Journal of Thermal Biology 74 (2018) 187–194

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

A new method of estimating thermal performance of embryonic development rate yields accurate prediction of embryonic age in wild reptile nests

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ARTICLE INFO

Keywords:
Thermal performance
Incubation
Reptile
Heat units
Fluctuating temperature
Turtle
Sex determination

ABSTRACT

Temperature has a strong effect on ectotherm development rate. It is therefore possible to construct predictive models of development that rely solely on temperature, which have applications in a range of biological fields. Here, we leverage a reference series of development stages for embryos of the turtle *Chelydra serpentina,* which was described at a constant temperature of 20 °C. The reference series acts to map each distinct developmental stage onto embryonic age (in days) at 20 °C. By extension, an embryo taken from any given incubation environment, once staged, can be assigned an equivalent age at 20 °C. We call this concept "Equivalent Development", as it maps the development stage of an embryo incubated at a given temperature to its equivalent age at a reference temperature. In the laboratory, we used the concept of Equivalent Development to estimate development rate of embryos of *C. serpentina* across a series of constant temperatures. Using these estimates of development rate, we created a thermal performance curve measured in units of Equivalent Development (TPC_{ED}). We then used the TPC_{ED} to predict developmental stage of embryos in several natural turtle nests across six years. We found that 85% of the variation of development stage in natural nests could be explained. Further, we compared the predictive accuracy of the model based on the TPC_{ED} to the predictive accuracy of a degree-day model, where development is assumed to be linearly related to temperature and the amount of accumulated heat is summed over time. Information theory suggested that the model based on the TPC_{ED} better describes variation in developmental stage in wild nests than the degree-day model. We suggest the concept of Equivalent Development has several strengths and can be broadly applied. In particular, studies on temperature-dependent sex determination may be facilitated by the concept of Equivalent Development, as development age maps directly onto the developmental series of the organism, allowing critical periods of sex determination to be delineated without invasive sampling, even under fluctuating temperature.

1. Introduction

Temperature has a strong effect on rates of physiological processes in plants and ectothermic animals (Gillooly et al., 2002; Kingsolver, 2009). The reaction norm that describes how a performance trait is related to temperature is typically referred to as thermal performance curve (TPC, Fig. 1a) (Huey and Stevenson, 1979). General biochemical principles govern the shape of TPCs in broad taxa (Schoolfield et al., 1981; Sharpe and DeMichele, 1977), such that the shape of this curve is conserved, being Gaussian and left-skewed (Kingsolver, 2009). Yet, TPCs are also under selection, such that their precise shape varies considerably among species and populations.

If the TPC for development rate can be accurately characterized, it can be used as a basis for a development model and applied to estimate developmental milestones under fluctuating temperature conditions (Georges et al., 2005). Indeed, development models of various types have proven very useful in agricultural and forensic sciences (Pedigo, 1996), for example in determining the suitability of different crop strains to local environments, controlling pests, and predicting the timing of flowering in plants and larval instar stages of insects (Got

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https://doi.org/10.1016/j.jtherbio.2018.03.008
Received 10 September 2017; Received in revised form 26 February 2018; Accepted 9 March 2018
Available online 13 March 2018

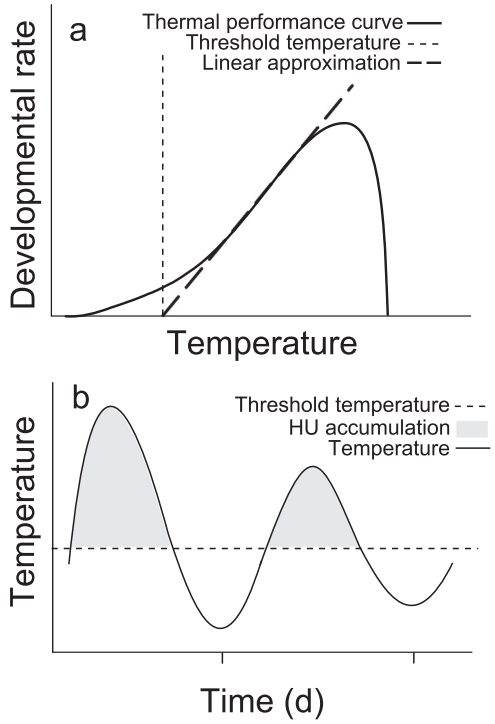

Fig. 1. Thermal performance curve and its linear approximation. (a) The relationship between development rate across a range of temperatures. The solid line represents the thermal performance curve, and the large dashed line shows the linear approximation of the curve and the lower threshold temperature below which no development is predicted to occur. In this depiction, the linear approximation predicts that development rate will continue to increase indefinitely with temperature. (b) The number of heat units accumulated (shaded area) is the area beneath the temperature profile above the threshold temperature, assuming temperature is linearly related to development (i.e., linear approximation in panel (a)). Degree days are heat units accumulated over time, expressed in degree-days.

et al., 1997; Manel and Debouzie, 1995). Development models have also proven critical in ecology and evolution, especially in predicting sex ratios of species with temperature dependent sex determination (Georges et al., 1994; Telemeco et al., 2013).

