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

Keywords: Temperature-dependent sex determination (TSD); Hatching success; Development; Incubation temperature; Thermal limits; Western pond turtle

What is already known: Incubation temperature is known to affect developmental processes in many ectotherms, including sex determination in some groups. Most studies have focused on temperature-dependent sex determination (TSD) under *ex situ* conditions to find the pivotal temperature for sex determination and optimal developmental ranges. More recently, studies that have looked at *in situ* conditions have found that fluctuating temperatures can have different effects on development in comparison to *ex situ* constant temperature procedures.

What this study adds: The methods used in this study enabled us to look at the incubation temperatures *in situ* throughout the incubation period of each egg. We found that incubation temperatures fluctuated more than what has previously been recorded in most other studies. Due to the wide range of temperature fluctuation we were able to assess possible thermal limits for this population in regards to hatching success, sex determination, and incubation duration.

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 a greater degree of climatic instability. Such changes could have potentially serious consequences for oviparous ectotherms, especially those with temperature-dependent sex determination (TSD). 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 on top of each layer of eggs within nests and recorded temperatures hourly through the first 2-3 months of incubation. These methods allowed us to look at *in situ* nest temperatures with high resolution. 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 greatly in both cases. The hatchling turtles were more likely to be female if they spent 30% or more of their sex-determining period of incubation above 29°C. Hatching success was best predicted by the maximum incubation temperature. We also found that incubation duration tended to be shorter as the mean temperature increased. However, exposure to either extremely high or low temperatures extended incubation times.

Introduction

Environmental perturbations, including habitat alterations and climate change, 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. Habitat loss and alteration can lead to increased surface temperatures locally through the addition of roads or loss of vegetation (Trombulak and Frissell 2000; Reese and 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 elevation 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 (Stebbins and Cohen 1995; Fuiman and Werner 2009). 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 and Lang 1995; Booth 2006). While there is evidence that embryos can use locomotion to thermoregulate inside the egg (Du et al. 2011), this effect is limited by the thermal gradient of the egg provided by the nest environment, making these organisms particularly vulnerable during their embryonic stage. Oviparous ectotherms that exhibit temperature-dependent sex determination (TSD), including all crocodilians, most turtles, and some lizard species (Ewert et al. 1994; Bowden et al. 2014), are especially sensitive to environmentally caused changes in incubation temperatures, as these species are at risk of potentially dire consequences due to the alterations in sex ratios that may result (Schwanz et al. 2010). Sex determination in TSD species occurs during the middle third portion of their development, also known as the thermosensitive period (TSP) (Georges et al. 1994). Many TSD species are currently experiencing population declines, a trend that is well documented in turtles, with more than half of turtle species listed on the spectrum from Vulnerable to Endangered on the 2015 IUCN Red List.

Embryonic development, including TSD, has been extensively studied in turtles under *ex situ* conditions, often using constant incubation temperatures (Rhen and Lang 1999; Kettlewell et al. 2000; Ligon et al. 2009; Geist et al. 2015). 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). Pivotal temperatures (i.e., the constant temperatures at which a 1:1 sex ratio is expected to occur) have also been determined in most of these experiments. Additionally, other important developmental factors have been examined in a number of turtle species, including hatching success and incubation duration. Studies investigating hatching success have shown that incubation temperature and the developmental rate have a positive curvilinear relationship. This includes an optimal developmental range (ODR) in which the developmental rate has an approximately positive linear relationship with temperature. As temperatures move beyond the ODR at either the low thermal limit

(LTL) or the high thermal limit (HTL), developmental rates are non-linear and quickly approach zero (Booth 1998; Georges et al. 2005; Les et al. 2009; Bowden et al. 2014). These data, while valuable, do not necessarily reflect the developmental and phenotypic responses to temperature fluctuation inherent to *in situ* incubation environments. Several studies have simulated a natural incubation environment by having the incubation temperatures fluctuate around a mean at various fixed rates (Wilhoft et al. 1983; Georges et al. 2005; Les et al. 2007; Micheli-Campbell et al. 2012). These studies have shown that fluctuating temperatures tend to produce more females than would be predicted by the mean temperature when compared to those incubated at constant temperatures. However, natural nest temperatures often do not show a consistent fluctuation around a mean, and 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 under laboratory conditions (Paitz et al. 2010; Micheli-Campbell et al. 2012), and others have recorded nest temperatures in relation to habitat types (Freedberg et al. 2011; Refsnider 2013). The degree-day model has been created to account for diel fluctuation and make predictions about effects on sex determination (Georges et al. 1989). While this model is useful in many situations, there are limitations. One such limitation can occur in shallow nests when temperatures reach well beyond the limit of those that support successful development (Georges et al. 2005; Warner and Shine 2011). It is becoming increasingly apparent that the fluctuations of incubation temperatures play a key role in phenotypic outcomes of the hatchlings (Georges 2013; Bowden et al. 2014; Miller and Ligon 2014).

