THERMAL STRESS DURING PRE-INCUBATION INDUCES SUBSEQUENT DEVELOPMENTAL PLASTICITY IN NORTHERN BOBWHITES

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Northern bobwhite populations have declined concurrent with global warming. The focal period of this study was the 12-d pre-incubation period, when bobwhite eggs remain in the nest without the thermal protection of the incubating parent. This study first established the storage and thermal limits of bobwhite eggs, then investigated how global warming may impact oviparous embryos and how bobwhite embryos react to acute and chronic doses of simulated drought temperatures during pre-incubation.

First, the maximum storage limit of bobwhite eggs was determined by storing eggs ≤21 d and measuring hatching success and pH of egg albumen and yolk. Hatching success of stored eggs declined after 14 d, when yolk and albumen pH reached levels detrimental to embryonic development.

Secondly, thermal limits were determined by exposing bobwhite eggs to hyperthermic temperatures (38–52 °C). Bobwhite embryos survived 50 °C for 1 h, 49 °C for 3 h and 46 °C for 6 h. Results indicate an adaptation to the naturally occurring temperature extremes that can occur in the bobwhite’s southern range during pre-incubation.

Subsequently, bobwhite eggs were exposed to either low constant (LC), low fluctuating (LF), high constant (HC), or high fluctuating (HF) temperatures
during pre-incubation to determine if the nature of temperatures differentially affected development. Although eggs exposed to high heat loads (HC and HF), and low heat loads (LF and LC) had equal heating degree-hours within groups, they exhibited differential growth during pre-incubation. Oxygen consumption, hatch timing, and hatching success were also affected by the thermal regimes. Eggs in simulated drought (HF) had a 47% lower hatch rate than eggs in simulated non-drought (LF) indicating that thermal stress during pre-incubation may contribute to population declines during drought.

Finally, northern bobwhite eggs were exposed to acute or chronic doses of simulated drought temperatures, which tested for critical periods of development during pre-incubation. Collectively, data indicated that the earliest stages of bobwhite development were more affected by hyperthermic temperatures. Indeed, a critical period of development exists during the first 2/3 of pre-incubation during which exposure to hyperthermic temperatures results in aberrant development, hatching plasticity, and reduced hatch rates.
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CHAPTER 1

GENERAL INTRODUCTION

Climate Change

Climate change is one of the many factors that threaten our environment and contribute to biodiversity loss (Root and Schneider, 2002). Climate exhibits few boundaries yet imposes limits on the activity, interaction, and livelihood of species. One of the major challenges of the 21st century is to understand the biological response to limits (if any) imposed by climate change (Hughes, 2000; Parmesan and Yohe, 2003; Root et al., 2003). The global average temperature has increased 0.6 °C ± 0.2 °C since the late 19th century (Root and Schneider, 2002). This value is 0.15 °C higher than previously predicted by the Intergovernmental Panel on Climate Change (IPCC) due mostly to a higher rate of increase since 1995 compared to previous years (IPCC, 2001). The global average temperature is projected to increase by 5 °C ± 1 °C in the United States by the period 2071–2100 relative to the period 1961–1990 (Root and Schneider, 2002; IPCC, 2007). Concurrent with higher temperatures, scientists predict changes in the magnitude of diurnal fluctuations will occur (Easterling et al., 1997) with an increased frequency of droughts for the southern portion of the United States (IPCC, 2007). In the southern United States, a number of species are uniquely adapted to their environment in ways that allow them to
seasonally acclimatize to conditions of drought and non-drought. Nevertheless, the unprecedented rate of temperature increase with a concomitant increase in drought frequency (experienced and predicted) is of major concern to the survival of wild species and their ecosystems (EPA, 1997; Root et al., 2003; IPCC, 2007). Several scientific reviews of biological responses to climate change (Hughes, 2000; Walther et al., 2002; Parmesan and Yohe, 2003; Root et al., 2003) predict that changes in temperature and drought frequency might directly affect metabolic and developmental rates in many animals, including both endotherms and ectotherms (Oppenheim and Levin, 1974; Decuypere and Michels, 1992; Hughes, 2000). In addition to the above physiological effects, climate change could alter competition and interaction among or within species, leading to population declines and possible extinctions as a direct result of physiological stress or altered species interactions (Hughes, 2000; Parmesan and Yohe, 2003; IPCC, 2007; Calosi et al., 2008).

A comprehensive understanding of the implications of environmental change on vertebrates will benefit from atypical combinations of disciplines in unique interdisciplinary efforts. One of these combinations—the focus of this dissertation—combines developmental physiology with wildlife biology in an attempt to answer important questions regarding the relationship between high temperatures (projected for climate change) and the vertebrate ontogeny. The model animal used for this study was the northern bobwhite (*Colinus virginianus*),
a precocial, commercially important game bird experiencing population declines coincident with global warming (Brennan, 1991).

**Temperature Drives Development**

Of the constituents of climate, temperature is one of the most important physicochemical forces acting on living organisms, especially on ectotherms which operate within a narrow range of environmental conditions to develop, persist, and reproduce (Schmidt-Nielsen, 1997). Most ectotherms have the ability to behaviorally control their body temperature by posturing or relocating to a preferred thermal environment. However, stenothermal ectotherms such as avian embryos (*in ovo*) cannot regulate temperature and are especially susceptible to developmental failure or death when even briefly exposed to temperatures outside a range of 35–40.5 °C (Dawson, 1984). Below physiological zero (approximately 25 °C for most avian species), development stops (Landauer, 1967; Miller and Wilson, 1975; Wilson, 1991b; Ono et al., 1994; Gill, 1999; Pendlebury et al., 2004). Temperatures above physiological zero yet below 35 °C can disrupt development and increase the occurrence of teratogens (Romanoff, 1960; Gill, 1999). Alsop (1919) and Romanoff et al. (1938) found that an internal egg temperature >41 °C resulted in developmental abnormalities and reduced hatchability in domestic chickens (*Gallus gallus*). Additionally, Gill’s (1999) review of avian thermo-tolerance found that most avian embryos begin having developmental abnormalities at 40°C and have an upper lethal temperature of 43°C.
Fluctuating Pre-Incubation Temperatures

The temperature of avian eggs during incubation is maintained relatively constant and close to optimal temperature (i.e., the range of temperatures producing the highest survivorship of embryos) by the incubating parent through behavioral and physiological manipulations (Webb, 1987). As a result, most studies dealing with avian nest or egg temperatures use a constant temperature (e.g., averaged daily-temperatures) for thermal treatments (see Webb, 1987 for a review). However, constant temperature treatments do not fully represent the thermal exposure that most eggs of precocial species encounter in the wild (i.e., diel thermal fluctuations). Many precocial birds which lay multiple eggs in a clutch (at a rate of approximately 1 egg per day) start their incubation with the penultimate or ultimate egg (Tazawa and Whittow, 2000). Thus, there is an extended pre-incubation period—the focal period of this study—where eggs that were laid first are unattended and remain in the nest without the thermal protection provided by an incubating parent.

During the pre-incubation period, eggs are often subjected to fluctuating, potentially harmful, high heat loads (Decuyper and Michels, 1992; Gill, 1999; Guthery et al., 2004) that could alter development (Oppenheim and Levin, 1974; Decuyper and Michels, 1992), effect the timing of hatch (Stoleson and Beissinger, 1999), decrease hatchability (Yeatter, 1950; Wilson et al., 1979; Stoleson and Beissinger, 1999), reduce the fitness of hatchlings (Webb, 1987),
or kill the embryo. The magnitude of the results is dependent upon the thermal intensity of the heat load, timing, and duration of exposure.

Critical Periods of Development

Early studies that examined the effects of hyperthermic conditions on avian development (typically with chickens) did so during short segments of time immediately before incubation (e.g., 30 min) or for only one brief period during incubation (Decuypere and Michels, 1992). However, environmental temperature manipulations may have different effects on an animal’s ontogeny depending on the timing of exposure or the age of the embryo at the time of exposure, even with the same amount of heating degree-hours (Romanoff, 1949; Romanoff, 1960; Burggren, 1998; Christensen, 2001; Olson et al., 2006; Tzschentke, 2008). For example, between incubation day 10 and 14 of the chicken, hypothalamo-hypophyseal-thyroid development occurs. Decuypere et al. (1988) found that periodic cooling during this critical period resulted in a sustained thyroxine (T4) response to thyrotropin-releasing hormone (TRH) injections up to 7 days after hatching. Periodic cooling during other stages of embryogenesis resulted in only transient T4 responses to TRH injections.

Time or age dependent results suggest that critical periods of development exist (Romanoff, 1949; Burggren, 1998; Dzialowski et al., 2002; Spicer and Burggren, 2003) in which embryos are most susceptible to experiencing developmental change (morphological or physiological) or death due to exposure to environmental stressors (e.g., high-fluctuating heat loads).
Put another way, by changing exogenous factors such as temperature during early sensitive or critical stages of development, the developmental trajectory of an embryo might be altered (Burggren, 1998). Romanoff (1949) reviewed several studies evaluating thermal tolerance of chicken embryos and suggested that the embryos were more susceptible to thermal stress during early and late incubation, and were more tolerant of thermal stress during the middle third of incubation. Similarly, Christensen (2001) and Tzschentke (2008) both demonstrated that the effects of increased incubation temperature were time dependent. Prior research has not tested for critical periods of development during pre-incubation.

**Oxygen Consumption: Indicator of Development**

One method to gauge developmental responsiveness to stress in avian embryos is to measure metabolism, or an index of metabolic rate such as oxygen consumption (Romanoff, 1940). The rate of oxygen consumption (\( \dot{V}_{O_2} \)) is directly proportional to the wet mass and metabolic rate of an avian embryo (Romanoff, 1940), qualifying its measurement as a non-invasive technique for monitoring embryonic development under normal or stressful conditions (Romanoff, 1940; Vleck et al., 1980; Schmidt-Nielsen, 1997). The rate of whole animal oxygen consumption of developing precocial birds (in ovo) typically increases until approximately 80% of incubation time (Visschedijk, 1968; Vleck et al., 1980). Thereafter, the rate slows (gradually increasing, remaining constant, or decreasing) until the embryo internally pips into the air cell (Vleck et al., 1980).
when the rate increases, followed by another increase at the time the chick pips the shell and hatches (Visschedijk, 1968). Research in later chapters analyzes landmarks in the pattern of avian \( \dot{V}o_2 \) to investigate whether exposure to high-fluctuating temperatures during pre-incubation changes the timing of the onset of air breathing (internal pip), external pipping, or hatching during embryonic development.

**Hatching Plasticity**

Hatching is a developmental event and life history switch point (Warkentin, 2007). For birds that typically have multiple eggs in their clutch, hatching occurs either asynchronously (eggs hatch sequentially over an extended period of time) or synchronously (all eggs hatch within a 24-hr period) fundamentally depending on the timing of incubation commencement (Vleck et al., 1980). Waterfowl, for example, begin incubation before the clutch is complete resulting in asynchronous hatching (Caldwell and Cornwell, 1975). Other precocial birds (e.g., northern bobwhites) do not begin incubation until the last egg is laid (Stoddard, 1931) resulting in synchronous hatching. Although both approaches to hatching have advantages and disadvantages, experiments in this dissertation focus on synchronous hatching which beneficially allows the incubating parent to quickly transition behavior from incubating sessile eggs to brooding motile hatchlings (Vleck et al., 1980; Warkentin, 2007).

Pre-incubation temperature may influence hatching synchrony in wild species (Twomey, 1936). In temperate climates, cold torpor suspends
development of earlier laid eggs in a clutch so that once incubation begins, development and hatching occur synchronously (Drent, 1975; Ewert, 1992). In warmer climates, where spring temperatures often exceed physiological zero, earlier-laid eggs are assumed by the author (Chapter 4) to be more developed than late-laid eggs at the onset of incubation. What mechanism triggers these asynchronously developed chicks to hatch synchronously?

Hatching synchrony requires that eggs remain in contact with each other, especially in the last 20% of incubation (Vince, 1964). Regardless of relatedness or order of laying, bobwhite eggs of the same age hatch synchronously if viable eggs are touching (Pani et al., 1969) and, when isolated their time of hatch is spread over a longer period (≤70 h; Vince, 1968). Vince (1968) found that bobwhite eggs accelerated hatching when placed in contact with more-advanced eggs (24 h advanced) and delayed hatching when placed in contact with more time-retarded eggs (24 h delayed), suggesting that both an acceleration and delay of hatching time occur between earlier laid and later laid eggs in the wild.

