Do seasonal changes in developmental temperature have season-specific consequences in a lizard?

by

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Abstract

Phenotypic plasticity enables individuals to develop phenotypes that are suited to their immediate environment. Seasonal shifts in environmental conditions are particularly important because they provide predictable cues to which organisms can respond in adaptive ways. For example, seasonal changes in temperature can induce phenotypes at different times of the year that have season-specific fitness benefits. A previous study has shown that temperature during different times of the season can affect phenotypes and performance. Here, I assessed whether the timing of oviposition is adaptively matched to the thermal environment that embryos experience at a given time of the reproductive season. I used the brown anole lizard (*Anolis sagrei*), which has an extended reproductive season (April-October), to address this question. Eggs were collected from two temporally-separated breeding colonies and exposed to two incubation treatments that mimicked the natural fluctuations in nest site temperatures during early and late periods of the reproductive season. Hatchlings were measured, and their locomotor performance was assessed in the lab, and then released on an island to quantify growth and survival. Hatchlings from the late season were larger and faster overall than those from the early season. Late-season incubation temperatures also produced larger, faster hatchlings. Early season hatchlings had higher survival than late season hatchlings regardless of incubation temperature. These results show that the timing of oviposition and incubation temperature can differentially affect phenotype and fitness.

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List of Abbreviations

ANOVA	Analysis of variance
SVL	Snout-vent length
TSD	Temperature-dependent sex determination
UAB	University of Alabama at Birmingham

Chapter 1

Plasticity in reptiles: A literature review and thesis overview

Introduction to plasticity

Environmental conditions play a very important role in shaping phenotypes of animals at different life stages (Shine & Elphick 2001). Conditions that individuals experience during early stages of development can potentially affect gene expression, which are likely to have a significant influence on important phenotypes (DeWitt & Scheiner 2004; Gilbert & Epel 2009). The effects of these changes due to the developmental environment often benefit the organism's fitness, but the adaptive significance of environmentally-induced changes is often difficult to determine. The ability of a single genotype to produce multiple phenotypes in response to variable environmental conditions is known as phenotypic plasticity (Beldade et. al 2011; West-Eberhard 2003). Phenotypic plasticity has the potential to 'rescue' a maladaptive phenotype in a changing or unpredictable environment. Perhaps the most well-known example is the response of *Daphnia* to predator cues present in the water. These tiny crustaceans can quickly change their body shape to form armor-like structures that can deter predators from preying upon them (Spitze 1992; Boersma *et al.* 1998).

There are many hypotheses behind the underlying mechanisms of plasticity, and it could be argued that they are all intertwined. Some potential mechanisms include hormonal regulation, transcriptional regulation, direct induction, and epigenetic silencing (Gilbert & Epel 2009). The Western spadefoot toad is good example of how hormone

regulation can induce plastic phenotypes. This frog reproduces in ephemeral pools formed during the short rainy season of the dry areas of western North America. The tadpoles typically have only a short amount of time to develop before these pools begin to desiccate. Fortunately, they have the ability to accelerate their rate of morphogenesis as the water levels begin to decline due to changes in their stress hormone axis, which acts synergistically with thyroid hormones that induce metamorphosis in amphibians (Denver 1997). Another example involves diet-induced changes in the insulin-signaling pathway of larval male horned beetles, *Onthophagus nigriventris*. Their larval diet can give rise to two possible phenotypes, one with large horns and one without horns that play important roles in mate selection (Snell-Rood & Moczek 2012). *Daphnia pulex* exhibits the morphological changes in the presence of the phantom midge larva *Chaoborus*. The genome of this species of daphnid has now been fully sequenced allowing for determination of the exact pathway that produces these altered phenotypes. Miyakawa et al. (2010) found that several morphogen and endocrine coding genes were upregulated in young daphnids when predator cues were present including a novel gene that was only upregulated during embryonic development. Epigenetic modification of the DNA can also affect phenotypes. For example, DNA methylation and histone modifications often linked to diet and nutrition (Waterland & Jirtle 2003), and these changes could cause some genes to be suppressed while others to be expressed giving the potential for phenotypic plasticity to occur. Overall, these different mechanisms likely work together to produce a plastic trait.

This change in phenotype during early development could potentially make them better suited for the environment that they will experience at other life stages. Therefore,

plastic responses during early development should be favored by selection. Many studies have produced altered phenotypes in response to a multitude of environmental factors; however, few have shown how they can affect an organism in a natural setting (Shine et. al, 1997).

It is often difficult to determine the adaptive value of plasticity (Gotthard & Nylin 1995; DeWitt *et al.* 1998). A plastic trait is considered adaptive when it benefits the organism and selection acts in favor of the phenotypic response to the environment (Via *et al.* 1995). Though, increasing empirical research has shown that many modes of plasticity are adaptive (Mousseau & Fox 1998; Yeh & Price 2004). It has also been increasingly accepted that the adaptive significance of plasticity is that the developmental environment provides cues to embryos about the conditions they will be experienced later in life and physiologically primes them for those conditions (Stearns 1989).

One way to assess the adaptive value of plasticity is by empirically testing the environmental matching hypothesis. This hypothesis states that environmental cues experienced during early development serves to prepare organisms for the environments they experience later in life. The phenotypic changes during development should prove to be beneficial if similar environments are experienced during adulthood (West-Eberhard 2003). Conversely, if the organism's environment during early development differs substantially and unpredictably from that during adulthood, there could be detrimental effects because the "wrong" phenotype for that particular environment might develop (Uller 2008).

Phenology

Annual seasonal shifts in environmental conditions play a strong role in an organism's life cycle. Phenological processes are often mediated by changes in photoperiod, food availability, precipitation, and temperature (Walther *et al.* 2002). These phenological processes can indicate to an organism when it is time to reproduce, hatch, molt, migrate, hibernate, etc. (Fitzgerald *et al.* 1999; Carey 2009).

These seasonal shifts provide predictable cues to which organisms can respond in adaptive ways, *e.g.* seasonal changes in temperature can induce phenotypes at different times of the year that have season-specific fitness benefits (Hazel 2002; Warner *et al.* 2009; Pearson & Warner 2016). Seasonal changes in temperature and hydric conditions can give cues to some reptiles to hatch (Spencer & Janzen 2011). For example, some turtles, crocodilians, and varanid lizards will delay their emergence until the dry season is over (Doody 2011). Furthermore, changes in seasonal photoperiod have shown to create drastically different phenotypes when embryos experience a phenological mismatch. For instance, when early-season eggs of the Franklin's gull (*Leucophaeus pipixcan*) are exposed to late season photoperiods (and vice versa), the hatchlings produced from this mismatch have reduced fitness-associated phenotypes compared to offspring produced from eggs that experience the typical season-specific conditions (Clark & Reed 2012). These results suggest that embryos are well adapted to the specific conditions that they normally experience during a given time of year. It is evident that variation between seasons can cause season-specific fitness benefits in many species. Changing seasons can also affect the diet of animals, which can, in turn, induce changes in phenotype. For example, the larvae of the moth *Nemoria arizonaria* exhibits two different morphs

depending on the time of year the caterpillars are feeding (Greene 1989). Early in the year their main host, oak trees, have catkins present on which the caterpillars feed, and the caterpillars will mimic these catkins as a form of crypsis. However, when later in the season when there are no catkins present, the caterpillars feed on the leaves of the oak trees and resemble twigs rather than catkins. This change in phenotype is due to the levels of tannins in their diet, where the catkins are low in tannins and the leaves are high in tannins. Greene (1989) found that when the caterpillars are fed catkins with high levels of tannins they will resemble twigs rather than catkins.

Reptiles as models

Reptiles make excellent models for studying developmental plasticity due to their sensitivity to variation and changes in their environment. Many reptiles are oviparous and provide little to no maternal care of their young. Therefore, the embryos are at the will of their environment throughout the entirety of incubation (Shine 2005). Depending on the timing or location of oviposition, embryos can experience highly variable environments and are, therefore, easily exposed to wide-ranging fluctuations in thermal and hydric conditions making them very sensitive to slight changes (Packard *et al.* 1985; Shine 2004; Reedy *et al.* 2013).

As poikilotherms, reptiles are dependent on their thermal environment to regulate their internal temperature and physiological processes across all life stages (Shine 2005). Ambient temperature plays a key role in the development of reptilian embryos (Deeming & Ferguson 1991). Various studies have shown that temperature and other environmental conditions during development can have long-lasting effects on survival rates,

developmental rates, growth, sex, locomotor performance, and thermoregulatory behaviors (Van Damme *et al.* 1992; Shine & Harlow 1993; Andrews *et al.* 2000; Shine & Elphick 2001; Blumberg *et al.* 2002; Andrews 2008; Warner & Shine 2008; Warner *et al.* 2012). For example, when Northern Water Snakes (*Nerodia sipedon*) experience relatively warm developmental conditions, they prefer warm basking areas as neonates (Blouin-Demers *et al.* 2000). Warm incubation conditions also produce larger, faster offspring in the brown anole lizard (*Anolis sagrei*) than do cool incubation temperatures (Pearson & Warner 2016).