In this study, we looked at a population of western pond turtles, *Emys* (aka *Clemmys, Actinemys*) *marmorata*, a TSD species that is currently experiencing population declines throughout much of its range (Hays et al. 1999; Bury and Germano 2008). The objectives of this study are to investigate the *in situ* incubation temperatures experienced by this population of western pond turtle and examine how aspects of the incubation temperature regime (e.g., mean, fluctuation, maximum) affect sex determination, hatching success, and incubation duration.

Methods

Data were collected during active nesting of western pond turtles in the months of June and July in 2010, 2011 and 2012 at a vernal lake in Lake County, CA, with an elevation of approximately 800 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. Work was authorized under the California Department of Fish and Wildlife Scientific Collecting Permit #801005-02 to Nick Geist.

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 (Advanced Telemetry Systems, Model R2000) and returned to the edge of the pond. Females typically returned to nest within the next two days, occasionally longer. All captured females were marked with a unique identification number filed on the marginal scutes in order to track nesting behavior during the season and in the following years. Each day from approximately 1600 hours to dusk, radio telemetry was used to determine if females were out of the water. If so, females would be located initially and either watched from a distance if in plain view or subsequently located approximately every 20 minutes until nest completion. 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 ibutton temperature sensors (Thermochron Temperature Logger, Model DS1921G, Temperature Resolution 0.5°C), to record temperatures every hour throughout the *in situ* portion of the incubation period. A sensor was placed above the top layer of eggs in 41 nests, 10 of which from 2012 had sensors at both the top and at lower levels within the nest. Only eggs that were directly next to or below the temperature sensors were included in the analyses. Photographs were taken and diagrams were drawn from different angles of each nest to record the positions of the eggs and temperature sensors within the nests.

After approximately 80 days (past the TSP), eggs were collected from the field and brought to Sonoma State University to be maintained for the duration of their incubation in an incubator set at 29°C, the calculated pivotal temperature for this population of western pond turtle (Geist et al. 2015). 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 dedicated facilities at 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 (Geist et al. 2015) in which the gonads were visually identified. All procedures were performed by the veterinary staff of the San Francisco and Oakland zoos.

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, temperature variance (to calculate temperature fluctuation), maximum incubation temperature, and time spent above 29°C, the calculated 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 duration 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 determined to be not significant in these analyses; thus, 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

All nests were laid between 2-300 meters from the pond in the northwest direction. Nest sites were typically located in areas with annual grasses several feet from trees or shrubs. Nest depths were found to range between 6 cm. to 13 cm. The top eggs in each nest were generally located 1-4 cm. below the surface.

Mean incubation temperatures only ranged approximately 5°C between nests (24.4°C-29.9°C) and were virtually the same within nests, regardless of the position (i.e. top or bottom). However, maximum incubation temperatures were very different between and within nests, with the maximums ranging from 32.6 °C to 54.6 °C. An example of three temperature traces from different positions in one nest is shown in Figure 1.

157 eggs were included in this analysis, with 94 being viable. Eight individuals that hatched did not grow large enough to be sexed via endoscopic procedures 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 (LR χ^2=48.79, $p<0.0001$) and the temperature fluctuation (variance of nest temperatures) during the calculated middle third of the incubation period (LR χ^2=4.60, $p=0.0320$). During this period, embryos that spent 30% or more of their TSP above 29°C were more likely to be female than those that spent less time above 29°C (Fig. 2). Also, embryos that developed into males experienced relatively small temperature fluctuations while those that became females experienced both large and small temperature fluctuations (Fig. 3).

