The Effect of Incubation Temperature on Sex and Morphology in the African Redhead Agama (*Agama picticauda*)

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 16, 2017

Keywords: *Agama agama*, egg incubation, lizards, temperature-dependent sex determination, morphology, Charnov-Bull model

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Abstract

The first demonstration of temperature-dependent sex determination (TSD) in a vertebrate was in the African redhead agama (*Agama agama*) nearly 50 years ago. The original study was generally overlooked until it was later identified in turtles, which attracted more interest into this peculiar sex determining mechanism. Since then, numerous studies have investigated the physiological mechanisms behind TSD, the evolutionary transitions between genetic sex determination and TSD, and the adaptive significance of TSD, however, research on the original species has not been conducted. My research aims to revisit the original study to evaluate the effect of incubation temperature on sex ratio and fitness-relevant phenotypes, and to identify the sex-specific effects of incubation temperature in *Agama picticauda* (formerly *A. agama*). Eggs were incubated under constant and ecologically-relevant fluctuating temperatures to better characterize the pattern of TSD in this species, provide insight into the ecological relevance of TSD, and quantify the effects of incubation temperature on fitness-relevant phenotypes and sex-specific effects. Hatchling sex ratios varied with incubation temperature in a pattern similar to a female-male-female pattern of TSD. Fitness-relevant phenotypes were also affected by incubation temperature, although the effects were not sex-specific. Eggs incubated at male-biased temperatures hatched earlier than eggs at female-biased temperatures, which may have sex-specific consequences later in life as predicted by models for the adaptive significance of TSD.

Acknowledgments

First, I would like to thank my advisor, Dr. Dan Warner, for his constant support, guidance, patience, and encouragement throughout my studies. I would also like to thank my lab mates who have helped with brainstorming ideas, providing feedback and edits on my thesis, and support during my time at Auburn University: Phil Pearson, Jenna Pruett, Putter Tiatragul, Josh Hall, Dr. Tim Mitchell, and Dr. Renata Brandt. I would also like to thank the undergraduates that worked in the lab for their help with animal care and feedback: Robin Lloyd, Austin Hulbert, Dani Douglas, Cassie Guiffre, Kayla Wilson, David Quinn, Ryan Weesner, and Lauren Boggs. A special thank you goes out to Josh Hall and Tim Mitchell for helping me locate and collect the lizards for my research in Miami, FL. I would like to thank Dr. Thane Wibbels for his valuable help on verifying the sex of my hatchlings at the University of Alabama at Birmingham, and for his vast knowledge in all things TSD. Thank you to all of my friends, both within graduate school and outside of graduate school, for your constant support and understanding, you all kept me grounded during my graduate studies, and I honestly couldn’t have done it without all of you! Finally, I would like to thank Auburn University and the Alabama Academy of Sciences for funding my research.

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List of Abbreviations

TSD	Temperature-Dependent Sex Determination
SVL	Snout Vent Length
TL	Tail Length
HL	Head Length
HW	Head Width
HD	Head Depth
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
SDM	Sex Determining Mechanism
GSD	Genetic Sex Determination
ESD	Environmental Sex Determination
FM	Female-Male
MF	Male-Female
FMF	Female-Male-Female

Temperature-Dependent Sex Determination in Reptiles: A Thesis Overview

Environmental factors experienced during embryonic development can influence several phenotypes in reptiles, such as morphology, behavior, locomotor performance, growth, and even sex (Deeming, 2004; Booth, 2006). An individual's sex already has major influences on life history, behavior, physiology and morphology, thus the mechanisms that determine one's sex can have indirect consequences throughout an individual's life as it pertains to fitness relevant traits. Reptiles display a variety of sex determining mechanisms, ranging from genotypic (both XX/XY and ZZ/ZW systems) to temperature-dependent sex determination (Valenzuela & Lance, 2004), and evolutionary transitions along the sex determining continuum have been identified throughout reptile lineages and even within family groups (Gamble, 2010; Gamble et al., 2015; Pokorna & Kratochvil, 2009; Quinn et al., 2011). Moreover, the patterns of TSD can vary greatly among taxa.

Temperature-dependent sex determination (TSD) is defined as the phenomenon where offspring sex is irreversibly determined by incubation temperatures experienced during the middle-third of embryonic development (Valenzuela & Lance, 2004). TSD has been studied extensively in reptiles and is present in all crocodilians and tuatara, several species of turtles, and some species of lizards. A variety of TSD patterns have been described in these taxa and these reaction norms have been categorized based on the sex ratios produced as a function of constant incubation temperature (Figure 1). TSD Ia, also referred to as male-female (MF), produces males at low temperatures and females at high temperatures, whereas the opposite is true for TSD Ib (also referred to as female-male (FM)). TSD II, which is referred to as female-male-female (FMF), produces females at cool and warm temperatures and males at intermediate temperatures.

All three patterns have a pivotal temperature that produces a 1:1 sex ratio, but TSD Ia and TSD Ib have one pivotal temperature whereas TSD II has two (Valenzuela & Lance, 2004). Sex is determined during a small window of time during development called the temperature sensitive period (TSP). The exact timing of the TSP varies across taxa, but it is generally during the middle-third of embryonic development.

TSD reaction norms have been characterized based on constant incubation experiments in the laboratory, and thus do not accurately reflect the thermal environment embryos experience in natural nests. Temperature fluctuations experienced in nature add a level of complexity to categorizing TSD patterns and can create a bias towards one sex depending on the amount of time spent above or below the pivotal temperatures (Georges, 1989). Therefore, experiments that assess the effect of natural nest temperatures on sex ratio are needed to understand how sex ratios are influenced in nature.

The variety of TSD patterns and multiple origins of this peculiar sex determining mechanism has raised numerous questions about its evolution and adaptive significance. The leading hypothesis for the adaptive value of TSD proposes that natural selection should favor TSD when temperature differentially affects the fitness of males and females (Charnov-Bull model; Charnov & Bull, 1977). One hypothesis within the Charnov-Bull framework predicts that there should be sex-specific reaction norms for fitness-related phenotypes, which in turn would confer sex-specific fitness consequences of incubation temperature (Shine, 1999; Warner & Shine, 2005). Although incubation temperature affects fitness-relevant traits of offspring (Deeming, 2004), the sex-specific effects of incubation temperature are largely unknown except for a few cases (Rhen & Lang, 1995; Elphick & Shine, 1998; Warner & Shine, 2008). However, some empirical tests on short-lived species support this hypothesis (Conover 1984; Warner &

Shine, 2008). Warner and Shine (2008) found that incubation temperature had a differential effect on the reproductive fitness of male and female jacky dragons (*Amphibolurus muricatus*), showing that a male or female had higher reproductive success when they developed at the temperature most optimal for the production of that sex. They were able to test the effects of sex and incubation temperature by applying an aromatase inhibitor to eggs in order to produce both sexes at each incubation temperature, thereby decoupling the confounded effects of sex and incubation temperature. The relationship that followed showed that the males produced at the male-determining temperature had higher reproductive success and larger body sizes than males produced at the female-determining temperatures. This relationship was true for the females as well, and showed how each sex was differentially affected by incubation temperature. Although this study supports the Charnov-Bull Model in the short-lived lizard species that was studied (*A. muricatus*), it is difficult to draw general conclusions that apply to other taxa with TSD that differ substantially in their life history traits, especially for species with long reproductive lifespans (e.g. turtles and crocodilians).

Study Organism

The first demonstration of TSD in a vertebrate was in the African redhead agama (*Agama agama*), but research on this species has not been conducted since the original study (Charnier, 1966). *Agama agama* is native to the semi-arid and tropical regions of West Africa, and has been introduced to Florida via the pet trade. This species is highly abundant where it occurs in Florida, and occupies a range of habitat types (e.g., urban areas, city parks). Moreover, a recent taxonomic study of the *Agama agama* species complex in Africa has split this species into several distinct species within its native range (Leache et al., 2014, 2016). Although the original study of TSD in *A. agama* (Charnier, 1966) does not clearly state the source population of the animals used, the location of that research in Dakar suggests that *A. picticauda* (formerly *A. agama*) was likely the subject of that study. Coincidentally, recent work on introduced populations in South Florida were also shown to be *A. picticauda* (Nunez et al., 2016).

Although TSD in *A. picticauda* (*agama*) has not been studied since the original description nearly 50 years ago (Charnier, 1966), this species has numerous characteristics that make it suitable for studies of TSD. For example, their high fecundity facilitates egg incubation experiments (Enge et al., 2004), offspring sex is easily and harmlessly identified (Harlow, 1996), and these lizards mature at a relatively young age, but are long-lived (~20 years). In Florida, *A. picticauda* has been reported to lay 3 clutches annually with approximately 3-9 eggs per clutch for a maximum of 27 eggs per reproductive season (Blunden & Krysko, 2007). *Agama picticauda* is also highly territorial, and live in large groups with a dominant breeding male, numerous females, and a small number of subordinate non-breeding males (Enge et al., 2004). In addition, sexual dimorphism in size and color suggests that these traits impact fitness differently in males and females, especially since studies have shown a relationship between sexual size dimorphism, territoriality, and incubation temperature (El Mouden et al., 2001). Invasive populations in Florida within the last 10-20 years enable extensive research on this species in the United States (Enge et al., 2004; Nunez et al., 2016).

Thesis Objectives

For my thesis research, I evaluate the effects incubation temperature on sex ratio and fitness-relevant phenotypes of the African redhead agama (*Agama picticauda*). My research has three primary goals. For the first goal, I aim to characterize the pattern of TSD in *A. picticauda* and determine the effects of ecologically relevant fluctuating temperatures on offspring sex-ratio. For the second goal, I aim to characterize the reaction norms for fitness-relevant phenotypes using a broad range of incubation temperatures. In addition, I quantify the effect of ecologically relevant incubation temperatures on phenotype and survival in *A. picticauda*. For the third goal, I aim to quantify the interactive effects of incubation temperature and sex on fitness-relevant phenotypes and survival and examine the onset of sexual size dimorphism in sub-adult *A. picticauda.*

Significance of this Research

The long-term effects of developmental environments are of considerable interest to many biological fields, but experimental assessments are rare. This study will directly address this issue. By incubating eggs at different temperatures and raising offspring in large enclosures, this research provides a rigorous assessment of how developmental plasticity shapes juvenile phenotypes and survival. Moreover, this study will advance our understanding of the effects of the environment on the development of sexually dimorphic traits, and test a long-standing hypothesis for the adaptive significance of TSD, specifically during early life stages in a relatively long-lived species of lizard. Given vastly different life-history strategies of males and females, understanding the independent and synergistic effects of incubation temperature and sex could provide insight into the evolution of sexual dimorphism in morphology and life-history strategies. Populations of *A. picticauda* in Florida not only provide an opportunity to address these broad conceptual topics, but also enabled me to revisit TSD in this species, which has not been studied in nearly 50 years.

