

## RESEARCH ARTICLE

# Impact of fluctuating developmental temperatures on phenotypic traits in reptiles: a meta-analysis

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## ABSTRACT

During the vulnerable stages of early life, most ectothermic animals experience hourly and diel fluctuations in temperature as air temperatures change. While we know a great deal about how different constant temperatures impact the phenotypes of developing ectotherms, we know remarkably little about the impacts of temperature fluctuations on the development of ectotherms. In this study, we used a meta-analytic approach to compare the mean and variance of phenotypic outcomes from constant and fluctuating incubation temperatures across reptile species. We found that fluctuating temperatures provided a small benefit (higher hatching success and shorter incubation durations) at cool mean temperatures compared with constant temperatures, but had a negative effect at warm mean temperatures. In addition, more extreme temperature fluctuations led to greater reductions in embryonic survival compared with moderate temperature fluctuations. Within the limited data available from species with temperature-dependent sex determination, embryos had a higher chance of developing as female when developing in fluctuating temperatures compared with those developing in constant temperatures. With our meta-analytic approach, we identified average mean nest temperatures across all taxa where reptiles switch from receiving benefits to incurring costs when incubation temperatures fluctuate. More broadly, our study indicates that the impact of fluctuating developmental temperature on some phenotypes in ectothermic taxa are likely to be predictable via integration of developmental temperature profiles with thermal performance curves.

**KEY WORDS:** Developmental stability, Phenotypic plasticity, Phenotypic variance, Squamate, Systematic review, Turtle

## INTRODUCTION

Many organisms are exposed to the vagaries of local environmental conditions during early development (Refsnider and Janzen, 2010). Specifically, because embryos are largely immobile, they are


restricted to the environmental conditions at their oviposition site or the conditions they experience inside their mother (but see Du and Shine, 2015). This includes the main abiotic factor responsible for driving physiological reactions: temperature. Developmental temperature affects nearly every organismal trait, including sex determination, developmental rate, morphology, performance and behaviour (Booth, 2006; Colinet et al., 2015; Noble et al., 2018a; Massey and Hutchings, 2020). As a result, understanding the nature of this organism–environment interaction during early development is of fundamental importance in evolutionary ecology (Refsnider and Janzen, 2010).

Much of our insight into thermal developmental plasticity has been generated through experiments conducted at constant developmental temperature (Booth, 2006; Bowden et al., 2014; Colinet et al., 2015; Booth, 2018; Noble et al., 2018a; While et al., 2018; Massey and Hutchings, 2020). However, in the wild, developmental temperature is rarely constant, varying across both short and long time periods (Colinet et al., 2015; Booth, 2018; Bowden and Paitz, 2018). For example, oviposition sites experience irregular variation associated with seasonal changes, heat waves, cold snaps and rainfall events, as well as consistent variation associated with time of day (Colinet et al., 2015). The extent to which oviposition sites experience these temperature fluctuations depends on whether the sites are sheltered (e.g. shaded, under cover, buried underground; Grossmueller and Lederhouse, 1985; Janzen, 1994; Booth, 2006; 2018; Doody et al., 2006; Potter et al., 2009; Colinet et al., 2015). Moreover, across and within underground nests, embryos often experience different levels of diel fluctuation around the same mean temperature, depending on nest depth or whether they are at the bottom or the top of the nest (Georges, 1992; Booth, 2006; 2018). Thus, embryos are likely to be exposed to considerable variation in thermal conditions across both space and time (Bowden et al., 2014). Such fluctuations in the thermal environment may themselves have significant implications for offspring phenotype over and above the mean temperature (Folguera et al., 2011), creating selection on oviposition behaviour and embryonic thermal sensitivity. For example, lizard eggs exposed to a 2 week period of ‘warm’ temperatures (22°C), in an otherwise ‘cool’ (17°C) treatment, had higher survival and shorter incubation periods than those incubated at a constant ‘cool’ temperature (Shine and Elphick, 2001). Given these important developmental effects of embryonic temperature, the thermal microenvironment chosen for oviposition can have significant impacts on offspring phenotype. Unfortunately, we still have a limited understanding of how phenotypes are affected by fluctuating developmental temperatures across ectothermic taxa (Bowden et al., 2014; Colinet et al., 2015; Booth, 2018; Noble et al., 2018a; Massey and Hutchings, 2020).

While empirical tests of the effects of fluctuating temperature on phenotypic development have been growing over the past 10 years,

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there is also long-standing work on the mechanistic links between temperature and developmental physiology that allows us to make predictions for how these effects may play out (reviewed in Bowden et al., 2014; Colinet et al., 2015; Massey and Hutchings, 2020). Developmental rate increases with mean temperature, until it hits an upper thermal limit, when development slows and survival decreases (Sharpe and DeMichele, 1977; Angilletta, 2009; Massey and Hutchings, 2020). Based on this, the manner in which temperature fluctuations affect the phenotype probably depends on both the mean temperature and the magnitude of fluctuations (Georges et al., 2005; Massey and Hutchings, 2020). The importance of combining temperature fluctuations with temperature-dependent developmental rates has previously been demonstrated by the improved predictive ability of heat summation approaches and degree-hour and non-linear developmental models (Sharpe and DeMichele, 1977; Georges et al., 2005; Colinet et al., 2015; Massey and Hutchings, 2020); for example, the constant temperature equivalent (CTE; Georges et al., 1994, 2005) daily duration with CTE (Carter et al., 2018), weighted cumulative temperature units (wCTU; Valenzuela et al., 2019). These metrics may provide a biologically relevant substitute for the mean temperature in fluctuating regimes. The heat summation approach predicts that fluctuations at cool temperatures should benefit embryos by decreasing incubation duration and improving survival and phenotypic measurements (assuming cool mean temperatures are suboptimal). Furthermore, for reptile species with temperature-dependent sex determination, where degree-hour approaches have been explored, fluctuations at cool temperatures are predicted to increase the proportion of the sex produced at warm temperatures (Georges et al., 1994; 2005; Bowden et al., 2014). In contrast, temperature fluctuations around a warm mean are more likely to push ectotherms into suboptimal upper temperatures, where developmental rate and stability are hypothesized to decline (Georges et al., 2005; Angilletta, 2009; Noble et al., 2021). Thus, we predict that fluctuations around warm mean temperatures will increase incubation duration (Georges et al., 2005), have little effect on sex ratio, and decrease survival and phenotypic outcomes. However, experimental work has produced mixed results, leaving us without a clear picture of how fluctuating thermal conditions affect phenotypic outcomes over and above the effects of mean temperature per se.