Hatching Success

Maximum incubation temperature had a significant effect on hatching success (LR χ^2=47.8, p<0.0001), while mean incubation temperature did not (LR χ^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 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. The LT_{90} was also calculated and found to be 45.0°C

Incubation Duration

Incubation duration was significantly affected by both mean incubation temperature (LR χ^2=60.47, p<0.0001) and maximum incubation temperature (LR χ^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 (LR χ^2=45.44, p<0.0001). Eggs that experienced the combination of relatively 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 means and high maximums.

Discussion

Our data show that different variables of *in situ* incubation temperatures had significant effects on sex determination, hatching success, and incubation duration in this population of western pond turtle. Typically, studies of the effects of *in situ* incubation temperatures use data from one sensor per nest and performed their analyses by nest (Schwanz et al. 2010; Warner and Shine 2011; Freedberg et al. 2011; Miller and Ligon 2014). Recording temperature data from ibutton temperature sensors placed at several levels within the nests provided a unique look at the differences in the temperature regimes experienced both between and within nests. Since our analysis was performed on temperatures experienced by individual eggs, and not by whole nest averages, this allowed us to investigate which variables of natural temperature regimes affect sex-determination, hatching success, and incubation duration with a high level of resolution.

Temperature Data

The incubation temperatures in our study commonly fluctuated more than 20°C on a daily basis, with nearly half of the eggs reaching maximums of 39°C or above. Few *ex situ* fluctuating incubation temperature experiments have explored the effects of incubation temperature maximums near or above 35°C (Georges et al. 2005; Micheli-Campbell et al. 2012; Miller and Ligon 2014). Few studies have reported extreme maximum temperatures similar to those found in our study (Warner and Shine; Miller and Ligon 2014). We suspect that with future recordings of *in situ* incubation temperatures from different species and locations, it will likely reveal many more turtle populations experiencing temperature regimes similar to those we found.

Sex Determination

The results of our *in situ* data are typical for reptile development, showing that the proportion of time spent above the pivotal temperature, as well as the degree of fluctuation were significant in determining sex (Georges 1989). Specifically, turtle embryos that spent more than approximately a third of their TSP above the estimated pivotal temperature were more likely to be female (Fig. 2). Also, the degree to which the temperature fluctuated was significant, with males developing from eggs that experienced lower fluctuation and females from those experiencing both high and low fluctuation (Fig. 3).

From 2009-2012, the hatchling sex ratio for this population (including those not used in this study) was 69% female, on average (122 females, 177 total). These years experienced relatively typical summer temperatures for this site, suggesting that a female bias in the hatchling sex ratio would likely occur in most years. Other *in situ* studies of turtle hatchlings have shown a similar female bias (Georges 1992; Booth 2006; Correa-H et al. 2010; Miller and Ligon 2014). 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 and Avise 2001; Roques et al. 2006). However, with a relatively rapid rise in annual temperatures and a decrease in viable nesting habitat, sex ratios could conceivably be skewed further toward females (Janzen 1992). Over time, such a sex ratio is likely not stable and reproductive success would be reduced (Nelson 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 on this population demonstrated a low hatching rate above of 30°C at constant temperatures under laboratory conditions (Geist et al. 2015). However, our results indicate that mean nest temperature was not significant in determining hatching success. This is consistent with prior observations 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.

Previous constant incubation temperature studies performed in the laboratory on this population of western pond turtle showed that the HTL was approximately 31°C, with little to no hatching success at or above this constant temperature (Geist et al. 2015). The lowest *in situ* maximum incubation temperature was recorded as 32.6°C, which means that all of the eggs in this study spent time near or above the HTL. While it is widely known that embryos can tolerate spending relatively short periods outside of the ODR (Demuth 2001; Bowden et al. 2014), we also wanted to approximate the thermal limits that could not be tolerated.

Our analysis calculated that the LT_{50} was a maximum of 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 (LT_{90}). 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 this population is experiencing temperatures approaching the limits of their thermal tolerance during development. Thus, even relatively minor increases in maximum temperatures due to environmental changes would likely have seriously deleterious effects on viability for this population.

