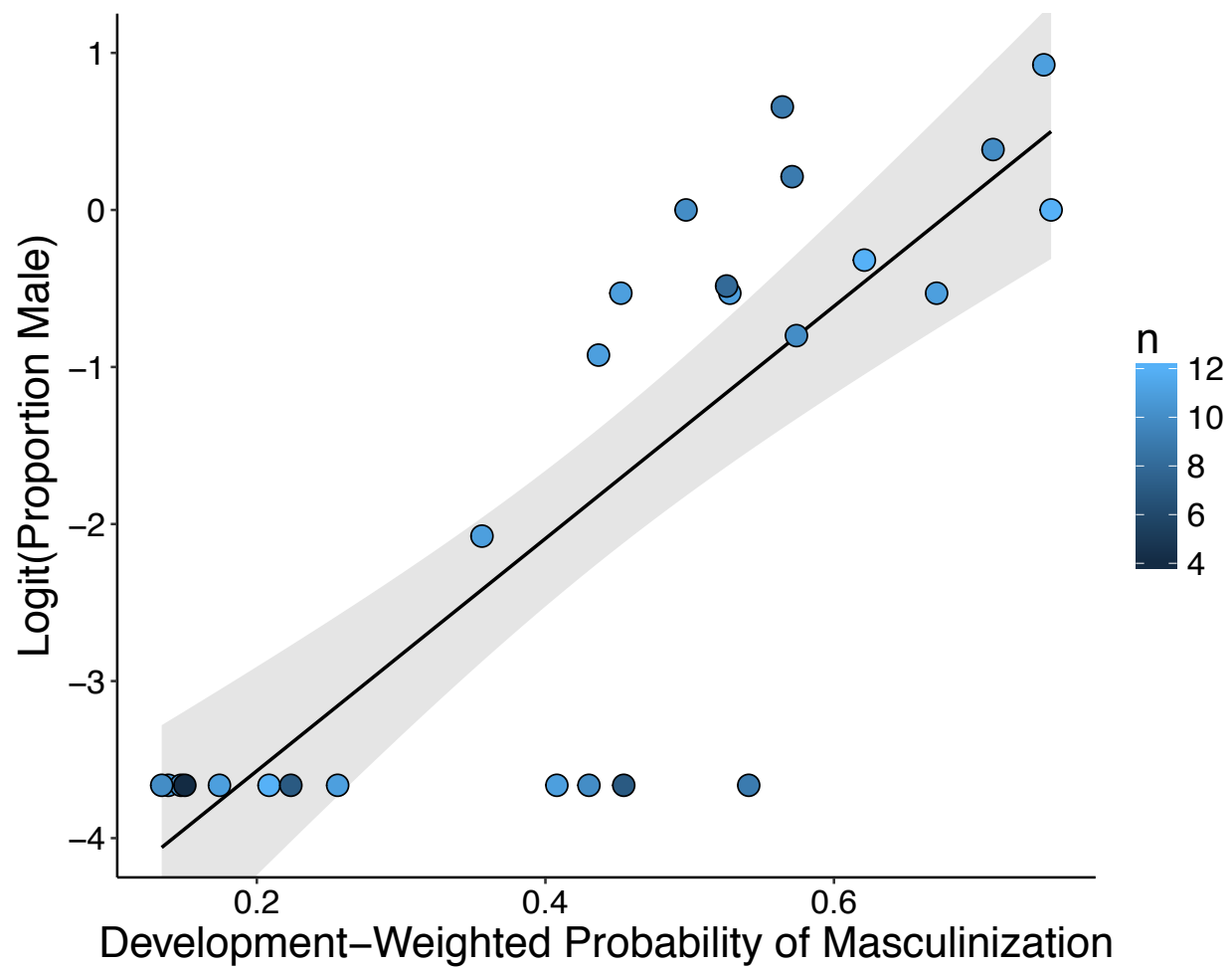


Figure 6. The linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in semi-natural nests. Point size reflects sample size (weight).