Temperature fluctuations may not only impact the average phenotype but also the phenotypic variance. Exposure during development to constant extreme temperatures or heat shocks has been shown to increase phenotypic variation and heritability of trait variation in *Drosophila* and other ectothermic taxa (Waddington, 1953; Hoffmann and Merilä, 1999; Rowiński and Rogell, 2017). In cold or warm oviposition sites, fluctuating temperatures may frequently reach extreme levels, thereby potentially increasing developmental instability and variation in temperature-sensitive traits. Thus, in a natural setting, variation in oviposition site characteristics and developmental temperature could create a landscape of phenotypic variation (altered means and variance) upon which selection can operate. Despite this potential, phenotypic variance in relation to fluctuations in developmental temperature is rarely investigated (St. Juliana and Janzen, 2007; Colinet et al., 2015; Rowiński and Rogell, 2017; Noble et al., 2021).

To address these shortcomings, we collated data on studies that manipulated temperature fluctuations during incubation across a range of reptile species. We used a meta-analytic approach to investigate how the phenotypic outcomes (means and variances) of embryos incubated under fluctuating temperatures differ from those

incubated under constant temperatures. Furthermore, we explored the extent to which the effects of fluctuations were mediated by both the magnitude of fluctuation and the mean temperature. Studies on fluctuating temperatures in reptiles have taken diverse experimental approaches (e.g. heat shock, irregular thermal regimes and natural thermal regimes; reviewed in Bowden et al., 2014; Massey and Hutchings, 2020). To establish valid comparisons, the present study focused on studies that have examined differing amplitudes of daily fluctuating temperatures around a stationary mean in direct comparison to a constant temperature of the same mean.

## MATERIALS AND METHODS

### Data collection

Data were primarily obtained from the Reptile Development Database (REPDEVO; version 1.0.2 downloaded from www.repdevo.com; Noble et al., 2018a, 2018b). The REPDEVO database is a comprehensive database describing the effects of egg incubation temperature on reptile phenotype, comprising 300 laboratory-based manipulative studies published between 1974 and 2016. In total, this represents nearly 10,000 phenotypes from 155 reptile species (Noble et al., 2018a, 2018b). Details of how these data were collected and extracted can be found in Noble et al. (2018a, 2018b). We complemented the data contained within the REPDEVO database with data extracted from studies published more recently (2016–2019) which, as a result, are not available in the REPDEVO database. To achieve this, we used the same search protocol (Noble et al., 2018a). Briefly, we conducted searches in Web of Science using the search terms: TOPIC:(reptil\*) AND TOPIC:(temperature\*) AND TOPIC:(incubat\*). In total, this generated an additional 140 studies, resulting in a total sample size of 440 studies.

The dataset gathered from the REPDEVO database and Web of Science was then narrowed down to studies that included fluctuating temperature treatments. To be included in the analysis, studies must have had a ‘constant’ temperature treatment ( $\leq \pm 0.5^\circ\text{C}$ ) and a daily ‘fluctuating’ temperature treatment, with the two treatments at the same mean temperature (Fig. 1). In addition, each study must have measured one or more of the following post-hatching phenotypes: incubation duration, survival (hatching success), sex ratio (proportion male), morphology (mass or length) or performance (speed – swim speed for turtles and snakes and terrestrial speed for lizards). These traits were chosen because they are widely considered as functionally – and biologically – important in reptiles (Noble et al., 2018a). This process resulted in a total of 48 papers.

We then excluded any studies where (1) the fluctuating treatments used only irregular programmes (not diel fluctuations) that

Control	25±0	ES1.1	28±0	ES2.1
Treatment A	25±2		28±2	
Treatment B	25±4	ES1.2	28±4	ES2.2

**Fig. 1. Example experimental design.** This example experiment has two mean temperatures, each with a constant temperature control and two levels of temperature fluctuation. This design allows four effect sizes (ES) that vary in the moderators of ‘mean temperature’ and ‘temperature fluctuation magnitude’. The grey area represents the minimum design requirement needed to be included in the meta-analysis: one constant control and one fluctuation temperature treatment.

prevented treatment summary as a daily mean and range, (2) temperature was not manipulated for the entire egg stage, (3) we were not able to extract all summary information for each treatment (mean, error and sample size). We contacted authors for missing data or for additional data if it seemed likely that it was collected but not presented (e.g. hatching success and sex ratio data). This resulted in a total of 22 studies included in the final analyses (Table S1; Andrewartha et al., 2010; Ashmore and Janzen, 2003; Bull and Vogt, 1979; Carter et al., 2017; Días et al., 2012; Du and Feng, 2008; Du et al., 2009; Georges et al., 1994; Les et al., 2007, 2009; Li et al., 2013a,b; Löwenborg et al., 2012; Ma et al., 2018; McGaugh and Janzen, 2011; Micheli-Campbell et al., 2012; Mullins and Janzen, 2006; Paitz et al., 2010; Patterson and Blouin-Demers, 2008; Qu et al., 2014; Shine and Harlow, 1996; Warner and Shine, 2011). The dataset included 19 different species: 9 turtles, 8 lizards and 2 snakes. Most species were unique to a single study; however, some species were the subject of several studies: *Chrysemys picta* (5 studies), *Trachemys scripta* (2 studies), *Apalone mutica* (2 studies). Studies were conducted in the USA (9 studies), China (6 studies), Australia (5 studies), Spain (1 study) and Sweden (1 study).

The sex ratio dataset was further filtered to include only species with temperature-dependent sex determination (TSD) because of our interest in examining how fluctuating temperatures affect sex ratio. There are two main types of TSD: type 1, where one sex is produced at cool temperatures and the other is produced at warm temperatures; and type 2, where one sex is produced at cool and warm temperatures and the other is produced at intermediate temperatures (Bull and Vogt, 1979). Sex determination data (genetic sex determination/TSD) was collected via the Tree of Sex database (Bachtrog et al., 2014), except in the case of two species (*Plestiodon chinensis* and *Takydromus septentrionalis*), where information was not available. Information on sex determination of these species was obtained from the author of the study publication (Weiguo Du, personal communication). Our final sex ratio dataset included 20 effect sizes from 8 studies. Sixteen effect sizes were for turtles that develop into males at cool temperatures and females at warm temperatures (TSD type 1); 4 effect sizes came from one species of lizard with males produced at intermediate temperatures (TSD type 2).