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 most of the incubation temperature maximums found at this site are outside of the ODR and thus too high to support normal development. This retardation of development at higher temperatures leads to a longer incubation period overall, a pattern typically found in reptilian development (Deeming and Ferguson 1991; Andrewartha et al. 2010). This species is known to overwinter in the nest and emerge the next spring. Since embryos typically use more energy during an extended incubation period compared to a shorter one (Gutzke 1987; Booth 1998), longer incubation periods could leave hatchlings with low energy reserves, possibly making it more difficult for them to survive.

Conclusion

It is clear that most embryos in this population are developing at temperatures close to their thermal limits. Increases in incubation temperature caused by climate change or lack of proper nesting habitat could lead to skewed sex ratios, decreased hatching success, and possibly lower energy stores for hatchings due to increased incubation duration. Research has shown that turtle embryos in eggs similar to the size of the eggs in this population 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, would likely result in 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, suggests that incubation temperature means and maximums are likely to rise. Continuing anthropogenic environmental perturbation may also add to climactic instability (e.g., altered shade regimes, etc.). Any consistent increases in temperatures, especially maximums, could have serious consequences for western pond turtles and, potentially, for other TSD species. Almost half of crocodilian species are considered endangered or critically endangered. There has been a call to collect more *in situ* incubation temperature data to aid laboratory experiments and add to our understanding of thermal limits (Bowden et al. 2014). Our methods can potentially be applied to other listed turtle species and other oviparous ectotherms with TSD to see if they are approaching their predicted thermal limits regarding hatching success and sex determination, as well as countless other developmental effects.

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Figure Legends

Figure 1. Temperature Trace Example. The temperature traces for the top, middle, and bottom sensor from nest 1 in 2012 show similar mean temperatures (24.4°C-25.4°C) and differing maximums.

Figure 2. Sex Determination vs. Time Above Pivotal Temperature. This plot shows the proportion of time spent above 29°C during the middle third of the incubation period for female and male western pond turtles.

Figure 3. Sex Determination vs. Incubation Temperature Variance. This plot shows the *in situ* incubation temperature variance during the middle third of the incubation period for female and male western pond turtles.