Estimation of TPCs for development rely on accurate characterization of development at constant temperatures (Georges et al., 2005), yet characterization of development rate can be difficult in some taxa. For instance, in ectothermic vertebrates, development rate at a given temperature is often estimated by applying constant temperature throughout the entire incubation period, then measuring time-to-hatch in days (Ewert, 1985; Lang and Andrews, 1994; Niehaus et al., 2012; Shine and Harlow, 1996). This method is likely used because development rate is easy to estimate when rates can be delineated within obvious developmental milestones. However, rates estimated over the entire incubation period may be subject to bias. For example, if egg hatching is environmentally-cued (e.g., by oxygen levels or social factors), then the number of days between oviposition and hatching is not a good proxy for development rate, as embryos are not developing for the entire period they are in the egg (Webb et al., 1986). Environmentally-cued hatching is not uncommon among reptiles (Doody, 2011), amphibians (Mills and Barnhart, 1999), fish (Czerkies et al., 2001), and ectotherms in general (Warkentin and Caldwell, 2009). Alternative methods of estimating development usually rely on enlargement of a single morphological feature across ontogeny, such as head width (Beggs et al., 2000; Georges et al., 1994). Although embryonic enlargement may provide a good estimate of developmental progression under some conditions (e.g. Webb et al., 1986), it is generally recognized that development (passing through life stages) is a process that is distinct from growth (increasing in size), and these processes have different thermal sensitivities (Forster et al., 2011; van der Have and de Jong, 1996). Specifically, development is more sensitive to temperature than growth (Forster et al., 2011), which helps explain why full-term embryos are often relatively small when incubated under relatively warm conditions (Janzen and Morjan, 2002; Van Damme et al., 1992). Given that the size of the term embryo itself may depend on temperature (Janzen and Morjan, 2002; Packard et al., 1984), it follows that embryonic growth expressed as a fraction of term embryo size may be an imprecise method of estimating development rate (Georges et al., 2005). More broadly, estimates of development obtained by measuring enlargement of a morphological feature must be considered a proxy for development, as enlargement is a measure of growth and an indirect measure of development (Forster et al., 2011).

A method that is more strongly rooted in the process of development would allow rates of development to be compared unambiguously across a diversity of environments, including that created by the mother (e.g., egg size). Webb et al. (1983) developed such a method by leveraging a reference series of development stages for embryos of the crocodile *Crocodylus porosus,* which was quantified at 30 °C. Importantly, the reference series acts to map each distinct developmental stage onto embryonic age in days at 30 °C. The implication is that an embryo arbitrarily collect from an incubation environment of 30 °C could be aged accurately, as developmental stage corresponds to a particular embryonic age, in days, within the reference series. Next, Webb et al. (1983) assumed that temperature would affect rate of development, and introduced a correction factor that allowed a '30 °C age' of embryos to be estimated at different temperatures. For example, an embryo incubated at 33 °C for a given number of days could be collected and dissected to determine developmental stage. Then developmental stage could be converted to the number of days at 30 °C necessary to reach that developmental stage, resulting in a '30 °C age'. Herein, we refer to this method of aging embryos with the term "Equivalent Development", as it maps the development stage of an embryo to its equivalent age at a reference temperature.

The goal of the present study is, first, to apply the concept of Equivalent Development (Webb et al., 1983) in a new way: to create a TPC for embryonic development rate. Given that we modify the methods of Webb et al. (1983), we provide validation of the modified method by testing whether the TPC for Equivalent Development (TPC_{ED}) can accurately predict embryonic age and stage in wild nests. We find that our TPC_{ED} explains a majority of the variation in developmental age and stage in the wild, performing better than alternative development models. Second, we emphasize some strengths of the Equivalent Development concept, and outline why it may be particularly useful in the field of temperature dependent sex determination.

2. Methods

The present study is part of a long-term research program on the biology of snapping turtles, which was initiated in 1972 in the Algonquin Wildlife Research Station (AWRS) in Algonquin Provincial Park, Ontario, Canada (45°30′ N, 78°30′ W). Snapping turtles in this population nest from late May through early July, and clutch size varies between 19 and 69 eggs (Armstrong et al., 2017; Edge et al., 2017; Rollinson et al., 2012).

2.1. Quantifying development

Yntema (1968) described 26 embryonic stages for the snapping turtle based on embryos incubated at 20 °C. The embryonic stage of dissected embryos that were examined herein was determined following Yntema (1968) by E.G. Nancekivell, S. Holt, or M.D. Massey. In some cases, more than one embryo characteristic did not match Yntema's description of a specific stage. For instance, eye development might have suggested the embryo had reached Yntema stage 15,

whereas carapace development suggested stage 14. In these cases, stage was estimated from the average stage of four characters: digits, eyes, carapace, and pigmentation.