### Data extraction

For each of the 22 studies in our final dataset, we extracted sample size and trait level data from constant and fluctuating treatments. Paired treatments consisted of a constant mean temperature and a fluctuating treatment of the same mean temperature but a differing level of daily temperature fluctuation. For each study, we considered the constant temperature treatment to be the control for the study. Some studies provided multiple levels of fluctuation. For these studies, a comparison with the control was made for each level of fluctuation (Fig. 1). Treatment fluctuation was tabulated as the full daily range in temperature. For example, if a paper reported the treatment as mean±2°C, the fluctuation was noted as 4°C. In some studies, multiple mean temperatures were included in the experimental design, providing multiple controls (Fig. 1). This protocol resulted in a dependence structure between effect sizes in the same study, and those with the same temperature, which we accounted for in our analyses (discussed further below). Data on traits were extracted as either treatment-level proportions (e.g. survival and sex ratio) or trait means with their associated standard error or standard deviations (e.g. incubation duration, mass, length and speed). In one instance, data were collected across two parts of a

breeding season, and so were subsequently pooled (Carter et al., 2017). Standard errors were converted to standard deviation.

### Effect size calculation

We investigated the effect of temperature fluctuation on: (1) the treatment-level proportions and mean value of quantitative traits, and (2) the variation in quantitative traits. For treatment-level proportions, i.e. proportion survival (Eqn 1) and proportion male (Eqn 2), we calculated the log odds ratio (lnOR), which gives the relative odds that an outcome will occur in the treatment compared with a control group:

$$\ln\text{OR} = \ln\left(\frac{\text{Survive}_{\text{fluct}}/\text{Die}_{\text{fluct}}}{\text{Survive}_{\text{control}}/\text{Die}_{\text{control}}}\right), \quad (1)$$

$$\ln\text{OR} = \ln\left(\frac{\text{Male}_{\text{fluct}}/\text{Female}_{\text{fluct}}}{\text{Male}_{\text{control}}/\text{Female}_{\text{control}}}\right). \quad (2)$$

We calculated sampling variance,  $s_{\ln\text{OR}}^2$ , for proportion survival (Eqn 3) and proportion male (Eqn 4) using the following formulas:

$$s_{\ln\text{OR}}^2 = \frac{1}{\text{Survive}_{\text{fluct}}} + \frac{1}{\text{Die}_{\text{fluct}}} + \frac{1}{\text{Survive}_{\text{control}}} + \frac{1}{\text{Die}_{\text{control}}}, \quad (3)$$

$$s_{\ln\text{OR}}^2 = \frac{1}{\text{Male}_{\text{fluct}}} + \frac{1}{\text{Female}_{\text{fluct}}} + \frac{1}{\text{Male}_{\text{control}}} + \frac{1}{\text{Female}_{\text{control}}}. \quad (4)$$

Because cells with 0 counts cause infinite errors, we used the adjusted Woolf (1955) method, adding 0.5 to each count in the contingency table.

To estimate the mean value of quantitative traits (mass, length, performance and incubation duration), we calculated the log response ratio (lnRR), which represents the log of proportional difference in the means between the treatment and control group (Hedges et al., 1999; Lajeunesse, 2011):

$$\ln\text{RR} = \ln\left(\frac{\bar{x}_{\text{fluct}}^2}{\bar{x}_{\text{control}}^2}\right). \quad (5)$$

We calculated the sampling variance,  $s_{\ln\text{RR}}^2$ , using the following formula:

$$s_{\ln\text{RR}}^2 = \ln\left(\frac{(s_{\text{control}})^2}{N_{\text{control}}\bar{x}_{\text{control}}^2} + \frac{(s_{\text{fluct}})^2}{N_{\text{fluct}}\bar{x}_{\text{fluct}}^2}\right). \quad (6)$$

Here,  $\bar{x}$  is the mean value of the trait,  $s$  is the standard deviation and  $N$  is the sample size.

To look at differences in phenotypic variance for mass, length, speed and incubation duration, we calculated the log coefficient of variance ratio (lnCVR). lnCVR is the log of the ratio of the coefficients of variation for each group. The use of the coefficient of variation accounts for a potential association between the mean and SD, assuming that the standard deviation is proportional to the mean (Senior et al., 2020):

$$\ln\text{CVR} = \ln\left(\frac{\text{CV}_{\text{fluct}}}{\text{CV}_{\text{control}}}\right) + \frac{1}{2}\left(\frac{1}{N_{\text{fluct}} - 1} - \frac{1}{N_{\text{control}} - 1}\right) + \frac{1}{2}\left(\frac{s_{\text{control}}^2}{N_{\text{control}}\bar{x}_{\text{control}}^2} - \frac{s_{\text{fluct}}^2}{N_{\text{fluct}}\bar{x}_{\text{fluct}}^2}\right). \quad (7)$$



We calculated its sampling variance,  $s_{\ln\text{CVR}}^2$ , using the following formula:

$$s^2(\ln\text{CVR}) = \frac{s_{\text{control}}^2}{N_{\text{control}}\bar{x}_{\text{control}}^2} + \frac{s_{\text{control}}^4}{2N_{\text{control}}^2\bar{x}_{\text{control}}^4} + \frac{1}{2(N_{\text{control}} - 1)} + \frac{1}{2(N_{\text{control}} - 1)^2} + \frac{s_{\text{fluct}}^2}{N_{\text{fluct}}\bar{x}_{\text{fluct}}^2} + \frac{s_{\text{fluct}}^4}{2N_{\text{fluct}}^2\bar{x}_{\text{fluct}}^4} + \frac{1}{2(N_{\text{fluct}} - 1)} + \frac{1}{2(N_{\text{fluct}} - 1)^2}. \quad (8)$$

In all cases, effect sizes were calculated such that a positive value indicates an increase in that trait (proportion male, proportion survived, mean trait value, or variance) in the fluctuating treatment compared with the control treatment. Effect sizes were calculated in R (version 3.5.1; <http://www.R-project.org/>) using the function *escalc* in the *metafor* package (version 3.0-2; Viechtbauer, 2010).  $\ln\text{CVR}$  was calculated using the developmental version (version 2.4-0) of the *metafor* package, which contains an update to the effect size and sampling variance calculations following Senior et al. (2020). All data are available from figshare (<https://doi.org/10.6084/m9.figshare.17304599.v1>).