Temperature plays a major role in the developmental rate of embryos of oviparous species (Deeming & Ferguson 1991). Many of the biochemical and physiological processes occurring during development are reliant on the embryo's environmental temperatures (Birchard & Marcellini 1996). For instance, many species of reptiles have temperature-dependent sex determination; therefore, sex ratios of clutches may vary due to the fluctuations in nest temperatures during incubation (Shine 1999). Multiple studies have shown that when incubation temperatures are increased, the incubation duration is decreased (Huey 1976; Andrews 2004; Georges *et al.* 2005). Cooler incubation temperatures have shown to slow developmental rates and in some species arrest development until temperatures are raised (Deeming & Ferguson 1991; Deeming DC. 2004; Díaz-Paniagua 2007). Though the temperature effects of incubation are well documented, few studies use ecologically relevant thermal regimes during laboratory incubation experiments (Göth & Booth 2005; Warner & Shine 2007). Most use a constant temperature that will give the highest hatching success or a minimal fluctuating regime with only a few set temperatures (Georges *et al.* 2005). These experimental designs are

useful in answering many questions, but they do not give insight to the potential effects of the fluctuating temperatures that a developing embryo would experience naturally (Bowden *et al.* 2014).

Most studies use unrealistic treatments to test the fitness consequences of egg incubation environments (e.g. locomotor performance, body mass, etc.) or assess growth and survival solely in laboratory conditions. However, several studies have used alternative means to evaluate the consequences of egg incubation environment on offspring fitness under natural or semi-natural conditions (Andrews & Warner 2000; Warner & Andrews 2002; Oufiero & Angilletta 2006; Oufiero & Angilletta, Jr. 2010; Warner *et al.* 2012). These experimental designs are informative, but they may not give insight into the effects of incubation environment on the overall fitness of the offspring and consequently fail to evaluate the adaptive value of developmental plasticity. This is because they only assess the fitness consequences under a single environment or across a relatively short timeframe.

Climate change and phenology

The global climate is increasing at an alarming rate. The global average surface temperature is predicted to rise about 2.6 – 4.8 degrees Celsius by the year 2100 (Carey 2009; IPCC 2014). This huge spike in surface temperatures is likely to have major impacts on the overall weather patterns on the planet. Extended droughts may begin to occur more frequently, and the polar sea ice will continue to be depleted leading to rising sea levels. Organisms rely on many factors including temperature and precipitation to begin phenological processes; thus, as global temperatures continue to rise, the

phenological processes of organisms are being disrupted (Ljungström *et al.* 2015). This has become an increasing concern for many populations of organisms as these shifts have the potential to affect the timing of food availability and offset reproduction, migration, emergence, and hatching. For example, the warming temperatures are shifting the timing of insect emergence such that it is not corresponding with the arrival of migrating birds, which are reliant on these insects to prepare for reproduction, in turn, offsetting the timing of reproduction (Visser *et al.* 2006; Bonal *et al.* 2010). Hovick et al. (2016) shows that 52 percent of 277 bird species analyzed have shifted their range poleward in the past 43 years in response to warmer air temperatures. Extensive long-term research has been conducted on these shifts in birds because they have such a well-defined annual cycle, but some studies have shown that this could also have detrimental effects on ectotherm phenology (Deutsch *et al.* 2008).

Herpetofauna are highly susceptible to shifts in climatic conditions (Gibbons *et al.* 2000). Many amphibian species are reliant on seasonal rains that provide ephemeral pools where they reproduce; thus, as temperatures rise, increased chances of drought are becoming more likely which could reduce the ability of many species to reproduce (Bickford *et al.* 2010; Klaus & Lougheed 2013). Many species of reptiles are also dependent on the hydric conditions of their incubation environment for proper development (Deeming & Ferguson 1991; Deeming DC. 2004). Many species lay their eggs with only a fraction of the water needed for development (Deeming DC. 2004). These eggs are subject to desiccation and embryo death during higher temperatures and drought. Increased temperatures are also likely to cause shifts in sex ratios in reptiles with temperature-dependent sex determination (Janzen 1994; Mitchell *et al.* 2008).

Temperature shifts also have the potential to shift timing of reproduction of reptiles and amphibians in a way that could mismatch the timing of food abundance just as it has done with birds causing detriment to the fitness of offspring. Sinervo et al. (2010) predict that local extinctions of lizard populations in Mexico could rise to 39 percent by 2080 due to the changes in temperature. In a 20-year study of a temperate species of snake, *Vipera aspis,* the snakes have shifted their annual onset of above ground activity, feeding, and date to hibernation in response to shifts in climatic changes (Rugiero *et al.* 2013). A study of the distribution of reptiles in Spain has shown that some species have shifted their historic range north by about 15.2 km over 65 years (Moreno-Rueda *et al.* 2012). Lu et al. (2013) experimentally tested the effects of warming climates on an oviparous species of lizard and found a reduction in the onset of reproduction in females exposed to warmer temperatures, decreased incubation duration and increased embryonic mortality in eggs subjected to warmer incubation temperatures. Overall, reptiles must adapt to the changing climate via phenotypic plasticity or genetic mutations or face detrimental declines in populations or potential extinction.

The changing environment has the potential to induce plastic responses in developing embryos and behavior in adults. These plastic responses have increasingly become a focus of research that could provide valuable insights into how organisms may cope with climate change. Du and Shine (2015) provide evidence that embryos themselves can have a plastic behavioral response to extreme temperatures to ensure survival, which could be beneficial when dealing with the foreshadowed global temperature spikes. Some birds like yellow warblers (*Dendroica petechia*) and the marsh tit (*Poecile palustris*) have become very flexible with their timing of clutch initiation

(Mazerolle *et al.* 2011; Wesołowski *et al.* 2015). Moreover, long term studies on a Dutch great tit population have shown that plastic traits correlated with climate change are being favored by selection, which could lead to new coping mechanisms (Visser *et al.* 1998; Nussey *et al.* 2005). However, a long-term study on British great tits suggests that this population is no longer showing plastic responses (Charmantier *et al.* 2009). The cold-adapted tuatara (*Sphenodon guntheri*), which has temperature-dependent sex determination, is predicted to have nests that produce all male clutches by the year 2080 in response to the warming temperatures if females continue to select similar nest sites (Mitchell *et al.* 2008). To keep sex ratios from becoming biased, female tuatara would have to change their historically preferred nesting areas to those with cooler ground conditions or offset the timing of oviposition (Mitchell *et al.* 2008). This plasticity would have to become widespread through species with TSD. However, a population of western painted turtles (*Chrysemys picta*) is not exhibiting individual plasticity in timing of oviposition that could offset the negative effects of climate change (Schwanz & Janzen 2008).

As the global climate continues to change, the need for conservation efforts and further ecological research are growing (Loarie *et al.* 2009; Urban *et al.* 2014). More empirical research is required to better understand the potential effects caused by global climate change (Merilä & Hendry 2014). New modeling techniques are being utilized to help understand ecological patterns as well as advancements molecular ecology. General Circulation Models are now being used to predict ecological outcomes of climate change and which species may be most vulnerable (Baker *et al.* 2016). Clock genes associated with circadian rhythm have been shown to play a major role in migration phenology of

birds (Saino *et al.* 2015). Further research into genes such as these could give insight into whether they could be affected by the environmental changes occurring.

Study Organism

The genus *Anolis* (Dactyloidae) is one of the most speciose genera of terrestrial animals with nearly 400 species (Losos 2009). Most species inhabit subtropical to tropical climates in the new world and have shown an amazing amount of convergent evolution across species that reside on many Caribbean islands. Across the Caribbean, anoles have evolved to fill the same niches but at different time points. Those species that share the same niche but are on different islands are said to have the same ecomorphology or ecomorph, which means that their body shape is suited for their ecology (Losos 2009). The diversity within the genus has allowed for many studies involving evolution and ecology, and now anoles are becoming important in the biomedical sciences since the genome of green anole (*Anolis carolinensis*) was fully sequenced in 2011 (Alföldi *et al.* 2011).