Yntema (1968) provided data on the time in days for embryos to reach a particular developmental stage at 20 °C. Using the values provided by Yntema, we divided time (d) to reach each Yntema stage (1 through 26) by a value of seven. This procedure created a continuum of values, with a maximum of 20 (i.e., it took 140 days to reach Ynetma stage 26 in Yntema, 1968, so 140d/7d wk^{-1} = 20wks), with each value representing the number of weeks of development that would have occurred at a temperature of 20 °C. We call this general approach to quantifying development "Equivalent Development", and in the present study, it represents Equivalent Development in weeks at 20 °C, or ED_{20} (Table S1). For example, when the ED_{20} value is 10.6, the developmental age of the embryo reflects 10.6 weeks of development at 20 °C. The notation used in the present study will therefore be $ED_{20}n$, where n is the number of weeks of development. For instance, $ED_{20}10.6$ indicates that the embryo is at an age equivalent to 10.6 weeks of development at 20 °C. Assuming that hysteresis of development rate is absent, i.e., rate of development at a given temperature does not depend on anatomical stage (Worner, 1992), then age data converted to Equivalent Development can be analyzed in a biologically-meaningful manner (See Supplemental Material for an experiment that supports an absence of hysteresis). We note that dividing values by seven so that age is expressed in weeks is simply so that age values remain within a smaller range and hence are more manageable (i.e., 1 – 20 weeks *vs* 1 – 140 days).

2.2. Estimating the TPC for equivalent development (TPC_{ED})

In the summer of 2004, three clutches were collected from turtles that nested in the same week (female #ID[Julian nest day]: #K13[169]; #782[174]; #940[176]). Clutches were placed in a plastic container with equal parts lake water and vermiculite and returned to our field laboratory at the AWRS. Although substrate moisture was not tightly controlled after initial collection, all containers had a plastic lid perforated with small holes to reduce evaporation, and tap water was added if and when substrate moisture appeared to be low. Embryos remained at room temperature (≈15–20 °C) in our field lab until Julian day 180, when they were transported to the University of Guelph and placed in a temperature-controlled wetlab. All clutches were then incubated at a temperature that varied between 21.5 °C and 27.0 °C (mean ± 1 SD = 25.1 ± 1.1 °C) until we estimated that embryos had reached approximately $ED_{20}14.0$.

The experimental procedure described below involved allocating embryos to a prescribed constant temperature, then estimating the amount of development that occurred over specific time intervals, measured in ED_{20}. For instance, if an $ED_{20}14$ embryo was placed at 28 °C and progressed to $ED_{20}15$ over three days, then development rate at 28 °C is (16 wks – 15 wks)/3 d = 0.33 wk•d^{-1}, indicating that an equivalent of 0.33 weeks of development at 20 °C are experienced for every day the embryo is held at 28 °C. The Yntema stage of embryos was assessed immediately prior to each experimental run, by staging one to three embryos from each female, therefore providing a baseline upon which developmental advancement could be estimated. Yntema stages were then converted to ED_{20}, and in all cases, initial embryos were between $ED_{20}13.0$ and $ED_{20}15.0$. In total, four different initiation dates were used, where one or more temperature treatments was initiated: 12 July, 19 July, 26 July, and 27 July 2004. Experimental runs consisted of placing 4–6 embryos in one of 14 temperature treatments (14 °C, 16 °C, 18 °C, 20 °C, 22 °C, 24 °C, 26 °C, 28 °C, 30 °C, 31 °C, 32 °C, 34 °C, 36 °C, 38 °C), where constant temperatures were maintained either in Koolatron™ Thermoelectric Digital Precision Incubators, or in temperature-controlled wetlabs at the University of Guelph Aqualab facility. To help ensure incubation method did not affect development rate, we replicated the 26 °C treatment with turtle #K13.

Each run was therefore nested within each constant temperature treatment. For each run, a sample of embryos from a given female was assigned to a particular temperature treatment, such that each run was ultimately represented by a sample of embryos from one female. When embryos in a run were estimated to have developed approximately 0.5, 1.0, and 1.5 equivalent weeks, between one and three embryos was removed from the run, frozen, and subsequently staged. For example, staging of embryos in cool temperatures was performed 9 days, 20 days, and 26 days after runs began, whereas staging in warmer temperatures was generally performed 4 days, 5 days, and 6 days after runs began (Table S4). Embryos were staged blindly with respect to female and temperature treatment, and the average increase in ED_{20} age of embryos at each time point was calculated in wk d^{-1}, where day was a continuous value measured to the nearest hour. As we discuss below, freezing embryos is not the ideal method of preserving specimens for staging, but this method was most amenable to our experimental protocol.