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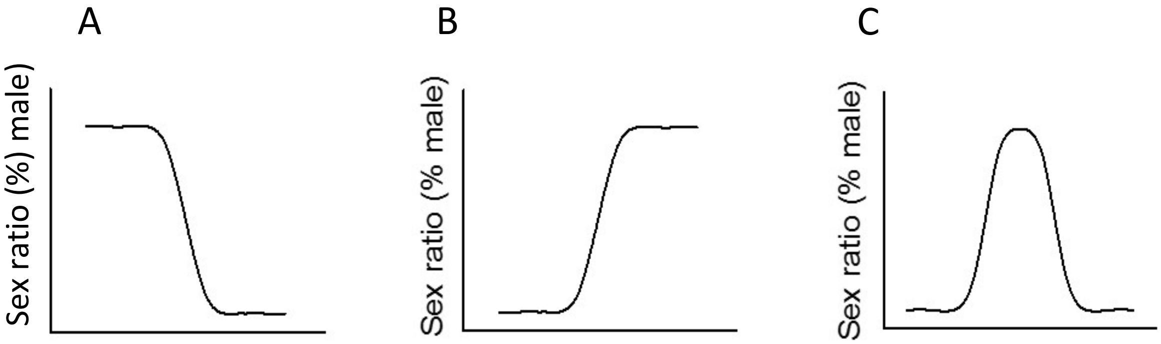

Figure 1: Sex ratio reaction norms as a function of constant incubation temperature most commonly found in reptiles. A) TSD Ia (Male-Female), cool temperatures produce 100% male, warm temperatures produce 100% female. B) TSD Ib (Female-Male), cool temperatures produce 100% female, warm temperatures produce 100% male. C) TSD II (Female-Male-Female), cool and warm temperatures produce 100% females, intermediate temperatures produce 100% male.

Chapter 1: Revisiting the first report of temperature-dependent sex determination in a vertebrate, the African redhead agama (*Agama picticauda*)

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Keywords: *Agama agama*, Charnier, egg incubation, lizard, temperature-dependent sex determination, evolution

Abstract

Vertebrates display a wide variety of sex determining mechanisms (SDMs). Identifying SDMs for different taxa is important for understanding the origins and phylogenetic history of sex-determining mechanisms. Temperature dependent sex determination (TSD), is found in several vertebrate and invertebrate species, but has been studied most extensively in reptiles. The initial discovery of TSD was in a lizard, the African redhead agama (*Agama agama*), over 50 years ago. The original study reported *A. agama* as having TSD consistent with pattern Ia (females produced at cool temperatures and males at warm temperatures), although only two incubation temperature treatments and a small sample size were used. The goal of this study is to revisit TSD in this species (now *A. picticauda*) from an introduced population in South Florida to better characterize the pattern of TSD. We also assess the effect of fluctuating incubation temperatures on offspring sex ratio to provide insight into the ecological relevance of TSD in this species. After incubating eggs across six constant temperature treatments, we demonstrate that females are produced at relatively cool temperatures and males at warmer temperatures (30 ºC). However, some females are again produced at even warmer temperatures, although the sex ratio is still male-biased, indicating the possibility of a female-male-female pattern of TSD. Fluctuating temperatures produced female-biased sex ratios at cooler mean temperatures and male-biased sex ratios at warmer mean temperatures, and may provide further insight into the sex-ratios produced from natural nests in the field.

Introduction

Vertebrates exhibit a wide diversity of sex determining mechanisms (SDM), ranging from systems that are purely determined by genetic factors to systems that are highly sensitive to the environment. Although an individual's sex is influenced by contributions from both genetic and environmental factors (Sarre et al., 2004), SDMs have traditionally been categorized as either genotypic sex determination (GSD) or environmental sex determination (ESD). This dichotomous classification oversimplifies the complexity of SDMs, but it is still useful for understanding the phylogenetic history of SDM evolution (Janzen & Krenz, 2004; Pokorna & Kratochvil, 2009; Mank & Uller, 2014). Despite recent advances in our understanding of SDM evolution and diversity in vertebrates (Bachtrog et al., 2014; Beukeboom & Perrin, 2014), we still lack basic knowledge of SDMs in specific taxa. For example, although ESD has evolved multiple times within fish and reptiles, overall conclusions about phylogenetic patterns are weak because <5% of the species have been examined for their SDM (Pokorna & Kratochovil, 2009). Thus, studies that characterize the SDM of individual species are of great value because they will continually fill in major gaps needed for a comprehensive understanding of SDM evolution.

Environmental sex determination has been characterized in some species of fish and many reptiles (i.e., some lizards, most turtles, and all crocodilians). The form of ESD in these taxa is temperature dependent sex-determination (TSD), where offspring sex is determined by the incubation temperatures embryos experienced during the middle third of development (Valenzuela & Lance, 2004; Shine et al., 2007). These vertebrate groups have evolved TSD multiple independent times (Mank et al., 2006; Gamble, 2010), and the patterns of TSD also exhibit considerable diversity. For example, in some species, eggs incubated at constant low temperatures yield 100% females, whereas eggs incubated at constant high temperatures results in 100% males (type Ia [FM] pattern seen in some lizards, tuatara; Kraak & Pen, 2002). In other species, the pattern is reversed where males are produced at low constant temperatures and females at high constant temperatures (type Ib [MF] pattern seen in many turtles). A third pattern results in female production at both high and low constant incubation temperatures, and males at intermediate temperatures (type II [FMF] pattern; e.g., few turtles, crocodilians, some lizards) (Kraak & Pen, 2002; Harlow & Taylor, 2000; El Mouden et al., 2001). Importantly, the level of sex ratio bias at different temperatures can vary considerably across different species, among populations, and even among family groups within populations (Rhen & Lang 1995; Warner 2011; Bull 2015; Inamdar Doddamani et al., 2012). Thus, the TSD patterns described above are generalizations of sex-determining reaction norms based on constant temperature incubation in the laboratory, but there is considerable variation at different levels.

Natural nest temperatures are rarely constant and add a level of complexity to categorizing TSD patterns. Thermal fluctuations can create a bias towards one sex depending on the mean nest temperature and the variation around that mean. Models suggest the proportion of development spent above or below the male-producing temperature or female-producing temperature will determine the offspring's sex, creating a sex ratio bias (Georges, 1989), and studies have shown empirical support for those models in turtles (Les et al., 2007; Miller & Ligon, 2014). However, in other cases, models of sex determination under fluctuating temperatures poorly predict empirical patterns (Warner et al., 2011; Schwanzkopf & Brooks, 1985). Thus, experiments that include fluctuating regimes that mimic natural nest temperatures should provide a more realistic approach for evaluating the effects of incubation temperature on offspring sex ratio and other phenotypes.

Among reptiles, squamates (i.e. snakes and lizards) express a variety of sex determining mechanisms, especially since only a few species of lizards have been described to have TSD (Harlow 2004; Pokorna & Kratochovil, 2009; Tokunaga, 1985). The lizard family Agamidae includes species with both GSD and TSD, with a variety of patterns and multiple origins (Harlow 2004). Indeed, TSD was first described in an agamid lizard (*Agama agama*) over 50 years ago (Charnier, 1966). Although the original study reported that *A. agama* has TSD consistent with the FM pattern, only two incubation temperatures and a small sample size were used. Since this initial discovery of TSD, the original study has not been revisited despite recent research showing TSD in other species of agamid lizards (El Moulden et al., 2001; Harlow & Shine, 1999; Harlow 2004). Unfortunately, the small sample sizes and limited number of incubation temperature treatments used by Charnier could lead to errors in descriptions of SDMs; this could lead to a misinterpretation of SDM evolution if erroneously classified in phylogenetic studies. For example, TSD was reported in the agamid *Ctenophorus pictus* based on relatively small sample sizes (Harlow 2004), but a more extensive study revealed a pattern consistent with GSD in this species (Uller et al., 2006). As a result, the incorrect SDM (i.e. TSD) for this species has been propagated in the literature (Pokorna & Kratochovil, 2009).

Indeed, the TSD pattern in *A. agama* has been inferred to be FMF based on research of other African agamid species (Mitchell et al., 2006), but empirical studies have not been conducted to demonstrate this. Another complication is that the original report of TSD in *A. agama* (Charnier 1966) does not indicate where the lizards were collected, but given that the research laboratory was based in Dakar one could assume that this was the source of the study population. Nevertheless, this is problematic because recent taxonomic changes have split the West African *A. agama* clade into 7 distinct species (Leache et al., 2014, 2016), and the species examined by Charnier is not known with complete certainty. Given that *A. picticauda* is the most common species of *Agama* in West Africa (Leache et al., 2016) and around Dakar, it is likely that *A. picticauda* was the subject of Charnier's research. Thus, we will be working under this assumption in our study.

The goal of this research is to revisit the original study and further characterize the pattern of TSD in *A. picticauda*. This re-evaluation will be of historical interest since *A. pictiauda* (formerly *A. agama*) was the first vertebrate for which TSD was described. Our research focuses on a population of *A. picticauda* that was introduced to Miami, FL (Enge et al., 2000; Nunez et al., 2016) via the pet trade. Although the precise date of the introduction to Miami is unknown, records suggest that it occurred around 1976 (Nunez et al., 2016; Enge et al., 2000). We predict that by increasing the number of incubation treatments to include more temperature treatments than those used by Charnier (1966), *A. picticauda* will exhibit a FMF pattern of TSD, which is a common pattern described in other species of agamid lizards (El Mouden et al., 2001; Harlow & Taylor, 2000; Mitchell et al., 2006). In addition, to further understand the effect of the thermal environment on sex ratios we also investigate how ecologically-relevant temperature fluctuations influence sex ratio; we hypothesize that temperature fluctuations will skew sex ratios depending on the overall average incubation temperature (Warner & Shine, 2010; Newald & Valenzuela, 2010). Specifically, we predict that temperatures that fluctuate around low and high mean temperatures will produce a female-biased sex ratio while fluctuations around an intermediate mean temperature will produce a male-biased sex ratio.