### Meta-analysis

We first ran a multi-level meta-analysis (MLMA) containing three random effects (study ID, phylogeny and effect size ID), using residual maximum likelihood (REML) to obtain an overall mean estimate of the effect size of fluctuating temperatures and an estimate of the degree of heterogeneity as  $I^2$ .  $I^2$  is the percentage of variance among effect sizes not explained by sampling error alone: estimates over 75% are considered high, 50% medium and 20% small (Higgins et al., 2003), although in multispecies analyses such as ours  $I^2 > 90\%$  is common (Senior et al., 2016). Effect size identity was included as a random effect to account for within-study effect size variance additional to sampling error. Because the trait datasets contain shared controls within a study (i.e. Fig. 1), we created a variance/co-variance matrix for each trait to account for sampling variances based on shared controls (Noble et al., 2017). For the  $\ln\text{RR}$  analyses of mass, length and speed, the matrix was not positive definite as is required for analysis, so was subject to a 'bending' procedure. The original and derived matrices were plotted against one another and were highly correlated. Our dataset included studies that contributed multiple effect sizes, species repeated across studies and species with varying phylogenetic relatedness. These could lead to biased estimates if not properly controlled for. Accordingly, we included study identity and phylogenetic relatedness as random effects. To create the phylogenetic tree containing all the species in the dataset, we used the Interactive Tree of Life online tree generator (<http://itol.embl.de/>) via the *rotl* package (Michonneau et al., 2016). The phylogenetic tree contained only taxa topology without branch lengths; therefore, this topological tree was converted to an ultrametric tree with simulated branch lengths using the *compute.brLen* function from the *ape* package (Paradis and Schliep, 2019).

### Meta-regression

We used meta-regressions to determine the effect of our moderators of interest: temperature fluctuation magnitude (continuous, range: 3–19.5°C), mean temperature (continuous, range: 23–32.5°C), and taxonomic Order (categorical, Testudines/Squamata). We also included the interaction between fluctuation magnitude and mean

temperature when sample size permitted ( $N > 40$ ). To place our continuous moderators on the same scale for comparison, 'temperature fluctuation magnitude' and 'mean temperature' were Z-transformed using the function *scale* (<http://www.R-project.org/>). We examined meta-regression models in the *metafor* package (Viechtbauer, 2010) using maximum likelihood. The models included the same random effects as above. We performed model selection using Akaike's information criterion for small sample sizes (AICc) to find which moderators were retained in the best-fit model. For this, we used the function *dredge* from the *MuMIn* package (<https://CRAN.R-project.org/package=MuMIn>) to generate an AICc table. Moderators in the top AICc model were included in the final model. The effect of each retained moderator in the final model was estimated in a meta-regression using the REML method.

### Publication bias

To test for publication bias over time in each dataset, we ran a model using the function *rma.mv* from the *metafor* package (Viechtbauer, 2010) that included all moderators and random effects in each response variable's full model along with Z-transformed publication year and precision ( $w_i$ , the inverse of the variance). Publication year was a significant predictor of  $\ln\text{CVR}$  for incubation duration (year=0.261,  $P=0.025$ ) and  $\ln\text{OR}$  for sex ratio (year=1.367,  $P=0.006$ ). In these datasets, larger effect sizes are being reported in more recent studies compared with older studies, possibly indicating selective publication in more recent literature.

## RESULTS

### MLMA: the effect of fluctuating temperature on average phenotype

We found the strength of the effect of fluctuating temperature on embryonic development varied among trait categories. Fluctuating temperature had a small, but significant overall effect on the sex ratio (Table 1) for TSD species. Specifically, embryos in the fluctuating temperature treatment had a higher chance of developing as female compared with those in the constant temperature treatment at the same mean temperature. Fluctuating temperature had no overall effect on the phenotypic mean in any other trait (Table 1). By conventional measures, overall heterogeneity in the data was high (Table 1).

### Meta-regression of moderators explaining effect of fluctuating temperature on average phenotype

Despite the low overall impact of fluctuating temperature, we found that the two main sources of variation in experimental design – the mean temperature and the magnitude of fluctuation – were important predictors of how strongly fluctuating temperature impacted mean phenotype compared with control treatments. The mean temperature at which the constant and fluctuating treatments were compared was an important moderator of the effect of temperature fluctuation on incubation duration and the odds of surviving (hatching success). At low mean temperatures, embryos in fluctuating temperature treatments had shorter incubation durations and increased survival compared with those in constant temperature treatments. At high mean temperatures, fluctuations had the opposite effect; fluctuations increased incubation duration and decreased survival rate compared with the constant temperature treatment (Table 1, Fig. 2). For example, at a mean temperature of 24°C, a 10°C temperature fluctuation (i.e.  $\pm 5^\circ\text{C}$ ) resulted in a 3.82% reduction in incubation duration compared with the constant treatment, and the odds of surviving in the fluctuating treatment were 54.05% higher than the odds of surviving in the constant treatment. In contrast, at a mean temperature of 30°C and 10°C

**Table 1. Effect of fluctuating (versus constant) incubation temperature treatments on phenotypic means**

Trait	$N_e$	$N_{st}$	$N_{sp}$	$I^2$ (%)	MLMA		Meta-regression		Fluctuation magnitude:mean temperature
					Mean	Order	Fluctuation magnitude	Mean temperature	
Incubation duration	47	18	16	99.60	InRR=-0.006 CI=-0.042, 0.029 PI=-0.145, 0.132	-	InRR=0.017 CI=-0.001, 0.034 ( $P=0.071$ )	<b>InRR=0.019</b> <b>CI=-0.002, 0.036</b> <b>(<math>P=0.030</math>)</b>	<b>InRR=0.039</b> <b>CI=0.024, 0.054</b> <b>(<math>P&lt;0.0001</math>)</b>
Survival	42	16	14	64.61	InOR=-0.315 CI=-0.833, 0.202 PI=-2.48, 1.85	-	<b>InOR=-0.401</b> <b>CI=-0.743, -0.059</b> <b>(<math>P=0.022</math>)</b>	<b>InOR=-0.704</b> <b>CI=-1.12, -0.293</b> <b>(<math>P=0.0008</math>)</b>	-
Sex ratio	20	8	7	75.63	<b>InOR=-1.192</b> <b>CI=-2.14, -0.245</b> <b>PI=-3.87, 1.48</b>	NA <sup>a</sup>	<b>InOR=-0.681</b> <b>CI=-1.28, -0.078</b> <b>(<math>P=0.027</math>)</b>	-	NA <sup>a</sup>
Mass	48	17	15	95.36	InRR=-0.014 CI=-0.046, 0.017 PI=-0.156, -0.127	-	-	-	-
Length	47	16	14	95.97	InRR=-0.006 CI=-0.021, 0.010 PI=-0.076, 0.065	-	-	InRR=-0.010 CI=-0.021, 0.001 ( $P=0.072$ )	-
Speed	37	9	8	97.48	InRR=0.279 CI=-0.219, 0.777 PI=-0.812, 1.370	InRR=0.636 CI=-0.394, 1.66 ( $P=0.226$ )	-	-	NA <sup>a</sup>