Sample size was restricted by the clutch size of each turtle, such that female #782 (n = 24 embryos) was assigned to three different temperatures (18 °C, 31 °C, 34 °C), female #940 (n = 36 embryos) was assigned to five different temperatures (22 °C, 24 °C, 32 °C, 36 °C, 38 °C), and female #K13 (n = 37) was assigned to six different temperatures (14 °C, 16 °C, 20 °C, 26 °C, 28 °C, 30 °C). All temperatures were verified using a Thermochron DS1921G iButton programmed to record temperature every 30 mins throughout incubation. During the experiment, mean incubation temperatures in each unit were on average within ± 0.45 °C of the desired temperature, and mean observed temperature (not desired temperature) was subsequently used to estimate the TPC_{ED}. The resultant data from this experiment was a set of development rates, expressed in wk d^{-1}, each measured at a constant temperature. To reduce the non-independence in our development data that arises from using multiple estimates of development rate from the same incubation room/chamber and female, we calculated the median development rate of embryos in each run (i.e., at each constant temperature) and used these median values in subsequent analyses (n = 14 medians in total).

We constructed the TPC_{ED} using the R environment (R Development Core Team, 2016). To model the TPC_{ED}, we attempted to fit the Sharpe-Schoolfield model of development (Schoolfield et al., 1981). However, convergence could be achieved only by constraining at least one parameter to a range of user-specified values (See Supplemental Material), and there were large error terms associated with parameter estimates (Table S5). To avoid constraining parameters and to hence reduce observer bias in the shape of the TPC_{ED}, we instead modelled the TPC_{ED} using cubic splines (Schluter, 1988), using a value of lambda that minimizes the cross-validation score. Cubic splines are a form of general additive model that links a series of polynomials to the data, and they provide an unbiased and unconstrained estimate of the relationship between development rate and temperature (Schluter, 1988).

2.3. Field validation of new methods

Between 1988 and 1994, nesting turtles on the Sasajewun dam in the AWRS were monitored, and clutches laid by individually marked turtles were excavated within 8 h of oviposition. Eggs were removed from the nest, and covered in moist vermiculite, and transported to the field lab at the AWRS. Clutches were kept in the laboratory at room temperature (≈15–20 °C) for 1–11 days after oviposition (mean = 5.1 days), then each clutch was reburied in its original nest. Clutches were not reburied in the field immediately after oviposition because other nesting turtles might accidentally dig them up, or turtles become entangled in leads connecting temperature probes to the data logger. Given that all embryos were generally kept below 20 °C, we expected that minimal development would occur (Ewert, 1985). Each clutch was reburied with a temperature probe next to the centre-most egg, and hourly temperatures were logged for all nests throughout the

incubation period using temperatures logged from a Squirrel 16-channel data logger (Grant Instruments Ltd.). All nests were left undisturbed for at least two weeks after being reburied. Thereafter, 1–3 eggs from each nest were removed approximately every two weeks. Embryos were immediately frozen at − 4 °C and subsequently thawed and staged.

Once the embryos from natural nests were staged, we explored whether the TPC_{ED} estimated in the laboratory could be used to predict developmental stage (and hence, ED_{20} age) in natural nests. Instantaneous rate of development in wk d^{-1} was estimated by mapping observed hourly incubation temperature (unique to each nest in each year) to the TPC_{ED}. ED_{20} age was estimated by summing development across time (see also Georges et al., 2004). This resulted in a predicted value of ED_{20} for each natural embryo (predicted development from the TPC = "PD_{TPC}") at each time point, as well as an observed value of ED_{20} at specific time points (i.e., the staged embryo from the wild).

To estimate whether PD_{TPC} is a good predictor of observed ED_{20} age in wild nests, we compared the predictive accuracy of PD_{TPC} to standard degree-day model, and to a model based solely on time elapsed since egg-laying. The degree-day (°D) approach is based on the assumption that the relationship between development rate and temperature is linear (Fig. 1a). Degree days are calculated as cumulative exposure to heat above a lower threshold temperature over a certain length of time (Pedigo, 1996). For instance, the °D accumulation for an embryo incubated at a constant temperature of 22 °C with a threshold of 20 °C would be 2°D per day. Using the degree-day approach, the number of °D accumulated under fluctuating temperatures is represented by the area beneath the temperature profile over time (Fig. 1b), above a threshold temperature (Georges et al., 1994; Lewis et al., 2015; Morrison et al., 2014). We calculated °D accumulation using a threshold temperature of 20 °C, given that Yntema (1978) showed that eggs incubated at a constant temperature of 20 °C developed slowly but did not hatch. Degree-day accumulation for each staged embryo in natural nests over the relevant incubation period was calculated using hourly incubation temperatures above 20 °C, using the trapezoid rule of integral approximation. Each embryo incubated in a natural nest was therefore associated with a specific °D value. Note that we did not use an upper threshold temperature (above which development rate is zero) when estimating °D accumulation, as we are at the northern range limit of snapping turtles and incubation temperatures are generally low (see Results).

Finally, we built a model based solely on time elapsed since egg laying, as our assumption is that both the °D method and the PD_{TPC} method should outperform a model solely based on time elapsed. In turtles, oviposition occurs when embryos are at the very earliest stages of development (Andrews and Mathies, 2000), and for a viable embryo that has been oviposited, development will increase with time elapsed since fertilization. Thus, time elapsed in days since embryo reburial (Time) was used as a predictor variable of ED20 stage in natural nests.