Figure 1

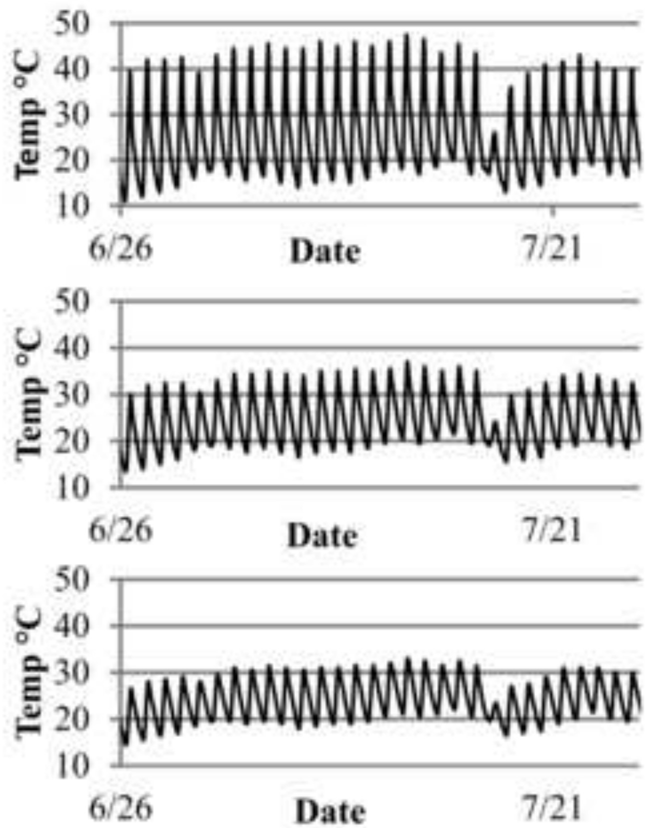

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Figure 2

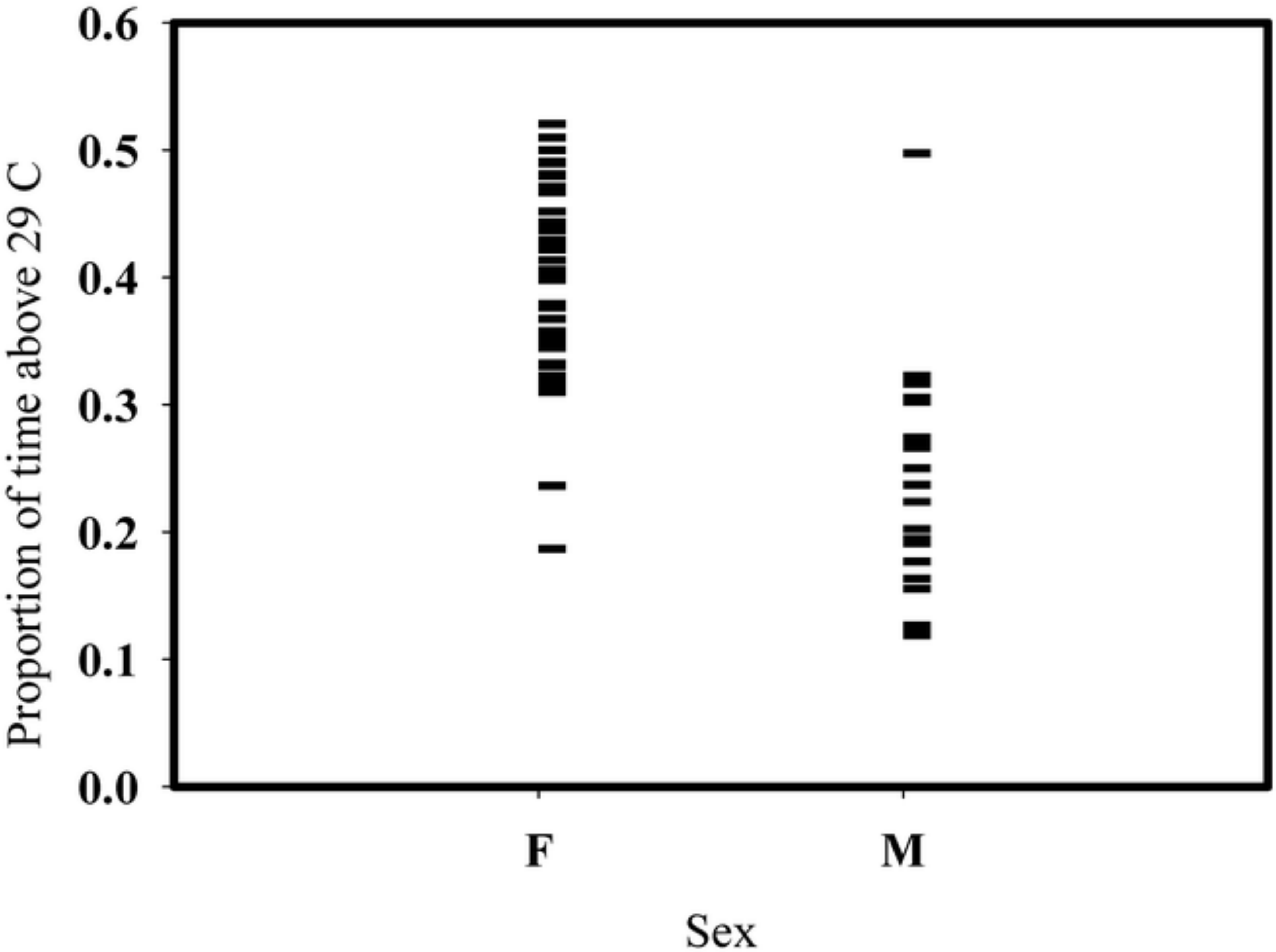
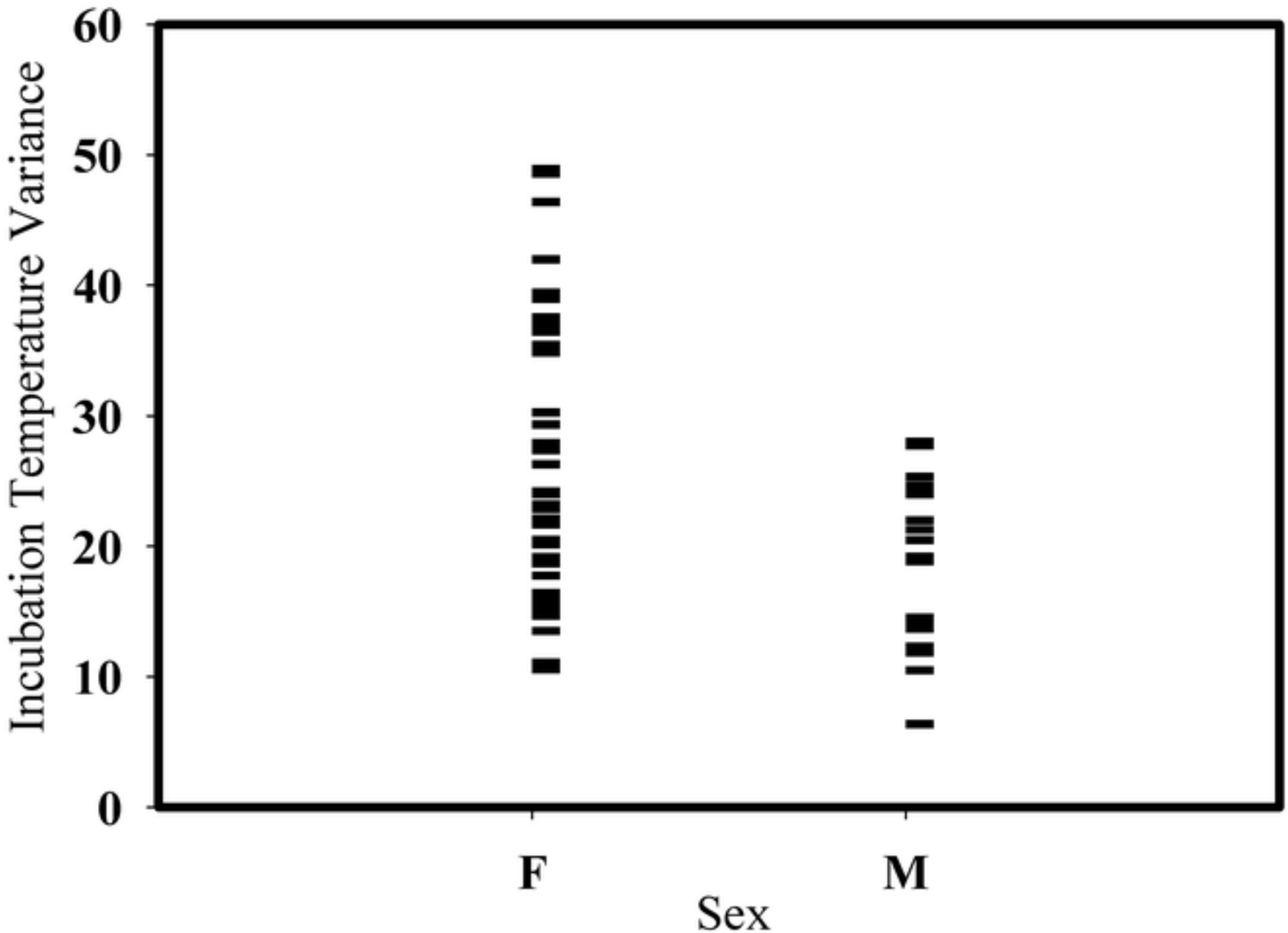


Figure 3



Response to Reviewers: We would like to thank the reviewer for taking the time to review our responses to the previous comments.

Reviewer Comments

1. To my reading, the authors responded thoroughly and conscientiously to all of the reviewers' comments and recommendations. In light of comment #4 of reviewer #1, I think it would be wise to mention the resolution of the temperature loggers, perhaps near lines 133,134, where the use of temperature loggers is described. I do not think the relatively low resolution of the temperature loggers is a major issue -- it certainly has no bearing on the acceptability of the paper. Please make this change.

Response 1: Thank you for this suggestion. We agree that the resolution of the temperature sensors should be included. We have added the resolution to the sentence on lines 132-135, which now reads, "All eggs were then returned to the nest along with ibutton temperature sensors (Thermochron Temperature Logger, Model DS1921G, Temperature Resolution 0.5°C), to record temperatures every hour throughout the in situ portion of the incubation period."