Table 3. 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. These parameters describe the linear relationship between the probability of masculinization statistic and actual sex ratios.

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

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, I 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 to the modified CTE ($w_i=0.03$). Similarly, when I 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 ($w_i=0.13$).

3.3 Explaining sex ratio variation in natural nests

In 2016 and 2017, I sampled a total of 14 natural nests ranging from 10-32 surviving eggs (mean=21.4). I 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), I applied the short PM method (the simplest model, as it requires less temporal thermal data) to natural nest sex ratios from 2016-2017, 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 (Fig. 7, Table 4).

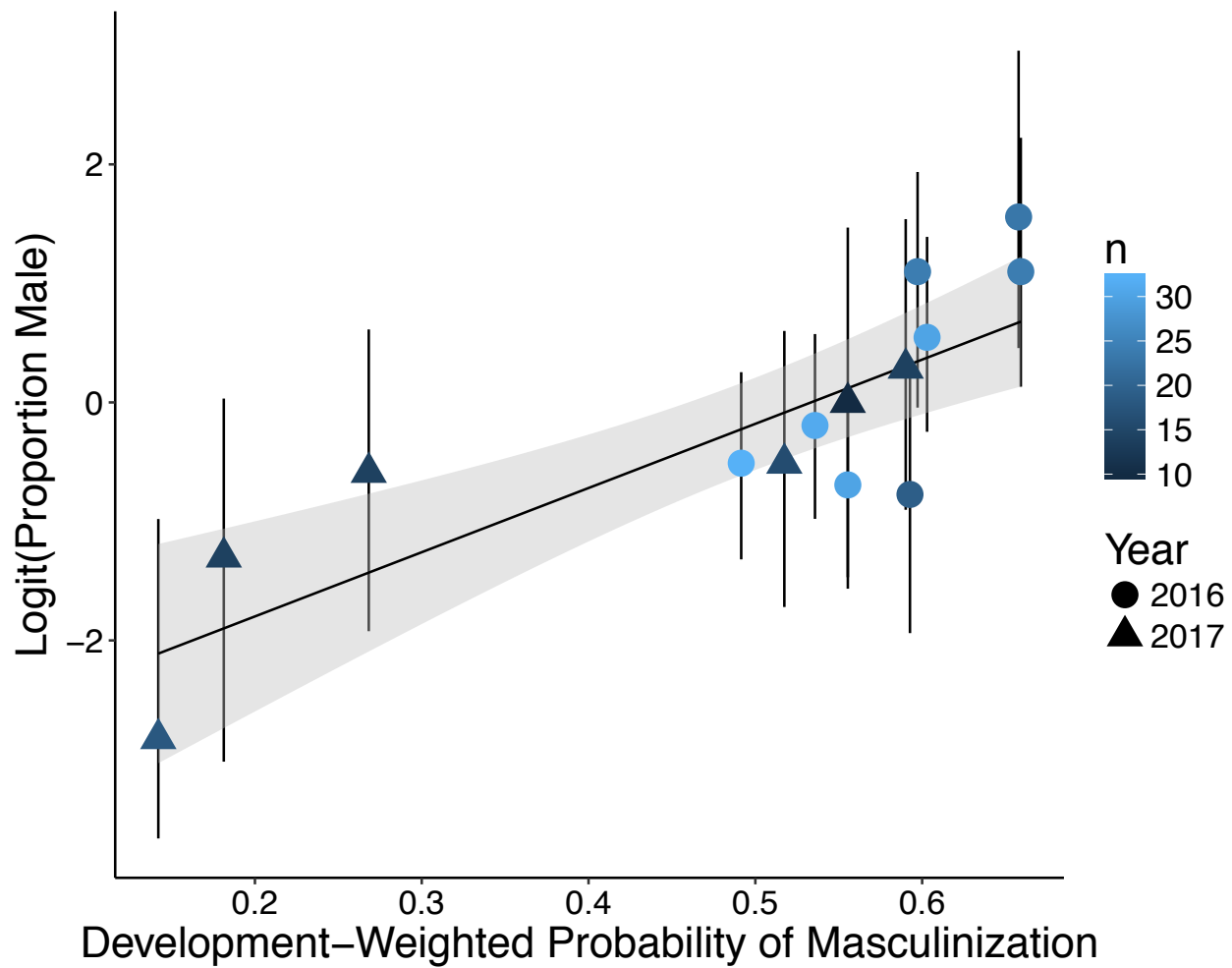


Figure 7. The linear relationship between the development-weighted probability of masculinization (PM) and the logit-transformed sex ratios found in natural nests from 2016-2017. Point size reflects sample size (n).

Table 4. Summary information for the PM (Short TSP) model applied to natural nest data (model $r^2 = 0.671$).

	Estimate	SE	t	P
Intercept	-3.12	0.632	-4.93	<0.001
Slope	5.82	1.17	4.94	<0.001

4 Discussion

4.1 Summary of findings

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-1980's (*e.g.*, Davenport, 1989; Girondot, Delmas, Prevot-Julliard, & Godfrey, 2004; Hulin et al., 2009; Janzen, 1992, 1994; Mitchell et al., 2010; Mitchell et al., 2008; Mrosovsky & Provancha, 1991; Ospina-Álvarez & 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, López-Luna, & Cupul-Magaña, 2016; Fuentes et al., 2009; Fuentes et al., 2010; Hays, Broderick, Glen, & Godley, 2003; Mitchell et al., 2008; Poloczanska, Limpus, & Hays, 2009; Stubbs et al., 2014; Telemeco et al., 2013; The Chu, Booth, & Limpus, 2008). Further, no existing method has been purported to predict sex ratios in natural nests of species with Type II TSD, such as the snapping turtle.

In the present study, I 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, I compared it to 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, I found that the modified CTE method was also able to explain a respectable amount variation in

sex ratios in semi-natural nests, and that both the PM and CTE methods outperform the use of a mean nest temperature, even when 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, and also provides the first thorough and quantitative test of the CTE outside of a laboratory setting. Further, I 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.