The process of synchronization is thought to occur as a result of both audible sounds and inaudible vibrations (low frequencies) emitted from eggs in contact with one another beginning as early as 6 d before and continuing until hatching (Freeman and Vince, 1974). Earlier in this period (3–6 d before hatch), the sounds are produced by small, irregular movements occurring throughout the incubation period (Hamburger, 1968; Freeman and Vince, 1974). Approximately 1–3 days before hatching (<2 d for chicken; approximately 3 d for bobwhite),
sharp and short higher frequencies are produced from beak clapping, followed by low frequency pulses introduced at the onset of pulmonary respiration (Freeman and Vince, 1974). After regular breathing has been established (about 10–15 hours before hatching), short, intermittent click-like signals of higher amplitude occur and $\dot{V}O_2$ increases. These clicks, associated with $\dot{V}O_2$ increases, are thought to be the primary accelerating stimuli in bobwhites that promote hatching synchrony (Vince, 1968; Freeman and Vince, 1974; Vleck et al., 1980; Martin, 1999).

Northern Bobwhites: a Naturally Important Animal Model

When attempting to answer large-scale questions such as determining the physiological response of developing vertebrates to climate change, animal models are often used based on their ease of study and relevance to the question at hand (Krogh, 1929; Burggren, 2000; Huss et al., 2008). Rather than repeat studies with each vertebrate organism, results from experiments conducted on relevant animal models can be inferred (cautiously) to similar species or similar species during a particular life stage. The utility of animal models for studying development, the primary use in this dissertation, is greatest in early development where vertebrate embryonic physiology is highly conserved (Burggren, 1998).

Since Aristotle, the first known embryologist, opened his first chicken egg and wondered how complex organ systems emerged from such simple beginnings (Aristotle, 350 B.C.), the chicken has been a prominent model for
studying development. Chicken eggs are self-contained, readily available, easily manipulated with modern incubation equipment, and require a relatively short incubation of 21 days (Romanoff, 1960). For similar reasons, two species of quail have emerged as popular animal models, the Japanese quail (*Coturnix japonica*) and the northern bobwhite (*Colinus virginianus*). Incubation periods are similar to chickens at 17-d and 23-d respectively. Eggs of both species are readily available and are small, meaning similar quantities can be stored in smaller spaces relative to chicken eggs. Since quail eat seeds and small arthropods, researchers have been increasingly using these birds to determine effects of agricultural herbicides and pesticides on wildlife (Huss et al., 2008).

Studies in this dissertation used northern bobwhites as animal models to determine the developmental response of vertebrates to climate change, not only due to their ease of use but also because of their biological importance and natural history. In particular, as described below, bobwhite populations fluctuate with local and regional changes in climate; increasing in rainy years, and decreasing in droughty years. Additionally, the embryonic stage of the bobwhite life-cycle presents itself as a plausible stage that limits production during droughty years. As a species that is often exposed to hyperthermic conditions during development, and is declining concurrent with climate warming, the northern bobwhite was selected as a natural animal model in the following studies to investigate how developing vertebrates respond to climate warming, and to examine how northern bobwhite embryos respond to thermal stress.
Natural History of Northern Bobwhites

Northern bobwhites (Colinus virginianus) belong to the order Galliformes (megapodes, curassows, pheasants, quail, and grouse), family Odontophoridae (new world quail), and are the northern most inhabitants within the Colinus (bobwhite) genus (Guthery et al., 2000b), hence the common name “northern” bobwhite. They are native to the United States and Mexico, primarily east of the 100th meridian. Their northern boundary is likely determined by winter temperatures, while their western limit is probably established by reduced precipitation (Rosene, 1969).

Bobwhites inhabit a variety of habitats (e.g., grassland prairies, shrublands, coastal marshes, and timbered areas), elevations (sea level to >2000 m; Rosene, 1969), and climates. Generally, bobwhites have been described as a lower succession species, meaning that in the series of transitory plant communities that emerge during ecological succession (ranging from bare ground, “early”, to the climax stage, “late”), bobwhites prefer early seral stages identified by more open ground and less ground cover (Rosene, 1969). This generalization has been based on the diet of bobwhites, a granivorous species, that feed primarily on seeds produced from early successional forbes and grasses; and arthropods residing within (Stoddard, 1931). However, Spears et al. (1993) showed that bobwhites preferred early successional stages only in geographical areas with moderate to high precipitation, and they inhabited more mature seral stages (more cover, less open areas) in sites with lower
precipitation. Seemingly, in areas with less precipitation, bobwhites may prefer larger, more advanced vegetation and woody cover that provide cooler microclimates; better combating associated increased body temperatures and reductions in evaporative cooling.

Because northern bobwhites are found in various habitats, they are also important ecologically. They serve as a “canary in the coal mine” for sympatric species, meaning they are an biological indicator species that reflects the overall health of an ecosystem (Brennan et al., 2005). Thus, as changes in climate and habitat occur, biological responses of the more sensitive bobwhite may serve as precursors to interspecific responses, further making it an important “natural” animal model.

Bobwhites are not only good for the local ecosystems and biodiversity, they are also very important for sportsman and those employed by the multimillion dollar recreation industry. Northern bobwhites are economically important game birds generating much needed income for rural landowners, state and federal governments, and communities. Burger et al. (1999) found that 500,000 northern bobwhite hunters in 11 southeastern states spent nearly $95 million in one year (1991). Average retail expenditure was $180.00 per hunter, average economic impact was $368.00 per hunter, and quail hunting supported approximately 3,000 jobs during that year (Burger et al., 1999). The subsequent year (1992), a drought year in much of the southern United States, less hunters took the field (6.9% reduction) resulting in a decrease in economic impact of
$13.3 million across those 11 states. Since, bobwhites are important both ecologically and economically, any decline in their population numbers could be a warning sign for the ecosystem and be damaging to state and local economies.

Northern Bobwhite Population Decline

Bobwhite population numbers have exhibited a declining trend, concurrent with global warming, since population records were first established in the 1930’s (Brennan, 1991; Guthery et al., 2000a; Sauer et al., 2008). As a result, the northern bobwhite was listed as a near-threatened species on the International Union for Conservation of Nature (IUCN) red list of threatened species (IUCN, 2008) in 2004; clearly identifying the northern bobwhite as a species in conservation need.

Declining populations of northern bobwhites first attracted the attention of researchers in the 1930’s when urbanization and clean farm practices caused fragmentation of bobwhite habitat (Leopold, 1931; Errington, 1945). Concern regarding the decline persists today as populations of bobwhites have become more fragmented, reducing connectivity between populations, due to rural, suburban, and urban “development”. Additionally, concerns over the impact of global warming on bobwhite abundance have entered into the quail literature (Guthery et al., 2000a). Is there a cause-effect relationship between climate change and the population decline?

Population numbers have declined approximately 60% since 1967 and continue to decline at approximately 3% annually across the U.S. territories
(Figure 1.1; Sauer et al., 2008). In their southern climes, Texas in particular, bobwhite populations have declined at a similar rate (Sauer et al., 2008) and of further concern is the phenomenal annual changes exhibited, known as “boom” and “bust” population fluctuations (Figure 1.1; Peterson, 2001; Sauer et al., 2008).
Figure 1.1. Bobwhite population trends for the United States (closed circles) and Texas (open circles) between 1967–2007 from the North American Breeding Bird Survey (BBS; Sauer et al. 2008).
Although weather is implicated as the ultimate controller of much of the variation in year-to-year bobwhite abundance, the actual mechanism(s) governing population fluctuations is unclear (Hernandez and Peterson, 2007). For decades, biologists believed precipitation alone was the driving factor behind the population variations (Lehmann, 1946; Jackson, 1947; Kiel, 1976; Rice et al., 1993) until Guthery et al. (2001) recorded temperatures during the nesting season that exceeded 39 °C, the critical thermal maximum for adult bobwhites (Case and Robel, 1974). According to the chicken literature, these temperatures were high enough in nature to suppress bobwhite production (Guthery, 1997), accelerate the onset of incubation (Romanoff, 1960), and disrupt synchronous hatching (Christensen, 2001; Guthery et al., 2001).

Since precipitation and heat are both factors that contribute to bobwhite population declines, Bridges et al. (2001) mathematically modeled the relationship between northern bobwhite populations and drought conditions, and showed the Palmer Drought Severity Index (Palmer, 1965) correlated ($r = 0.78$, $P<0.001$) with the mean number of bobwhites observed per biological survey route. Subsequently, Reyna (2008) showed that drought conditions correlated with a decline in bobwhite populations in 65 Texas counties and that the percentage of juveniles in the local populations was lower during drought years (Figure 1.2).
Figure 1.2. Mean (± 1 S.E.) percentage of juvenile bobwhites in the fall population across 7 study sites in Texas. Severe drought conditions occurred across all study sites in 2006 resulting in a lower percentage of juveniles in the population (P = 0.008; Reyna, 2008). Letters indicate statistical groupings.
Heat Hypothesis

The majority of research that examined the bobwhite’s response to temperature during the reproductive period challenged Guthery’s (1997) heat hypothesis which states that a large portion of the variation in annual bobwhite reproduction is associated with annual variation in heat loads in the epigeal (i.e., near-ground) environment. Specifically, operative temperatures (air temperature less cooling effect of the wind plus the heating effect of the sun) drive the length of the laying season and the number of females that go into reproductive condition (Klimstra and Roseberry, 1975; Guthery et al., 1988; Forrester et al., 1998; Guthery et al., 2001; Guthery et al., 2004).

The length of the laying season and the number of females in reproductive condition are both reduced coincident with higher than normal (hyperthermic) operative temperatures since hens will not typically enter reproductive condition or lay eggs when body temperature exceeds the upper critical temperature of approximately 35 °C (Case and Robel, 1974; Guthery, 2002; Hernandez et al., 2005). As a result of less hens laying and a shorter laying season (Guthery et al., 1988), the heat hypothesis suggests less juveniles or lower recruitment would result in the population, contributing to lower population numbers in that year.

The above heat hypothesis is most applicable to drought conditions in the semi-arid portions of the bobwhite’s range (e.g., Texas) and suggests that during droughty years, heat acts upon the hen to suppress or prohibit reproduction. However, Hernandez et al. (2005) compared the reproductive variables of hens...
and the subsequent age ratios of same-site populations between rainy and
droughty years and found confounding results. The length of the laying season
and number of hens laying eggs was reduced in drought years as compared to
wet years (as expected) although the percentage of juveniles in the population
during drought slightly dipped from 78% to 69%. Hernandez et al. (2005) did not
report temperatures from the study areas; thus, we can only assume that
ambient moisture during drought (or lack thereof) affected survival and
reproductive behavior of adult bobwhites and did not significantly impact
juveniles. What then is the aspect of drought that results in a lower percentage
of juveniles in the population?

In Kansas, Robinson and Baker (1955) found that bobwhite production is
a function of the interaction of annual rainfall and ambient temperature.
Robinson and Baker (1955) observed low juvenile production in rainy years when
temperatures were high and moderate production in droughty years when
ambient temperatures were relatively lower. They concluded that the effect of
rainfall on bobwhite production often masked the effect of temperature.
However, in years with minimal rainfall (e.g., drought), variations in bobwhite
productivity were a function of temperature (Robinson and Baker, 1955).
Perhaps Hernandez et al. (2005) experienced relatively low temperatures in the
droughty year which minimally affected juvenile production.
The Overlooked Pre-Incubation Period

Studies that challenged Guthery’s heat hypothesis focused on the response of adult bobwhites to heat loads and overlooked the critical period of pre-incubation. Prior to the nesting season, typically May–August, (Lehmann, 1984) bobwhites exhibit an extended pre-incubation period during which bobwhite hens lay one egg per day for approximately 12–14 d (Stoddard, 1931; Lehmann, 1984; Roseberry and Klimstra, 1984). The hen’s clutch of eggs remains largely unattended during that time until incubation begins ≤7 d after laying is complete (Stoddard, 1931).

