TEMPERATURE EFFECTS ON DEVELOPMENT AND PHENOTYPE IN A FREE-LIVING
POPULATION OF WESTERN POND TURTLES (*EMYS MARMORATA*)

By

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Date

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ABSTRACT

Changes in temperature regimes are occurring globally due to climate change as well as habitat alterations. Temperatures are expected to continue to rise in the future, along with more frequent heat waves and extreme weather events. This could have potentially serious consequences for oviparous ectotherms, especially those with temperature-dependent sex determination. In order to investigate the effects of temperature on a range of developmental phenomena in a population of western pond turtles (*Emys marmorata*), we placed temperature sensors at several egg levels within the nests and recorded temperatures at one hour increments. We found that mean incubation temperatures were similar between different nests and at different levels within nests, but that incubation temperature fluctuations and maximum incubation temperatures differed wildly in both cases. We also found that sex determination, hatching success, and incubation duration were all significantly affected by incubation temperature.

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Table of Contents

Abstract.....	iv
Acknowledgement:	v
Introduction	1
Methods	1
Results	7
Discussion	9
Tables and Figures	21

INTRODUCTION

Environmental perturbations, including climate change and habitat alterations, are considered a potential threat to many organisms on a global scale (Gibbon, et al., 2000; McCarty, 2001; Rosenzweig et al., 2008; Quesnelle et al., 2013). While these factors can have a variety of effects on the environment, one commonality is a change to the temperature regime. According to the 2013 report from the Intergovernmental Panel on Climate Change (IPCC), global mean surface temperature is likely to increase by 2°C, with more frequent heat waves very likely as we move into the late 21st century. Habitat loss and alteration can also lead to increased surface temperatures locally through the addition of roads or loss of vegetation (Trombulak & Frissell, 2000; Reese & Harvey, 2002). Along with their individual effects, a synergistic deleterious effect of the combination of climate change and habitat loss/alteration has been suggested (Mantyka-Pringle et al., 2012). While it is hard to predict how a small rise in temperature will affect most animals, it is clear that those living close to their thermal limits are most at risk (Stillman, 2002; Somero, 2010).

All living organisms have upper and lower thermal limits that vary between taxa and habitat type (Gates, 1980). In many cases, early life stages are the most sensitive to these limits due to the occurrence of a suite of critical developmental processes (Fuiman & Werner, 2009; Stebbins & Cohen, 1995). For oviparous ectotherms, almost every aspect of development is affected by incubation temperature, with many of these effects lasting into adulthood (e.g., sex, growth, locomotor

performance) (Rhen & Lang, 1995; Booth, 2006). This makes these organisms particularly vulnerable as they cannot regulate temperature during their embryonic stage. One group of oviparous ectotherms that is especially sensitive to changes in incubation temperature are those with temperature-dependent sex determination (TSD), as these species are at risk of potentially dire consequences due to alterations in sex ratios that may result from environmentally caused temperature changes (Schwanz et al., 2010). TSD occurs when sex is determined by the incubation temperature, not by genetics. Sex-determination is known to be affected by the temperature during the middle third of the incubation period, also known as the thermosensitive period (TSP) (Bull and Vogt, 1981). Turtles, of which the majority of the approximately 300 turtle species are known to exhibit TSD (Ernst & Lovich, 2009), are currently experiencing severe population declines, with 134 of the 221 species considered critically endangered, endangered, or vulnerable according to the IUCN Redlist (Version 2013.2).