Methods

Breeding Colony

Adult lizards were captured either by hand or by noose at two locations in Miami, Florida from 4-6 May 2016 (Krysko, 2000). Some adults were captured at Mattheson-Hammock Park in Coral Gables, FL while others were captured about 35 km away in Homestead, FL. Age class (juvenile vs adult) of individuals was estimated based on body size, and sex of the individual was identified by color, since *A. picticauda* is sexually dimorphic. We captured 6 males and 24 females and brought them to Auburn University. Females were given a unique toe-clip for identification. Adults were housed in six large outdoor aviaries (3.65m long x 1.22m wide x 2.13m tall) with 1 male and 5 females per cage. This sex ratio mimics the social group structure of *A. picticauda*, which typically consists of a dominant male and several females (Enge et al., 2000). Cinder blocks (stacked about 0.5 m tall) provided hiding places and basking rocks, and landscaping logs were used as climbing and perching surfaces. Animals in neighboring cages could see each other, but could not physically interact. Large planter containers (0.61 m diameter) filled with a 50/50 mixture of moist potting soil and peat moss were used for nest boxes. Holes were drilled into the bottom of the planters and set upon wood planks for drainage. A small plant saucer was used as a water dish. All cages were sprinkled with water daily and lizards were fed large crickets dusted with calcium every other day. Lizards were kept under these conditions from 7 May to 30 September 2016, at which point they were humanely euthanized and deposited in the Auburn University Museum of Natural History.

Egg Collection and Incubation

Females were regularly monitored for gravidity. If a female was gravid, nest containers were checked for eggs every other day. When eggs were found, females were captured to identify the individual that laid the clutch. Eggs were brought to the laboratory, individually weighed to the nearest 0.0001 gram, and given an identification number. Eggs were placed individually in glass jars containing moist vermiculite (-150 Kpa) and covered with plastic wrap. The date of oviposition was recorded and the jar was randomly assigned to one of 9 incubation treatments. To characterize the pattern of TSD, six constant temperature incubation treatments of 24, 26, 28, 30, 32, and 34ºC were used. To assess the effects of temperature fluctuations on sex ratio, three fluctuating treatments were used. These temperature treatments fluctuated about ± 2 degrees around means of 26, 28, and 30ºC (Figure 1). The pattern of temperature fluctuation was based on temperature logger (iButton) data collected from potential nest sites near the Matheson Hammock collection site in Coral Gables, FL (Tiatragul et al., 2017) and uploaded into programmable incubators.

Hatchling Sex Identification and Measurements

Incubators were checked daily for hatchlings. If a hatchling was found, it was removed and given a unique toe-clip for identification, and measurements of mass, snout-vent length (SVL), tail length (TL), head depth, width, and length were taken. All individuals were immediately sexed via hemipene eversion (Harlow, 1996). These lizards were then raised in captivity for 6-8 months, and humanely euthanized on 17 March 2017. To verify the sex of each lizard, all individuals were dissected for gonad inspection, and small individuals whose gonads were difficult to distinguish had their gonads removed for histological cross sectioning (Figure 2).

Statistical Analyses

Statistical analyses were performed in R (R Core Team, 2016). Generalized linear mixed models with a binomial error structure were used to assess the effects of incubation treatment on egg hatching success; egg mass was included as a covariate and maternal identity was a random effect. Logistic regression models were used to assess the effects of incubation treatment on offspring sex ratio. Because one treatment produced all females, a firth correction was used to account for separability (i.e., when all observations in treatment have the same status). To characterize the pattern of TSD, sex ratio data were compared across the constant incubation temperature treatments. To quantify the effects of fluctuating incubation temperatures, independent variables included the mean temperature (i.e., comparison among 26, 28, and 30 ºC treatments), thermal regime (comparison between constant vs fluctuating treatment), and their interaction. To identify TSD using egg incubation studies, the potential for sex-biased mortality across incubation treatments must be ruled out. In this conservative approach, all eggs that had died during incubation were scored as the opposite sex of what was expected for that treatment and then analyzed using logistic regression. More specifically, dead eggs from the 24ºC, 26ºC, 28ºC and 34ºC treatments were scored as males, and all dead eggs from 30ºC and 32ºC treatments as females.

Results

Incubation

A total of 132 viable eggs were produced by females (91 eggs assigned to constant treatments, 41 eggs assigned to fluctuating treatments). Overall hatching success (constant and fluctuating) was 69%. There was no effect of constant incubation temperature on hatching success

($F_{5,68}$=0.41, p=0.8372; Table 1). Hatching success did not differ between constant and fluctuating thermal regimes ($F_{1,66}$=2.71, p=0.1043) and the interaction between mean temperature and regime was not significant ($F_{2,66}$=0.49, p=0.6151; Table 1).

Sex Ratio

Constant incubation temperatures influenced hatchling sex ratio (χ^2=21.866, p<0.001). Eggs incubated at low (24 ºC, 26 ºC, 28 ºC) temperatures produced female-biased sex ratios (0%, 10% and 37.5% males, respectively; Figure 3; Table 1). Eggs incubated at warmer (30ºC, 32ºC) temperatures produced male-biased sex ratios (90.91% and 63.64% males, respectively; Table 1). The treatment at constant 34 ºC produced a nearly balanced sex ratio of 55.56% male (Table 1). Fluctuating temperature treatments also influenced sex ratio (χ^2=21.86, p<0.05; Table 1) and produced female-biases at 26 ºC (0% male) and 28ºC (33.33% male) and a male-biased sex ratio at 30 ºC (72.73% male). Neither constant incubation treatments (χ^2=0.86, p=0.3531) nor fluctuating temperature treatments (χ^2=1.173, p=0.2787) affected sex ratio when the potential for sex-biased mortality was taken into account.

Discussion

In this study, we revisit the historical work of Charnier (1966) and provide a more robust characterization of TSD in *A. picticauda*. In addition, we explored the effects of fluctuating incubation temperatures on sex ratio to provide a realistic assessment of how natural nest temperatures affect variation in sex ratios in this species. We demonstrated that hatchling sex ratios in *A. picticauda* vary with incubation temperature in a pattern with similarities to a FMF pattern of TSD, which has been shown in other agamid lizards (El Mouden et al., 2001; Harlow, 2004; Mitchell et al., 2006). Although our warmest two incubation treatments (32 and 34 °C) showed a trajectory towards a female bias, our incubation temperatures may not have been warm enough to induce that bias. Thus, more research is warranted to quantify sex ratios produced at temperatures > 34 °C (albeit, constant temperatures >34 ºC might be lethal to embryos). Interestingly, sex ratios differed among the fluctuating temperature treatments, but this variation in sex ratio did not differ between incubation regimes (constant vs fluctuating).

To identify TSD using egg incubation studies, one must rule out the potential for sex-biased mortality of embryos across incubation treatments (Valenzuela et al., 2003). Indeed, sex biases in embryo sensitivity to incubation temperature (Goth & Booth 2005, Burger & Zappalorti, 1988; DuRant et al., 2016) could provide results similar to TSD, yet this does not demonstrate that sex is actually determined by temperature. In the present study, egg mortality was 40 out of 132, which could raise concern about our ability to detect TSD if it exists in this species. Egg mortality was not affected by incubation temperature, but to assess the likelihood of differential mortality to influence sex ratio, we scored all dead eggs as the opposite sex expected from each treatment. Using this conservative approach, sex ratio was not influenced by incubation temperature, and therefore we cannot entirely rule out the possibility of differential mortality. However, given that TSD has been described in African agamids previously (Charnier 1966; El Mouden et al., 2001) and is common in other agamid lizards (Harlow 2004), the most parsimonious explanation is that *A. picticauda* also has TSD.

Although *A. picticauda* is likely the species of Charnier's (1966) work, our subjects were from an invasive population in Florida. Whether sex determining mechanisms differ among native and non-native populations is unknown, but it is possible that our study population exhibits a different pattern from native populations. Previous studies exhibit considerable geographic variation in TSD reaction norms (Conover, 1984; Ewert et al., 1994; Bull et al., 1982) in other organisms. The tropical climate in Miami (NOAA, 2017) is considerably different from the hot semi-arid climate of West Africa (Cotillon, 2017), and these novel conditions in a non-native range could potentially induce rapid shifts in sex determining mechanisms. Indeed, rapid transitions from TSD to GSD have been demonstrated in an Australian agamid lizard (Hollelley et al., 2015), and recent molecular work has uncovered several transitions between sex-determining mechanisms in geckos (Gamble et al., 2015). Invasive populations of *A. picticauda* in South Florida provide a unique opportunity to investigate shifts of TSD reaction norms in a novel environment using comparative studies between native and non-native populations.

The ecologically relevant fluctuating incubation temperatures mimic some potential nest sites in Miami, FL and may represent sex ratios produced in natural nests. Treatments that fluctuated around the means of 26 ºC and 28 ºC produced a female-biased sex ratio while temperature fluctuations around the mean of 30 ºC produced a male-biased sex ratio. Our results do not support our predictions that high fluctuating temperatures produce a female-biased sex ratio, but future studies can use a wider range of temperature fluctuations to assess the effect of ecologically relevant temperatures on sex ratios and determine if temperature fluctuations produce a reaction norm similar to constant temperatures. Field studies of natural nests in South Florida will provide insights into TSD patterns and sex ratio biases in *A. picticauda* under natural conditions.

Descriptive studies of the patterns of TSD in reptiles will help continuously fill in gaps needed for a more comprehensive understanding on the evolution of sex determining mechanisms. Phylogenetic studies of SDM evolution rely on robust characterizations of SDMs, and thus we urge researchers to use a broad range of incubation conditions, and when needed, revisit past studies to provide accurate characterizations of TSD. Reptiles display a variety of sex determining mechanisms, and classifying taxa-specific SDMs will provide important insights into the transitions between SDMs within families and broaden our understanding for the adaptive significance of environmental sex determination.