The table shows parameter estimates with 95% confidence intervals (CI) and 95% prediction intervals (PI) from the multi-level meta-analysis (MLMA) model and the top Akaike's information criterion (AIC) model of the meta-regression for each trait analysis. We also report the heterogeneity percentage ( $I^2$ ; MLMA model). Effect size for incubation duration, speed, mass and length is log risk ratio (InRR) and effect size for survival and sex ratio is log odds ratio (InOR). Bold indicates significance; 'NA' denotes a factor not included in the full model; '-' denotes a factor was included in the full model but not in the AIC-selected model.  $N_e$  represents the number of effect sizes,  $N_{st}$  represents the number of studies and  $N_{sp}$  represents the number of species for each dataset. <sup>a</sup>Low sample sizes prevented inclusion of all predictor variables.

fluctuation, there was a 4.61% increase in incubation duration, and the odds of hatching were 67.32% lower in the fluctuating treatment compared with the odds of hatching in the constant incubation treatment. The mean temperature at which fluctuations switched from being a benefit to a cost (assuming a fluctuation of 10°C) were very similar for these two traits: the effect sizes were predicted to be 0 at 26.78°C for incubation duration and 25.70°C for survival. The importance of mean temperature for incubation duration was amplified when fluctuation magnitude was high (i.e. a significant interaction term; Table 1).

The fluctuation magnitude significantly affected survival and the sex ratio. As the magnitude of temperature fluctuations increased, the odds of surviving decreased and the odds of producing a male decreased (odds of female increased) in the fluctuating treatment compared with in the control treatment (Table 1, Fig. 2F).

Overall, taxonomy was not an important moderator of the effect of fluctuating incubation temperatures across the phenotypic traits measured. While Order was included in the AIC-preferred model of speed, it was not a significant predictor in the meta-regression (Table 1).

#### **MLMA: the effect of fluctuating temperature on phenotypic variance**

Temperature fluctuation had a relatively weak overall effect on phenotypic variance, with confidence intervals overlapping zero for all traits measured (Table 2). Overall heterogeneity within the data was high (Table 2).

#### **Meta-regression of moderators explaining effect of fluctuating temperature on phenotypic variance**

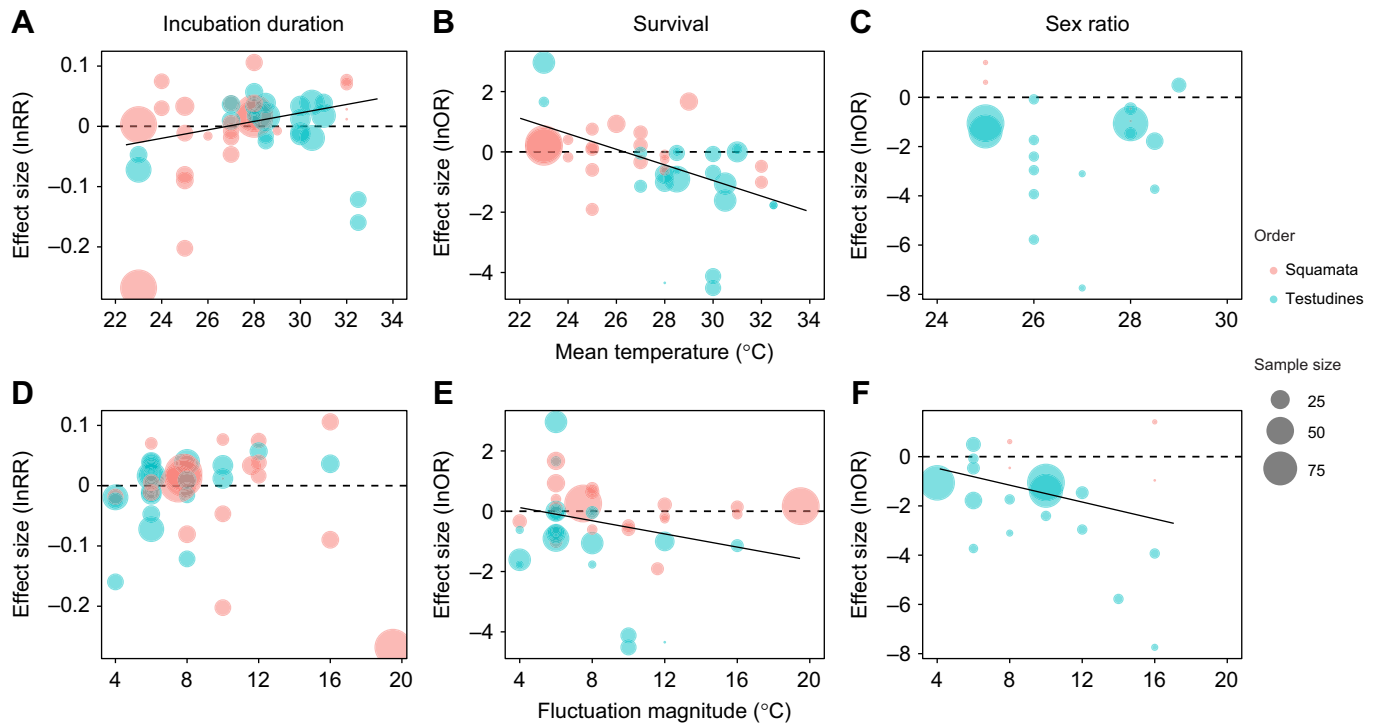
Our moderators provided little explanatory power with respect to the impact of fluctuating temperature on phenotypic variance. As mean temperature increased, there was a non-significant trend for phenotypic variance in incubation duration to become increasingly

reduced in the fluctuating treatment compared with the constant treatment. We also found that temperature fluctuation (compared with constant) caused a greater reduction in the variance of speed in turtles than in squamates (Fig. 3, Table 2).