Most studies of turtle development, including TSD, have been done in *ex situ* incubation conditions, under constant temperatures (Rhen & Lang, 1999; Kettlewell et al., 2000; Ligon et al., 2009; Geist et al., in press). These studies have shown that the majority of turtle species exhibit type 1A TSD, in which females develop at warmer temperatures and males at cooler temperatures (Bull, 1980; Ewert et al., 1994). They have also shown that there is a pivotal temperature at which a 1:1 sex ratio will occur. Along with TSD, there are a variety of other important developmental factors that have been examined in a number of turtle species, including hatching success and incubation duration. Studies investigating hatching

success have shown that there is a range of temperatures in which embryos develop successfully, with hatching success dramatically declining at extremes of the temperature range. The same studies have found that within the range of temperatures that produce viable hatchlings, development tends to proceed more rapidly at higher temperatures (Booth, 1998; Les et al., 2009; Ligon & Lovern, 2009). These data, while valuable, do not necessarily reflect the developmental and phenotypic responses to temperature variation inherent to *in situ* incubation environments. Models have been created to account for diel fluctuation and make predictions about effects on sex determination (Georges, et al. 2005), and several studies have simulated a natural incubation environment by having the temperature fluctuate around a mean at various fixed rates (Wilhoft et al., 1983; Georges et al., 1994; Les et al., 2007). Results of these studies show that fluctuating temperatures tend to produce more females than would be predicted by the mean temperature. However, natural nest temperatures often do not show a consistent fluctuation around a mean, and thus several recent studies have focused on the phenotypic effects of temperature in naturally incubated nests. Some of these studies looked at the effects of *in situ* temperature fluctuations compared to those that were incubated in under laboratory conditions (Paitz et al., 2010; Micheli-Campbell et al., 2012), others have recorded nest temperatures in relation to habitat types (Freedberg et al., 2011; Refsnider 2013, J. M). It is becoming increasingly apparent that the fluctuations of incubation temperatures play a key role in phenotypic outcomes (Georges 2013). With environmental conditions around the world being altered dramatically due to various types of human activities, it is prudent to

understand how natural temperature regimes currently affect development in turtles, and how predicted changes to temperatures may affect developmental patterns.

The western pond turtle (WPT), *Emys* (aka *Clemmys*, *Actinemys*) *marmorata*, is a TSD species and is the only native freshwater turtle along the entire Pacific Slope of the United States. Historically, *E. marmorata* was abundant throughout its range, from northern Washington to northern Baja California (Storer, 1930; Stebbins, 1985). In recent times, habitat loss/fragmentation, introduced predators, non-native competitors and commercial exploitation for food (Hays, et al. 1999) have contributed to their decline. In response to these declines, this species is now listed by the respective state Departments of Fish and Wildlife as endangered in Washington, threatened in Oregon and a species of special concern in California. The objective of this study is to investigate the *in situ* incubation temperatures of a population of (WPT) and examine how they affect hatching success, sex determination, and incubation duration. It is important to understand the effects of current temperatures in order to hypothesize about how predicted changes in temperature could potentially affect WPT.

METHODS

Data were collected during active nesting of WPT in the months of June and July between 2010 and 2012 at a vernal lake in Lake County, CA with an elevation of

approximately 1000 meters. The nesting ground is part of an approximately 62-hectare preserve managed by The Nature Conservancy and The California Department of Fish and Wildlife. Western pond turtle nesting activity has been noted at this site since 2004 (Geist et al., in press).

Female western pond turtles were identified through visual surveys during terrestrial nesting forays. Those found nesting were watched from a distance as to not disturb the nesting process. Gravid females found not nesting were fitted with radio transmitters and returned to the edge of the pond. All such females were returned to nest within the next several days. All captured females were given unique carapace notches according to the Holland method (1991) in order to record nesting behavior during the season and in subsequent years. Radio telemetry was used to determine if females were out of the water and to track them to their nesting sites. After nest construction was complete, eggs were removed and marked with a pencil to denote the nest number and given a unique letter for identification during removal. Morphometric data were recorded from each egg (mass, length, width). All eggs were then returned to the nest along with Thermochron I-button temperature sensors to record temperatures every hour throughout the *in situ* incubation period. A sensor was placed on the top layer of eggs in 31 nests, while 10 nests from 2012 had sensors at both the top and at lower levels within the nest as diagramed in figure 1. Only eggs that were directly next to or below the temperature sensor were included in the analyses. Pictures were taken and diagrams were drawn from different angles of each nest to ensure the final positions of the eggs were clearly known.