I propose that the PM method provides a stepping stone between using 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 interactions between sex-determining genes and gene products in the sex-determining cascade, by multiplying the observed probability of masculinization with the proportion of differentiation occurring at instantaneous points in time during the TSP.

4.2 Criticisms of the PM method and other sources of variation in sex ratio

Despite the strong model support for the PM method, a third of the variation in sex ratios remains unexplained. Other studies in reptiles with TSD have found that sex is also influenced by many factors other than temperature. Painted turtles (*Chrysemys picta*) have been shown to increase their allocation of the feminizing yolk steroid oestradiol, and decrease their allocation of the masculinizing yolk steroid testosterone as the nesting season progresses, modifying the sex ratios produced at constant temperatures (Bowden et al. 2000). Additionally, in *C. picta*, older females produce a higher proportion of daughters, representing an maternal ontogenetic factor that affects

the outcome of sex (McGaugh et al. 2011). As variation in sex ratio production at constant temperature among individuals exists, even within a population, there is also a strong genetic component affecting the outcome of sex (Warner et al. 2008; Rhen et al. 2011). Further, nutrient-deprived mothers have been shown to produced male-biased offspring sex ratios in the lizard *Amphibolurus muricatus*, a species with TSD (Warner et al. 2007). Lastly, environmental factors can also affect the outcome of sex; in *T. scripta*, elevated CO₂ has been shown to feminize embryos (Etchberger et al. 2002). In sum, sex determination in animals with TSD is complex, and also affected by a suite of environmental, genetic, and maternal factors; it seems unlikely that any quantitative method based solely on temperature can explain an overwhelming majority of variation in sex ratios among nests.

I 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 my methodology; similarly, for ethical reasons, I sampled a subset of 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, I suggest it may be widely applicable to highly seasonal environments and all types of TSD.

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. 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, Equation 2 could be applied.