#### **DISCUSSION**

Embryos of ectothermic organisms rarely experience constant temperatures during development. Instead, they are typically exposed to considerable fluctuation in the thermal environment (Bowden et al., 2014; Colinet et al., 2015). While the impacts of these fluctuations on embryonic development have been reviewed qualitatively (Bowden et al., 2014; Colinet et al., 2015; Booth, 2018; Massey and Hutchings, 2020), this is the first quantitative meta-analysis examining the strength of evidence across studies for an impact of fluctuating temperature on phenotypic means and variance. We show that temperature fluctuation impacts embryonic development (developmental time, egg hatching success and sex ratio) in a manner that has been predicted qualitatively for diverse ectothermic taxa (Sharpe and DeMichele, 1977; Georges et al., 2005; Colinet et al., 2015; Massey and Hutchings, 2020). Specifically, the fundamental physiological phenomenon of thermal performance curves (temperature-dependent developmental rates) causes fluctuations to impact animals differently at cool mean temperatures (generally beneficial) compared with warm mean temperatures (generally costly). Moreover, greater fluctuations often lead to greater impacts.

Our findings for incubation duration and survival (hatching success) provide support for developmental rates in reptiles increasing with warmer temperatures up to a maximum, then declining at sublethal and lethal hot temperatures. This pattern has been difficult to demonstrate with constant temperature experiments owing to mortality at constant hot temperatures. In our study, temperature fluctuation at cool mean temperatures caused shorter incubation duration and higher survival (particularly when



**Fig. 2. Effect sizes of mean phenotype in fluctuating versus constant incubation treatments as a function of mean temperature and fluctuation magnitude for incubation duration, sex ratio and survival.** For the log response ratio (lnRR) for incubation duration ( $N=47$ ), plotted versus mean temperature (A;  $P=0.030$ ) and fluctuation magnitude (D), values above zero reflect animals from the fluctuating treatment having longer incubation durations than those from the control treatment; values below zero indicate fluctuating treatments had shorter incubation durations. For the log odds ratio (lnOR) for survival ( $N=42$ ), plotted versus mean temperature (B;  $P=0.0008$ ) and fluctuation magnitude (E;  $P=0.022$ ), values above zero reflect animals from fluctuating treatments having higher survival rates than control; values below zero indicate fluctuating treatments had lower survival rates. For the log odds ratio (lnOR) for sex ratio ( $N=20$ ), plotted versus mean temperature (C) and fluctuation magnitude (F;  $P=0.027$ ), values above zero represent an increased chance of developing as male in the fluctuating treatment compared with the control; values below zero represent a reduced chance of developing as male in the fluctuating treatment compared with the control. Regression lines were estimated from meta-regression models at the average level of fluctuation difference or mean temperature present across studies in each dataset.

fluctuations were large), owing to development accelerating when fluctuations reach warm temperatures. In contrast, incubation duration was extended and survival decreased when fluctuations occurred around warm mean temperatures, presumably owing to diminished development at extreme hot temperatures. Indeed, our findings for incubation duration show striking congruence with predictions arising from non-linear developmental models (Sharpe–deMichele model), and do not resemble predictions from linear models (Georges et al., 2005). This pattern across mean temperatures has also been described in insects, fish and amphibians (Arrighi et al., 2013; Colinet et al., 2015; Massey and Hutchings, 2020). For example, in the green peach aphid, *Myzus persicae*, fluctuating temperatures increased survival at the coolest mean temperature (15°C) but decreased survival at all warm mean temperature treatments (20, 30 and 32°C) (Davis et al., 2006). Clearly, these physiological processes are fundamental to ectothermic developmental biology and deserve more cross-taxon, quantitative treatment to measure the strength of the effects.

Importantly, our novel cross-species, quantitative approach allowed us to estimate the key temperatures whereby fluctuations transitioned from being beneficial to being costly for phenotypic development. We found that the temperatures where the switch between benefit and cost occurs (~26–27°C, with 10°C fluctuation) were slightly cooler than the average optimal constant temperatures for hatching success (27–29°C; Du and Shine, 2015; Noble et al., 2021) and cooler than the common range of pivotal temperatures for

turtles with TSD (27–28°C; Hulin et al., 2009). Therefore, our results suggest that natural fluctuations in nest temperature might decrease the optimal mean temperature for hatching success and cause extra embryonic mortality and delayed incubation for whichever sex develops at warm temperatures under TSD (often females).

Given that fluctuations around a warm mean temperature led to reduced developmental rate and decreased survival, it seems likely that these conditions involve exposure to ‘extreme’ temperatures that are stressful to normal development. Stressful conditions often increase phenotypic variation in addition to impacting the phenotypic mean, typically attributed to developmental instability (Hoffmann and Hercus, 2000; Rowiński and Rogell, 2017; Scharloo, 1991; but see Noble et al., 2021). In insects, heat shock or extreme temperatures can lead to increased phenotypic variation and even novel phenotypes (Hoffmann and Merilä, 1999; Rowiński and Rogell, 2017; Suzuki and Nijhout, 2006; Waddington, 1953). However, in our study, variance in all trait types we examined was, on average, lower (non-significantly) in the fluctuating treatment compared with the constant treatment, opposite to our prediction. Phenotypic variance in reptiles also appears to be minimally impacted by constant hot and cold temperatures (Noble et al., 2021), with variance only increasing at extreme temperatures in one trait category (post-hatching growth) that we were not able to include in the present study owing to insufficient data. One interpretation of previous work is that extreme and constant temperatures are simply