The brown anole lizard (*Anolis sagrei*) is a small anole of the trunk-ground ecomorph. They exhibit sexual dimorphism where the males are much larger than females with different dorsal patterns depending on the sex. The males also possess a large, brightly-colored throat fan or dewlap, which they use in mate attraction and territorial displays, which are greatly reduced in females. These lizards are native to Cuba and the Bahamas but are invasive throughout the Caribbean, Central America, states along the Gulf of Mexico, Hawaii, and Taiwan. Many studies have been performed using the *A. sagrei* as models for testing various ecological questions including development,

sexual selection, behavior, and plasticity (Tokarz 1995; Sanger *et al.* 2008; Cox *et al.* 2009; Warner 2014; Warner *et al.* 2015). They make excellent models for both laboratory and field studies as they readily reproduce in captivity, are abundant in their invasive range, and are relatively short-lived. Like other anoles, *A. sagrei* lay a single egg clutch over a reproductive period that spans from March through October. This long reproductive period exposes developing embryos to a variety of environmental conditions that could influence the phenotype of the offspring.

Thesis objectives

For my thesis research, I wanted to evaluate the effects that timing of oviposition and incubation temperature can have on developing reptilian embryos. As mentioned previously, temperature plays a major role in shaping the phenotype of developing reptiles. I tested the hypothesis that there is an adaptive match between the time of year an egg is laid and the temperature in which it develops. *Anolis sagrei* made an excellent model for testing these questions. Their long reproductive season allowed me to collect eggs from different time periods with differing temperatures. I incubated eggs produced early and late in the reproductive season under early season and late season temperatures to test the environmental matching hypothesis. I quantified the phenotypes of the hatchlings and then released them onto a small study island to assess their survival in a natural setting.

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Chapter 2
Do seasonal changes in developmental temperatures have season specific consequences in a lizard?

Introduction

Environmental variation that accompanies changes in seasons (e.g., temperature, precipitation, photoperiod) are often highly predictable and provide cues for many organisms to initiate a variety of phenological processes such as reproduction, migration, emergence, and hibernation (Jonzen *et al.* 2006; Doody 2011; Ljungström *et al*. 2015). Accordingly, schedules of key life-history events are often well adapted to seasonal changes in environmental conditions. In addition, variations in environmental conditions have the potential to induce plastic phenotypes in organisms (Mazerolle *et al*. 2011; Clark & Reed 2012; Wesołowski *et al.* 2015), resulting in season-specific phenotypes that are well matched to the environment at different times of the year. Plastic responses to seasonal changes in the environment could occur across different life-history stages and have important fitness consequences. Even phenotypic changes during embryonic development can have long-lasting effects, in turn, affecting the fitness of individuals (Pigliucci 2005).

Like most ectotherms, reptiles are especially sensitive to their thermal environment throughout all life stages as they must thermoregulate to carry out many physiological processes (Angilletta 2009). As environments change across season, reptiles shift their daily habits to prepare for reproduction. Many oviparous species lay their eggs in nests and rarely exhibit parental care, thereby leaving eggs at the will of the environment (Shine 2005). With little ability to move during incubation, the embryos

must endure whatever conditions their environment provides (Georges *et al.* 2005; Du *et al.* 2011; Telemeco *et al.* 2013). Many studies have shown that variations in incubation temperature have significant effects on growth, behavior, morphology, locomotor performance, fecundity, and sex of individuals (Janzen 1994; Shine *et al.* 1997; Elphick 1998; Andrews *et al.* 2000; Blouin-Demers *et al.* 2000; Warner & Andrews 2002; Warner & Shine 2008). These plastic traits that are induced during development could have the potential to prepare the individual for the environment that it will experience at later life stages (West-Eberhard 2003). Therefore, developmental responses to the environment could be crucial for the fitness of offspring.

Unfortunately, the adaptive value of environmentally-induced phenotypes is often poorly understood because measurements of fitness in the wild are rarely obtained and ecologically-relevant conditions that induce phenotypic variation are almost never assessed (Shine & Elphick 2001; Du & Ji 2006; Oufiero & Angilletta 2006; Oufiero & Angilletta, Jr. 2010; Pearson & Warner 2016). Most studies that have tested the effects of developmental temperature have used either constant or arbitrary fluctuating temperatures, which are not what an embryo would experience in the wild as the eggs may experience a broad range of temperatures within a single day, and thermal fluctuations across days are rarely, if ever, the same. Some studies have looked at how the timing of oviposition can affect the growth and survival of individuals many showing hatching early is beneficial (Olsson & Shine 1997; Shine 2004; Warner & Shine 2007). Though, none have looked at the timing of oviposition in conjunction with natural, fluctuating temperatures to assess how hatchling phenotypes vary across the reproductive season and whether any varying phenotypes are adaptive. In addition, although several

studies provide important insight into the fitness consequences of environmentally-induced phenotypic variation (*e.g*., Janzen 1993; Warner & Andrews 2002; Warner & Shine 2005, 2008; etc.), most studies only assess phenotypic proxies of fitness (*e.g*., body size, locomotor performance) rather than directly assessing survival or reproductive success.

To assess the effects of seasonal variation in developmental temperatures, we used the brown anole (*Anolis sagrei*), a relatively short-lived, invasive lizard that lays a single egg every 7 to 10 days throughout a long reproductive season (March-October). This species of lizard is ideal for laboratory and field studies as they have genetic sex determination, are abundant in their native and invasive range, and readily breed in captivity (Sanger *et al*. 2008). Importantly, their extensive reproductive season encompasses a broad range of conditions that shift through spring and summer. Most notably, temperature rises substantially from the early to late periods of the egg-laying season, resulting in large and predictable differences in thermal environments of nest sites at different times of the year. Moreover, the differences in seasonal temperatures significantly influence phenotypic variation; indeed, eggs incubated under warm, July conditions produced larger offspring with faster running speed than those incubated under cool, April conditions (Pearson & Warner 2016). Although these seasonal differences in temperature may have fitness consequences, the adaptive value of this seasonally-driven plasticity is unknown.

My primary aim was to determine if early and late produced embryos are adaptively matched to their respective seasonal thermal environments (i.e., environmental matching hypothesis). My specific hypotheses were (1) that embryos produced early in

the season that are exposed to early-season temperature will have better fitness-related phenotypes than those exposed to late-season temperatures (and vice versa for late-season embryos), and (2) survival of offspring will be relatively high when there is a match between timing of oviposition and the season-specific incubation temperature, and survival will be low when there is a mismatch. Support for these hypotheses will provide evidence that plastic responses of embryos are adaptively matched to seasonal environmental trends.

Methodology

Animal housing

Two temporally separated breeding colonies of wild *Anolis sagrei* consisting of 80 females and 40 males were collected from Palm Coast, Florida. The first colony was established in early February 2015 and the second colony collected in early July 2015. This enabled collection of eggs produced at different times of the reproductive season. Using two temporally-separated breeding colonies (rather than one colony) also eliminated confounding maternal effects associated with housing mothers in the laboratory for unequal periods for early versus late eggs.

All animals were housed at the University of Alabama at Birmingham. Adult females were housed individually in a single cage where males were rotated between the females weekly to encourage frequent mating. Cages were composed of plastic sweater boxes (29 cm tall x 26 cm wide x 39 cm deep) with a screen top that securely locks. Two bamboo perches were provided in every cage, and the floor of each cage was covered with reptile ‘cage carpet,’ a product specifically designed for reptile cages to prevent

mold and for easy cleaning. A plastic plant pot (4-inch diameter, 4-inch-tall) filled with moist potting soil and an artificial plant was placed in each cage to provide an area for the female lizards to oviposit as well as additional perching and shelter beneath the plant. Artificial plants, rather than live plants, were used as frequent uprooting of live plants while searching for eggs could kill them. All cages were illuminated with Reptisun 5.0 UVB bulbs (ZooMed Inc.) and plant-grow bulbs (model F40, General Electric Co.), which is necessary for vitamin D3 synthesis in reptiles. Room and cage lights were set on timers so that the photoperiod gradually goes from light to dark (and vice versa) each day (14:10 hour light:dark cycle; similar to the summer photoperiod at the collection site). The photoperiod was adjusted throughout the experiment to recreate the seasonal changes in photoperiod. The animal rooms were kept at approximately 28 degrees Celsius. Each cage was misted with de-chlorinated water daily. Lizards were fed crickets (dusted with vitamins and calcium) three times per week (3 crickets per lizard at each feeding) through the entirety of the experiment.

Egg incubation and experimental design

Each flowerpot was searched for eggs three times a week (Monday, Wednesday, and Friday) for each cage, which began April 1 for the first cohort and July 15 for the second. Once collected, eggs were weighed and randomly assigned to a treatment so that both early and late-produced eggs were incubated under early and late-season temperatures. Eggs were incubated individually in glass jars (59 ml) filled with organic substrate collected from the field sites (-150kPa) and covered with plastic wrap with a rubber band to seal and prevent moisture loss.

Thermal data from 24 potential natural nests sites were used to establish two 45-day incubation treatments that mimic daily fluctuating thermal regimes that occur from April through mid-May and mid-July through August (Figure 1). This combination of early season and late season collection of eggs and varied incubation temperatures (early vs. late) created a 2 x 2 experimental design.