After approximately 80 days, eggs were collected from the field and brought to Sonoma State University to be maintained in an incubator set at 29°C, the calculated pivotal temperature (Geist, et al., In Press), for the duration of their incubation. Eggs were monitored several times a day for pipping and hatching activity. Hatching success was recorded and incubation duration was calculated from the date of oviposition. Once hatched, hatchling morphometrics were recorded and the turtles were transported to the Oakland and San Francisco zoos for captive husbandry. At approximately nine months of age, the sex of each hatchling was determined using an endoscopic procedure in which the gonads were visually identified (figure 2). All procedures were done by the veterinary staff of the San Francisco and Oakland zoos.

Thermochron I-button temperature sensors were recovered along with the eggs and data from the sensors were downloaded for analysis. Temperature summaries were performed for the entire incubation period, as well as the middle third of the incubation period (TSP) including the mean, standard deviation (to measure amount of temperature fluctuation), maximum incubation temperature, and time spent above 29°C (the predicted pivotal temperature). Specifically, it has been demonstrated that the thermosensitive period occurs during the middle third of embryonic development of the incubation period, and thus is based on the stage of development, rather than the time/day of incubation *per se* (Georges, et al., 1994). However, since we could not accurately determine the embryonic stage for each turtle while eggs were incubating *in situ* we were limited to approximating the thermosensitive period by using time since oviposition.

Generalized linear models with logit linking functions were used to examine the effects of the *in situ* incubation temperature on sex determination and hatching success. A general linear model was used to test the effects of incubation temperature on incubation duration. The effects of maternal identity, egg size, and clutch size were not significant in any analysis so they were removed from all models. Model effects were assessed for multicollinearity using variance inflation factors. If collinear, only the best predictor for each analysis was included in the models.

RESULTS

The overall ranges of the temperature summaries are shown in table 1. Incubation temperature fluctuations and maximum incubation temperatures were both very different between and within nests. However, the mean temperatures only ranged approximately 5°C between nests and were virtually the same within nests, regardless of the position (i.e. top or bottom).

157 eggs were included in this analysis with 94 being viable. Eight individuals that hatched either did not survive long enough or grow large enough to be sexed thus they were not included in the sex determination analysis. Of those sexed, 59 were female and 27 were male. Incubation durations ranged from 75 to 134 days.

Sex Determination

Sex determination was significantly affected by the time spent above 29°C during the sex-determining middle third of the incubation period (LR $\chi^2=48.79$, $p<0.0001$)

and the temperature fluctuation (standard deviation of nest temperatures) during the middle third of the incubation period ($\text{LR } \chi^2=4.60$, $p=0.0320$). During this period, the more time the embryo spent above 29°C and the higher the temperature fluctuation, the more likely it was to be female (Figure 3).

Hatching Success

Maximum incubation temperature had a significant effect on hatching success ($\text{LR } \chi^2=47.8$, $p<0.0001$), while mean incubation temperature did not ($\text{LR } \chi^2=0.63$, $p=0.43$). Higher maximums were more likely to render eggs inviable. Since mean temperature did not impact viability, it was removed from the model and a logistic regression was performed to make an inverse prediction (Figure 4) to identify the maximum temperature at which we would expect a 50% rate of inviability (LT_{50}). This temperature was calculated to be 40.3°C.