4.3 Evaluation of the modified CTE

In the present study, the modified CTE method also resulted in a reasonable quantitative estimate of sex ratios for semi-natural nests of the snapping turtle. I 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 the assumptions of homogenous thermal variance, and a stable thermal mean. Surprisingly, the modified CTE method used herein also appears to overcome the restriction of the CTE model to Type I TSD (Warner and Shine 2011; Georges et al. 2004), as predictions were reasonable for my Type II study species. As I found in the PM method, the modified CTE method did not explain all of the variation in sex ratios, likely due to the reasons aforementioned for the PM method. Additionally, I 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 a sex ratio 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, I conclude the modified CTE method developed herein represents a reasonable method for estimating sex ratios.

4.4 Summary of the modified mean nest temperature approach

There are several explanations for why the development-weighted mean nest temperature explains less variation in sex ratio than the PM and CTE methods, and why its usefulness more generally may be limited. Within a day, the mean nest temperature does not account for the developmental dynamics experienced by eggs when thermal variance occurs, as higher temperatures increase the rate of poikilotherm development (Sharpe and DeMichele 1977); the embryos therefore incur more sexual differentiation in a given increment of time at higher temperatures (Georges et al. 2004). Thus, the mean nest temperature was a relatively poor predictor of sex ratio because the large range of thermal variation experienced in the nests is not captured by averaging, even when daily mean temperatures are weighted by development.

4.5 The thermosensitive period

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 (Hewavisenanthi and Parmenter 2002; Yntema 1979). This 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 this study, I compared the two extreme windows of the thermosensitive period described by Yntema (1979) for snapping turtles. Importantly, I 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). I 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 this is the first study that develops tools to explore how prediction accuracy of empirical models varies under different assumptions of TSP duration.

4.6 Applications of the PM method

The PM method is a promising tool, as it is relatively accurate, non-destructive, and can accommodate various assumptions regarding TSP duration. The PM method is of immediate benefit to researchers conducting long-term monitoring studies of at-risk species, for which estimation of primary sex ratios may be of interest, but are difficult to consistently obtain due to the destructive, expensive, or labor-intensive nature of sexing juveniles. Initial calibration must be done without a proxy for sex ratio, but data such as the temperature-sex reaction norm, and a degree-day development model, are frequently available in the literature. After calibration is complete, the PM model can then be applied using only temperature logs on a year-to-year basis for the purpose of monitoring primary sex ratios.

Further, if combined with projections of future climate change effects on soil temperature, it is possible to adapt the PM method to make sex ratio predictions. For example, Stubbs et al. (2014) made sex ratio projections in the flatback turtle (*Natator depressus*) in the years 2030 and 2070 under various emissions scenarios, using air temperature predictions and soil temperature modelling software. Their model produced hourly temperature projections, which were converted to a CTE statistic that estimated sex ratios. This methodology could easily be applied using the PM method, once hourly sand temperatures are predicted.

Likewise, by using historical soil temperatures to estimate sex ratio, researchers can look into the past and infer what a population's naturally occurring sex ratios were before a selected disturbance (*e.g.*, post-industrial climate change). For instance, Hays et al. (2003) reconstructed 150 years of sex ratios for a population of *C. mydas* in the South Atlantic Ocean by regressing historical water temperatures against soil temperatures, which were then converted into sex ratios using the mean temperature statistic. The resolution of such sex ratio estimates could be vastly improved by the use of the PM method, and researchers would be able to acquire new information regarding the changes, or lack thereof, in historic sex ratios.

4.7 Additional conclusions

In addition to the insights into TSD and practical applications gleaned from the PM method, I also note some interesting implications of my 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 produce mixed clutches. According to the PM model, a probability

of masculinization value of only 0.17 is needed to produce males, and my results suggest clutches of 100% males are unlikely to occur in Algonquin Park. As a result, in the focal population, mixed clutches are likely to occur as long as expected fluctuations in incubation temperature continue. Therefore, I 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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