Two incubators equipped to perform fluctuating temperatures were set to the treatments, which carried out two 45-day cycles beginning April 1 and July 15 (Figure 1). Due to temporal variation in egg production within and among females, not all eggs within treatments experienced the exact same temperature regimes. However, the early and late temperature regimes were substantially different from each other; therefore, thermal variation between the two treatments was greater between treatments than within treatments. These temperature regimes maintained ecologically relevant conditions and captured the potential thermal effects experienced in the field. Because eggs were placed in these treatments at different times during the 45-day period, eggs that had not hatched by the end of the 45-day cycle were cycled back through the regime until hatched.

Hatchling Phenotypes and Survival

Hatchling morphometrics were measured (snout-vent length, tail length, and mass) and sexed (males have two enlarged post-anal scales present) immediately after hatching. Each lizard was uniquely marked by toe clip and housed individually in captivity for a brief period. Hatchling cages were smaller than the adult cages (23 x 19 x 17 cm) and consisted of one perch (wooden dowel), an artificial plant, and cage carpet

covering the floor. Ambient conditions and feeding/watering regimes were the same as those described above for adults.

Sprint speed of individuals was assessed as a measure of in-lab fitness between 7 and 9 days after hatching. Race trials were performed by chasing hatchlings along a meter-long, racetrack with an artists' paintbrush. The racetrack was placed at a 20-degree angle, and lizards fell into a container after being chased to the end of the track. Each individual was raced 5 times with a 2-minute resting period between running trials. Photocells, connected to a stopwatch, were placed at 25-cm intervals along the track. The number of stops was recorded across the full meter for each racing trial.

Hatchlings were eligible for field release after they had been raced, and release dates varied between 1 and 3 weeks post hatching. Prior to release, the morphometrics of each animal were taken to quantify any growth that occurred in the laboratory. They were transported from UAB and released on a single small island approximately 50 meters in diameter (Figure 2) in the Matanzas River near Crescent Beach, Florida (19.63 km from the collection site of their parents). The habitats on this island consisted primarily of two areas: an open area with sparse mangrove trees and other small plants and a central, shaded forested area comprised mostly of cedar trees with little underbrush. The island served as a natural enclosure limiting dispersal, and thereby facilitating survival estimates. Recapture efforts allowed for the quantification of growth, movement, habitat use, and survival in a natural setting. All trees on the island were tagged with a unique number, which were recorded to follow the location of recaptured lizards.

The first recapture effort was performed on 10 October, 2015 to assess survival and growth prior to winter. At this point, not all hatchlings had been released, as many of

the late season produced eggs incubated under the early temperature treatment had yet to hatch. A team of five people collected for 8.33 hours. Non-experimental resident *A. sagrei* were also collected, measured, marked, and released back onto the island where they could act as a naturally occurring control group. All lizards captured in October were measured for SVL, tail length, and mass. A second recapture effort was performed to assess the overwinter survival of both the experimental animals as well as the naturally occurring animals marked in October. Only individuals with a mark were measured for size. A team of five people collected animals March 14, 2016- March 17, 2016, a total of 32.82 hours. At the end of the March recapture trip, all captured animals were transported to Auburn University and preserved as museum specimens.

Statistical Analysis

All analyses were performed using SAS software (SAS Institute, Inc., Vers. 9.4, 2016).

Two-way mixed model ANOVAs were used to assess the effects of the timing of oviposition (early vs late), incubation temperature (early vs late seasonal regimes), their interaction, and sex on fitness-related phenotypes of offspring (morphology, sprint speed, growth). Generalized linear mixed models were used to assess the effects of the timing of oviposition, incubation temperature and offspring phenotypes (and interactions) on survival in the lab. Tail length, sprint speed, and average number of stops were log-transformed to normalize the data. Mothers were included in all models as a random effect. Egg mass was used as a covariate when testing the effects on SVL and hatchling mass. SVL was used a covariate when analyzing tail length and hatchling mass (body

condition). Mass at running time and log-transformed average number of stops were used as covariates for analyzing sprint speed. Body condition was determined by residual scores of the analyses between SVL and hatchling mass.

Survival analyses in the field were assessed by using generalized linear mixed models to assess the timing of oviposition, temperature, and their interaction on the presence in October and survival to March. Individuals that were recaptured in March but not in October were marked as present in the analyses. The growth of recaptured individuals was assessed by mixed model ANOVAs. Growth rates were calculated by dividing the difference in mass at hatching and mass at recapture by the difference between hatch date and recapture date. These values were calculated for both "early growth" (October recapture) and "late growth" (March recapture). Because no late produced hatchlings from the early temperature incubation treatment were released at the time of the October recapture efforts, only the effect of temperature was used to look at early growth.

Comparisons of survival between the lab-raised and field-caught hatchlings were performed with Chi-square tests. Field-caught lizards were labeled "hatchlings" if their SVL was less than 22mm. From this, October presence and overwintering survival was determined. Only hatchling field-caught lizards (not adults) were used in these analyses to ensure meaningful comparisons with hatchling lab-raised lizards.

Results

Incubation

A total of 1031 viable eggs were produced by the two cohorts of females (493 from the early season and 538 from the late season). Egg survival was affected by seasonal cohort, incubation temperature, and their interaction (Table 1, Figure 3) with total egg survival at 77.78 %. Eggs produced by females in the late cohort had overall higher survival than early produced eggs, and eggs exposed to late season incubation temperatures had higher survival than early season temperatures. Eggs produced by late cohort females that were incubated under the corresponding late season temperatures had the highest survival (89.26%). Incubation period was significantly affected by seasonal cohort, incubation temperature, and their interaction (Table 1, Figure 4). Late produced eggs had shorter incubation periods than early eggs, while late season incubation temperatures had substantially shorter incubation periods than those in the early temperature treatment (Figure 4).

Hatchling phenotypes

Offspring snout-vent length was affected only by timing of oviposition, where late season hatchlings were larger than early-hatched lizards (Table 1, Figure 5). Sex also had a significant effect on offspring SVL (Table 2, Figure 6); however, when looking at the actual measurements, there was only a minimal (0.23 mm) difference between males and females, which questions its biological relevance. Tail length was affected by seasonal cohort, incubation temperature, and their interaction (Table 1, Figure 7). Late produced hatchlings had longer tails than early, and hatchlings from late season incubation

temperatures had longer tails than those from the cooler early season temperature treatments. Late produced hatchlings that experienced late season temperatures had the longest tails, and early produced hatchlings that experienced early season temperatures had the shortest tails. Body mass of the hatchlings was affected by timing and incubation temperature when egg mass was used as a covariate (Table 1, Figure 8). Late produced hatchlings had a higher mass than early, and late incubation temperatures produced heavier hatchings than early temperatures. Body condition was affected by both seasonal cohort and incubation temperature where early produced hatchlings had lower condition than late, and hatchlings from the early produced, early temperature treatment had the lowest body condition. Late produced hatchlings had faster running speed than early produced hatchlings (Table 1, Figure 9), and late incubation temperature hatchlings were faster than those incubated at the early-season temperatures. The number of stops over 1m was affected by timing, incubation temp, and their interaction (Table 1, Figure 10). Late-season hatchlings made more stops than early-season hatchlings, but hatchlings incubated at late-season temperatures had fewer stops than those from early-season temperatures. In-laboratory survival was only affected by incubation treatment where offspring that experienced late-season incubation temperatures had relatively high survival (Table 1, Figure 12).

Field release

A total of 595 hatchlings were released onto the island. Only 19 experimental individuals were captured during the October recapture effort and 25 individuals in March. Those individuals captured in March but not in October were marked as present in October giving a total of 37 individuals present prior to March recapture efforts. Thus, a 7.86% recapture rate in October and 4.04% in March ($\chi 2=14.47$, DF=1, $p<0.0001$). The recapture rate of individuals from the early-season cohort was greater in October than those from the late-season cohort (Table 1, Figure 13). Overwinter survival was also affected by the seasonal cohort, where again early hatched individuals had high survival (Table 1, Figure 14). Early growth in the field was affected by incubation temperature and the interaction between timing of oviposition and incubation temperature (Table 1). Effects of season was probably not observed since no late produced offspring incubated under early season temperatures were released at the time of collection. We observed no differential changes in growth due to treatments in those individuals recaptured in March (Table 1).

Laboratory hatched individuals vs. field caught individuals

During the October recapture efforts, 285 wild *A. sagrei* were caught, measured, and marked. We determined that 49 individuals qualified as 'hatchlings' (SVL<22mm). Six of these were recaptured in March resulting in a 12.24% overwinter survival. The survival rate of field-caught hatchlings was significantly higher than that of lab-raised hatchlings (4.04%; $\chi 2=19.78$, DF=1, $p<0.0001$).