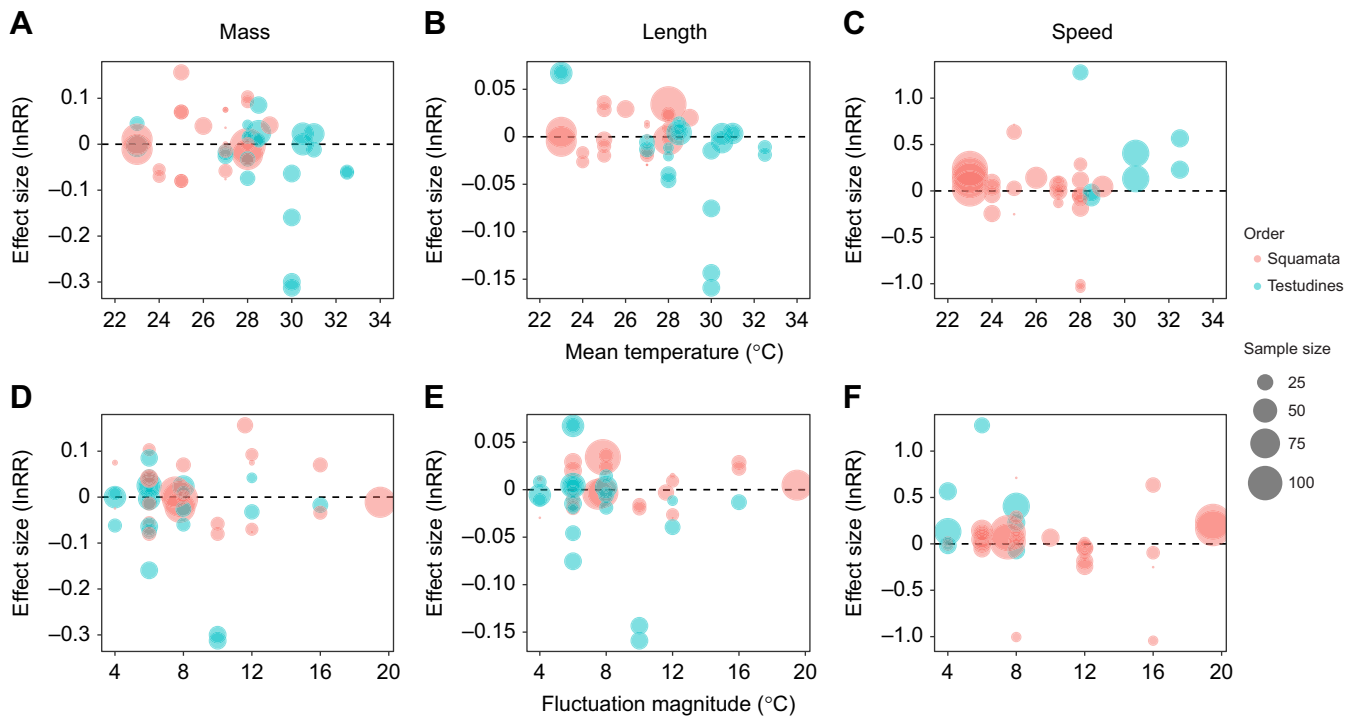
**Table 2. Effect of fluctuating (versus constant) incubation temperature treatments on phenotypic variance**

Trait	MLMA				Meta-regression			Fluctuation magnitude:mean temperature
	$N_e$	$N_{st}$	$N_{sp}$	$I^2$ (%)	Mean	Order	Mean temperature	
Incubation duration	47	18	16	92.36	InCVR=-0.171 CI=-0.432, 0.091 PI=-1.54, 1.20	-	InCVR=-0.187 CI=-0.400, 0.026 ( $P=0.085$ )	-
Mass	48	17	15	88.28	InCVR=-0.133 CI=-0.405, 0.140 PI=-1.25, 0.986	-	-	-
Length	47	16	14	91.15	InCVR=-0.110 CI=-0.424, 0.203 PI=-1.40, 1.18	-	-	-
Speed	37	9	8	92.36	InCVR=-0.195 CI=-0.836, 0.447 PI=-1.95, 1.56	<b>InCVR=-1.167</b> <b>CI=-2.08, -0.258</b> <b>(<math>P=0.012</math>)</b>	-	NA <sup>a</sup>

The table shows parameter estimates with 95% confidence intervals (CI) and 95% prediction intervals (PI) from the multi-level meta-analysis (MLMA) model and the top Akaike's information criterion (AIC) model of the meta regression for each trait analysis of phenotypic variance. We also report the heterogeneity percentage ( $I^2$ ; MLMA model). Effect size for incubation duration, speed, mass and length is log coefficient of variance (InCVR). Bold indicates significance; 'NA' denotes a factor not included in the full model; '-' denotes a factor was included in the full model but not in the AIC-selected model.  $N_e$  represents the number of effect sizes,  $N_{st}$  represents the number of studies and  $N_{sp}$  represents the number of species for each dataset. <sup>a</sup>Low sample sizes prevented inclusion of all predictor variables.

too extreme to support any developmental deviations (Noble et al., 2021). However, our congruent findings from even inconstant exposure to extreme temperatures supports the conclusion that extreme temperatures and fluctuating temperatures have little effect on developmental processes that increase viable variation in reptiles (St. Juliana and Janzen, 2007).

The trait that exhibited the greatest overall difference between fluctuating and constant temperature treatments was offspring sex. For species with TSD, the proportion of development occurring at a given temperature (i.e. the amount of sex-specific tissue generated) is expected to be a better predictor of sexual outcome than is the proportion of time spent at that temperature (Georges et al., 1994). Our results support this hypothesis across the taxa included. The



**Fig. 3. Effect sizes of mean phenotype in fluctuating versus constant incubation treatments as a function of mean temperature and fluctuation magnitude for mass, length and speed.** For the log response ratio (lnRR) for mass ( $N=48$ ), plotted versus mean temperature (A) and fluctuation magnitude (D), values above zero reflect animals from the fluctuating treatment having larger mass than those from the control treatment; values below zero indicate fluctuating treatments had smaller mass. For the log response ratio (lnRR) for length ( $N=47$ ), plotted versus mean temperature (B) and fluctuation magnitude (E), values above zero reflect animals from fluctuating treatments having longer length than control; values below zero indicate fluctuating treatments had shorter length. For the log response ratio (lnRR) for speed ( $N=37$ ), plotted versus mean temperature (C) and fluctuation magnitude (F), values above zero reflect animals from the fluctuating treatment having faster speed; values below zero represent animals in the fluctuating treatment having slower speed.



majority of reptiles in our sex ratio dataset are turtles, where sex ratios show a greater proportion of males at low temperatures and females at high temperatures (TSD) (Bull and Vogt, 1979). Thus, as embryos experience greater fluctuations, they experience more of their development at higher, feminizing temperatures, resulting in a greater proportion of females. However, non-linearity in developmental rates may counter-intuitively increase male production at warm, fluctuating temperatures if the hot extremes prevent development altogether while the permissive cool extremes are male producing (Neuwald and Valenzuela, 2011). Moreover, the timing of exposure to idiosyncratic temperature fluctuations can have a dramatic impact on sex ratio (Breitenbach et al., 2020). For example, two thermal regimes with similar CTEs but with different timing of exposure to warm temperatures can lead to different sex ratio outcomes (Breitenbach et al., 2020). Analogously, two thermal regimes with very different CTEs can produce the same sex ratio outcome if they experience similar temperatures during a critical phase of temperature sensitivity (Carter et al., 2018). Unfortunately, very few studies exist for other vertebrates or invertebrates to assess how well heat summation (e.g. a constant temperature equivalent) predicts sex ratios in these taxa (e.g. Baras et al., 2000). Importantly, the role of fluctuating temperature in sex ratios extends beyond species with TSD, as temperature fluctuation can impact sexual development in fish with genotypic sex determination (Baras et al., 2000; Coulter et al., 2015). The sex ratio is a crucial population demographic; thus, greater understanding of the effects of natural temperature fluctuations on sexual outcome is imperative for understanding the evolutionary and ecological dynamics of thermally sensitive species (Bowden and Paitz, 2018; Schwanz and Georges, 2021).