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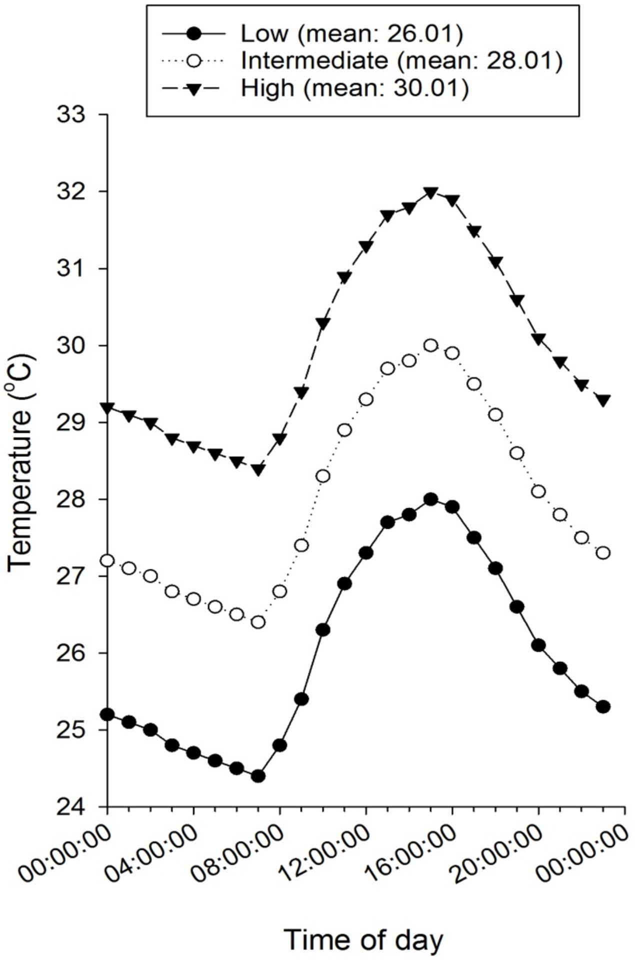

Figure 1.1: Daily temperature fluctuations based on potential nest sites from ibutton data collected at one of the field sites in Coral Gables, FL (Tiatragul et al., 2017). Temperature loggers were placed in potential nest sites at Matheson-Hammock Park. iButtons were placed at 4-6cm depth in the soil. The average temperature for each hour of the day was calculated based on thermal data collected from 1 June – 15 Sept 2014. This thermal profile was then uploaded into programmable incubators, and shifted up or down so that mean temperatures for each treatment were 26, 28, and 30 ºC.

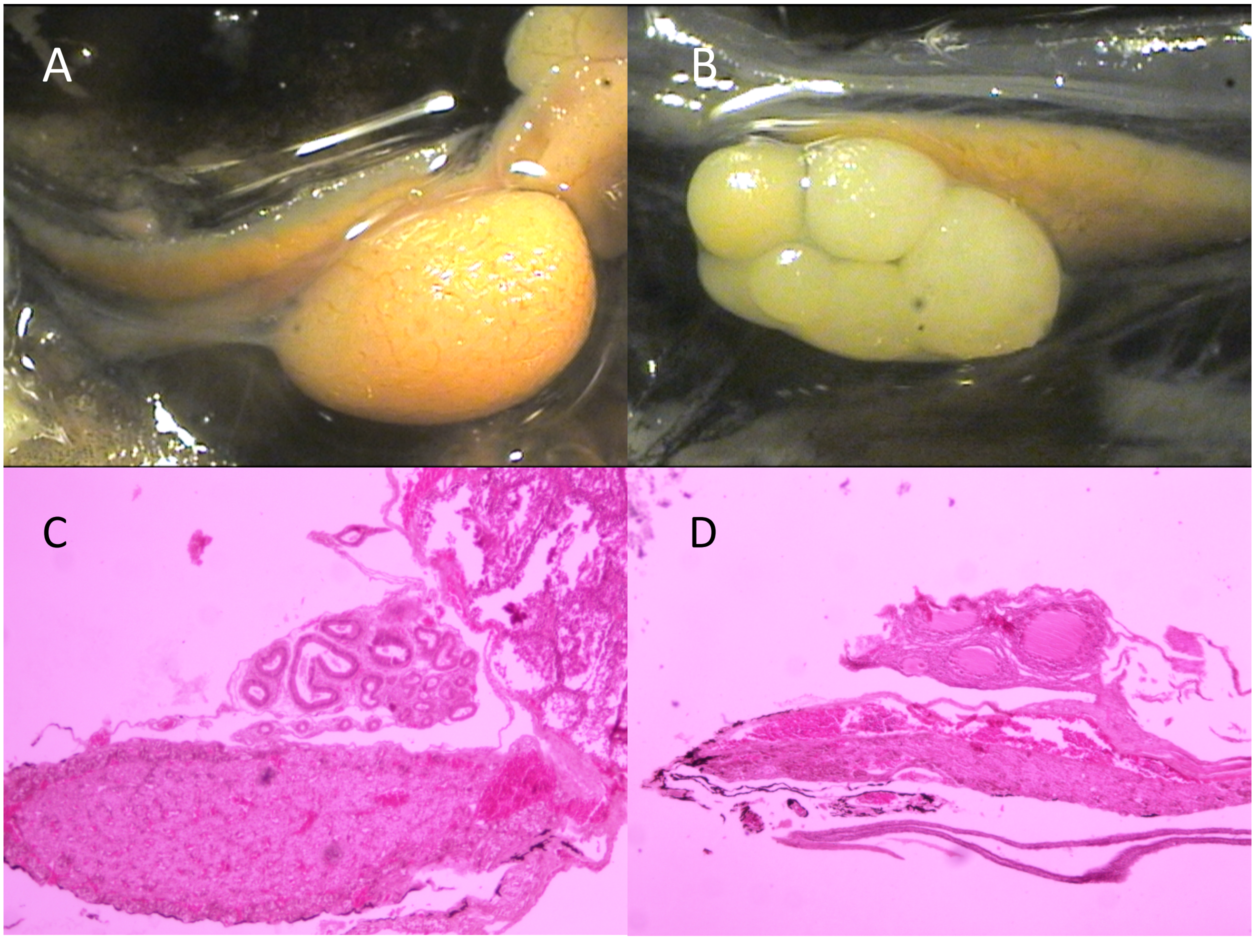

Figure 1.2: Features used to sex juvenile African redhead agama (*Agama picticauda*). (A-B) Gross morphology of older juvenile gonads. A) distinct testes with mesonephric tissue, epididymis, and seminiferous tubules. B) distinct ovary with mesonephric tissue, oviduct, and follicles. (C-D) Histological cross-section of gonads and adjacent reproductive ducts of smaller juveniles. C) testes with mesonephric tissue and epididymis. D) ovary with mesonephric tissue, follicles, and oviduct.

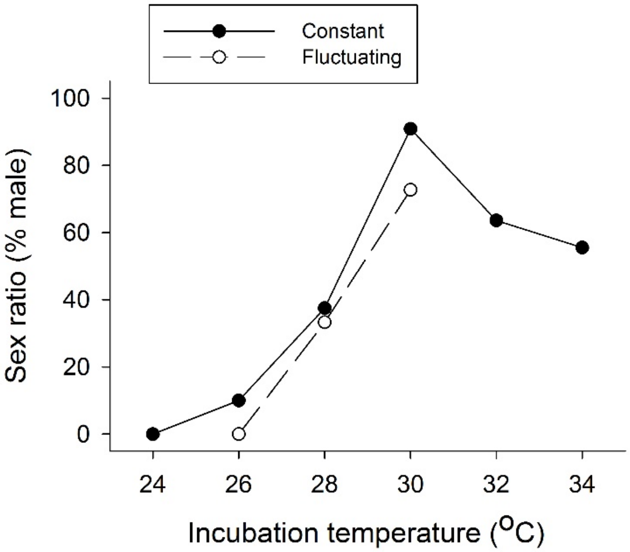

Figure 1.3. The effect of incubation temperature on sex ratio in *Agama picticauda*.

Table 1.1: Summary of hatching success and sex ratio in *Agama picticauda.*

	Incubation Temperature (Degree C)								
	Constant						Fluctuating		
	24	26	28	30	32	34	26	28	30
No. Eggs	14	15	15	16	16	12	15	13	13
Egg Survival (% Hatch)	57.14	66.67	60.00	68.75	75.00	75.00	73.55	76.92	91.31
No. Hatchlings	8	10	8	11	11	9	10	9	11
Sex Ratio (% Male)	0	10.0	37.5	90.91	63.64	55.56	0	33.33	72.73

Chapter 2: The Effect of Incubation Temperature on Morphology and Growth in the African Redhead Agama (*Agama picticauda*)

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Keywords: *Agama agama*, morphology, lizard, Charnov-Bull model, egg incubation, temperature-dependent sex determination

Abstract

The developmental environment plays a pivotal role in shaping fitness-relevant phenotypes of all organisms. Phenotypes are highly labile during embryogenesis, and environmental factors experienced early in development can have profound effects on fitness-relevant traits later in life. Many reptiles exhibit temperature-dependent sex determination (TSD), and studies have aimed to understand how the interaction between sex and temperature influences fitness in these species. The goal of this research is to assess the effects of incubation temperature on fitness-relevant phenotypes, and to identify the sex-specific effects of incubation temperatures on hatchling *Agama picticauda*, a lizard with TSD. By incubating eggs under constant and fluctuating temperatures, we demonstrated that incubation temperature affects fitness-relevant phenotypes in *A. picticauda*, but the effects were not sex-specific. However, eggs incubated at male-biased temperatures hatched earlier than eggs incubated at female-biased temperatures, which may have sex-specific consequences later in life as predicted by models for the adaptive significance of TSD.

Introduction

The environment plays a pivotal role in shaping phenotypes and fitness of all organisms. Phenotypic plasticity, which is the ability of an organism to change its behavior, morphology, or physiology in response to an environmental cue, can generate substantial amounts of phenotypic variation within populations (Gilbert & Epel, 2009). While phenotypes at any life stage can be influenced by environmental factors, embryos are especially sensitive to their surrounding environment whether it be conditions *in utero,* or abiotic conditions surrounding eggs in a nest. The phenotypic response to environmental cues during early developmental stages is referred to as developmental plasticity. Embryos are exposed to many environmental factors that can affect their phenotypic development, such as nutrition, light, stress, and temperature (Gilbert & Epel, 2009). Because phenotypes are highly sensitive and labile during the embryonic stage, these environmental agents experienced early in development can have profound effects on fitness-relevant traits later in life (e.g. morphology, behavior, physiology, growth, and reproductive success). Indeed, long-term phenotypic effects of the developmental environment are well established in many organisms (Deeming, 2004; West-Eberhard, 2003).

Oviparous organisms provide excellent models for studying the influence of developmental environments on offspring phenotypes. Eggs are subject to abiotic and biotic conditions during development, and these conditions are often easy to measure and manipulate in laboratory studies. Of particular interest is the influence of incubation temperature on offspring phenotype and fitness. Studies on the phenotypic effects of temperature during egg incubation have been shown to influence patterns of development, growth, pigmentation, behavior, and even sex (Deeming, 2004).