Discussion

The impacts of timing of oviposition and developmental temperatures on offspring phenotypes have been well studied, but the implications are poorly understood due to experimental designs that do not mimic ecologically relevant conditions (Bowden *et al*. 2014; Pearson & Warner 2016). Our novel approach allowed us to quantify the effects of the timing of oviposition and natural incubation temperatures on the phenotypes and fitness of a lizard with a long reproductive season.

I originally predicted that the timing of oviposition would be adaptively matched with the corresponding seasonal incubation temperature. Although support for this hypothesis was generally low, the patterns associated with egg survival were partially consistent with this prediction. For example, eggs that were produced late in the reproductive season and incubated under late season temperatures had higher survival than late produced eggs incubated under early temperatures. However, contrary to my predictions, the eggs that were laid early and incubated under early temperatures had lower survival than those incubated under late temperatures, resulting in relatively low survival under matched conditions. A multitude of studies have shown that incubation temperature can differentially affect incubation duration where warmer temperatures accelerate embryonic development (Huey 1976; Andrews 2004; Georges *et al.* 2005). We found similar results where eggs incubated under late-season temperatures had substantially shorter incubation lengths that those from the early-season temperature treatment. Interestingly, we also found that the timing of oviposition significantly affected incubation duration regardless of season-specific temperatures. Eggs that were laid late in the season developed faster than those laid early. The underlying mechanisms

of accelerated development for late-season embryos is unknown, but could reflect the effects of seasonal changes in maternal effects. Indeed, egg size and yolk testosterone have previously been show to increase over the reproductive season (Warner & Lovern 2014), and such effects may have consequences on development.

Hatchling morphology, locomotor performance, and behavior were also influenced by the timing of oviposition and incubation temperature. Eggs that were laid late in the season produced lizards that were larger overall and faster. Hatchlings that experienced late season incubation temperatures were heavier, had longer tails, faster, made fewer stops, and had higher in-lab survival than those from the early incubation treatment. These attributes follow a similar pattern to previous reports (Pearson & Warner 2016) where *A. sagrei* hatchlings incubated under warm, late season temperature conditions were relatively large and fast runners. These differences in phenotypes could compensate for any negative effects of late hatching given that those laid late will be entering an environment where they will be competing with individuals that hatched early in the season and have had time to grow.

Our recapture study showed that the potential beneficial traits of hatching late in the season do not outweigh the benefits of hatching early. Regardless of the temperature eggs were incubated under, eggs that were laid early produced hatchlings that had higher early-life post-hatching survival and over winter survival than those that were laid late. These results reinforce the importance of the timing of hatching (Olsson & Shine 1997; Warner & Shine 2007; Le Henanff *et al*. 2013), and particularly demonstrate that phenotypic effects of the developmental environment are relatively minor to that of the timing of hatching.

We found that field hatched lizards had a higher survival rate than lab-incubated hatchlings that were released in the field. This could be because selection had already operated on the phenotypic variation in the wild population prior to the capture efforts. Other studies show similar patterns, where field caught *Sceloporus undulatus* had higher fitness than lab raised animals (Warner & Andrews 2002). Since these lizards were hatched into their natural environment, they may have had an advantage by being more familiar with the environment than lab-raised individuals. Regardless of the difference in survival between laboratory and field caught animals, however, there was still a significant effect of timing of oviposition and temperature on survival of the experimental animals.

This study showed that the timing of oviposition and seasonal variation in incubation temperatures play an important role in shaping the phenotypes and survival of offspring in different ways. My experimental design utilized a unique approach that mimicked the thermal environments of natural incubation conditions, and enabled me to decouple the confounded effects of season and season-specific temperatures. Although general support for embryo adaptation to season-specific environments was weak, the incubation-induced phenotypic variation may have important fitness consequences. However, these potential consequences were relatively minor compared to the effects of the seasonal timing of oviposition. Although phenotypes of late-produced offspring are typically associated with relatively high fitness, the benefits of early hatching on survival exceed that of late-season phenotypes. Overall, these results provide new insights into the relative importance of incubation-induced phenotypes under natural environments.

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Table 1. Effect of treatments on phenotype. Traits denoted by '*' represent Egg Mass as a covariate and by '**' represent SVL as a covariate. Bold face values are statistically significant.

Trait	Season effect	Temperature effect	Interaction
Egg survival	F=10.31; **p=0.0014**	F=12.78; **p=0.0004**	F=7.52; **p=0.0062**
Incubation period	F=37.63; **p<0.0001**	F=25547.5; **p<0.0001**	F=24.10; **p<0.0001**
Snout-vent length*	F=82.35; **p<0.0001**	F=0.53; p=0.4676	F=0.58; p=0.4481
Hatchling Mass*	F=53.81; **p<0.0001**	F=10.40; **p=0.0013**	F=0.49; p=0.4846
Hatchling Mass**	F=21.37; **p<0.0001**	F=6.69; **p=0.0099**	F=3.08; p=0.0798
Log Tail length**	F=20.50; **p<0.0001**	F=113.63; **p<0.0001**	F=7.92; **p=0.0051**
LogSprint speed 25cm	F=4.21; **p=0.0407**	F=12.52**; p=0.0004**	F=0.34; p=0.5580
LogAverage stops 1m	F=81.93; **p<0.0001**	F=34.14; **p<0.0001**	F=5.34; **p=0.0212**
Survival to release	F=0.47; p=0.4954	F=37.10; **p<0.0001**	F=0.02; p=0.8768
October growth	F=2.51; p=0.1371	F=85.20; **p<0.0001**	F=75.95; **p<0.0001**
Overwinter growth	F=0.16; p=0.6936	F=0.04; p=0.8448	F=0.43; p=0.5184
October Presence	F=11.92; **p=0.0006**	F=0.05; p=0.8240	-
March Presence	F=9.83; **p=0.0018**	F=0.29; p=0.5875	F=0.19; 0.6621

Table 2. Effect of sex on phenotype. Traits denoted by '*'represent Egg Mass as a covariate and by '**' represent SVL as a covariate. Bold face values are statistically significant.

Trait	Sex effect
Incubation period	F=0.52; p=0.4693
Snout-vent length	F=14.10; **p=0.0002**
Hatchling Mass*	F=1.26; p=0.2611
Hatchling Mass**	F=1.30; p=0.2540
Log Tail length**	F=2.93; p=0.0876
LogSprint speed 25cm	F=2.58; p=0.1019
LogAverage stops 1m	F=0.55; p=0.4580
Survival to release	F=1.22; p=0.2690

Figure Captions

Figure 1. Forty-five-day fluctuating incubation regimes representing average 2.5-hour temperatures from 24 nests. Early temperatures (blue line) represent April 1, 2014-May 15, 2014 and late temperatures (red line) represent July 15, 2014-August 28, 2014.

Figure 2. Small study island in the Matanzas River near Crescent Beach, Florida where lizards were released with approximately 50 meters of forested area (denoted by red arrows).

Figure 3. Effects of seasonal cohort and incubation temperature treatment on egg survival.

Figure 4. Effects of seasonal cohort and incubation temperature treatment on incubation duration. Error bars represent standard error values.

Figure 5. Effects of seasonal cohort and incubation temperature treatment on hatchling snout-vent length. Error bars represent standard error values.

Figure 6. Effect of sex on snout-vent length of hatchlings. Error bars represent standard error values.

Figure 7. Effects of seasonal cohort and incubation temperature treatment on hatchling tail length. Error bars represent standard error values.

Figure 8. Effects of seasonal cohort and incubation temperature treatment on hatchling mass. Error bars represent standard error values.

Figure 9. Effects of seasonal cohort and incubation temperature treatment on hatchling body condition. Error bars represent standard error values.

Figure 10. Effects of seasonal cohort and incubation temperature treatment on hatchling sprint speed over 25cm. Error bars represent standard error values.

Figure 11. Effects of seasonal cohort and incubation temperature treatment on average number of stops made by hatchlings over 1 meter. Error bars represent standard error values.

Figure 12. Effects of seasonal cohort and incubation temperature treatment on hatchling in-laboratory survival.

Figure 13. Effects of seasonal cohort and incubation temperature treatment on hatchling presence in October 2015. No late produced, early temperature incubated hatchling were released at this time. Error bars represent standard error values.

Figure 14. Effects of seasonal cohort and incubation temperature treatment on hatchling over winter survival.