We are interested in predicting variation in ED20 under natural conditions from variation in $PD_{TPC,}$ or °D, or Time. Each predictor variable was substituted into the following model, each model was estimated using maximum likelihood,

$$\text{Model (1)} \qquad ED_{20ijk} = \beta_0 + \beta_1 x_{ijk} + \nu_{0j} + \varepsilon_{ijk}$$

Where ED_{20} is the equivalent age (Table S1) of embryo i at 20 °C, x is the relevant predictor variable (PD_{TPC}, °D, or Time), ν_0 is the random effect (intercept) of Nest Identity j, and ε is error; β_0 and β_1 are the fixed effect parameters to be estimated. The use of Nest Identity as the sole random effect is based on model selection of random effects using restricted maximum likelihood estimation (see Supplementary Material). Models were compared following Burnham and Anderson (2002), using Akaike Information criterion corrected for small sample sizes (AICc) and AICc weights (w_i). We also calculated $R2_{GLMM}$ for each model following Johnson (2014), where $R2_{GLMM}$ reflects an estimate of the amount of variation in the predictor variable explained by fixed effects in mixed-effect models. $R2_{GLMM}$ is roughly analogous to r^2 for least-square models, although it is an approximation.

3. Results

3.1. Estimating the thermal performance curve for Equivalent Development (TPCED)

In total, 79 embryos from the constant temperature experiment were staged in 14 experimental runs. Embryonic mortality at 38 °C was 100% (all 4 embryos sampled were dead), and 17% at 34 °C (1 of 6 dead), leaving 74 staged embryos. Dead embryos with zero development rate were not included in our analyses, such that 42 estimates of development rate across 14 set-point temperatures were ultimately collected. We note that two of the 74 embryos in our experiment appeared to experience negative development, both of which were in the same experimental run on the same day (1 d of exposure at 36 °C). Further, the remaining embryos at 36 °C were left for ca. 6 – 9 days prior to sampling, and it is unclear whether these embryos were dead or under developmental arrest at the time of sampling. We substituted a development rate of zero when a negative rate of development was observed. Ultimately, data on development rate at 36 °C has little bearing on thermal performance under wild conditions (see below), and we decided to include this experimental replicate in our analysis.

Using median development rate per experimental run, we used cubic spline analysis to estimate the TPC_{ED}. The spline explained the majority of variation in development rate (adjusted $r^2 = 0.961$, n = 14) and revealed the expected pattern of development rate with respect to temperature: a peak development rate at warm temperatures (30.0 °C), and a long tail at cooler temperatures (Fig. 2, Table S3). A spline fit using all available data (n = 42 estimates of development rate, Fig. S1) was very similar to the model fit using median development rate per run (n = 14, Fig. 2). Further, the TPC_{ED} estimated using the cubic spline was very similar to the TPC_{ED} estimated using the Sharpe-Schoolfield equation (Fig. S2), suggesting that the spline provided a biologically realistic fit to the data, and that our results are not sensitive to the method of TPC_{ED} fitting.

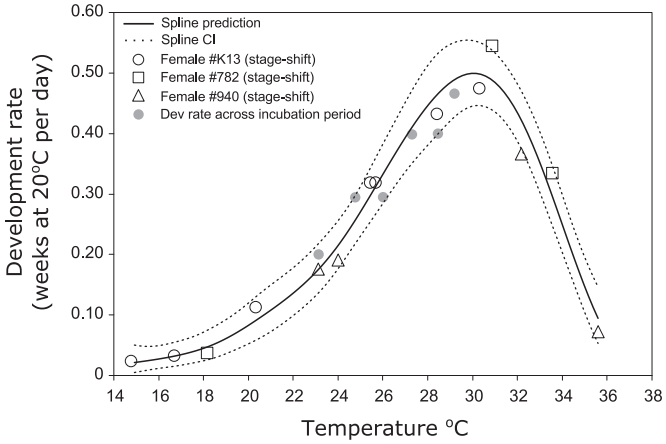

Fig. 2. Rate of development estimated at constant temperatures in the lab are fit with a cubic spline (± 95% CI), using median development rate per experimental run (n = 14 runs, see Fig. S1 for a spline using all available data). Dark circles reflect a separate experiment, where development rate was estimated at constant temperature throughout nearly the entire incubation period; the close match between dark circles and light circles suggests that development rate does not depend strongly on anatomical stage of development (see Supplemental Material).