The effect of incubation temperature on offspring phenotypes has been studied extensively in reptiles (Noble et al., 2017). Several studies show that temperature influences hatchling morphology, locomotor performance, behavior, thermal responses, growth, and sex ratios (Rhen & Lang, 2004). Many reptile species exhibit temperature-dependent sex determination (TSD), where the sex of offspring is determined by the temperature embryos experience during the middle-third of development. Since sex is already an important determinant of life history traits and fitness of an organism, the interaction between incubation temperature and sex on offspring phenotypes and fitness in reptiles is of interest. Moreover, evolutionary explanations predict that TSD is favored by natural selection when incubation temperature differentially affects the fitness of males and females (Charnov & Bull 1977). Indeed, the Charnov-Bull model posits that the interactive effect of incubation temperature and sex on fitness enables each sex to develop at their optimal temperature. One hypothesis within the Charnov-Bull framework predicts that there should be sex-specific reaction norms for fitness-related phenotypes, which in turn would confer sex-specific fitness consequences of incubation temperature (Shine, 1999; Warner & Shine, 2005). Although incubation temperature affects fitness-relevant traits of offspring (Deeming, 2004), the sex-specific effects of incubation temperature are largely unknown except for a few cases (Rhen & Lang, 1995; Elphick & Shine, 1998; Warner & Shine 2008).

The goal of this research is to assess the effects of incubation temperatures on offspring morphology, growth, performance, and survival, and to identify sex-specific effects of incubation temperature on hatchling *Agama picticauda.* This species is native to much of west central Africa, but invasive populations are well established in south Florida, USA. *Agama picticauda* exhibits TSD whereby constant cool (24-26 ºC) incubation temperatures produce mostly female offspring, intermediate constant temperatures (30 ºC) produce nearly all-male sex ratios, and warmer constant temperatures (32-34 ºC) produced mixed sex ratios with a slight male-bias (Charnier 1966, Chapter 1). This species also exhibits sexual dimorphism in color and body size, with adult males reaching a larger size than females, as well as developing bright coloration during the breeding season (Enge et al., 2004). Males are highly territorial and aggressively defend their territories, which often contain multiple females, subordinate non-breeding males, and juveniles (Enge et al., 2004). Females produce clutch sizes of 3-9 eggs, and invasive populations in Miami, Florida have been observed to have up to 3 clutches per reproductive season from February to August (Enge et al., 2004).

Our first objective is to characterize reaction norms for developmental rate, offspring morphology, performance, and survival in *A. picticauda* by using a broad range of incubation temperatures that span 10 ºC. Based on previous work in reptiles, we predict that incubation temperature will positively relate to developmental rate, and significantly shape variation in morphology and performance (Noble et al., 2017). Secondly, by using incubation treatments that produce mixed sex ratios, we will quantify the interactive effects of incubation temperature and sex on these phenotypic traits and survival. We will also examine the onset of sexual size dimorphism by raising offspring to sub-adult stages. In accord with the Charnov-Bull model and pattern of sexual size dimorphism in *A. picticauda*, we predict that thermal reaction norms for body size will differ between the sexes, or eggs incubated at male-biased temperatures will produce large offspring and eggs incubated at female-biased temperatures will produce small offspring. Alternatively, offspring from male-biased temperatures will grow faster than those from female-biased temperatures. Support for any of these predictions should generate male-biased SSD and provide evidence in support of the Charnov-Bull model.

In addition to the above goals, our third objective is to quantify the effects of ecologically relevant incubation temperatures on phenotype and survival of *A. picticauda*. Although reaction norms for different phenotypes are traditionally characterized using constant incubation temperatures, such conditions do not simulate the environments that eggs experience in nature. By using constant and ecologically-relevant fluctuating temperature treatments to test the effect incubation temperature has on fitness-relevant phenotypes in hatchlings, we will be able to better interpret how incubation temperatures in nature might generate variation in phenotypes and fitness in the wild.

Methods

Egg Collection and Incubation

Adult *A. picticauda* were collected from South Miami on 4-6 May 2016. Six males and 24 females were housed in outdoor enclosures at Auburn University and used for breeding. Details on adult housing and care are in Chapter 1. Females were regularly monitored to determine gravidity. If a female was gravid, nest boxes were checked for eggs every other day. When eggs were found, the female was captured for identification. Eggs were brought to the laboratory, individually weighed to the nearest 0.0001 gram, and given an identification number. Eggs were placed individually in glass jars containing moist vermiculite (-150 pKa); jars were covered with plastic wrap and sealed with a rubber band. The date of oviposition was recorded and the jar was randomly assigned to one of 9 incubation treatments. To characterize the pattern of TSD, six constant temperature incubation treatments of 24, 26, 28, 30, 32, and 34ºC were used. To assess the effects of temperature fluctuations on sex ratio, three fluctuating treatments were used. These temperature treatments fluctuated around the means of 26, 28, and 30ºC. The pattern of temperature fluctuation was based on temperature logger (iButton) data collected from potential nest sites near one of the collection sites in Coral Gables, FL (Chapter 1; Tiatragul et al., 2017) and uploaded into programmable incubators.

Hatchling Measurements and Care

Incubators were checked daily for hatchlings. If a hatchling was found, it was removed and given a unique toe-clip for identification, and measurements of mass, snout-vent length (SVL), tail length (TL), head depth, width, and length were taken. All individuals were immediately sexed via hemipene eversion (Harlow, 1996). Hatchlings were randomly assigned to one of ten cages using a random number generator, and done such that only one hatchling per treatment was in each cage (up to nine lizards per cage). Hatchling cages (1.106m long x 0.5461m wide x 0.4572m tall) were illuminated with Reptisun 5.0 UVB bulbs and 100 watt light bulbs for heat. The bottom surface of the cage was covered in a sand substrate, and rocks were placed throughout for basking perches and hiding places. Leaves and halved PVC pipes were also used for hiding places. Two small petri dishes were used for water dishes. Hatchling lizards were fed three small crickets three times per week and cages were misted with water every day. Hatchlings were housed from the date they hatched (from 6 July – 28 October 2016) to 13 March 2017.

To measure sprint performance, one-week old hatchlings were chased with a paintbrush down a 1-meter long wooden racetrack at room temperature (25 ºC). The racetrack consisted of photocells (placed every 25 centimeters) connected to a laptop computer. As lizards passed through the photocells, the split times were recorded on the program TrackMate ScL Timer v. 6.8.1 (Trackmate Racing, Surrey, British Columbia, Canada). Hatchling mass was taken prior to the sprint trials. Each individual was chased five times, and allowed to rest for 2 minutes between each trial. The fastest 25-cm time (in m/s) was considered the maximum sprint speed and was used for the analysis. The experimenter was blind to the treatment group of each individual raced at the time of the sprint performance trials.

Body size measurements (SVL, TL, mass, head width, depth, and length) were taken at 6-week age intervals for each individual to assess the effect of incubation temperature on growth and the onset of sexual size dimorphism. These measurements were taken until the last individual reached 18 weeks of age; by the time the youngest lizard reached 18 weeks, the oldest individual was 36 weeks of age. Dead lizards found during the experiment were identified, their date of death was recorded, and they were formalin fixed and preserved in 70% ethanol. On 18 March 2017, all lizards were humanely euthanized, formalin fixed and deposited in the Auburn University Museum of Natural History. The sex of each individual was verified by surgical inspection of gonads and subsequent gonadal histology (Chapter 1). All analyses that involved sex was based on the sexual identification from gonad inspection and histology since the sex of some individuals was misidentified based on hemipene eversion.

Statistical Analysis

Statistical analyses were performed in R (R Core Team, 2016) and SAS. Mixed-model analyses of covariance (ANCOVA) were used to quantify the effects of incubation treatment on incubation duration, date of hatching, morphology and performance at hatching. The analysis of hatching date included oviposition date as a covariate. Egg mass was used as a covariate for SVL and body mass, while SVL was used as a covariate for sprint speed, tail length, and head size (length, depth, width). For sprint performance, hatchlings incubated at 24 ºC were removed from the analyses because of a small sample size due to mortality in this treatment. Maternal identity was a random effect in all models. The first set of analyses compared the six constant incubation treatments, and the second set of analyses quantified the effect of incubation temperature, regime (constant vs fluctuating), and their interaction using only data from the 26, 28, and 30 ºC treatments. Our third set of analyses quantified the interaction between incubation temperature and sex to assess the sex-specific effects of incubation temperature, using only temperatures that produced a mixed sex ratio (i.e., 28, 30, 32, 34 ºC); for this analysis, fluctuating and constant temperature regimes were combined to ensure sufficient sample sizes for sex-specific comparisons. Sex differences in incubation period and hatch date were assessed using a mixed model ANOVA that included all temperature treatments; constant and fluctuating treatments were combined since there were no differences in incubation period and hatch date between regimes.

The effects of incubation temperature on growth rate was quantified with a repeated measures linear mixed-effects model using incubation temperature, age, and their interaction as fixed factors with cage and hatchling identity nested within maternal identity as random factors. The dependent variable was body size (mass or SVL) at 6-week age intervals up to 18 weeks old; although many individuals survived longer than 18 weeks, growth was assessed only to this age. The model also included body size at hatching and the level of competition with other individuals within the cage as covariates. The level of competition was assessed as the number of days spent in the cage relative to the first hatchling that entered the cage; this value was standardized to a mean of zero and unit variance per cage prior to analyses. Sex-specific effects of incubation temperature on growth was not assessed due to small sample sizes.

Treatment effects on survival were analyzed with a generalized linear mixed model with a binomial error distribution. Survival was quantified as those that survived vs died by the end of the study. This analysis included hatching date, hatchling mass, and level of competition (as described above) as covariates.

Results

Incubation Duration and hatchling phenotypes

Incubation duration declined with increasing incubation temperature (Fig. 1a). At the coolest incubation temperature (24 °C) eggs took 102 days to hatch, whereas incubation length was 40 days at the warmest temperature (34 °C), resulting in about a 60% difference in incubation length. Accordingly, the date of hatching was negatively related to incubation temperature (Fig. 1b). When adjusted for date of oviposition, average hatching date for individuals from the coolest treatment (7 October ± 2.4 days SE) was 62 days later those from the warmest treatment (6 August ± 2.3 days SE). There was no difference in incubation period or hatching date between eggs from the constant and fluctuating temperatures (Table 1).