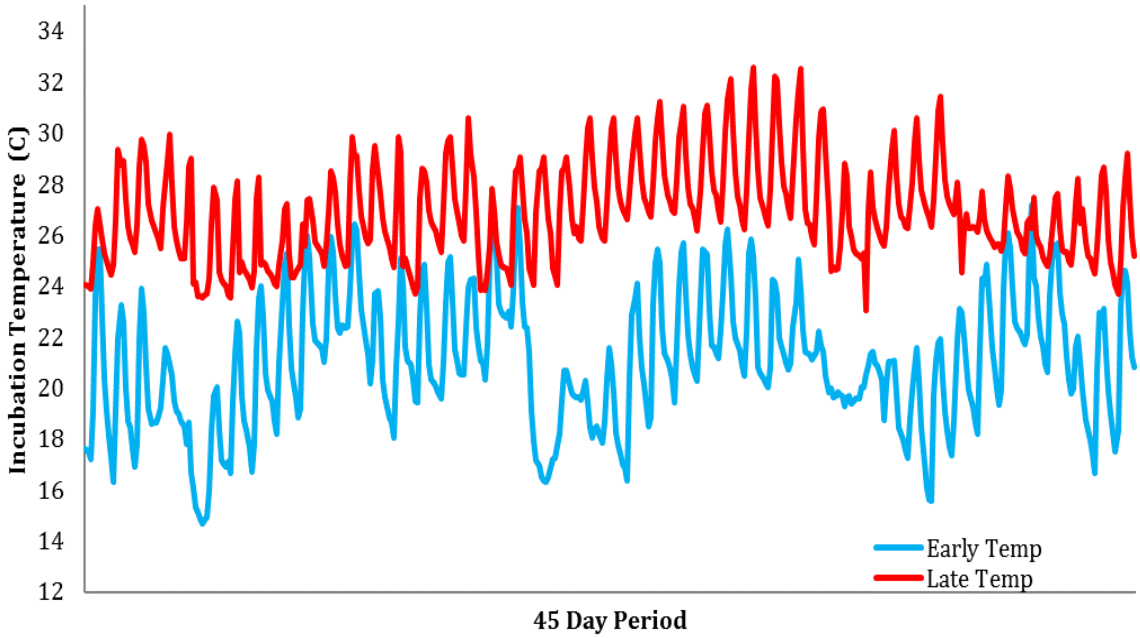
34
32
30
28
26
24
22
20
18
16
14
12
Incubation Temperature (C)
Early Temp
Late Temp
45 Day Period

Figure 1

Figure 2.

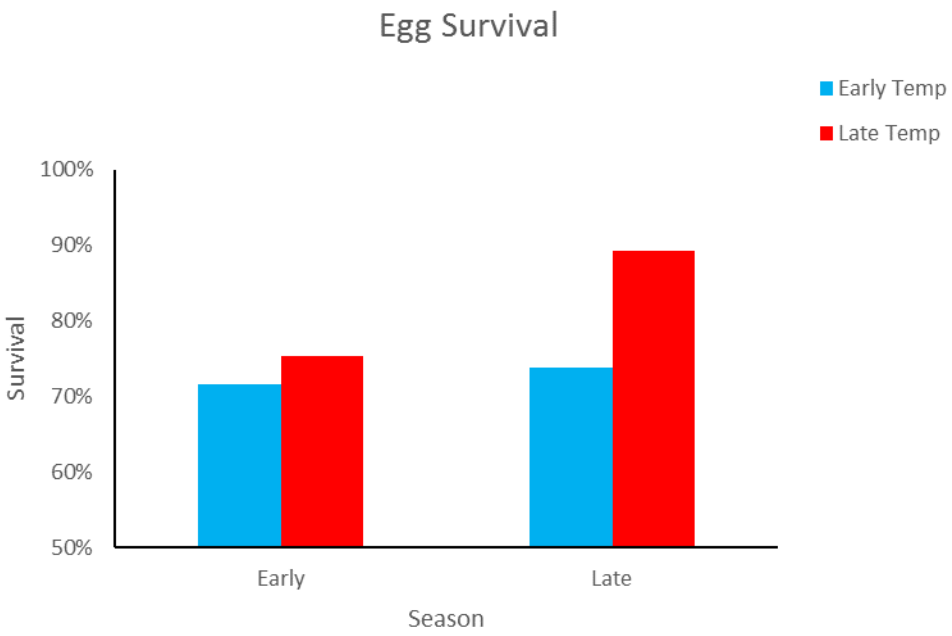
Egg Survival
Early Temp
Late Temp
100%
90%
80%
70%
60%
50%
Survival
Early
Late
Season

Figure 3

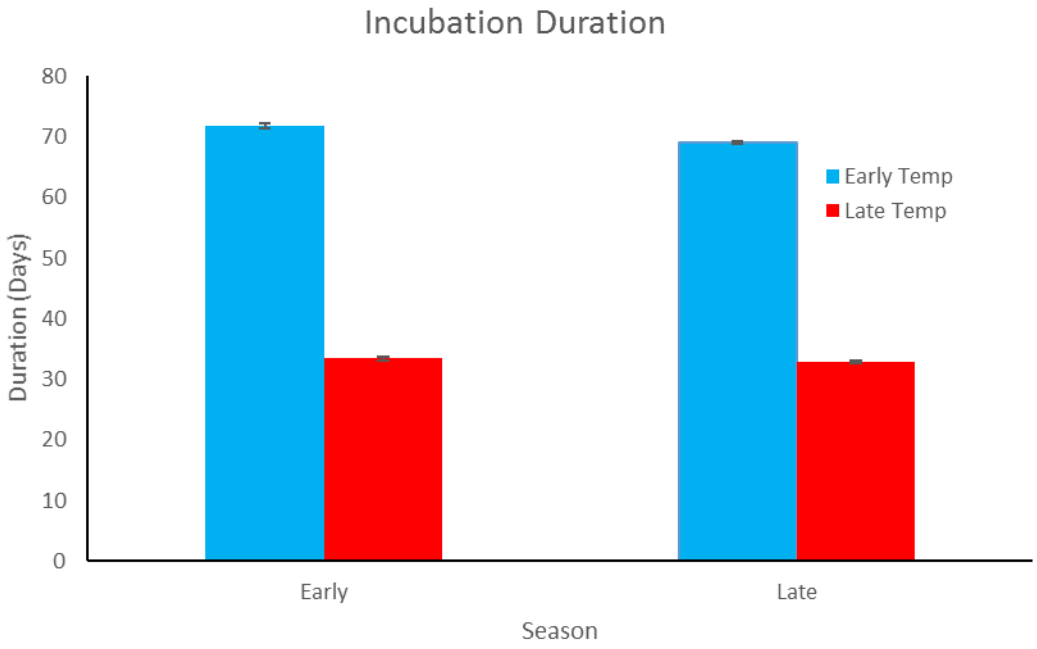
Incubation Duration
80
70
60
50
40
30
20
10
0
Duration (Days)
Early Temp
Late Temp
Early
Late
Season

Figure 4

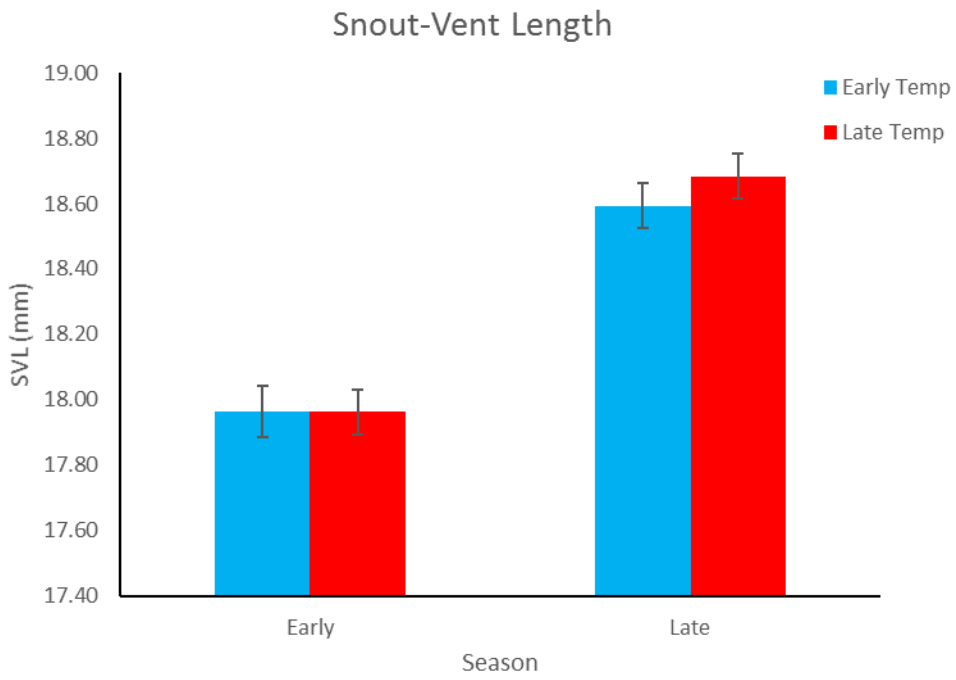
Snout-Vent Length
19.00
18.80
18.60
18.40
18.20
18.00
17.80
17.60
17.40
SVL (mm)
Early Temp
Late Temp
Early
Late
Season