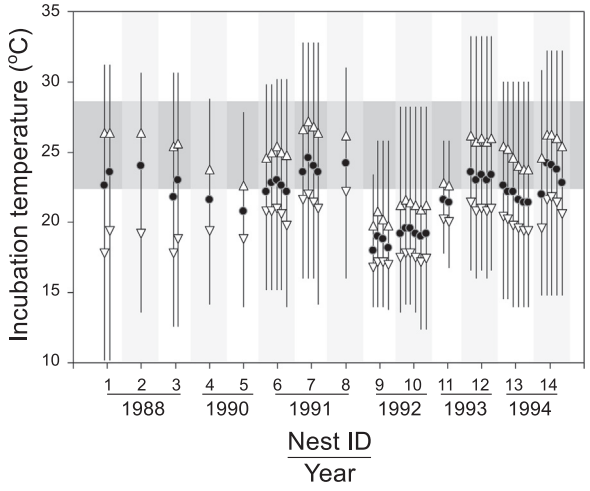

Fig. 3. Incubation temperatures in the field experienced by embryos sampled in the present study, by nest and by year. Vertical lines are the range of temperatures experienced, triangles are upper (75th percentile) and lower (25th percentile) quartiles, black dot is the median (50th percentile). Horizontal shading represents the approximate range of temperatures in which the relationship between development rate and temperature is linear (cf. Fig. 2).

3.2. Field validation of new methods

In general, incubation temperatures were relatively cool across the 6 years in Algonquin Park (Fig. 3). The vast majority of incubation temperatures fell between 18 °C and 27 °C, and temperature rarely exceeded 30 °C. If the range of temperatures in which development rate is linear with respect to temperature is approximately 23–28 °C (cf. Fig. 2), then only the upper quartiles of incubation temperatures experienced fell in the linear range (Fig. 3), and for some nests (e.g., in 1992), the vast majority of temperatures were well below the linear range.

A total of 75 embryos were staged from natural nests, belonging to 14 clutches produced by 10 females over 6 years (Table S2). Of all staged embryos, ED_{20} age ranged from ED_{20}3.57 to ED_{20}17.0. Because these 75 embryos were sampled in groups of one to three embryos from the same nest on each sampling date, samples were averaged, creating 46 estimates of ED_{20} for our analysis. We found that PD_{TPC} was more strongly supported (w_i = 0.94) than either °D or Time (Table 1, Fig. 4), suggesting PD_{TPC} is able to predict variation in ED_{20} age better than other candidate predictors. We found that the slope of PD_{TPC} was not different from 1.0, as would be expected for a 1:1 prediction (Fig. 4, Table 2). We also found that r^2_{GLMM} was high for both PD_{TPC} and °D, around 0.85 (Table 1). We note that coefficients of determination should not be used in model selection (Johnson and Omland, 2004), and we have not done so here (Table 1), but the high r^2_{GLMM} and conjunction with strong model support (w_i = 0.94) suggests that PD_{TPC} predicts ED_{20} in wild nests with reasonable accuracy (Fig. 4).

Table 1
Rankings of models predicting variation in ED_{20} in wild nests over six years, fit with different fixed effect predictors, where *K* is the number of model parameters, *LogLik* is the log likelihood of the model, w_i is the Akaike weight, r^2 is the coefficient of determination from a least-squares model, and r^2GLMM is the coefficient of determination estimated while accounting for the random effect of nest.

Model	K	LogLik	ΔAICc	w_i	r^2	r^2GLMM
ED_{20} = PD_{TPC} + Nest. ID	4	− 81.76	0	0.94	0.787	0.849
ED_{20} = °D + Nest. ID	4	− 84.47	5.42	0.06	0.708	0.837
ED_{20} = Time + Nest. ID	4	− 92.21	20.89	0.00	0.475	0.665

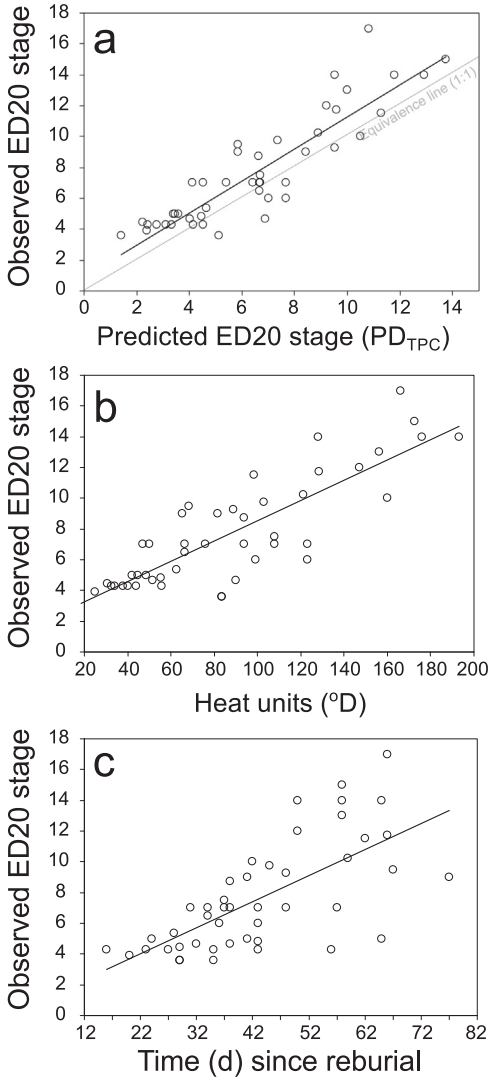

Fig. 4. Least squares regressions predicting ED_{20} age in field nests using (a) ED_{20} age predicted from thermal performance curve (see Fig. 2), (b) degree days, and (c) time elapsed since embryo reburial. The equivalence line in panel (a) represents one-to-one prediction, i.e., perfect prediction.