Constant incubation temperatures affected hatchling snout-vent length and tail length but not mass at hatching (Table1, Figure 2a, b, c). Eggs incubated at 28 ºC produce longer hatchlings than those incubated at any other temperature. Eggs incubated at 32 ºC produced hatchlings with shorter tails than those from the 24 ºC and 30 ºC treatments. Hatchlings from the 34 ºC treatment had shorter tails than hatchlings incubated at 24, 26, and 30 ºC. Hatchling body condition (mass relative to SVL) was not affected by constant incubation temperature. Phenotypes were generally unaffected by fluctuating incubation regimes, except for tail length where those incubated at a fluctuating temperature around 26 °C had long tails compared to other treatments.

Constant incubation temperature had a significant effect on head depth and width, but not head length (Figure 2d, e). Generally, hatchling head size increased with temperature. For example, hatchlings incubated at 32 ºC had wider heads than hatchlings incubated at 24, 26, 28, and 30 ºC. Sprint performance was not influenced by incubation temperature (Figure 2f; Table 1). Fluctuating incubation regimes had no effect on head size or sprint performance (Table 1).

Incubation temperature did not differentially affect male and female phenotypes (Table 2). However, males and females differed in incubation period and hatch date (Table 2). Across all treatments, incubation length of male eggs (mean = 48.9 days ± 1.26 SE) was on average 20.5 days shorter than female eggs (mean = 69.4 days ± 2.70 SE; $F_{1,73}$=37.86, P<0.001); this difference was generated by the male-biased sex ratio produced at warmer temperatures (see Fig. 1.3). Accordingly, this sex difference in incubation length generated sex differences in hatching date ($F_{1,72}$=40.54, P<0.0001). The average hatching date for males and females was 17 August (±4.79 days SE) and 9 September (±4.23 days SE), respectively.

Hatchling Growth and survival

Lizard growth rates varied substantially over 18 weeks, but constant incubation temperature did not explain this variation (mass: $F_{4,52}$=0.1, p=0.968; SVL: $F_{4,51}$=0.42, p=0.792; Fig. 3a). Growth rate across each six-week interval also did not vary among constant incubation treatments (growth x time interaction: mass: $F_{12,52}$=0.78, p=0.665; SVL $F_{12,51}$=1.6, p=0.120). The level of competition within cages was negatively related to growth rate (mass: $F_{1,52}$=5.3, p=0.026; SVL: $F_{1,51}$=7.07, p=0.012). Lizards that hatched from eggs exposed to fluctuating thermal regimes grew faster over 18 weeks than those from constant incubation treatments (mass: $F_{1,67}$=8.5, P=0.005; SVL: $F_{1,67}$=7.71, p=0.007), particularly during the last 6-week interval (age 12-18 weeks) (regime x time interaction for growth in mass: $F_{3,67}$=5.12, p=0.003; growth in SVL: $F_{3,67}$=4.23, p=0.0085; Fig. 3b). Males and females did not differ in growth rate up to 18 weeks of age (mass: $F_{1,60}$=3.89, P=0.0533; SVL: $F_{1,61}$=1.11, P=0.2968). However, due to a relatively long growing period, individuals that hatched early (i.e., mostly males) were larger by the end of the study that those that hatched late (r=-0.423, P=0.0126; Fig. 4); consequently, males tended to be larger than females at this time period, but this difference was marginally non-significant (mass: $F_{1,32}$=4.04, P=0.0531; SVL: $F_{1,32}$=3.95, P=0.0554; Fig. 4).

The survival rate of hatchlings from the coolest incubation treatment was relatively low, but this difference was not statistically significant ($F_{5,36}$=0.6, p=0.700; Fig. 5). Survival rates did not differ between constant and fluctuating incubation regimes ($F_{1,41}$=0.06, p=0.814), nor was there a significant interaction between mean temperature and regime ($F_{2,41}$=1.51, p=0.234). Males and females did not differ in survival ($F_{1,47}$<0.01, P=0.999), and the interaction between sex and incubation temperature on survival was not significant ($F_{1,47}$<0.01, P=0.999).

Discussion

The primary objective of this research was to assess the effect of constant and ecologically-relevant fluctuating incubation temperatures on fitness relevant phenotypes of *A. picticauda*. Our results demonstrated that constant incubation treatments influence phenotypes at hatching, and that the effects of fluctuating temperature treatments are largely similar to that of constant temperatures. Our second objective was to investigate sex-specific effects of incubation temperature on hatchling phenotypes and survival in order to test hypotheses within the Charnov-Bull framework for the adaptive significance of TSD. Although we found no evidence for sex-specific effects of incubation temperature on phenotypes at hatching, some of the phenotypes (as well as egg hatching date) that were influenced by incubation temperature could have sex-specific fitness consequences.

As in other reptiles, incubation duration showed a negative exponential relationship with incubation temperature (Van Damme et al., 1992; El Mouden et al., 2001; Harlow & Taylor, 2000). Although this thermal effect on developmental rate is well established (Noble et al., 2017), this effect can have serious fitness consequences. For example, eggs exposed to relatively warm temperatures hatch sooner, and thus eggs will have reduced exposure to predation or adverse weather conditions. Moreover, offspring that hatch early in the season typically enter a less competitive environment and have greater survival than those hatching late (Olsson & Shine, 1997; Warner & Shine, 2007). The reduced competition early in the season and a longer growing season prior to the onset of winter will lead to relatively large body sizes prior to the first reproductive season (Warner & Shine, 2005). In our study, the effect of temperature on incubation duration in *A. picticauda* was large (e.g., 60% difference between the coolest and warmest temperatures) and would likely generate substantial variation in body size prior to the first reproductive season. Indeed, lizards hatched from warm incubation temperatures were significantly larger in body size at the end of the study (13 March 2017) compared to those from cool incubation treatments (Fig. 4), even when adjusted for their size at hatching. Moreover, individuals that were exposed to less competition within their enclosures grew more rapidly that those that hatched late and entered an environment with competitors already present.

Incubation temperature also generated variation in many (but not all) phenotypes of offspring at hatching. Hatchlings from eggs incubated at an intermediate temperature (28ºC) were longer on average than those from the other treatments, yet body mass was not statistically different among treatments. This pattern contrasts that of other studies on lizards where relatively warm incubation temperatures produce small hatchlings (Angilletta et al., 2000; Warner & Shine, 2005; Brana & Ji, 2000; El Mouden et al., 2000; Radder et al., 2002). The size advantage (~2 mm) from those produced at the intermediate incubation temperature might benefit these individuals (Sinervo et al. 1992; Uller & Olsson, 2010), but any benefit of a slightly larger size at hatching may not outweigh benefits of hatching earlier in the season (Warner & Shine 2007). Warm incubation temperatures produced hatchlings with large heads relative to their SVL. The benefit of a large head size at hatchling stages is unknown, but this might confer an advantage by enabling individuals to consume diverse sizes and types of prey (Verwaijin et al., 2002; Herrel et al., 2001) or possibly aid in competitive interactions with conspecifics (e.g., aggressive interactions involving biting; Lailvaux et al., 2004; Perry et al., 2004; Kratochvil & Frynta, 2002). Relatively large head sizes coupled with early hatch dates due to warm incubation temperature might provide these individuals major advantages over those produced from cooler incubation temperatures.

Performance, growth and survival were generally unaffected by constant incubation temperatures. The lack of an effect of incubation temperature on hatchling locomotor performance is consistent with research on another agamid lizard (Warner & Shine, 2005), but contrasts with studies on other lizards (Elphick & Shine, 1998; Brana & Xi, 2000; Van Damme et al., 1992; Booth, 2006). Growth rate was not directly impacted by constant incubation temperature, but was significantly reduced by increased levels of competition within enclosures. Competition, as discussed above, changes over the season as eggs continue to hatch. Indeed, those that hatched late (i.e., from cooler incubation temperatures) in the season experience greater levels of competition in ways that can reduce growth and survival (Boone et al., 2002). Consistent with this was the reduced (though not statistically significant) survival of hatchlings from our coolest incubation treatment (24 °C, Fig. 5); the lack of statistical significance was likely due relatively low sample sizes.

Thermal fluctuations mimicked the average daily conditions that eggs are likely to experience in this invasive population of *A. picticauda*. Thus, our fluctuating temperature treatments were designed to gain an ecologically-relevant evaluation of how incubation temperatures generate variation in offspring phenotypes. In general, the phenotypic effects of fluctuating incubation regimes differed very little from those of constant incubation regimes. These results were surprising given recent findings that show substantial differences in how fluctuating and constant temperatures affect phenotypic development in other reptiles (e.g., Warner & Shine, 2011; Neuwald & Valenzuela, 2011; Bowden & Carter, 2014; Pearson & Warner, 2016). Although we show that tail length was influenced by the fluctuating incubation treatment, the effect was only at a mean temperature of 26 °C and the biological significance of such an effect is questionable. Intriguingly, however, offspring from fluctuating incubation temperatures grew significantly faster during their first 18 weeks of life. Thus, natural thermal regimes may provide some benefits to hatchlings.

Adaptive significance of TSD in Agama picticauda

The Charnov-Bull model predicts that TSD is favored by natural selection when incubation temperature differentially affects the fitness of males and females such that the interactive effect of incubation temperature and sex enables sons and daughters to develop at their optimal temperature (Charnov & Bull, 1977). This differential effect may be realized via several different mechanisms (Shine, 1999). For example, one hypothesis within the Charnov-Bull framework suggests there should be sex-specific reaction norms (e.g., sex x incubation temperature interaction) for fitness-relevant phenotypes. Sex-specific reaction norms could ensure that each sex develops optimal phenotypes at the temperature they are produced. Sex-specific reaction norms have been demonstrated for some phenotypes in TSD species (Shine et al., 1997), but is often difficult to demonstrate because sex and temperature are typically confounded in species with TSD. Importantly, studies that have experimentally decoupled the effects of sex and incubation temperature often show little evidence of sex by incubation temperature interactions for offspring phenotypes (Rhen & Lang, 1995; Warner & Shine 2005). Consistent with this, we also provide no evidence of sex-specific effects of incubation temperature on hatchling phenotypes. Importantly, one caveat of our study is that our constant and fluctuating incubation temperatures were combined to ensure proper sample sizes for statistical analyses; but even then, the number of male and female offspring for each temperature treatment was less than ideal for a robust evaluation of sex by temperature interactions. Moreover, mortality during the 18 weeks of growth precluded our ability to test for interactions on growth rates. Thus, future studies that use larger sample sizes are greatly needed to fully rule out this hypothesis for the adaptive significance of TSD.