During the pre-incubation period, bobwhite eggs are potentially exposed to extreme, fluctuating temperatures of the environment without the thermal buffer of the incubating parent, especially during drought years. Guthery et al. (2004) illustrated the thermal stress eggs are exposed to during pre-incubation by simultaneously recording internal temperatures of incubated and non-incubated eggs in situ (Figure 1.3). The inner-temperatures of the incubated eggs were maintained within the approximate thermoneutral zone of an adult bobwhite, 30–35 °C (Case and Robel, 1974), while the non-incubated eggs experienced large diel fluctuations in temperatures, consistent with ambient fluctuations, and exceeded 40 °C in the non-drought year (Figure 1.3).
Figure 1.3. Smoothed dynamics of nest-content temperatures for incubated and control nests of northern bobwhites, Tallahone Pasture, Mesa Vista Ranch, Roberts County, Texas, 21–29 July 2002. The dashed horizontal lines bound the approximate thermoneutral zone for adult bobwhites (Guthery et al. 2004).
In an attempt to better understand heat loads on reproducing bobwhites, Guthery (2002) examined the micro-climate of simulated northern bobwhite nests in simulated drought and rainy periods. Soil moisture and shade were maintained in simulated non-drought periods, while dry soil fully exposed to the sun served as simulated drought. Temperature fluctuations peaked ≥45 °C during simulated drought and were recorded as high as 60 °C in a similar experiment (Guthery et al., 2000a).

The relationship between heat and the decline of northern bobwhite populations is likely complex. However, the fluctuating, high temperatures of the pre-incubation period (the focal period of this dissertation) during drought conditions could act upon autonomous bobwhite eggs, influence the ontogeny of embryos (*in ovo*), and disrupt or alter their hatching strategy (Christensen, 2001).
Objectives and Hypotheses

Understanding the impacts of thermal stress on the vertebrate ontogeny is the overarching goal of this investigation. By using the northern bobwhite as a natural animal model, insight may be gained into the effects of thermal stress experienced during pre-incubation on the subsequent embryonic development and the extent of embryonic involvement in the population decline. The first two studies are independent studies developed to understand how long eggs could be stored at room temperature (Chapter 2) and to determine the upper lethal temperature of northern bobwhite eggs. These initial studies were critical components to subsequent studies (Chapters 4 and 5) that evaluated the developmental response of northern bobwhite embryos to thermal stress experienced during pre-incubation.

In the first study (Chapter 2), the objective was to determine the effects of prolonged egg storage on the pH of egg albumen and yolk (indicators of egg quality and embryonic viability), and subsequent hatchability of northern bobwhite eggs. I compared the results with data published in the poultry literature on chickens. I hypothesized that bobwhite eggs that are often stored for 12–21 days in their native environment, could be stored longer than chicken eggs which experience a reduction in hatchability after 7 days.

In the second study (Chapter 3), the objective was to determine the thermal properties and lethal temperature doses of fresh northern bobwhite eggs. I hypothesized that bobwhite eggs would reach thermal equilibrium with high heat
loads faster than chicken eggs (due to their smaller size) and have a higher thermal tolerance since they are often subjected to temperatures exceeding 43°C (lethal temperature for chickens) in nature.

In the third study (Chapter 4), the objectives were to determine: 1) if bobwhite embryos develop differently when exposed to diurnally fluctuating temperatures or a constant temperature equal to the mean of the fluctuating regime during pre-incubation, and 2) if development or hatching is altered as a result of increased heat loads equal to simulated drought conditions or conditions predicted as a result of global warming. I hypothesized that bobwhite embryos exposed to fluctuating temperature regimes would develop at a different rate and exhibit a reduced hatching success compared to eggs exposed to a constant temperature equal to the mean of the fluctuating regime.

The fourth study (Chapter 5) tested for critical periods of development during pre-incubation—seemingly the first to do so—to determine whether a time or stage dependent response to cyclic hyperthermic temperatures existed by exposing fertilized bobwhite eggs to chronic and acute doses of high-fluctuating heat during three different periods of pre-incubation. I hypothesized that equal doses of heat given in different periods of pre-incubation would induce different rates of development, and effect hatchability and hatching synchrony.
CHAPTER 2
PRE-INCUBATION EGG STORAGE EFFECTS ON ALBUMEN PH
AND HATCHABILITY OF NORTHERN BOBWHITE EGGS

Introduction

Pre-incubation Egg Storage

Pre-incubation egg storage occurs in nature with several precocial avian species, and is a necessity in the commercial egg industry. Many precocial birds which lay multiple eggs in a clutch (primarily ducks such as mallards, *Anas platyrhynchos*, and Galliformes such as northern bobwhites, *Colinus virginianus*) begin incubation upon or shortly after the last egg of the clutch is laid (c.f. Tazawa and Whittow, 2000). Thus, there is an extended pre-incubation period, based on clutch size, where eggs that were laid first are unattended in the nest until the clutch is complete. In the poultry industry, it is cost effective to store eggs that are laid daily to provided hatcheries with the flexibility to meet market fluctuations and to reduce the amount of incubations (Fasenko, 2007). Ultimately, pre-incubation egg storage is used in nature and in commercial settings to obtain the highest possible hatching rate in the most efficient manner.

Pre-incubation egg storage, although inevitable, reduces hatchability of eggs (Proudfoot, 1969; Mather and Laughlin, 1977; Wilson et al., 1984). The optimum storage condition to achieve the highest hatch rate is a topic that has
received much attention among poultry researchers. Numerous studies have indicated that the hatchability of stored avian eggs is influenced by the length of storage, ambient temperature, humidity, gaseous environment, and orientation of the eggs (Romanoff, 1960; Proudfoot, 1969; Meijerhof, 1992; Brake et al., 1997; Fasenko, 2007).

Although an egg is at its maximum hatching potential the moment it is laid, avian eggs can generally be stored for ≤7 days without a significant loss of hatchability when appropriate conditions are met (Romanoff, 1960; Proudfoot, 1969; Meijerhof, 1992; Brake et al., 1997; Fasenko, 2007). For example, in experimental and industrial settings, eggs are typically stored below physiological zero (the temperature at which development is arrested; Landauer, 1961), at a relative humidity (RH) of 75–90% (Proudfoot, 1969), in normoxia (approximately 150 ± 10 mmHg), and with the large end of the egg positioned upward (Romanoff, 1960). However, in natural settings, laying hens store eggs for 1–21 days (much longer than in commercial settings) in various environmental conditions during pre-incubation.

Storage Temperature

The most widely studied factor in determining storage period effects on egg hatchability is the interaction of storage time and holding temperature (Proudfoot, 1969; Meijerhof, 1992; Fasenko, 2007). Chicken eggs (Gallus gallus), when stored at physiological zero (24–26 °C; Funk and Biellier, 1944), begin to lose hatchability after 7 days (Waite, 1919). Proudfoot (1969) obtained
higher hatchability of chicken eggs when stored at 20–25 °C for <4 days, 15–16 °C for 4–7 days, and 10–12 °C for storage exceeding 7 days. Turkey eggs (Meleagris gallopavo) stored at physiological zero also displayed a reduction in hatchability after 7 days, although eggs could be stored for 8–14 days without significant loss of hatchability at 11.7 °C, if eggs were warmed for 5 hours at 37.6 °C prior to storage (Fasenko et al., 2001). Similarly, Shom and Abbott (1974) stored northern bobwhite eggs at 12.8 °C and did not observe a significant reduction in hatchability until a storage duration of 15 days was reached. Miller and Wilson (1976) showed conflicting results indicating that northern bobwhites could be stored for ≤ 21 days at a temperature range of 12.8–15.5 °C without a significant reduction in hatchability. Wilson (1984), in an attempt to resolve the relationship between storage duration and temperature for the northern bobwhite, stored eggs below physiological zero (24.4–25.6 °C; Miller and Wilson, 1975) at 7.2 °C, 12.8 °C, 18.3 °C, and 23.9°C, and recorded significant loss of hatchability after 7 days when eggs were stored in the range of 18.3–23.9 °C, and after 13 days when eggs were stored at 12.8°C.

**Embryonic Viability**

The decline in embryonic viability and egg quality during prolonged storage (>7 days) might be caused by changes in the embryo—e.g., necrosis (Funk and Biellier, 1944; Mather and Laughlin, 1979) or physical changes in egg components—e.g., albumen (Dawes, 1975; Brake et al., 1997; Lapao et al., 1999; Kirunda and McKee, 2000; Scott and Silversides, 2000). This study
focused on the changes in albumen pH which is known to change during egg storage and development (Dawes, 1975; Tazawa et al., 1983; Gillespie and MacHanwell, 1987; Stern, 1991). Accordingly, albumen pH is often used in the poultry and commercial egg industry as an indicator of egg quality and embryonic viability, particularly in relation to egg storage (Scott and Silversides, 2000). Albumen pH of fresh chicken eggs is about 7.6, but increases during pre-incubation egg storage to a maximum of 9.5 due to the release of carbon dioxide from the egg (Romanoff and Romanoff, 1929; Brooks and Pace, 1938; Romanoff, 1944; Dawes, 1975; Tazawa et al., 1983; Stern, 1991). This is caused by the disruption in equilibrium between dissolved carbon dioxide, bicarbonate ions, carbonate ions, and proteins in the albumen (Romanoff and Romanoff, 1929; Brooks and Pace, 1938; Romanoff, 1944). Gillespie and MacHanwell (1987) found that optimum development of chicken embryos was achieved when mean albumen pH was 8.3, which is quite alkaline compared to adult tissues and fluids (Stern, 1991). Additionally, Stern (1991) found that a large hydrogen ion concentration gradient between the yolk and albumen (approximately 1000-fold) is necessary for embryonic development. Yolk pH of fresh chicken eggs is about 6.0 and gradually climbs during pre-incubation to 6.5 (Dawes, 1975). However, yolk pH does rise to an alkaline level of 8.0 after 14 days of incubation (Romanoff and Romanoff, 1929). Egg characteristics of the domestic turkey were reported to be similar to that of the domestic chicken (Bain and Deutsh, 1947).
Northern Bobwhite

Information regarding changes in albumen and yolk pH of northern bobwhite eggs in relation to pre-incubation storage is lacking in the scientific literature. Not surprisingly, most information on egg storage has been gathered for the domestic chicken. Such information would be useful since the northern bobwhite is an important game-bird that is commercially produced as part of a multi-million dollar industry to meet the demands of hunting preserves, restocking programs, and gourmet food outlets. In nature, northern bobwhites exhibit an extended pre-incubation period during which bobwhite hens lay one egg per day for approximately 12–14 d (Stoddard, 1931; Lehmann, 1984; Roseberry and Klimstra, 1984). The clutch of eggs (typically 12–14 eggs) remains unattended and stored in the nest until incubation begins ≤7 d after laying is complete (Stoddard, 1931), meaning eggs could be stored in the nest for ≤ 21 days. In the commercial industry, bobwhite eggs are collected multiple times per day to reduce cracking and exposure to bacteria and disease. As a result, they must be stored prior to incubation. Understanding the effects of pre-incubation storage on albumen pH and hatchability could contribute to the management and production of northern bobwhites.

Objectives and Hypotheses

The objectives of this study were to determine the maximum storage duration achievable without experiencing significant reductions in egg viability and subsequent hatchability of northern bobwhite eggs stored at 20–22 °C, a
temperature range below physiological zero and common, in the wild, during the egg-laying period. Additionally, I examined the effects of prolonged egg storage on the pH of egg albumen and yolk. I hypothesize that bobwhite eggs can be stored for longer periods than chicken and turkey eggs, since in their native environment they are stored for 12–21 days prior to incubation. Moreover, I suspected that albumen pH would rise above the optimum pH of 8.3 after 12–14 days as a result of degradation of albumen, indicating that clutch size is a function of egg viability.

Materials and Methods

Fertilized northern bobwhite eggs were collected from wild-type breeding pairs at Lake Cumberland Game Bird Farm (Mill Springs, KY, USA). Eggs were packaged and shipped to the University of North Texas (Denton, USA) on the day of collection. Eggs arrived on-site within 2–3 business days with a written record of the date and time of egg collection. Lake Cumberland Game Bird Farm was approved for egg production by the United States Department of Agriculture (USDA) and certified by the USDA National Poultry Improvement Plan. This research was approved by the University of North Texas Institutional Animal Use Care Committee, protocol # 0808.

Pre-incubation Egg Storage

Upon arrival, bobwhite eggs were randomly divided into 6 groups of 15 eggs and labeled with an indelible marker according to group. Each group corresponded to a storage duration of 0 d (control), 3 d, 7 d, 14 d, 17 d, or 21 d.
Each egg was weighed to the nearest 0.01 g with a digital scale (Ohaus Explorer Pro, Pinebrook, NJ, USA) and placed onto a plastic rack, blunt end up (Proudfoot, 1969), and stored in 20–22 °C with a RH of 50%.