Table 2
Parameter estimates from the mixed effect model predicting variation in ED_{20} in the field, fit by REML (n = 46 staged embryos).

Parameter	Effect Type	Estimate[a]	SE
Nest Identity	Random	1.33	
Residual	Random	1.46	
Intercept	Fixed	0.554	0.610
PD_{TPC}	Fixed	1.07	0.0765

[a] Parameter estimates for random effects are variance estimates.

4. Discussion

In the present study, we refine a method developed by Webb et al. (1983) to quantify development; here we call this method "Equivalent Development". We use the principle of Equivalent Development to help estimate a thermal performance curve (TPC_{ED}) for embryonic development rate of snapping turtles, and we provide evidence that our TPC_{ED} accurately characterizes development rate. Specifically, we demonstrated that development rate estimated at an arbitrary ED_{20} age (ca. ED_{20}14.0) provides estimates that are similar to those obtained by estimating development over nearly the entire incubation period (see

Supplemental Material and Fig. 2), suggesting that hysteresis of development rate is weak or absent. Further, we demonstrated that the TPC_{ED} estimated using advancement of ED_{20} ages can explain about 85% of variation in ED_{20} age under wild conditions, and we demonstrated that the TPC_{ED} performs better than alternative models (Table 1, Fig. 3). Below, we discuss how the concept of Equivalent Development can be useful (and improved) in future studies.

A goal of the present work is to promote renewed interest in concept of Equivalent Development (Webb et al., 1986, 1983), a concept that has received little attention since its inception. We argue that the use of the developmental series of the organism as a reference for development rate has three significant strengths. First, the use of Equivalent Development results in consistency in reference stages across studies (e.g., Cordero and Janzen, 2014; Yntema, 1968), providing an avenue for a biologically meaningful comparison of development rate both within species (Webb et al., 1983) and across related species (Webb et al., 1986). On the other hand, the use of anatomical enlargement across ontogeny cannot be easily compared between studies, even when studies are of the same species. This is because anatomical size at the end of the embryonic development is affected by the temperature experienced during incubation (Janzen and Morjan, 2002; Van Damme et al., 1992; Webb et al., 1986) likely because development is more sensitive to temperature than growth (Forster et al., 2011). The differential sensitivity of growth and development also relates to a second strength of the Equivalent Development method: advancement of development based on the developmental series of an organism necessarily captures the process of development, and the Equivalent Development method linearizes the developmental series so that it can be converted to embryonic age. On the other hand, rate of enlargement of size traits across ontogeny may not capture the true temperature-sensitivity of development, particularly when size enlargement across ontogeny is measured as a fraction of final embryo size (Georges et al., 2005).

A third strength of the Equivalent Development method is that embryonic age maps directly onto the developmental series of an organism, allowing estimation of the timing of key differentiation events, even under fluctuating temperatures. The Equivalent Development method may therefore prove extremely useful for species with temperature-dependent sex determination (TSD), where particular developmental stages within the developmental series comprise the thermosensitive period (Yntema stages 14 – 19, or equivalently $ED_{20}6$ – $ED_{20}11$ in *C. serpentina*, Table S1), which is when temperature influences sex (Lang and Andrews, 1994; Yntema, 1976). In other words, the timing and duration of the thermosensitive period can be estimated with accuracy, and non-destructively, from the thermal profile of a nest. Further, because sex in TSD species is thought to be determined by the amount of development that occurs above and below specific ("pivotal") temperatures (Georges et al., 1994), the methods used to create a TPC_{ED} herein could prove valuable in estimating sex ratios under fluctuating conditions. Specifically, the proportion of development that occurs above and below pivotal temperatures can be calculated using the TPC_{ED} for the duration of the thermosensitive period, regardless of the magnitude of temperature fluctuations. This could mark an enhancement of methods proposed by Georges et al. (1994), which rely on the assumption that temperature is linearly related to development rate, as well as other strict assumptions about the nature of temperature variation. Alternatively, estimating the timing of the thermosensitive period from morphological enlargement (Georges et al., 2005, 1994) and/or by candling the embryo to estimate developmental stage (Beggs et al., 2000) are less convenient, as the former involves destructive sampling and the latter requires that embryos undergo multiple examinations throughout incubation, which can be difficult under field conditions.

Equivalent Development is also amenable to the estimation of development rate over any number of stages at any point of development, such that rate can be quantified for large numbers of embryos at several set-point temperatures using relatively few incubation units. Equivalent Development also allows for reduced exposure to high incubation temperatures, and thus can perhaps minimize embryonic mortality observed at high temperatures (Demuth, 2001; Yntema, 1976; Yntema and Mrosovsky, 1980), and ultimately allow areas of the TPC to be estimated that are otherwise experimentally inaccessible for large embryos that develop slowly. Although the present study froze embryos prior to dissection, rendering assessment of temperature-induced mortality difficult (at least at 36 °C), we suggest future studies examine embryos immediately upon removal from the experimental temperature, allowing mortality to be assessed directly.