A second hypothesis within the Charnov-Bull framework posits that incubation temperature affects phenotypes regardless of sex, but those incubation-induced phenotypes have sex-specific fitness consequences (Shine, 1999). Support for this hypothesis has been provided in a few studies of species with TSD. For example, in leopard geckos, aggressive behaviors are induced by warm temperatures that produce male-biased sex ratios (Gutzke & Crews, 1988), and females produced at these temperatures develop aggressive behaviors similar to males. However, given that aggressive behaviors are more beneficial to male vs female fitness, selection should favor a match between incubation temperature and sex so that males are predominantly produced at warm temperatures. Similar to this argument, incubation length, and hence, timing of hatching could also have sex-specific benefits, as described in fish and lizards with TSD (Conover,1984; Warner et al., 2009; Warner & Shine, 2008). Indeed, males may benefit more from hatching early in the season because it provides them with an opportunity to grow larger and improve their competitive ability before the first reproductive season begins. Though we did not measure fitness, our data on *A. picticauda* are consistent with this hypothesis and general trends suggest males and females may be differentially affected by incubation temperature. Whether the effects of incubation temperature on offspring hatch date or hatchling phenotypes translate to phenotypic effects later in life at maturity is unclear, and future studies that investigate sex-specific effects of incubation temperature on phenotypes at different life stages up to sexual maturity are needed.

Previous authors have suggested an association between TSD and sexual dimorphism (Webb & Smith, 1984; Ewert & Nelson, 1991; Ewert et al., 1994; Harlow & Shine, 1997), whereby incubation temperature differentially affects males and females in ways that contribute to sex differences in adult body size. In *A. picticauda*, females are predominately produced at cool temperatures, whereas males are predominately produced at warmer incubation temperatures (albeit, even warmer temperatures produce a nearly balanced sex ratio; Chapter 1). Male *A. picticauda* are highly territorial, and this species exhibits strong sexual dimorphism as adults; males are larger and have brighter head and tail coloration than females (Enge et al., 2000). Similar to that described above, male-biased sexual size dimorphism may be achieved because males from warm incubation temperatures hatch earlier than males from cooler temperatures and thus have considerably more time to grow prior to the first reproductive season. While this is not a direct effect of incubation temperature on body size, since initial body size at hatching was smaller at warmer temperatures, it is an indirect consequence of males hatching sooner than females. Other studies have shown that incubation temperature influences sexual dimorphism in other agamid species (El Mouden et al, 2001).

We showed that incubation duration at male-biased temperatures (30-34 °C) was 27.2 days shorter than that for female-biased temperatures (24-28 °C). Moreover, by the end of the study lizards that hatched early in the season (due to warm incubation) were considerably larger by the end of this study, and males tended to be at the "large-size" end of the distribution (Fig. 4). Such an effect would benefit males more so than females because males compete for territories and access to females (Andersson, 1994). Thus, an increase in body size will benefit reproductive success (Warner & Shine 2008; Olsson & Shine, 1996). In addition, individuals incubated at the male-biased temperatures generally had larger heads relative to body size than those incubated at female-biased temperatures. This, in conjunction with early hatching, would also benefit males more so than females because males that hatch earlier in the season would reach an adult body size earlier and would compete with other males. Head width is positively correlated with bite force (Lappin & Husak, 2005; Herrel et al., 2001; Verwaijin et al., 2002), which is an important performance trait in agonistic interactions within territorial lizard species (Lailvaux et al., 2004; Kratochvil & Frynta, 2002).

Late hatching due to cold temperatures could put females at a disadvantage because they are entering a more competitive environment. This is reflected in the low survival of females from the 24 °C treatment, since they hatched into a competitive environment with individuals that had hatched out earlier. If warmer incubation temperatures benefit males more than females, then why do sex ratios shift back towards 1:1 when temperatures exceed 30 °C? Indeed, male production at these high temperatures could enhance fitness for the reasons described previously.

Perhaps production of some females this temperature also increases fitness; for example, as argued for other agamid lizards, hatching early for females (due to high incubation temperatures) might also be favored by selection because the head start can increase body size, and hence fecundity at an early age (Warner et al., 2009). On the other hand, this trend towards female production at extreme high temperatures could be an artifact of phylogeny, as many other agamid lizards exhibit the FMF pattern of TSD (Harlow, 2004). Research that addresses these hypotheses is warranted.

Hypotheses within the Charnov-Bull framework (Charnov & Bull, 1977; Shine, 1999) attempt to describe the adaptive significance of TSD in reptiles. Studies that have addressed this topic have generally used short-lived species of reptiles (Harlow & Shine, 1997; Warner & Shine, 2008). However, the majority of reptile species with TSD (i.e. turtles, crocodilians, and tuatara) are long-lived and have different life-histories from short-lived species, making it difficult to test long-term reproductive success and thus the adaptive significance of TSD in these species. *Agama picticauda*, however, provides a potential model for studies of the adaptive significance of TSD that eliminates some of the logistical problems associated with long-lived reptiles. For instance, *A. picticauda* is a long-lived species, but it matures at a relatively young age and researchers can feasibly house individuals for longitudinal studies of survival and reproductive success. Future studies aimed at evaluating the long-term fitness effects of incubation temperature in *A. picticauda* could be useful in extrapolating the adaptive significance of TSD in this species to other long-lived species with TSD.

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Table 2.1: Summary statistics of phenotypes at hatching of *A. picticauda*. Significant effects denoted by *

Hatchling Phenotypes	Constant Temperature Treatments		Fluctuating Temperature Treatments					
	Temp Effect		Temp		Regime		Interaction	
Incubation Period	$F_{5,42}=128.1637$	p<0.0001*	$F_{2,46}=144.853$	p<0.0001*	$F_{1,46}=0.046$	p=0.8313	$F_{2,46}=0.509$	P=0.6047
Hatch date	$F_{5,41}=119.6$	P<0.001	$F_{1,45}=107.4$	P<0.001	$F_{1,45}=0.05$	P=0.828	$F_{2,45}=0.48$	P=0.623
SVL	$F_{5,41}=3.803$	p=0.0064*	$F_{2,45}= 3.202$	p=0.0501*	$F_{1,45}= 0.925$	p=0.3412	$F_{1,45}= 2.829$	p=0.0696
Tail Length	$F_{5,41}=3.710$	p=0.0073*	$F_{2,45}= 1.916$	0.1590	$F_{1,45}= 3.232$	0.0789	$F_{2,45}= 3.853$	0.0285*
Mass	$F_{5,41}=0.9868$	p=0.4376	$F_{2,44}= 0.9807$	p=0.3829	$F_{1,45}= 0.4476$	p=0.5069	$F_{1,45}= 0.4341$	p=0.6505
Body Condition	$F_{5,41}=1.244$	p=0.3065	$F_{2,45}= 1.554$	P=0.2226	$F_{1,45}= 0.072$	p=0.7892	$F_{2,45}= 1.408$	p=0.2553
Head Depth	$F_{5,37}=4.269$	p=0.0036*	$F_{2,41}= 4.205$	0.0218*	$F_{1,41}= 1.304$	0.2600	$F_{2,41}= 2.316$	0.1114
Head Width	$F_{5,37}=3.747$	p=0.0076*	$F_{2,41}= 1.42$	0.2530	$F_{1,41}= 0.60$	0.4434	$F_{2,41}= 0.56$	0.5777
Head Length	$F_{5,37}=0.668$	p=0.6504	$F_{2,41}= 0.172$	0.8429	$F_{1,41}= 0.499$	0.4841	$F_{2,41}= 0.008$	0.9920
Sprint Performance	$F_{5,18}=1.17867$	p=0.3534	$F_{2,25}=0.44665$	0.6448	$F_{1,25}=1.72323$	0.2012	$F_{2,25}=1.44938$	0.2538

Table 2.2: Summary statistics of sex-specific effects of incubation temperature on hatchling phenotypes in *Agama picticauda*. Significant effects denoted by *

	Sex Specific Effects of Incubation Temperature					
Phenotype	Temp Effect		Sex Effect		Temp*Sex Interaction	
Incubation Period	$F_{3,40}=90.4$	$p<0.0001$ *	$F_{1,40}=8.25$	$p=0.0065$*	$F_{3,40}=1.76$	$p=0.1696$
Hatch Date	$F_{3,39}=88.52$	$P<0.0001$ *	$F_{1,39}=8.34$	$P=0.0063$*	$F_{3,39}= 1.70$	$p=0.1822$
SVL	$F_{3,39}=5.52$	$P=0.0029$	$F_{1,39}=0.27$	$P=0.6051$	$F_{3,39}=1.75$	$P=0.1728$
Tail Length	$F_{3,39}=4.68$	$P=0.0069$	$F_{1,39}=0.04$	$P=0.8491$	$F_{3,39}=0.43$	$P=0.7331$
Mass	$F_{3,39}=0.46$	$P=0.7134$	$F_{1,39}=0.36$	$P=0.5518$	$F_{3,39}=0.15$	$P=0.9285$
Body Condition	$F_{3,39}=1.93$	$P=0.1404$	$F_{1,39}=0.01$	$P=0.9132$	$F_{3,39}=1.81$	$P=0.1617$
Head Depth	$F_{3,38}=8.79$	$P=0.0001$	$F_{1,39}=0.09$	$P=0.7659$	$F_{3,38}=0.88$	$P=0.4626$
Head Width	$F_{3,38}=6.25$	$P=0.0015$	$F_{1,38}=0.02$	$P=0.8834$	$F_{3,38}=0.97$	$P=0.4157$
Head Length	$F_{3,38}=0.36$	$P=0.7852$	$F_{1,38}=2.32$	$P=0.1360$	$F_{3,38}=0.52$	$P=0.6717$
Sprint Performance	$F_{3,26}=0.05$	$P=0.9831$	$F_{1,26}=0.06$	$P=0.8135$	$F_{3,26}=0.79$	$P=0.1508$

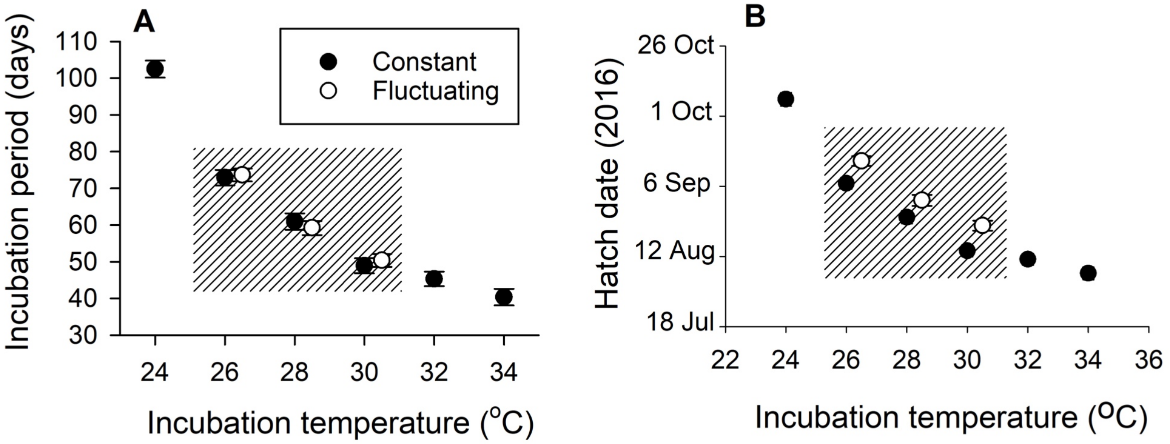

Figure 2.1. Effect of egg incubation temperature on incubation duration and date of hatching in *Agama picticauda*. For incubation duration (A) means ± 1 standard error are reported. Hatch date (B) was adjusted for oviposition date, and least squares means ± 1 standard error reported. Values within the cross-hatched areas illustrate treatments compared to test interactions among thermal regime (constant vs fluctuation) and mean temperature (26, 28, 30 °C). Statistical results are reported in Table 1.