We also provide the first examination of the effect of temperature fluctuation for traits that are less closely tied to developmental rates – morphology and performance – and show that fluctuations have little impact on these traits. These trait differences are consistent with other work in reptiles that has shown that while constant temperature treatments can have a significant impact on both morphology and performance (Booth, 2006; Deeming, 2004; Noble et al., 2018a), they have a much stronger effect on incubation duration, survival and sex ratio (Noble et al., 2018a). In non-reptile taxa, there are very few studies that explore how fluctuating temperatures affect morphology, and none, to our knowledge, that investigate the impact on performance (Colinet et al., 2015; Massey and Hutchings, 2020). In fruit flies, large fluctuation magnitudes reduce wing size, thorax size and mass (Colinet et al., 2015). For fish, fluctuating water temperatures can lead to smaller larvae (Scoppettone et al., 1993; Schaefer and Ryan, 2006; Kupren et al., 2011), or have no effect on morphology (Steel et al., 2012). Similarly, body size in frogs has been shown to increase (Arrighi et al., 2013) or decrease (Niehaus et al., 2011; 2012) in response to fluctuating temperatures. Thus, it is clear that while temperature fluctuations impact a variety of morphological traits, the effect varies among taxa, specific traits or experimental conditions.

High variation among taxa (i.e. high heterogeneity) may reflect adaptation to fluctuation. Populations that have less-sheltered oviposition sites naturally experience more embryonic temperature fluctuation (Booth, 2018; Tiatragul et al., 2019), and may be less sensitive to fluctuations. Within reptiles, turtles tend to construct deeper nests than squamates (e.g. sea turtle nests experience low diel temperature fluctuations; Booth, 2018), so we might have predicted that turtles are more sensitive than squamates to fluctuations. This was not the case in our dataset. Testing this prediction rigorously requires population-specific information on natural temperature

fluctuations, which we could not find for a sufficient number of the species in our dataset.

What are the potential consequences of our findings for ectotherm fitness and evolution? In cooler to average mean temperatures, increased fluctuations are advantageous because incubation duration decreases and survival increases. In general, reptiles that develop in hotter conditions develop quicker, hatch sooner and are smaller with a larger residual yolk reserve than those that are incubated for longer periods of time (reviewed by Deeming, 2004; Kingsolver and Huey, 2008; Noble et al., 2018a). While bigger is usually considered better (Kingsolver and Huey, 2008), a smaller hatchling with more residual yolk could, in some scenarios, survive longer in an environment with a scarcity of food, and have faster post-hatching growth (meaning they spend less time exposed to gape-limited predators) (Booth, 2006). Despite our finding that fluctuations reduce incubation duration (at cool temperatures), we did not find a matching pattern of effect on body size and length between fluctuating and constant incubation temperatures. Thus, interestingly, fluctuations in temperature have the potential to decouple the links often observed between developmental time and body size, potentially allowing the two traits to evolve independently in a way that constant temperatures do not allow.

Currently, our understanding of how thermal developmental plasticity actually plays out in the wild may be relatively poor. Our study demonstrates that the wealth of knowledge about thermal performance curves under constant developmental temperatures may be leveraged to make quantitative predictions of phenotypic outcomes under natural temperature fluctuations (e.g. Georges et al., 2005). However, there are likely to be limitations to the predictive ability for natural nests, which fluctuate more idiosyncratically (Booth, 2018; Bowden and Paitz, 2018). To examine how fluctuations per se impact phenotypes, we need more studies that specifically compare constant, fluctuating and idiosyncratic regimes with the same heat summation metric (e.g. Carter et al., 2017; 2018; Hall and Warner, 2020; see metrics in Georges et al., 2005; Massey et al., 2019; Valenzuela et al., 2019). In particular, the timing and duration of exposure to fluctuations and heat waves is known to have a large impact on offspring phenotype (Carter et al., 2018; Breitenbach et al., 2020). In ectotherms, short durations of extreme temperature fluctuations outside the optimal developmental range often do not significantly affect embryo development, but repeated or prolonged exposures can be lethal (Overall, 2014; Davis et al., 2006; Les et al., 2009; Colinet et al., 2015; Hall and Warner, 2018; 2021). In addition, reptile embryos are likely to become less sensitive to temperature and heat waves as they develop (Howard et al., 2014).

While thermal developmental biology is studied intensely in invertebrate taxa, reptiles are excellent models for developing a quantitative framework of the impacts of temperature fluctuation owing to the relative ease of recording natural developmental temperatures. Terrestrial nests can be easy to locate and large enough to fit temperature loggers, and emergent offspring can be relocated for trait measurement. Examining the isolated impacts of altered fluctuations is ecologically meaningful because eggs deposited at different depths experience different fluctuations even with the same mean temperature across depths (Georges, 1992; Booth, 2006; 2018). Moreover, among natural nests, particularly shallow ones, increased fluctuations are likely to occur concomitantly with increased mean temperature (Weisrock and Janzen, 1999; Pearson and Warner, 2016; Tiatragul et al., 2019; but see Warner and Shine, 2011), so the negative effects observed under these conditions are particularly relevant in a wild setting.



With climate change predicted to bring increased temperature means, seasonally increased fluctuations and extreme weather events (such as drought, excessive precipitation and heatwaves) (Pachauri et al., 2014; Vázquez et al., 2017), a change in thermal variance could impact on organismal fitness as much as or more than an increase in mean temperature (Bozinovic et al., 2011). This has fundamental implications for our ability to make predictions regarding how natural variation and directional changes in thermal environments mediate key ecological and evolutionary processes.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: L.E.S., D.W.A.N., A.M.S., R.S.R.; Methodology: L.E.S., D.W.A.N., A.M.S., R.S.R.; Validation: D.W.A.N.; Formal analysis: R.S.R., D.W.A.N., A.M.S.; Resources: L.E.S.; Data curation: R.S.R., D.W.A.N., J.L.R., A.M.S., D.A.W.; Writing - original draft: R.S.R.; Writing - review & editing: R.S.R., D.W.A.N., J.L.R., A.M.S., D.A.W., G.M.W., L.E.S.; Supervision: L.E.S.; Project administration: R.S.R., L.E.S.

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#### Data availability

Data are available from figshare (<https://doi.org/10.6084/m9.figshare.17304599.v1>).

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