We note that about 85% of the variation in ED_{20} in natural nests was explained by our TPC_{ED} (Table 1) suggesting that the methods used herein may be broadly useful for predicting thermal performance of embryos in the field (see also Supplementary Material). It is tempting to attribute unexplained variation in development rate to differences in the abiotic environment, such as moisture availability in the nests. Moisture has indeed been shown to affect development in several species of turtles (Packard et al., 1987; Paukstis et al., 1984), but previous studies on snapping turtles show that even large differences in water potential do not seem to strongly influence development rate (Packard et al., 1984). Nevertheless, future studies may wish to more rigorously monitor substrate hydration in experiments involving Equivalent Development, as our experimental protocol did not rigorously monitor moisture levels in our experimental units.

Another source of unexplained variation in wild nests may also be related to our inability to eliminate time-dependent effects when estimating the TPC at set-point temperatures. Several studies have demonstrated that TPCs estimated from constant temperature in the laboratory result in poor predictions under fluctuating conditions (Kingsolver and Nagle, 2007; Niehaus et al., 2012). The problem can be traced back to the underlying concept of the TPC, which assumes that performance depends on current temperature alone (Kingsolver and Woods, 2016). In reality, there is ample evidence that thermal performance decreases with increasing duration of exposure to temperatures, particularly in the upper range of temperatures that are non-lethal (Kingsolver and Woods, 2016, 1997; Schulte et al., 2011). The mechanisms underpinning these time-dependent effects are not always clear, but may involve a variety of factors including induction of heat-shock proteins (e.g., Kingsolver and Woods, 2016) and hormone-mediated stress responses (e.g., LeBlanc et al., 2012). Regardless of the specific mechanisms, estimating thermal performance at set-point temperatures under constant temperature conditions cannot provide a perfect description of development rates under natural conditions (Kingsolver and Woods, 1997). Indeed, the ED_{20} age predicted from our TPC_{ED} is consistently lower than the observed ED_{20} stage in wild nests (Fig. 4), suggesting that development is faster in wild nests than predicted by our TPC_{ED}. If time-dependent effects occurred in our TPC experiment, then it is likely that brief incursions into elevated temperatures in wild nests would result in faster development rates than predicted by our TPC_{ED} (Kingsolver and Woods, 1997), and that these effects would appreciate over time, a possibility that is supported by supplementary analysis of our field data (see Supplementary material). We therefore suggest that when reference series are created (i.e., embryonic ages mapped onto a developmental series at constant temperature), they are based on moderate or low temperatures, as establishing embryonic age based on a series described under high temperatures may bias estimates of embryonic age, as stressed embryos develop relatively slowly (Kingsolver and Woods, 2016).

An additional source of error that we did not consider in the present study is that different snapping turtle populations exhibit different rates of development (Ewert, 1985). In the present study, we used a reference series (i.e., embryonic ages mapped onto a developmental series at constant temperature) described by Ynetma (1968), estimated at 20 °C for a population of snapping turtles in New York State (approximately 43.1°N, 76.2°W), about 350 km south of Algonquin Park (approximately

45.3°N, 78.3°W). Yet, if development is faster at 20 °C in Algonquin Park than in the populations Yntema studied, then the TPC_{ED} estimated in our study will suffer from bias. Our study therefore shows that Equivalent Development may be useful, but ideally the reference series upon which Equivalent Development is based should match the source population. We note, however, that reference series are not rare in the literature (e.g., see Table S9 for a sample of 29 reptile species for which a developmental series has been characterized at constant temperature), so for some species and populations, relevant data are readily available for conversion to Equivalent Development. Further, once a developmental series has been described for a given taxon (see Table S9), it is relatively straightforward to create a reference series for a given population: specifically, all that is required is incubation of embryos at a constant temperature, coupled with regular sampling and embryo dissection, using the developmental series as a guide for staging embryos.

In sum, the present study reintroduces the concept of Equivalent Development, an approach for quantifying development and development rate, originally conceived by Webb et al. (1983). We expand on Webb et al.'s methods and use Equivalent Development to estimate a TPC for snapping turtle embryos, and we provide good evidence that our TPC can produce accurate estimates of development rate in wild nests. We suggest that the Equivalent Development approach can be widely used in studies examining thermal performance of embryos, and it may be particularly useful in studies of temperature-dependent sex determination.

Acknowledgements

We thank the undergraduate and graduate students who participated on this project, and four anonymous reviewers who significantly improved and clarified this work. The Algonquin Wildlife Research Station provided field support, laboratory space, and accommodations, and Locke Rowe provided additional laboratory space. This research was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC RGPIN-2016-06469) Discovery Grants to RJB and NR. The University of Guelph Animal Care Committee approved our methods (AUPs #00R057 and #96R098).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtherbio.2018.03.008.

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