Incubation Duration

Incubation duration was significantly affected by both mean incubation temperature ($\text{LR } \chi^2=60.47$, $p<0.0001$) and maximum incubation temperature ($\text{LR } \chi^2=4.06$, $p=0.0471$). Higher mean temperatures led to shorter incubation periods and higher maximum temperatures led to longer incubation periods. The interaction of mean incubation temperature and maximum incubation temperature was also significant ($\text{LR } \chi^2=45.44$, $p<0.0001$). We determined the meaning of the significant interaction term by color-coding its leverage plot. We used the colors of the rainbow in order (i.e. red, orange, yellow, green, blue, and violet) starting with the hottest (red) down to the coolest temperatures (violet). Figure 5A shows mean color-coded and figure

5B has maximum color-coded. We found that eggs that had the highest mean and maximum incubation temperatures along with those that had the lowest mean and maximum incubation temperatures had the longest incubation duration. Those that had moderate means and maximums or a combination of high and low means or maximums had shorter incubation periods. Eggs that experienced the combination of low means and low maximums or high means and high maximums had longer incubation periods than those that experienced moderate means and maximums or a combination of high means and low maximums or low maximums and high means (figure 5).

DISCUSSION

Our data show that different aspects of *in situ* incubation temperatures had significant effects on sex determination, hatching success, and incubation duration in this population of western pond turtles. The incubation temperatures in our study regularly fluctuated approximately 20°C with maximums typically above 37°C, which is higher than incubation temperatures found in most other studies. Our maximum temperatures and temperature fluctuations are also considerably higher than those seen in *ex situ* fluctuating incubation temperature experiments.

Recording temperature data from I-button temperature sensors placed at several levels within the nests provided a unique look at the differences in the temperature regimes within and between nests in this population of western pond turtles. Since our analysis was performed on individual eggs, and not by nest, this allowed us to

investigate what aspects of natural temperature regimes affect sex-determination, hatching success, and incubation duration with a high level of resolution.

Sex Determination

The results of our *in situ* data show that time spent above the pivotal temperature, as well as the amount of fluctuation were significant in determining sex. More specifically, turtles that spent more than a third of their thermosensitive period above this pivotal temperature were more likely to be female (Figure 3). Also, the degree to which the temperature fluctuated above the pivotal temperature was significant, with higher fluctuation leading to more females. There were only three individuals that would have been predicted to be the opposite sex. This also may be due to individual differences within the population (Wilson 1998). This would potentially be selective in an evolutionary context because during times of climactic uncertainty, it would be advantageous to have some individuals in a population that vary from the thermal means in regard to temperature sensitivity. If these individual differences are heritable, it could lead to a shift in pivotal temperature under changing ambient conditions. For example, if temperatures increase to a level at which we would predict only females, the outliers that ended up male could still reproduce to pass on their alleles thus driving a change in the pivotal temperature for the population. This would potentially enable both females and males to persist in the population even under altered environmental conditions. Turtles, with a nearly 230 million year evolutionary history, have lived through many changes in

climate, and individual variation in the temperature sensitivity of the population may be a factor that aided them.

From 2010-2012, the hatchling sex ratio for this population was consistently in the range of 65%-70% female. These years experienced relatively consistent summer temperatures for this site, suggesting that most years would be expected to produce cohorts with a female bias in the hatchling sex ratio. Other studies of turtle hatchlings have shown a similar female bias (Georges, 1992; D. T. Booth 2006; Correa-H, et al., 2010). Considering that one male can fertilize the eggs of many females and female turtles are known to be capable of storing sperm until the next nesting season, this sex ratio may be sustainable short-term (Pearse & Avise, 2001; Roques, et al., 2006). However a consistent and relatively fast rise in temperature could negate the evolutionary potential of the individual differences in temperature sensitivity and the sex ratio could theoretically approach 1:0 (Janzen 1992). Over time, such a sex ratio is not stable and reproductive success would be reduced (Neslon, et al. 2004).

Hatching Success

Hatching success is arguably the most important aspect of development. If the embryos do not develop, all other temperature effects on phenotype are moot. We expected hatching success to be affected by the mean and the maximum temperature since prior studies demonstrated a low hatching rate above of 30°C at constant temperatures under laboratory conditions (Geist et al., in press). However, our results indicate that mean nest temperature was not significant in determining

hatching success. This makes sense when noting that the means showed little variation between multiple egg layers within the same nest and only ranged approximately 5°C between nests. Also, all means were lower than 30°C. Conversely, the maximums differed considerably within and between nests.