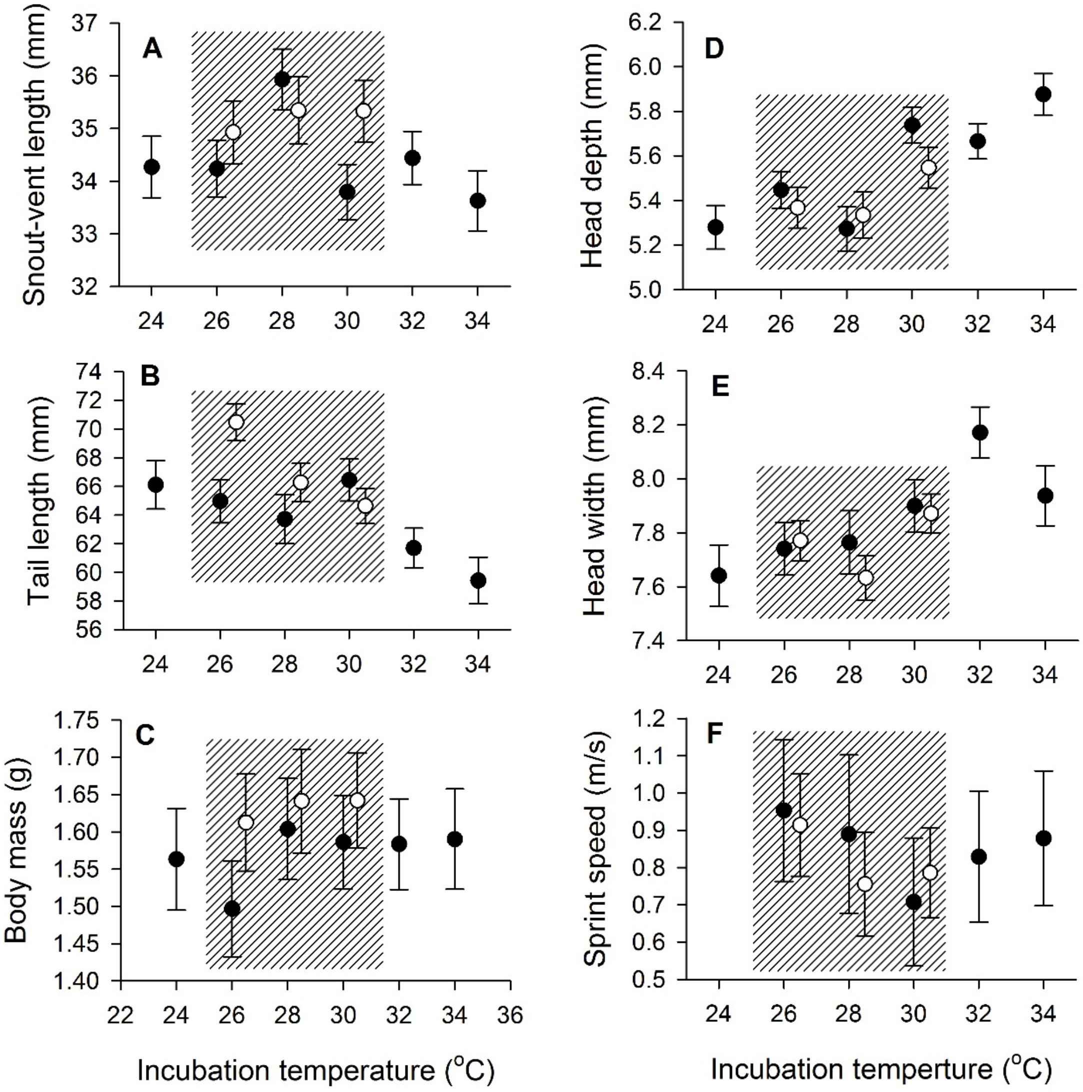

Figure 2.2. Variation among constant (solid circles) and fluctuating (open circles) egg incubation treatments in morphology and performance of hatchling *Agama picticauda*. A) Snout-vent length, B) tail length, C) body mass, D) head depth, E) head width, and F) sprint speed. Values within the cross-hatched areas illustrate treatments compared to test interactions among thermal regime (constant vs fluctuation) and mean temperature (26, 28, 30 °C). Data represent least squares means ± 1 standard error. Statistical results are reported in Table 1.

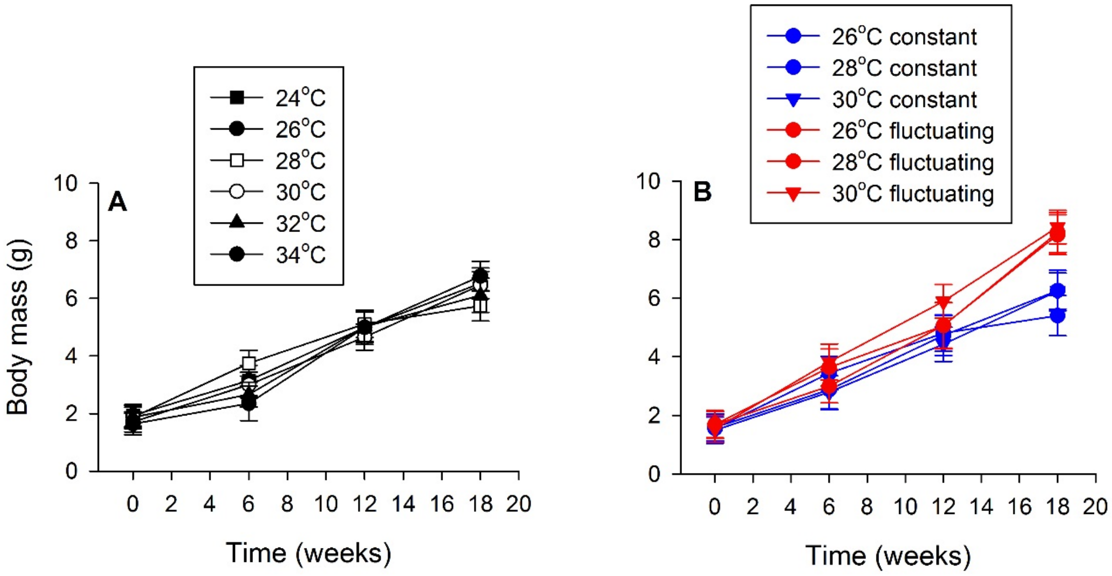

Figure 2.3. Growth of *Agama picticauda* over 18 weeks after hatching. A) Variation in growth among constant incubation treatments. B) Variation in growth among constant and fluctuating incubation treatments. Data presented illustrated growth in body mass, and the same patterns were observed for growth in snout-vent length. Data represent least squares means ± 1 standard error. Statistical results are reported in the text.

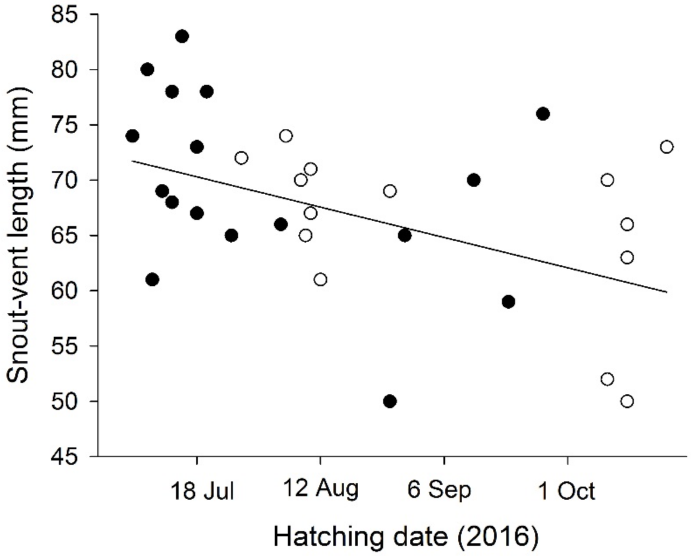

Figure 2.4. Relationship between hatching date and body size of *Agama picticauda* at end of the study. Open circles represent females and closed circles represent males.

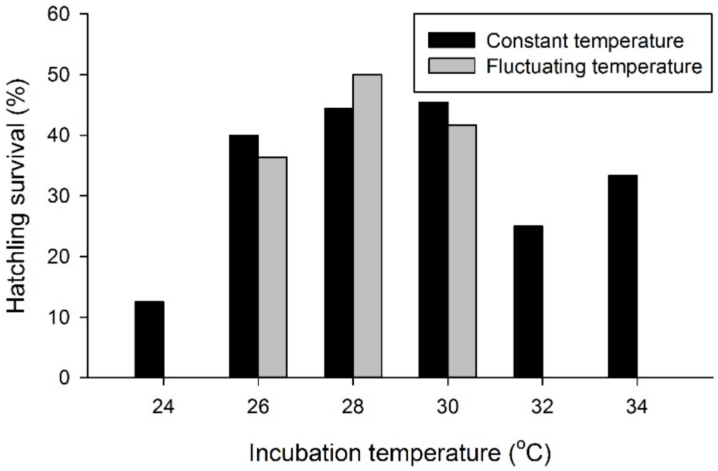

Figure 2.5. Variation in survival of hatchling *Agama picticauda* among egg incubation treatments