Figure 5


Sex Effect on SVL
Snout-vent length (mm)
18.60
18.50
18.40
18.30
18.20
18.10
18.00
Male
Female
Sex

Figure 6

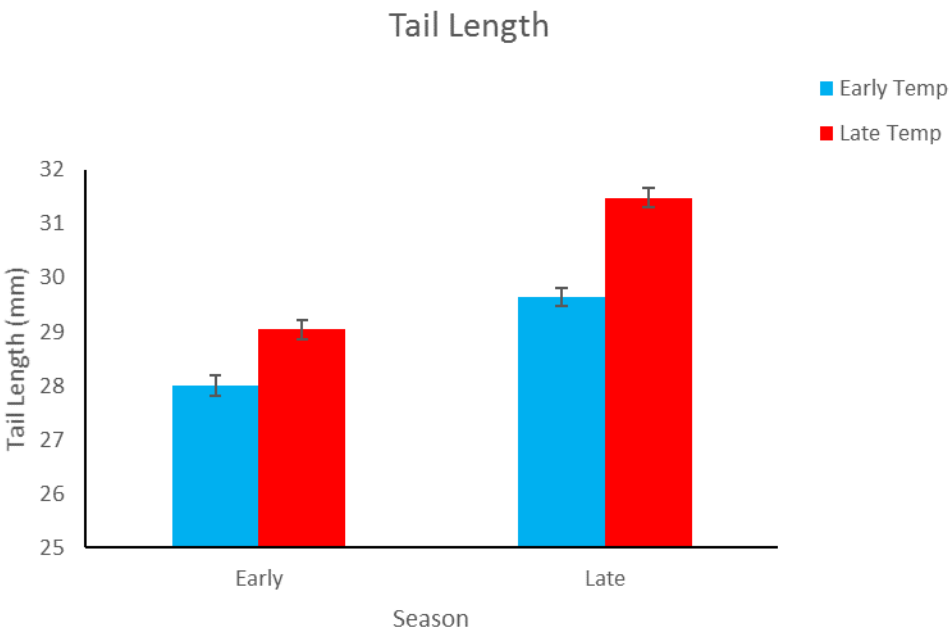
Tail Length
Early Temp
Late Temp
Tail Length (mm)
32
31
30
29
28
27
26
25
Early
Late
Season

Figure 7

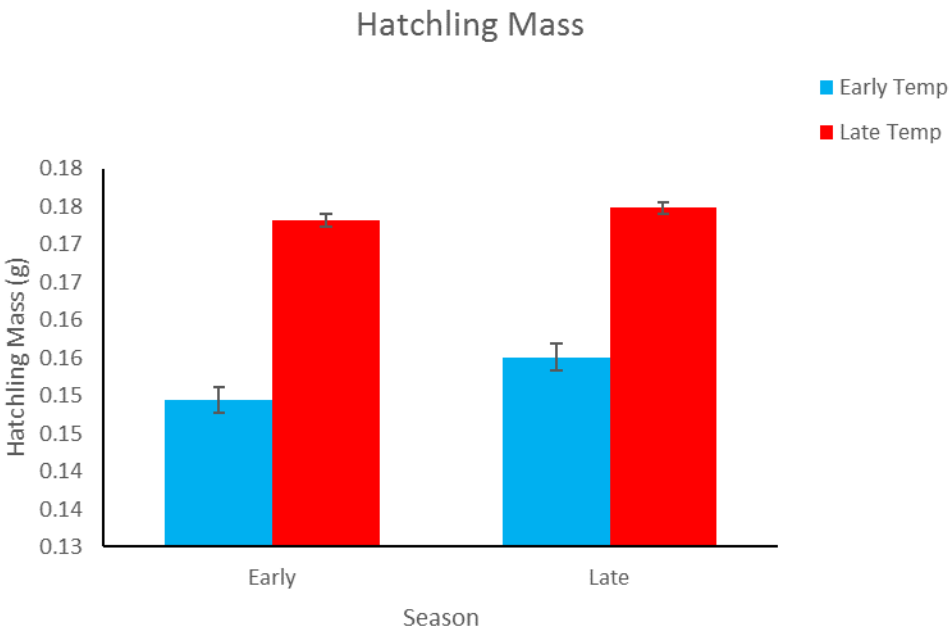
Hatchling Mass
Early Temp
Late Temp
Hatchling Mass (g)
0.18
0.18
0.17
0.17
0.16
0.16
0.15
0.15
0.14
0.14
0.13
Early
Late
Season

Figure 8

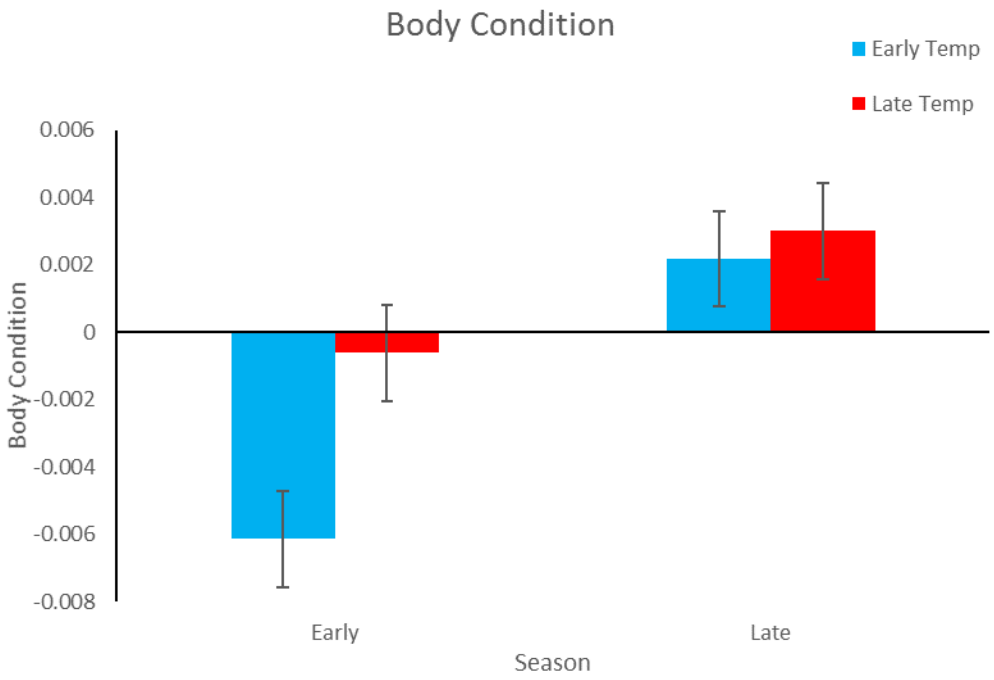
Body Condition
Early Temp
Late Temp
0.006
0.004
0.002
0
-0.002
-0.004
-0.006
-0.008
Body Condition
Early
Late
Season

Figure 9

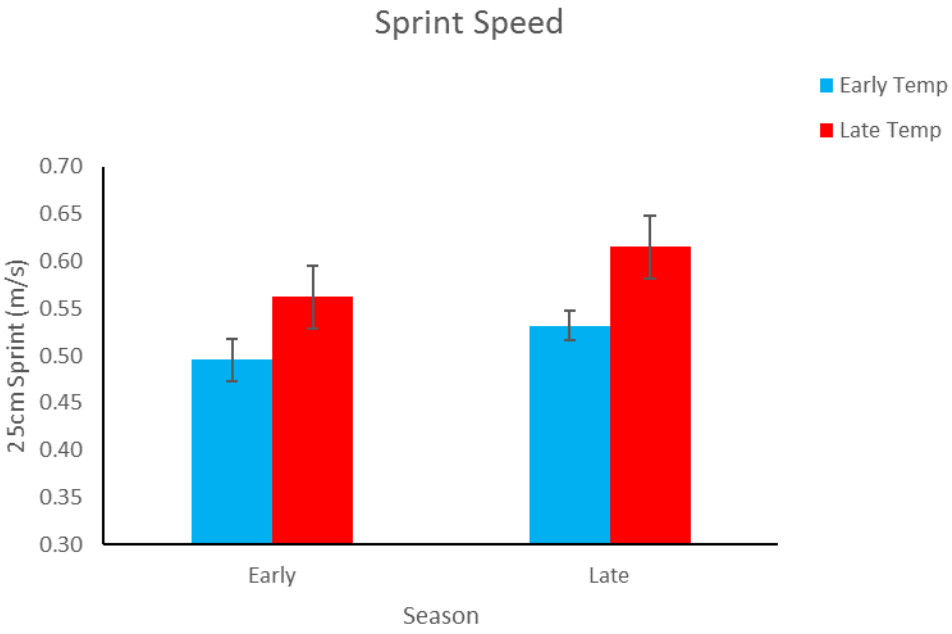
Sprint Speed
Early Temp
Late Temp
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
25cm Sprint (m/s)
Early
Late
Season