Hatchability of Stored Eggs

After the pre-incubation storage period was complete, egg groups were placed in a G.Q.F. 1502 Sportsman incubator (G.Q.F. Manufacturing Co., Savannah, GA, USA) with a temperature of 37 ± 0.5 °C and RH of 60%. Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, eggs were weighed to the nearest 0.01 g and egg groups were placed in separate hatching chambers, and no longer turned, at the same temperature and RH (after Romanoff, 1960). Hatching was determined when the eggshell was star-pipped, which was defined as an externally pipped egg where the embryo created a small hole (approximately 3 mm²) in the shell to initiate hatching (MacCluskie et al., 1997). This definition of hatching was used to compensate for any artificial hatching difficulties created by fluctuations in microclimate within the incubator. Upon star-pipping, the percentage of eggs hatched was recorded.

Albumen and Yolk pH

Fresh egg pH of albumen and yolk was obtained by a staff member at the hatchery who regularly records albumen height, a measure of albumen quality, since the 2–3 day shipping time prohibited recording fresh components. At the hatchery, egg albumen and yolk were separated and pH was recorded using pH
paper. In the laboratory, treatment egg characteristics were recorded following pre-incubation storage. Eggs were cracked and albumen and yolk were placed in separate plastic trays. Albumen and yolk pH were recorded by inserting a pH probe (Oakton Instruments, Vernon Hills, IL USA) into each egg component individually. The probe was standardized using known buffer solutions of 7.0 and 9.0.

Statistical Analyses

Albumen and yolk pH values were converted to hydrogen ion concentration \([H^+]\) using the formula:

\[
pH = -\log [H^+] \]

Statistics were performed on the \([H^+]\), and subsequently converted back to pH for applicability (after Davenport, 1974). The average of the hydrogen ion concentration for treatment replications was obtained and reported as a single pH value (Davenport, 1974; Boutilier and Shelton, 1980).

All data were tested with a Shapiro–Wilks normality test (Zar, 1999) and Hartley’s \(F_{\text{max}}\) test (Zar, 1999) before specific statistical analyses were performed. An ANOVA (Zar, 1999) was used to identify differences in mean values among treatment groups. A Holm-Sidak pairwise multiple comparison procedure (MCP) was subsequently used to test for significance between control (0 d of storage) and treatment groups (Zar, 1999). All statistical tests were conducted using SigmaStat 3.5 software (Systat Software Inc. San Jose, CA). Statistical decisions were made with a 0.05 level of probability.
Results

Hatchability of Stored Eggs

There was a significant difference (ANOVA; \( P=0.005 \)) in mean hatching success among groups of northern bobwhite eggs stored for 0, 7, 14, 17, or 21 days. Further, a Holm-Sidak MCP indicated that hatching success was not significantly affected by storage periods \( \leq 14 \) days, and eggs stored for 17 and 21 days had a reduced hatch rate when compared to control (Figure 2.1; \( P=0.03 \), and 0.02 respectively).

Albumen and Yolk pH

There was a significant difference in mean albumen and yolk pH among groups of northern bobwhite eggs stored for 0, 3, 7, 14, 17, or 21 d (ANOVA; \( P<0.001 \), \( N=15 \) eggs per treatment). The mean hydrogen ion concentration \([\text{H}^+]\) in albumen was converted to pH. For fresh bobwhite eggs (0 d of storage) the pH was 8.32. After 3 days of storage, pH had risen to 9.00. Albumen pH rose to 9.70 after 7 days of storage and peaked at 9.96 after eggs were stored 14 days. The pH level then stayed steady through 17 days (9.92) and 21 days (9.86) of pre-incubation storage (Figure 2.2). Yolk pH of fresh bobwhite eggs was 6.15 and gradually increased to 6.47 on day 3, 6.55 on day 7, and 6.54 on day 14 of storage. Yolk pH subsequently reached 6.99 on day 17, and 7.97 after 21 days of pre-incubation storage (Figure 2.2). A 1000-fold difference in \([\text{H}^+]\) (3 pH units) was observed between albumen and yolk pH between 7 and 14 days of pre-incubation storage (Figure 2.2).
Figure 2.1. Mean hatching success rate (%) of bobwhite eggs stored at 20–22 °C for 0, 7, 14, 17, and 21 d prior to incubation. Eggs stored for 17 and 21 d had a reduced hatch rate compared to control eggs (P=0.03, and 0.02 respectively). Error bars=1 S.E.; letters indicate statistical groupings; N=45 per group.
Figure 2.2. Mean albumen and yolk pH of northern bobwhite eggs stored at 20–22 °C for 0, 3, 7, 14, 17, and 21 d prior to incubation. A significant difference in pH of albumen and yolk was observed as storage duration increased (P<0.001; N=6 eggs per treatment; ANOVA). Letters indicate statistical groupings.
Discussion

Northern bobwhites delay incubation until after all eggs of the clutch are laid as part of an approach to achieve synchronous hatching. Bobwhites lay eggs at a rate of one egg per day for an average duration of 12–14 days until the clutch is complete. During egg-laying, eggs remain in the nest, unattended, until incubation begins ≤ 7 days after the last egg is laid. First-laid eggs must remain viable during the entire egg laying period (pre-incubation) to maintain synchronous hatching. During this study, hatching success of northern bobwhite eggs stored at 20–22 °C declined significantly after 14 days. These results coincide with Schom and Abbott, (1974) who observed a significant decline in hatchability after 15 d in bobwhite eggs stored at 12.8 °C; a typical storage temperature in commercial industries. The results indicate that average clutch size (12–14 eggs per clutch), and as a result the duration of pre-incubation, might be a function of egg viability—i.e., the benefit of additional eggs is offset by loss of egg viability.

Egg quality and viability are often judged by albumen pH which declines with storage (Romanoff and Romanoff, 1929; Romanoff, 1944; Proudfoot, 1969; Dawes, 1975). The albumen is positioned between the egg shell and perivitelline layer in such a way that external factors that affect hatching success also change the characteristics of the albumen (Brake et al., 1997). The pH levels for optimum chicken development consist of an albumen pH of 8.2–8.8 (Gillespie and MacHanwell, 1987; Stern, 1991; Brake et al., 1997), with a slightly acidic
yolk pH of about 6.2 (Dawes, 1975), creating an approximately 1000-fold hydrogen ion concentration gradient between the yolk and albumen that is necessary for embryonic development (Stern, 1991). In this study, albumen pH of bobwhite eggs stayed within the optimum pH range for chickens (8.2–8.8) for the first 2 days of storage, then exceeded the peak value for chickens (9.5) prior to 7 days of storage, and reached a peak value of 9.96 on day 14; subsequently turning less alkaline. The value of 9.7 reached after 7 days of storage is higher than the peak value of 9.5 observed in chickens after 7–10 days of storage (Kirunda and McKee, 2000; Scott and Silversides, 2000), or the equivalent value reached after 2 days of incubation (Romanoff and Romanoff, 1929). Additionally, the observed pH of 9.96 observed after 14 days has not appeared in poultry studies and could be the limiting factor for egg viability. The gradient between yolk and albumen pH was largest between 7 and 14 days of storage, due mostly to an excessively high albumen pH. Additionally, yolk pH rose to 7.0 after 14 d of storage and became slightly alkaline thereafter. This increase in yolk pH has been observed during incubation in chickens, reaching as high as 8.0 (Romanoff and Romanoff, 1929), but is lacking in the literature in regards to pre-incubation storage.

The increased albumen pH observed is expected due to the diffusion of dissolved carbon dioxide and the low buffering capacity of the albumen between the range of 7.5–8.5 (Stern, 1991). Additionally, Stern (1991) indicated that changes in albumen pH during early embryonic development could be due to the
secretion of hydrogen ions from the albumen to the sub-embryonic fluid, and removal of bicarbonate ions from the sub-germinal cavity into the albumen.

The observed increased albumen pH could be an adaptation to bacteria penetration of the wet cuticle post oviposition, often observed and reported in the poultry literature. At the time of oviposition, the cuticle is moist and slightly pliable to allow water passage in utero (Brake et al., 1997), which may also allow bacteria to penetrate the cuticle. Once laid, the egg-shell cuticle dries, preventing further penetration of bacteria. As observed in this study, the albumen pH increases over time to a value exceeding optimum conditions for bacteria growth, 6.5–7.5 (Tortora et al., 2009). The pH gradient between albumen and yolk is then established allowing optimum growth under incubation conditions (i.e., high temperatures), but prolonged storage causes degradation of the albumen and yolk which is detrimental to embryo development and subsequent hatching.

Success in prolonging egg storage has been achieved by lowering pre-incubation temperature to retard the degradation of albumen (Proudfoot, 1969; Wilson, 1991b; Ruiz and Lunam, 2002), and by briefly heating eggs to incubation temperature (37.5°C) for 30 min–2 h per day, during a prolonged storage period (Kosin, 1956; Fasenko, 2007). Heating eggs for a short period of time accelerates development of the embryo leading to increased carbon dioxide diffusing into the albumen, ideally lowering the pH of albumen.
The present study shows that northern bobwhite eggs can be stored ≤ 14 d without significant loss of viability which is greater than in chickens. This may be an adaptation for an extended pre-incubation period in nature. Not surprisingly, bobwhite hens lay clutches of 12–14 eggs in their natural environment (Stoddard, 1931) suggesting that egg viability may be a constraint on clutch size. Increases in albumen pH, although beneficial as an anti-microbial agent, may slightly impair development prior to 14 days of pre-incubation egg storage, yet prove detrimental after 14 days when extreme pH values are reached. Future studies could further investigate the effects of albumen pH in relation to pre-incubation storage and egg viability by manipulating holding temperature or experimenting with ways to limit carbon dioxide diffusion during pre-incubation.
CHAPTER 3

THERMODYNAMICS AND UPPER LETHAL TEMPERATURE OF
NORTHERN BOBWHITE EGGS

Introduction

One of the most important physicochemical forces ubiquitously acting on living organisms is temperature. Known to drive development, temperature plays a vital role in the ontogeny of avian species (Romanoff, 1960). Below a temperature defined as physiological zero, embryonic development is arrested. For most avian species, physiological zero occurs at or below 25 °C (see Kosin, 1964; Landauer, 1967; Wilson, 1991b for review). Funk and Biellier (1944) reported physiological zero in domestic chicken eggs (Gallus gallus) to be within the temperature range of 24–26 °C. More recently, Miller and Wilson (1975) observed that physiological zero for the northern bobwhite (Colinus virginianus) fell within the range of 24.4–25.6 °C. A surprisingly similar response was found by Weinrich and Baker (1976) who described physiological zero for the cold-dwelling Adélie penguins (Pygoscelis adeliae) as approximately 25 °C.

Above physiological zero, avian development begins—or resumes—and increases in rate and regularity as a function of increasing temperature, until a thermal optimum (i.e., the range of temperatures producing the highest survivorship of embryos) is reached (Romanoff, 1960). The thermal optimum for
domestic poultry eggs including chickens, northern bobwhites, turkeys (*Meleagris gallopavo*), and ducks (*Anas platyrhynchos*) falls within the range of 37–38 °C (French, 1997). This range is higher than egg temperatures experienced in natural settings for most avian species which lie in the range of 32–36°C (Drent, 1975; Webb, 1987; Wilson, 1991b). For instance, penguins and seabirds incubate eggs in the range of 32–36 °C (Webb, 1987). Additionally, ratites such as the ostrich (*Struthio camelus*), emu (*Dromaius novaehollandiae*) and rhea (*Pterocnemia pennata*) have a mean incubation temperature range of 35–36 °C (Hoyt, 1980; Vleck et al., 1980; Wilson, 1991b). This common range is most likely due to relatively similar inter-specific core body temperature of 40-41 °C of adult birds (Webb, 1987).

Although there are interspecific differences in incubation temperature, even slight deviations (± 1 °C) from optimum temperature within a species can disrupt embryonic development and affect hatchability of eggs (Romanoff, 1960; Dawson, 1984; Wilson, 1991b; Gill, 1999). An internal egg temperature >41 °C resulted in developmental abnormalities and reduced hatchability in chickens (Alsop, 1919; Romanoff et al., 1938). Indeed, most avian embryos exhibit developmental abnormalities when briefly exposed to 39 °C, just a few degrees out of the optimum range for incubation (for review see Webb, 1987).