Our analysis showed that the LT_{50} was approximately 40°C. We also found that being exposed to a 45°C maximum incubation temperature would lead to an expected 90% rate of inviability. In the three years included in this study, most nests had maximum temperatures close to or above the LT_{50} , and it is clear that most eggs are experiencing temperatures approaching the limits of their thermal tolerance. Thus, even relatively minor increases in average and maximum temperatures due to climate change and habitat alterations would likely have seriously deleterious effects on viability for this species.

Incubation Duration

Mean incubation temperature, maximum incubation temperature, and the interaction of mean and maximum incubation temperature all had significant effects on incubation duration. While higher means lead to shorter incubation durations, higher maximums actually have the opposite effect. We presume this is because some of the incubation temperature maximums found at this site are too high to support normal development. This retardation of development leads to a longer incubation period overall, a pattern typical in reptilian development (Deeming & Ferguson, 1991). This species is known to overwinter in the nest and emerge the next spring. Since embryos typically use more energy during a long incubation

period compared to a shorter one (Gutzke 1987). Longer incubation periods could leave hatchlings with low energy reserves, possibly making it difficult for them to survive.

Conclusion

It is clear that most eggs in this population are developing at temperatures close to their thermal limits regarding sex-determination, hatching success, and incubation duration. New research has shown that some turtle embryos have the ability to alter their position inside the egg to regulate their temperature up to 0.8°C (Du, et al. 2011). This ability, while remarkable, is likely too small of a temperature effect to make a notable difference in the outcome of sex-determination, hatching success, or incubation duration at the temperatures we recorded. The predicted increase in global temperatures of 2°C over the next 100 years (Stocker, et al. 2013), along with more frequent heat waves and extreme weather events, means that incubation temperature means and maximums are likely to rise. Continuing anthropogenic environmental perturbation is likely to add to the already changing climate (e.g., altered shade regimes, etc.). Any consistent increases in temperature means and maximums could have serious consequences for western pond turtles. Our methods can potentially be applied to other listed turtle species and possibly other oviparous ectotherms with TSD to predict if they are approaching their predicted thermal limits regarding hatching success and sex determination, as well as detecting temperatures ranges that successfully support development.

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TABLES AND FIGURES

	Low	High
Mean Incubation Temperature (°C)	24.4	29.9
Standard Deviation of Incubation Temperature (°C)	3.1	9.9
Maximum Incubation Temperature(°C)	32.6	54.6

Table 1. Temperature ranges of the temperature summaries used in the analysis.

Figure 1. Diagram of temperature sensor placement at different egg layers within a nest. The red discs are the temperature sensors and the white ovals are eggs.

Figure 2. Visualization of female and male gonads of *Emys marmorata* during an endoscopic procedure to determine sex.

Figure 3: Proportion of time spent above 29°C during the middle third of the incubation period for female and male *E. marmorata*.

Figure 4. Logistic regression showing maximum incubation temperature vs. the probability of non-viability (NV) in *Emys marmorata*. Also included is an inverse prediction showing LT_{50} of 40.3°C.

A - Mean is color coded

Mean (°C)	Color
> 29	Red
28>t≤29	Orange
27>t≤28	Yellow
26>t≤27	Green
25>t≤26	Blue
24>t≤25	Purple

B - Max is color coded

Maximum (°C)	Color
t≥41°C	Red
39≤t<41	Orange
37≤t<39	Yellow
35≤t<37	Green
33≤t<35	Blue
t<33	Purple

Figure 5. Leverage plot for the interaction of mean incubation temperature and maximum incubation temperature in relation to the incubation duration of *Emys marmorata*. Plot A is color coded by mean and plot B is color coded by maximum. The color coding helps to show the meaning of the interaction term.