Figure 10

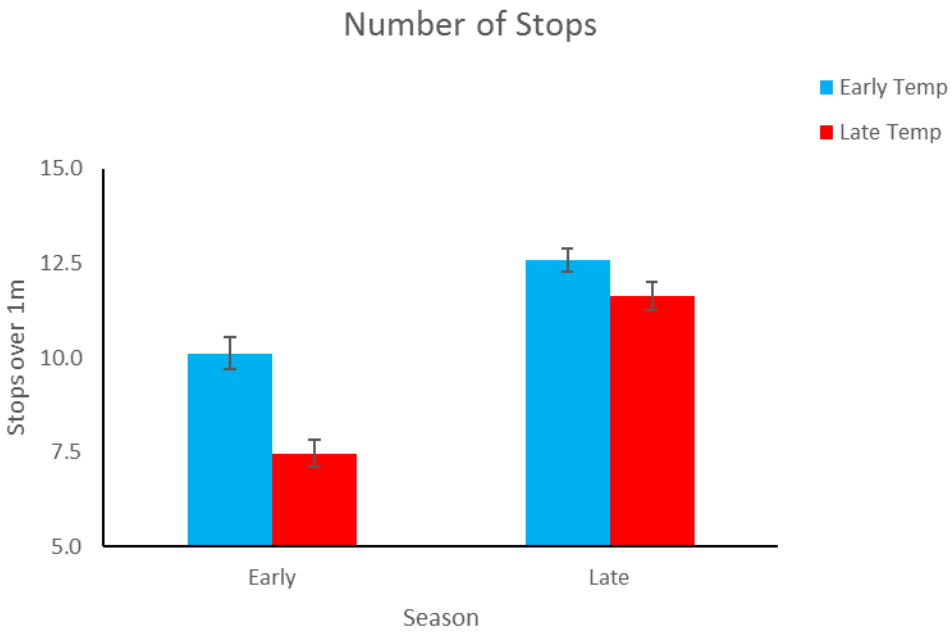
Number of Stops
Early Temp
Late Temp
15.0
12.5
10.0
7.5
5.0
Stops over 1m
Early
Late
Season

Figure 11

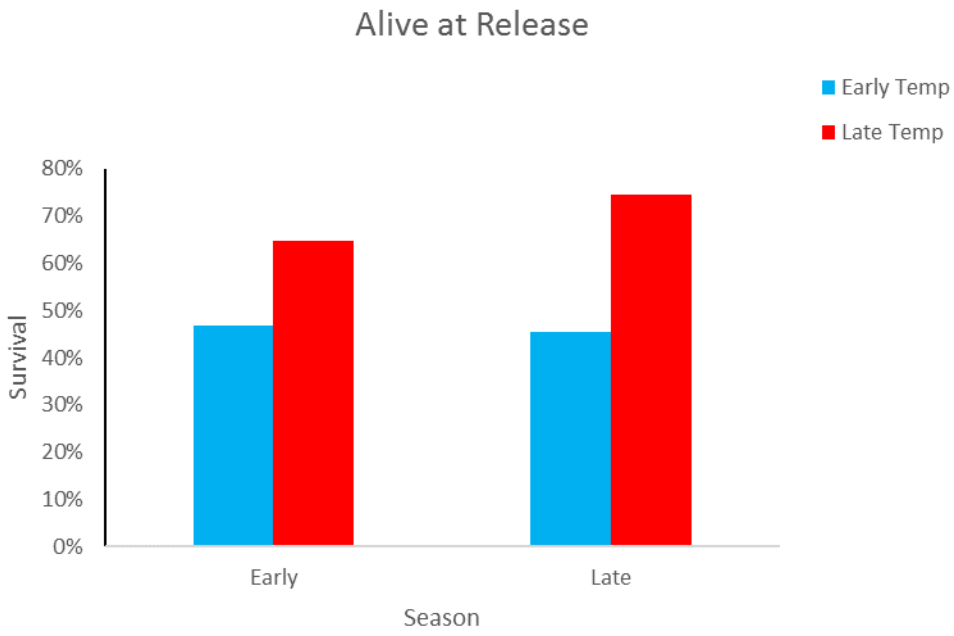
Alive at Release
Early Temp
Late Temp
80%
70%
60%
50%
40%
30%
20%
10%
0%
Survival
Early
Late
Season

Figure 12

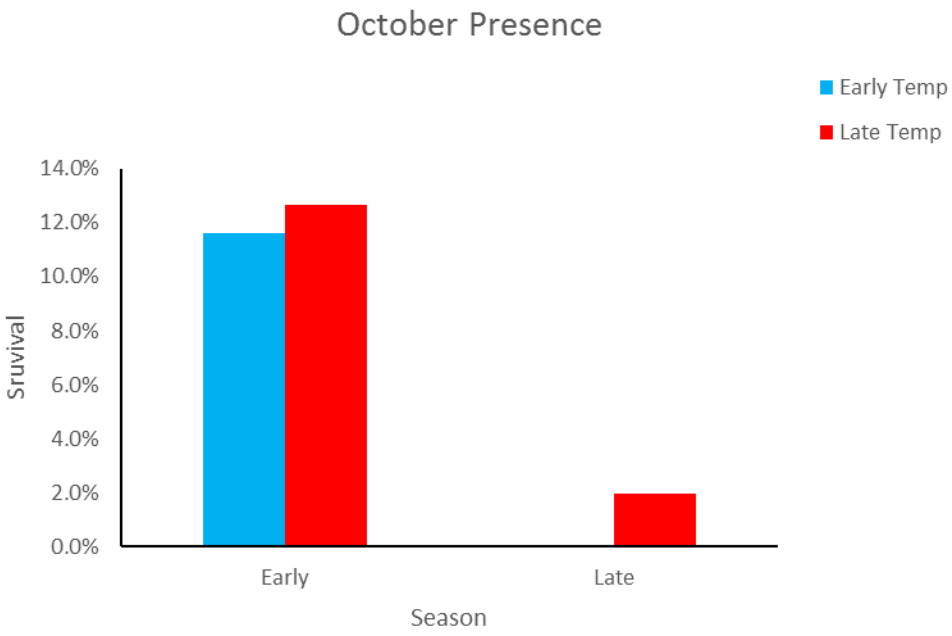
October Presence
Early Temp
Late Temp
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
Sruvival
Early
Late
Season

Figure 13

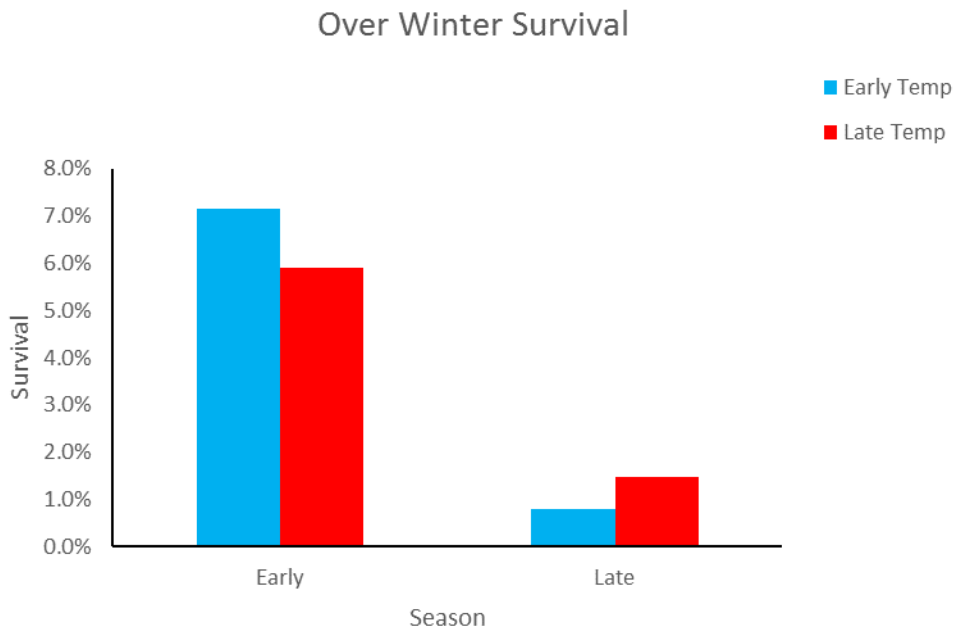
Over Winter Survival
Early Temp
Late Temp
8.0%
7.0%
6.0%
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
Survival
Early
Late
Season

Figure 14