For all avian species, an upper lethal temperature (ULT) exists, beyond which life is extinguished (Gill, 1999). Embryonic thermal tolerance is dependent on age, thus the value of the ULT changes throughout development, and
resultantly differs between embryos and adults. The interaction of age and exposure time comes into effect with older embryos (e.g., 18 d of incubation) that are more similar to adult birds than younger embryos and generate more metabolic heat (Webb, 1987). Muscovy duck (Cairina moschata) and chicken embryos in mid to late incubation exhibited a higher internal temperature than incubation temperature as incubator temperature was increased from 38–39 °C due to the addition of metabolic heat (French, 1997; Janke et al., 2002). This phenomenon could impact the perceived ULT of eggs.

The ULT of avian embryos (as measured by inner-egg or body temperature) is similar among embryos of the same species and like ages regardless of exposure time. However, since avian embryos reside within a cleidoic egg, the ambient temperature outside of the egg is not always equal to embryo inner-egg temperature. When ambient temperatures change, inner-egg temperature lags ambient temperatures until thermal equilibrium is reached. As a result the ULT for avian embryos of like ages is often listed as a combination of ambient temperature and duration of egg exposure (Lundy, 1969). For instance, the ULT for embryos in fresh chicken eggs is frequently reported as 43 °C at thermal equalization which takes approximately 2 h from room temperature (Kaplan et al., 1978). Thus, if fertile chicken eggs are transferred to 43 °C for <2 h, the embryos within may survive because they may not have experienced 43 °C. This unconventional method of acquiring ULTs is largely due to the
necessary transfer and storage of eggs in varying ambient temperatures by the commercial egg industry (Chapter 2).

The ULT for chicken embryos was reported as 43 °C and 44–45 °C (Dareste, 1891, and Dumas, 1824, respectively, as reported by Romanoff, 1960), although no age or exposure time was reported. Subsequently, the ULT temperature range for chicken embryos was reported as 42.4–48.3°C, depending on the exposure time and age of the embryo (Lundy, 1969). However, when internal egg temperature reached 46.5 °C in another experiment, chicken embryos of all ages died (Ono et al., 1994). Heerman gull embryos (Laris heermanni) exhibited a ULT of 43 °C for 1 h (Bennett and Dawson, 1979) and Adélie penguin embryos (Pygoscelis adeliae) expired after exposure to 42 °C for 96 minutes.

**Northern Bobwhite**

At the present time, no data appear to be available on the ULT of northern bobwhite embryos. The northern bobwhite is a ground-nesting gallinaceous game-bird that exhibits an extended pre-incubation period while laying 1 egg per day for approximately 12–14 d (Stoddard, 1931; Lehmann, 1984; Roseberry and Klimstra, 1984). The eggs remain in the nest, largely unattended during pre-incubation, until the hen begins incubation after laying is complete (Stoddard, 1931). During the pre-incubation period, bobwhite eggs are potentially exposed to extremely high, diurnally-fluctuating temperatures without the thermal buffer of the incubating parent, especially during drought years. In non-drought years,
internal temperatures of non-incubated bobwhite eggs followed ambient fluctuations and often exceeded 40 °C during peak thermal intensity (Guthery et al., 2004). Bobwhite nest temperatures in simulated drought regularly peaked at 45 °C and have been recorded as high as 60 °C (Guthery et al., 2000a).

Objectives

The effect of these extreme temperatures on the viability of bobwhite eggs is not known. Therefore, the objective of this study was to determine the thermal properties and lethal temperature doses of freshly laid northern bobwhite eggs. I hypothesized that bobwhite eggs would reach thermal equilibrium with high heat loads faster than chicken eggs (due to their smaller size) and have a higher thermal tolerance since they are often subjected to temperatures exceeding 43°C (lethal temperature for chickens) in nature.

Materials and Methods

Fertilized northern bobwhite eggs were collected from wild-type breeding pairs at Lake Cumberland Game Bird Farm (Mill Springs, KY, USA). Eggs were packaged and shipped to the University of North Texas (Denton, USA) on the day of collection. Eggs arrived on-site within 2–3 business days with a written record of the date and time of egg collection.

Upon arrival, bobwhite eggs were randomly divided into groups of 15 eggs, placed on plastic egg trays blunt end up, and labeled with an indelible marker as either control or treatment and assigned a number (e.g., C-1 = Control
group, egg #1). Each egg was weighed to the nearest 0.01 g with a digital scale (Ohaus Explorer Pro, Pinebrook, NJ, USA.

Lake Cumberland Game Bird Farm was approved for egg production by the United States Department of Agriculture (USDA) and certified by the USDA National Poultry Improvement Plan. This research was approved by the University of North Texas Institutional Animal Use Care Committee, protocol # 0808.

Control Incubation

Control eggs (no pre-incubation exposure) went directly into the control incubator, a G.Q.F. 1502 Sportsman incubator (G.Q.F. Manufacturing Co., Savannah, GA, USA). The temperature of the control incubator was 37.5 ± 0.5 °C with a relative humidity (RH) of 60%. Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, eggs were weighed to the nearest 0.01 g and placed in the hatching chamber of the same incubator where they were no longer turned (after Romanoff, 1960). Hatching was determined when eggs were star-pipped, which was defined as an externally pipped egg where the embryo created a small hole (approximately 3 mm²) in the shell to initiate hatching (MacCluskie et al., 1997). This definition of hatching was used to compensate for any artificial hatching difficulties created by fluctuations in microclimate within the incubator. Upon star-pipping, the duration of incubation and percentage of eggs hatched was recorded.
Upper Lethal Temperature

Treatment egg groups were placed into a thermal chamber (G.Q.F. 1583 Hova-Bator with circulated air; RH=60%) with an assigned treatment temperature, ranging from 39–51 °C, and a corresponding exposure time of either 1, 3, or 6 h. A class T thermocouple, connected to a digital converter (model bat-12; Physitemp Instruments, Clifton, NJ, USA), was placed approximately 1-mm above the eggs to record chamber temperature. Group exposure time began when chamber temperature reached intended temperature.

After the end of the assigned exposure time, eggs were removed from the thermal chamber and immediately placed into the control incubator (37.5 °C; RH = 60%). Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, treatment groups were placed in separate hatching chambers and were no longer turned (after Romanoff, 1960). Upon star-pipping, the duration of incubation and percentage of eggs hatched was recorded.

Thermal Dynamics of Fertile Eggs

An additional fresh bobwhite egg was placed into the thermal chamber with treatment eggs at 37.5, 40, 43, 45 and 50 °C, to monitor the heating and cooling times of northern bobwhite eggs. A small hole (1 mm²) was drilled into the air cell of the egg in which a class T thermocouple, connected to a digital converter, was inserted and held in place with modeling clay. The egg and embedded thermocouple were held at 22–25 °C (room temperature) for ≥3 h.
prior to the experiment to ensure a uniform temperature throughout the egg and common starting temperature among treatments. Inner-egg temperature and ambient temperature were recorded every 1–3 min to determine the time for the egg to reach chamber temperature.

**Statistical Analyses**

All data were tested with a Shapiro–Wilks normality test (Zar, 1999) and Hartley's $F_{\text{max}}$ test (Zar, 1999) before specific statistical analyses were performed. An ANOVA (Zar, 1999) was used to identify differences in mean values among treatment groups. A Holm-Sidak pairwise multiple comparison procedure (MCP) was subsequently used to test for significance between control and treatment groups (Zar, 1999). All statistical tests were conducted using SigmaStat 3.5 software (Systat Software Inc. San Jose, CA), with statistical decisions made with a 0.05 level of probability.

**Results**

**Thermal Dynamics of Fertile Eggs**

Egg temperature lagged behind chamber temperature by 30–50 min (Table 3.1) with a mean response time ($\pm$ S.E.) of 38 min ($\pm$ 1.9) for chamber temperatures between 37.5–50 °C and an initial egg temperature of 22–25 °C. Thus, eggs were fully exposed to the ambient temperature for 10–30 min of the nominal 1 h exposure, 2.2–2.5 h for the 3 h exposure, and 5.2–5.5 h for the 6 h exposure of eggs to chamber temperature regimes.
Table 3.1. Time to reach thermal equilibrium of northern bobwhite eggs transferred from 22°C to thermal chambers (37.5–50 °C).

<table>
<thead>
<tr>
<th>Chamber Temperature (°C)</th>
<th>Mean Response Time (min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.5</td>
<td>29.7 ± 0.76</td>
</tr>
<tr>
<td>40</td>
<td>31.7 ± 0.32</td>
</tr>
<tr>
<td>43</td>
<td>37.3 ± 0.88</td>
</tr>
<tr>
<td>45</td>
<td>42.7 ± 0.63</td>
</tr>
<tr>
<td>50</td>
<td>49.0 ± 0.57</td>
</tr>
</tbody>
</table>

* N=3 eggs per treatment

Upper Lethal Temperature

Eggs exposed for 1 h at the test temperature (approximately 10–30-min exposure for embryo) showed a decline in viability after 45°C, had 50% mortality (LT-50) ≥46 °C, and had a ULT of 51 °C (Figure 3.1). Eggs exposed for 3 h showed declining hatchability ≥ 40 °C, had a LT-50 ≥44 °C, and exhibited a ULT of 49–50 °C (Figure 3.2). Eggs exposed for 6 h displayed a decline in hatching success >38 °C, a LT-50 ≥ 40 °C, with death occurring at 47°C (Figure 3.3). Control eggs had a mean hatch rate (± S.E.) of 84.6% ± 2.1. Mean hatch rates were significantly different between groups for northern bobwhite eggs exposed to 1, 3, and 6 h of abnormally high temperature during pre-incubation between 44–47 °C (P=0.03; Figure 3.4). No difference between groups was observed between 38–42 °C (P>0.05), and ≥ 48 °C (P>0.05). Temperatures leading to 30, 50, 70, and 90% mortality (LT-30, LT-50, LT-70, and LT-90, respectively) are illustrated in Figure 3.5.
Figure 3.1. Mean hatching success rate (%) of bobwhite eggs exposed for 1 h to temperature treatments ranging from 38–52 °C prior to incubation. Letters indicate statistical groupings; error bars=± 1 S.E.
Figure 3.2. Mean hatching success rate (%) of bobwhite eggs exposed for 3 h to temperature treatments ranging from 38–52 °C prior to incubation. Letters indicate statistical grouping; error bars=± 1 S.E.
Figure 3.3. Mean hatching success rate (%) of bobwhite eggs exposed for 6 h to temperature treatments ranging from 38–52 °C prior to incubation. Letters indicate statistical grouping; error bars=± 1 S.E.
Figure 3.4. Mean hatching success rate (%) of bobwhite eggs exposed to temperature treatments of 38–52 °C for 1, 3, and 6 h prior to incubation. Boxes indicate no statistical difference; error bars = ± 1 S.E.
Figure 3.5. Estimated lethal temperatures at exposure times ranging from 1–6 h, for northern bobwhite eggs leading to 30, 50, 70, 90, and 100% mortality (LT-30, LT-50, LT-70, LT-90, and LT-100 respectively) from data illustrated in Figure 3.4.
Discussion

The thermal properties and lethal temperatures of chicken eggs and embryos are well described (Alsop, 1919; Romanoff et al., 1938; Romanoff, 1960; Wilson et al., 1979; Gill, 1999) but are lacking for northern bobwhites, since much of the data has been generated for the commercial industry and focused on optimum incubation conditions rather than extremes. This study describes the heating response time and ULT for bobwhite eggs to better understand the thermal limits imposed on avian embryos by climate change, nesting inattentiveness during hyperthermic conditions, or high temperatures experienced during pre-incubation.

Thermal Dynamics of Fertile Eggs

The time required for inner-egg temperature to equalize with outer-egg ambient temperatures, heating response time, is often not reported with upper lethal temperatures but is important when determining lethal heat loads or the effect of high temperatures on the embryo (in ovo). For instance, a northern bobwhite embryo (mean heating response time of 38 min) exposed to high temperatures for ≥2 h will experience high temperatures for approximately 1.4 h longer than a chicken embryo whose egg has a mean heating response time of 2 h. Future understanding of how egg surface area and volume interacts with heating response time of eggs, and the acid-base balance within the egg in response to thermal stress may further our understanding of temperature effects on embryos.
Northern Bobwhite ULT

Webb (1987) reported that egg temperatures >38 °C for prolonged periods of time may kill embryos and that most avian embryos perish when internal egg temperatures reach 41 °C for short durations. Comparatively, this research showed that pre-incubated bobwhite embryos exhibit a remarkable tolerance to brief hyperthermic exposures, surviving temperatures up to 50 °C for 1 h, 49 °C for 3 h and 46 °C for 6 h. The ability of bobwhite embryos to persist in a wide range of temperatures may be an adaptation to the naturally occurring temperature extremes that can occur in their naturally hot, semi-arid environment while awaiting incubation (Guthery et al., 2001; Guthery et al., 2004; Hernandez and Peterson, 2007).

The increased tolerance to high heat loads has costs. As pre-incubation temperature increased, mortality increased as a function of exposure time at a given temperature as indicated by declining LT-30, 50, 70, and 90 values (Figure 3.5). This phenomenon may describe differences in net productivity latitudinally, where the average number of juveniles per adult is 4.0 in northern U.S. populations (i.e., colder climates) and 2.3 in southern U.S. latitudes, or warmer climates (Guthery, 2002).

The thermal independence of embryonic function in temperatures ≤44 °C for short durations minimizes disruption of development associated with diel temperature extremes during pre-incubation, nest inattentiveness during incubation in hyperthermic conditions, and gives us insight into how avian eggs
and embryos may respond to changes in climate. Future research may be directed towards understanding the physiological mechanisms involved in embryo quiescence when exposed to temperature extremes. Investigations into the protein composition (e.g., the presence, quantity, or type of heat shock proteins) of surviving embryos compared to the deceased may provide a good start. Additionally, protein profiles of all embryonic stages and adult bobwhites may prove useful when examining the thermal limits imposed by climate change or drought conditions.
CHAPTER 4
DIFFERENTIAL INFLUENCE OF FLUCTUATING AND CONSTANT TEMPERATURES DURING PRE-INCUBATION ON SUBSEQUENT DEVELOPMENT OF NORTHERN BOBWHITE EMBRYOS

Introduction

Background

The global average temperature has increased 0.6 °C ± 0.2 °C since the late 19th century (Root and Schneider, 2002). This value is 0.15 °C higher than previously predicted by the Intergovernmental Panel on Climate Change (IPCC) due mostly to a higher rate of increase since 1995 compared to previous years (IPCC, 2001). Taking into account natural cycles in global climate and the unprecedented increase in greenhouse gasses in the atmosphere (Root and Schneider, 2002), scientists predict an increase in global average temperature of 5 °C ± 1 °C in the United States by the period 2071–2100 relative to the period 1961–1990 (Root and Schneider, 2002; IPCC, 2007). Concurrent with higher mean temperatures, scientists forecast increased variability in daily temperatures (Easterling et al., 1997; IPCC, 2001) and expect an increase in drought frequency for the southern portion of the United States (IPCC, 2007).

Species have responded to changes in climate throughout their evolutionary history; however, the unprecedented rate of temperature increase
with increased variability in daily temperature is of major concern to human health, wild species, and their ecosystems (EPA, 1997; Root et al., 2003; IPCC, 2007). Virtually everything an animal does is influenced by or dependent upon its thermal environment (Johnston and Bennett, 1996). Temperature influences the rates of physiological processes including metabolism, development, and growth. As such, it tremendously influences embryonic growth rate (Romanoff, 1960; Starck and Ricklefs, 1998; Tazawa and Whittow, 2000). Thus, it is not surprising that, within a zone of thermal tolerance (Chapter 3), higher mean temperatures result in higher embryonic growth rates than lower mean temperatures (French, 1997; Stoleson and Beissinger, 1999; Pendlebury et al., 2004). For viviparous species, the temperature during development is kept fairly close to optimal conditions (Nathanielsz, 1999), just as it is during incubation for oviparous species such as birds. Incubating birds maintain the temperature of their eggs relatively constant and close to optimal temperature (typically 35–38 °C) through behavioral and physiological manipulations (Webb, 1987). As a result, most studies evaluating temperature effects on avian development use a constant temperature equal to mean daily-temperatures of a particular species’ natural environment (see Webb, 1987 for review).

Constant temperatures, however, do not fully represent the daily thermal fluctuations that eggs of precocial species encounter. Many precocial birds which lay multiple eggs in a clutch (e.g., some ducks, gallinaceous birds, and ratites), deposit eggs in their nest at a rate of one egg per day, and do not begin
incubation until the penultimate or ultimate egg is laid (see Tazawa and Whittow, 2000 for review). Thus, there is an extended pre-incubation period—the focal period of this study—where eggs that are laid first are unattended and stored for the entire pre-incubation period without the thermal protection provided by an incubating parent. Egg temperatures during pre-incubation follow the diurnal thermal fluctuations of the environment (Guthery et al., 2004). The magnitude of the diurnal fluctuations is typically greater during drought years than non-drought years; in fact, potentially lethal temperatures have been experienced in ground-nests of northern bobwhites (Colinus virginianus) during drought conditions (Decuypere and Michels, 1992; Gill, 1999; Guthery et al., 2004). Eggs in ground nests could be severely impacted by further increases in temperature variability and magnitude (Chapter 3) as global warming continues (Easterling et al., 1997; IPCC, 2001; Parmesan and Yohe, 2003; Root et al., 2003).

Limited attention has been given to the effects of fluctuating temperatures on avian development. Avian studies have primarily evaluated the adult response to thermal fluctuations, finding that metabolism and energy requirements are increased under cyclic temperature regimes as compared to constant temperatures (Prinzinger, 1982; Pendlebury et al., 2004). For avian development, virtually no studies have evaluated diurnal thermal fluctuations, although a few studies have increased the pre-incubation temperature well above optimum to increase embryonic growth rate (Kosin, 1956) and discovered that constant hyperthermic temperatures alter metabolism and development (Alsop,
1919; Oppenheim and Levin, 1974; Decuypere and Michels, 1992), affect the
timing of hatch, and impact hatching success (Yeatter, 1950; Wilson et al., 1979;
Stoleson and Beissinger, 1999). Olsen et al. (2006) showed that periodic cooling
of zebra finch eggs (*Taeniopygia guttata*) experienced delayed development and
higher mass-specific metabolic rates than embryos incubated at a constant 37.5
°C. Since avian embryos act as poikilotherms *in ovo*, two studies of
poikilothermic response to fluctuating temperature revealed that larval mud crabs
(*Rhithropanopeus harrisii*) exhibit increased survivability in fluctuating
temperature regimes (Costlow Jr. and Bookhout, 1969), and skinks (*Bassiana
duperreyi*) develop at a higher rate and incur reduced incubation times in
fluctuating as compared to constant temperatures (Shine and Harlow, 1996).
These data suggest that cyclic temperature regimes influence development
differently than constant temperatures.

*Diurnal Temperature Variability in Northern Bobwhites*

This chapter investigates the impacts of diurnal temperature fluctuations
during pre-incubation on avian embryonic development (morphological and
physiological) using the northern bobwhite as the model species. The northern
bobwhite is a ground-nesting gallinaceous (chicken-like) game-bird that exhibits
an extended pre-incubation period of 12–14 d. During the entire pre-incubation
period, bobwhite eggs are potentially exposed to extremely high, fluctuating
temperatures without the thermal buffer of the incubating parent, especially
during drought years. Bobwhite egg and nest temperatures are scarce, but two
studies captured egg and nest temperatures in non-drought and simulated

drought years (Guthery et al., 2000a; Guthery et al., 2004). Internal
temperatures of non-incubated bobwhite eggs followed ambient thermal
fluctuations in non-drought years and often exceeded 40 °C during peak thermal
intensity (Guthery et al., 2004). Bobwhite nest temperatures in drought
conditions regularly peaked at 45 °C and were recorded as high as 60 °C
(Guthery et al., 2000a). Accordingly, this study uses 40 °C and 45 °C as the
peak of diurnal fluctuations in temperature to simulate non-drought and drought
conditions respectively.

Oxygen Consumption: Indicator of Development

Embryonic development is often monitored by measuring oxygen
consumption (\(\dot{V}O_2\)), an index of metabolism (Romanoff, 1940; Vleck et al., 1980).
Many factors influence \(\dot{V}O_2\), most notably body mass, oxygen availability, and
environmental temperature (Dejours, 1981). In precocial birds, the rate of whole
animal oxygen consumption (in ovo) typically increases until approximately 80%
of incubation time (Visschedijk, 1968; Vleck et al., 1980). Thereafter, the rate
slows (gradually increasing, remaining constant, or decreasing) until the embryo
internally pips into the air cell (Vleck et al., 1980) when the rate increases,
followed by another increase at the time the chick pips the shell and hatches
(Visschedijk, 1968). This study will further investigate whether the timing of the
initial peak in \(\dot{V}O_2\), pipping (internally or externally), or hatching is labile due to
exposure to fluctuating temperatures during the pre-incubation period.
Hatching Synchrony

Hatching typically occurs either asynchronously (eggs hatch sequentially over an extended period of time) or synchronously (all eggs hatch within a 24 h period) depending on the commencement time of incubation (Vleck et al., 1980). Northern bobwhites do not begin incubation until the last egg is laid (Stoddard, 1931), meaning all eggs receive equal amounts of incubation, resulting in synchronous hatching. This approach to hatching seems beneficial in that it allows the incubating parent to quickly transition behavior from incubating sessile eggs to brooding motile hatchlings (Vleck et al., 1980; Warkentin, 2007).

Pre-incubation temperature may influence hatching synchrony in wild species (Twomey, 1936). In temperate climates, cold torpor suspends development of earlier laid eggs so that once incubation begins, development and hatching occur synchronously (Drent, 1975; Ewert, 1992). Limited studies have investigated hatching synchrony in warmer climates where nest temperatures fluctuate during pre-incubation and often exceed physiological zero (the temperature above which development begins). In warmer climates, earlier-laid eggs could develop prior to incubation resulting in asynchronous development at the onset of incubation. In this case, eggs might hatch asynchronously (Stoleson and Beissinger, 1999) unless a synchronizing mechanism takes place (Vince, 1964; Vince, 1968; Vince et al., 1984; Stoleson and Beissinger, 1999). Hatching synchrony has been observed in the laboratory
among eggs that differed in developmental stages by 2 d (Vince et al., 1984), but not by 12-14 d; the probable result of northern bobwhites in warm climates.

For hatching synchrony to occur, eggs must remain in contact with each other, especially in the last 20% of incubation (Vince, 1964) when pipping occurs (Visschedijk, 1968). Regardless of relatedness or order of laying, bobwhite eggs of the same age hatch synchronously if touching (Pani et al., 1969) and, when isolated, their time of hatch is spread over a longer period (Vince, 1968). Vince (1968) found that bobwhite eggs accelerated hatching when placed in contact with more-advanced eggs (24 h advanced in development) and delayed hatching when placed in contact with more-retarded eggs (24 h retarded in development), suggesting that both an acceleration and delay of hatching time occur between earlier laid and later laid eggs in the wild.

The process of synchronization among neighboring eggs is thought to occur as a result of both audible sounds and low frequencies emitted from the eggs beginning as early as 6 d before and continuing until hatching (Freeman and Vince, 1974). Earlier in this period (3–6 d before hatch), the sounds are produced by small, irregular movements occurring throughout the incubation period (Hamburger, 1968; Freeman and Vince, 1974). Approximately 1–3 days before hatching (<2 d for chicken; 2–3 d for bobwhite), sharp and short higher frequencies are produced from beak clapping, followed by low frequency pulses introduced at the onset of pulmonary respiration (Freeman and Vince, 1974). After regular breathing has been established (about 10-15 hours before
hatching), short, intermittent click-like signals of higher amplitude occur and $\dot{V}O_2$ increases. These clicks, associated with increased $\dot{V}O_2$, are thought to be the primary accelerating stimuli in bobwhites that promote hatching synchrony (Vince, 1968; Freeman and Vince, 1974; Vleck et al., 1980; Martin, 1999). This study also investigates if fluctuating, hyperthermal treatments during pre-incubation compromises hatching synchrony.

**Objectives**

The objectives of the following experiments are to determine: 1) if avian embryos develop differently when exposed to diurnally fluctuating temperatures or a constant temperature of equal heating degree-hours, during pre-incubation; 2) if exposure to fluctuating temperature regimes during pre-incubation alters hatchability of eggs, hatching synchrony, or the duration of incubation as compared to eggs exposed to constant thermal regimes, and 3) if development or hatching is altered as a result of increased heat loads equal to simulated drought conditions and conditions predicted as a result of global warming. I hypothesized that bobwhite embryos exposed to fluctuating temperature treatments would exhibit a differential rate of development and hatching compared to those exposed to a constant temperature of the same mean value. Further, I hypothesized that an increased heat load would negatively affect development and hatching success.
Materials and Methods

Fertilized northern bobwhite eggs were collected from wild-type breeding pairs at Lake Cumberland Game Bird Farm (Mill Springs, KY, USA). Eggs were packaged and shipped to the University of North Texas (Denton, USA) on the day of collection. Eggs arrived on-site within 2–3 business days with a written record of the date and time of egg collection. Upon arrival, bobwhite eggs were randomly divided into 5 groups of 15 eggs, placed on plastic egg trays blunt end up, labeled with an indelible marker as either control or treatment and assigned a number (e.g., C-1 = Control group, egg #1). Each egg was weighed to the nearest 0.01 g with a digital scale (Ohaus Explorer Pro, Pinebrook, NJ, USA).

Lake Cumberland Game Bird Farm was approved for egg production by the United States Department of Agriculture (USDA) and certified by the USDA National Poultry Improvement Plan. This research was approved by the University of North Texas Institutional Animal Use Care Committee, protocol # 0808.

Control Incubation

Control eggs (no pre-incubation exposure) went directly into the control incubator, a G.Q.F. 1502 Sportsman incubator (G.Q.F. Manufacturing Co., Savannah, GA, USA). The temperature of the control incubator was 37.5 ± 0.5 °C with a relative humidity (RH) of 60%. Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, eggs were weighed to the nearest 0.01 g and placed in the hatching chamber of the
same incubator where they were no longer turned (after Romanoff, 1960). Hatching was determined when eggs were star-pipped, defined as an externally pipped egg where the embryo created a small hole (approximately 3 mm²) in the shell to initiate hatching (MacCluskie et al., 1997). This definition of hatching was used to compensate for any artificial hatching difficulties created by fluctuations in microclimate within the incubator. Upon star-pipping, the duration of incubation and percentage of eggs hatched was recorded. The age at the time of death was recorded for eggs that did not hatch.

**Constant and Fluctuating Treatment**

Treatment eggs were assigned to 1 of 4 groups (N=15 eggs): Low Constant (LC; 28.85 °C), Low Fluctuating (LF; 25–40 °C), High Constant (HC; 33.85 °C), or High Fluctuating (HF; 30–45 °C), based on the pre-incubation thermal regime (Table 4.1).

**Table 4.1.** Diel thermal regimes for fluctuating and constant treatments during a 12-d pre-incubation period.

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>Constant Temperature</th>
<th>Fluctuating Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low(LC)</td>
<td>High(HC)</td>
</tr>
<tr>
<td>0000–0759</td>
<td>28.85°C</td>
<td>33.85°C</td>
</tr>
<tr>
<td>0800–1059</td>
<td>28.85°C</td>
<td>33.85°C</td>
</tr>
<tr>
<td>1100–1359</td>
<td>28.85°C</td>
<td>33.85°C</td>
</tr>
<tr>
<td>1400–1659</td>
<td>28.85°C</td>
<td>33.85°C</td>
</tr>
<tr>
<td>1700–2359</td>
<td>28.85°C</td>
<td>33.85°C</td>
</tr>
</tbody>
</table>
The constant temperature chambers were kept at the intended temperatures throughout the entire 12-day pre-incubation period. Fluctuating chambers were kept at base temperatures, 25 °C (LF) and 30 °C (HF), from midnight (0000) to 0800 and were increased 5 °C at 0800, 1100, and 1400. At 1700, chamber temperatures were returned to their base temperature. These diurnal fluctuations were carried out each day of the 12-d pre-incubation period. Each treatment group remained in the assigned thermal chamber (G.Q.F. 1583 Hova-Bator with circulated air) for the entire 12-day pre-incubation, with a relative humidity (RH) of 60%. Eggs were not turned during this period, as they would not be turned in nature.

The peak temperatures of the low and high groups were selected based on thermal studies showing temperatures peaked ≥40 °C in non-drought years and ≥45 °C in simulated drought years (Guthery et al., 2000a; Guthery et al., 2004). The nature of each treatment was such that the value of the constant treatment was equivalent to the mean of the fluctuating treatment for the high and low groups. Additionally, each low group (LF and LC) received 92.4 heating degree-hours, and each high group (HF and HC) received 212.4 heating degree-hours per day for the entire pre-incubation. A heating degree-hour was defined as 1 °C above physiological zero (25 °C for northern bobwhites; Miller and Wilson, 1975) for 1 hour, such that 26 °C for 1 h = 1 heating degree-hour, and 27 °C for 1 h = 2 heating degree-hours.
Pre-incubation Development

To investigate the developmental differences during pre-incubation (if any), eggs were removed from the pre-incubation treatment the morning of the 13th day and weighed to the nearest 0.01 g to determine the amount of water loss during pre-incubation. Embryos were subsequently separated from the egg (yolk-free), weighed to the nearest 0.01 g, aged and staged according to morphological indicators of development (Hamburger and Hamilton, 1951; Hendrickx and Hanzlik, 1965; see appendix).

Treatment Incubation

Following pre-incubation, treatment groups were removed from the thermal chamber, weighed to the nearest 0.01 g, and immediately placed into the control incubator (37.5 °C; RH = 60%). Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, treatment groups were placed in separate hatching chambers and were no longer turned (Romanoff, 1960). Upon star-pipping, the duration of incubation, degree of hatching synchrony, and percentage of eggs hatched was recorded. The stage at the time of death was recorded for eggs that did not hatch.

Oxygen Consumption

On incubation days 10, 12, 14, 16, 18, 20, 22, and on 1-d post hatch, \( \dot{V}O_2 \) was recorded via flow-through respirometry as an indicator of development and timing of physiological processes (e.g., onset of pulmonary respiration). Northern bobwhite eggs (N=6) were placed in 6 of the 7 individual metabolic chambers
(Figure 4.1A) which were placed into a modified incubator (Figure 4.1B; 37.5° ± 0.5°C), as part of the flow-through respirometry system (Figure 4.2).

Figure 4.1. Metabolic chambers (modified Gerber® baby food jars) containing northern bobwhite eggs (A) were placed into a custom Plexiglas® incubator (B) as part of a flow-through respirometry system.
The system (Figure 4.2) was operated by pumping compressed normoxic air (159.22 mmHg) into a Sable System MF-8 airflow manifold (Sable Systems International, Las Vegas, NV, USA) which controlled the air flow through copper coils (to warm the air) and to the 7 metabolic chambers simultaneously. Chamber 1 was kept empty (the “blank”) and used as a reference for normoxic air, and chambers 2–7 contained bobwhite eggs. Air flow rates were adjusted to 100 ± 1 ml·min⁻¹ (Liu et al., 2008). From each chamber, air flowed to a Sable Systems multiplexor that regulated the sequence of air flow from the chambers to a column of drierite and soda lime, to remove excess water and carbon dioxide respectively. A sub-sample pump then pulled a low pressure air sample through the oxygen analyzer (Sable Systems FC-1B O₂ Analyzer). Each chamber was sampled at 5 sec consecutive intervals for 15 min. Oxygen measurements from the analyzer were sent to the computer via a Sable Systems Universal Interface II and processed by the Data acquisition system 2.0 and Datacan V data analyzer. \( \dot{V}_{O_2} \) was measured as the difference in oxygen concentration between the normoxic air flowing into the blank chamber (chamber 1) and the expired air flowing out of each treatment chamber (chambers 2–7).
Figure 4.2. Diagram of a flow-through respirometry system used for recording oxygen consumption of northern bobwhite embryos (*in ovo*). Air was pumped into 7 metabolic chambers simultaneously, and through a chamber of drierite and soda lime (DS) to remove water and CO₂. Oxygen consumption was recorded via the O₂ analyzer as the difference between the blank chamber (chamber 1) and each chamber containing an egg (chambers 2–7).
Statistical Analyses

All data was tested with a Shapiro–Wilks normality test (Zar, 1999) and Hartley’s $F_{\text{max}}$ test (Zar, 1999) before specific statistical analyses are performed. An independent t-test (Zar, 1999) was used to compare fluctuating to constant temperature groups. An ANOVA (Zar, 1999) was used to identify the relationship between treatment and control groups for all experiments. A Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to compare survival rates and time to mortality among groups. Significance between groups was determined with a Student–Newman–Keuls (SNK) multiple range post hoc test (Zar, 1999). All statistical tests were conducted using SigmaStat 3.5 software (Systat Software Inc. San Jose, CA), or by hand (Zar, 1999; Beitinger, 2006). Statistical decisions were made with a 0.05 level of probability.

Results

Pre-incubation Development

During pre-incubation, eggs of the high-constant temperature (HC) group developed the most, advancing to a mean (± S.E.) developmental stage of $21.4 \pm 0.2$, equivalent to 5.3 incubation days at 37.5 °C (Hamburger and Hamilton, 1951; Hendrickx and Hanzlik, 1965; c.f. Appendix). Eggs exposed to high-fluctuating temperatures (HF) developed to a mean stage of $13.7 \pm 0.2$ (2.2 incubation days), low-fluctuating (LF) embryos developed to a mean stage of $2.5 \pm 0.1$ (0.5 incubation days), and low-constant (LC) eggs developed the least during pre-incubation by progressing to a mean stage of $1.1 \pm 0.1$ (<0.5
incubation days). An analysis of variance indicated that mean stages of development were different among pre-incubation treatments (P<0.001). Further, a Holm-Sidak MCP confirmed that fluctuating and constant groups significantly differed (Figure 4.3) and that all groups were unique (P<0.05).

Figure 4.3. Mean developmental stages of northern bobwhite embryos after exposure to thermal regimes during a 12-d pre-incubation period. Numbers above bars indicate equivalent days of incubation. Letters indicate statistical grouping. Data are presented as mean ± S.E., N=23 eggs per regime.
Water Loss

Surprisingly, LF egg groups lost the highest percentage of fresh egg mass during the pre-incubation period with a percentage loss of 4.9% ± 0.2 (mean ± S.E.). HF eggs lost 4.5% ± 0.2, HC eggs lost an average of 4.1% ± 0.1, and LC eggs displaced the least amount of fresh egg mass during pre-incubation losing 3.2% ± 0.1. An ANOVA with a Holm-Sidak MCP indicated that mean weight loss among thermal regimes during the pre-incubation period was different between LF and LC (P<0.001), and HF and LF (P<0.001) but not between HF and HC (Figure 4.4).

Unlike pre-incubation, no difference in the percentage of fresh egg weight loss during incubation was detected between HF and HC (P=0.05) or LF and LC (P=0.88) regimes (ANOVA, P=0.88). However, there was a difference in egg weight loss between Control (11.5% ± 0.3) and HC eggs (14.9% ± 1.5) during incubation (Figure 4.4, ANOVA, P=0.01).

Combining the pre-incubation and incubation periods, all treatment groups lost a greater percentage of fresh egg mass than control (ANOVA, P<0.001). The mean percentage of fresh egg mass lost by control eggs was 11.5% ± 0.3, lower than HC (17.7% ± 1.9), HF (14.9% ± 1.5), LC (15.9 ± 0.6), and LF eggs (18.3% ± 0.7). No difference was detected in percentage of fresh egg mass lost between HF and HC (P=0.28), or LF and LC (P=0.12; Figure 4.4).
Figure 4.4. Mean (± S.E.) percentage of fresh egg weight loss for northern bobwhite eggs measured after exposure to fluctuating and constant thermal regimes for a 12-d pre-incubation period. N=45 eggs per regime. Letters indicate statistical groupings. Time periods are identified with lower-case, italicized, and capital lettering.
**Oxygen Consumption**

Oxygen consumption in LC and LF groups was similar in overall pattern throughout incubation, but absolute values differed significantly on days 10, 15, 18, 20, and 21 of incubation (Figure 4.5A; P<0.05). Slopes relating \( \dot{V}O_2 \) to development were not significantly different between LC and LF pre-incubation treatments (Figure 4.6A; t test for slopes, P>0.50). HC eggs did not survive past day 15 of incubation, so oxygen consumption was not compared between HC and HF eggs (Figure 4.5B). Slopes relating \( \dot{V}O_2 \) to development were significantly different between LF and HF pre-incubation treatments (Figure 4.6B; t-test for slopes, 0.02>P>0.001).
Figure 4.5. Oxygen consumption (mean ± S.E.) of northern bobwhite embryos as a function of development and pre-incubation thermal regimes. Statistically similar means are grouped within boxes. * No HC eggs survived past day 15.
Figure 4.6. Regressions relating oxygen consumption (mean ± S.E.) to development. No differences were exhibited between LC and LF (ANOVA; P>0.50) but were between HF and LF (B; 0.02>P>0.001). Coefficient of determination ($r^2$) and n values are shown.
**Stage at Mortality**

Mortality occurred in all groups prior to hatching. The survival rate and stage of development at the time of death was significantly different between groups (Log rank survival analysis; P<0.001). A Holm-Sidak Pairwise MCP statistically grouped the treatments receiving the lowest amounts of heating degree-hours (Control, Low-Constant, and Low Fluctuating) into one group. These low heating degree-hour groups were statistically different from the high heating degree-hour groups (High-Constant and High-Fluctuating; P<0.001, Figure 4.7). The mean survival time for LC eggs was 22.2 d ± 1.4. The low fluctuating group had a mean survival time of 22.1 d ± 0.7. These groups were not statistically different (P=0.25). The mean survival for HF eggs was 12.2 d ± 1.8 which was significantly different than HC eggs (4.47 d ± 0.3) which did not hatch during any trials (Figure 4.7).
Figure 4.7. Kaplan-Meier survival curves showing survival rate and time to mortality of northern bobwhite embryos exposed to thermal regimes during a 12-d pre-incubation. Stair-step declines show time of mortality. N=45 eggs per regime. Letters indicate statistical groupings.
Hatching Assessment

Mean hatch rates (± S.E.) for control eggs (80.8% ± 3.6) were significantly higher than all treatment groups (Figure 4.8; ANOVA, P<0.001). Hatching success of the LC treatment (63.3% ± 6.7) was not significantly different from the LF treatment (53.5% ± 6.4; P=0.24). HF hatch rates (6.0% ± 2.1) did not statistically differ (P=0.53) from HC hatch rates (0.0%). Hatch rates for LF and HF groups were statistically different (P<0.001)

The timing of specific hatching events (internally pip, externally pip, and hatch) was significantly different between control and treatment groups and within treatment groups (Figure 4.9; ANOVA, P<0.001). HF eggs internally pipped earlier (18.6 d ± 0.2) than LC eggs (19.7 d ± 0.2), LF eggs (20.5 d ± 0.2), and control eggs (20.6 d ± 0.2). Similarly, HF eggs externally pipped earlier (20.0 d ± 0.0) than LC eggs (21.5 d ± 0.3), LF eggs (21.5 d ± 0.2), and control eggs (21.7 d ± 0.2). HF eggs also hatch earlier (21.0 d ± 0.0) than control (23.0 d ± 0.0) and LF eggs (23.5 d ± 0.2), while LC eggs took longer to hatch on average than all other groups (23.8 d ± 0.1; Figure 4.9).

Also, the duration between internal pip and hatching was significantly different between groups (P<0.001). The hatching sequence was not significantly different between LC (3.7 d ± 0.4) and LF (3.2 d ± 0.1) treatments (P=0.16), but was between HF and LF (P<0.001). The duration between internal pip and hatch was the shortest for HF (2.4 d ± 0.2) and control eggs (2.4 d ± 0.2; Figure 4.9).
Finally, all eggs that hatched did so synchronously within groups. Although eggs of different groups experienced shorter or longer incubation periods (Figure 4.9), eggs within groups hatched within 24 h of each other.

Figure 4.8. Hatching success of northern bobwhite embryos exposed to thermal regimes during a 12-d pre-incubation. Mean (± S.E.) shown, N= 9 incubation trials, letters indicate statistical groupings with a 0.05 level of significance.
Figure 4.9. Time to internal and external pipping, and hatching for northern bobwhite eggs exposed to thermal treatments (LC, LF, and HF) or no treatments (Control) during a 12-d pre-incubation period. Data are presented as mean (± S.E.); sample size in parentheses. Events are statistically grouped (0.05 level of significance) with lower-case, italicized, and capital lettering.
Discussion

The present study was designed to answer two main questions: 1) Is development and hatching different between eggs exposed to fluctuating and constant temperatures during pre-incubation? 2) Is development and hatching different between groups exposed to simulated non-drought and drought conditions during pre-incubation?

Pre-incubation development

This study shows that embryos develop during pre-incubation when eggs are exposed to temperatures above physiological zero. As expected, more development was obtained with more heating degree-hours and less development occurred with fewer heating degree-hours, slightly above physiological zero. Even though eggs exposed to high heat loads (HC and HF), and low heat loads (LF and LC) had equal quantities of heating degree-hours within groups, they all exhibited differential growth during the pre-incubation. Constant regimes had the largest variation between high and low groups with about 5 d of development difference between HC and LC. The difference in fluctuating regimes was much less with about 1.7 d of development difference between HF and LF. This indicates that for avian development, the quantity of heat (e.g., number of heating degree-hours) is not the only driver of development and that the nature of the heat (constant or fluctuating) is also a major factor.
Oxygen Consumption

The nature of the thermal regime had important effects on oxygen consumption. Oxygen consumption was different between LC and LF thermal regimes during 5 different days during incubation showing differential development occurred within the incubation period (Figure 4.5A). The initial \( \dot{V}O_2 \) (day 5) and ending \( \dot{V}O_2 \) (1-day post hatch) were the same, the pattern of \( \dot{V}O_2 \) is typical of precocial species (Visschedijk, 1968), and the pattern is consistent with reports of developing northern bobwhites (Williams and Swift, 1988) and Coturnix quail, *Coturnix coturnix* (Vleck et al., 1980), a precocial bird with similar sized eggs. Additionally, the slopes of the normalized \( \dot{V}O_2 \) data were not different between LC and LF treatments (Figure 4.6A) showing no overall differences in the rate of development during incubation. Oxygen consumption in HC and HF eggs was similar between days 5–15, after which HC embryos expired (Figure 4.5B).

There were significant differences in \( \dot{V}O_2 \) between HF and LF groups logically due to HF embryos having developed more during pre-incubation (Figure 4.3), thus requiring more oxygen on any subsequent day. However, the lower slope of \( \dot{V}O_2 \) for HF eggs during incubation showed that HF eggs developed at a slower rate during incubation than LF eggs after a higher rate of development during pre-incubation (Figure 4.6B). Additionally, HF eggs did not exhibit the same fluctuating \( \dot{V}O_2 \) patterns in the last 20% of incubation as LF and LC eggs. Rather, \( \dot{V}O_2 \) of HF eggs stayed relatively constant during the pipping
process (Figure 4.5B). These results indicate differential development as a result of pre-incubation treatments.

**Hatching Assessment**

Eggs exposed to high fluctuating temperatures internally pipped, externally pipped, and hatched two days earlier than all other groups, ultimately showing a reduced incubation period (Figure 4.9). This should not be surprising since HF eggs developed approximately 2-days more than LC and LF groups during pre-incubation. Contrary to that reasoning, LC and LF eggs, which developed approximately 0.5 days during pre-incubation, exhibited an extended incubation time of 0.5 d (Figure 4.9). The reduced incubation duration of HF eggs is perhaps an adaptation to thermally stressful environments that northern bobwhites inhabit. However, the extended incubation period of 0.5 d exhibited by LC and LF eggs, while interesting, is probably not biologically important in nature.

Hatching success between constant and fluctuating groups was not statistically different; however HC groups did not hatch, dying at approximately 4–15 days of age (Figure 4.7). The death of HC embryos is noteworthy, since eggs were kept for 12-d at 33.85 °C, well within the natural incubating range of 30–35 °C witnessed in nature (Guthery et al., 2004). While the objective of this study was not to determine the cause of death in constant eggs, I hypothesize that constant temperatures near incubation temperature require eggs to be turned daily since studies have shown that turning of eggs is not required when eggs are stored at 12.8 °C, but is required during incubation temperatures
between 35–38 °C (Romanoff, 1960; Proudfoot, 1969; Wilson, 1991b). This anomaly was not experienced in LC groups (28.85 °C) presumably because the mass of the embryo was <0.5 d of age, a much smaller embryo, and thus was not affected by the absence of turning as turning prevents larger embryos from sticking to inner-egg membranes.

The difference in hatching success between HC (0.0%) and HF (6.0% ± 2.1), while not statistically significant, is certainly biologically important. A 6% hatch rate in nature is more desirable than a 0% hatch rate. Additionally, the high fluctuating temperature regime (HF) simulates nest temperatures during drought conditions. A 6% hatch rate during a drought would be much better than no hatching at all for purposes of sustaining the population.

Perhaps one of the most important findings is the difference in hatch rates between high-fluctuating (HF) and low-fluctuating (LF) groups. The HF group had 6.0% ± 2.1 while the LF group hatched 53.5% ± 6.4 (Figure 4.8). The intention of comparing LF (simulated non-drought temperatures) and HF (simulated drought temperatures) groups was to better understand the differences between drought and non-drought conditions as well as the potential impacts of global warming on avian development. The HF thermal regime is a +5 °C thermal regime as compared to the LF regime, coincident with the predicted increase in global average temperature of 5 °C ± 1 °C in the United States by the period 2071–2100 relative to the period 1961–1990 (Root and Schneider, 2002; IPCC, 2007). These results show that a +5 °C increase in pre-incubation
temperature, for just 12 days, reduces the hatch rate of northern bobwhites by approximately 47.5%. These findings indicate that reductions in the percentage of juveniles at different latitudes (Guthery, 2000) or during drought (Bridges et al., 2001; Reyna, 2008) could be caused by increased heat loads during the 12-d pre-incubation period. Additionally, these results suggest that the nature (constant or fluctuating) and magnitude of diel temperature should be considered when evaluating the biological response to predicted temperatures of global warming.

These results also suggest that fluctuating temperatures act differently on developing organisms than constant temperatures of equal heating degree-hours. The impact of these differences is increased as heat loads are increased, i.e., higher heating degree-hours result in more variation in development in embryos exposed to constant and fluctuating temperatures. Further, within fluctuating regimes, higher fluctuating heat loads caused an increase in the rate of development during pre-incubation, a decrease in rate of development during incubation and had more of a negative effect on hatching.

With increased variations in daily temperatures predicted with climate change, and more frequent droughts predicted in the southern U.S., understanding how developing vertebrates respond differently to fluctuating and constant temperatures may shed light on how avian species or vertebrates in general respond to thermal stress. Eggs in ground nests could be severely impacted by further increases in temperature variability and magnitude as global
warming continues (Easterling et al., 1997; IPCC, 2001; Parmesan and Yohe, 2003; Root et al., 2003). Additionally, the effects of thermal fluctuations should be considered in future investigations into the impact of predicted climate change on developing organisms.
CHAPTER 5
CRITICAL PERIODS OF PRE-INCUBATION IN DEVELOPING
NORTHERN BOBWHITES

Introduction

Pre-programmed Avian Development

From the instant of ovum fertilization, development of the genetically programmed zygote begins in the avian egg. As the fertilized egg passes through the oviduct it is coated with multiple layers of membrane that serve the embryo by providing a means of nourishment, protection, respiration, and segregation of waste products (Aristotle, 350 B.C.; Romanoff, 1960; Proudfoot, 1969; Webb, 1987; Gill, 1999). Shortly after the membranes are established and the hard outer-shell is in place, the egg is laid as an autonomous entity whose development and survival is directed by the interaction of its genome and inner- and outer-egg environments (Burggren, 1998; Gilbert, 2006).

The embryo’s genome provides the instructional program for its growth, and development (Newman and Muller, 2006). However, a multitude of environmental factors (physical and biotic) act upon the embryos genome, potentially causing aberrant development (see West-Eberhard, 1989; Burggren, 1998), changes in the timing of developmental processes
(Spicer and Burggren, 2003), or even death. The timing of environmental factors can be chronic or acute, and can change over time. When environmental perturbations vary temporally, species that invoke adaptive responses such as developmental plasticity (transformations in morphology, physiology, or behavior of the developing organism; West-Eberhard, 1989) might have a selective advantage over species that do not; results that could affect the life history and evolution of the species (West-Eberhard, 1989; Root and Schneider, 2002).

**Temperature Drives Development**

One of the most important environmental forces acting on living organisms is temperature (Chapters 3 and 4). Known to drive metabolic processes, temperature plays a vital role in the ontogeny of avian species (Romanoff, 1960). Aristotle, one of the first known embryologists, noticed environmental temperature affected the livelihood and duration of incubation of chicken eggs (*Gallus gallus*). Certainly, the thermal dependence of avian development is illuminated by artificial incubation, which has been practiced since 250 B.C. when Chinese villagers incubated bird eggs in dung heaps for personal use (referenced in Aristotle, 350 B.C.). The idea of artificial incubation and the thermal dependence of eggs was later expanded upon by Reaumur (1750) and Bucknell (1839) who were able to produce chickens in mass quantities by incubating eggs year-round in clay and wood-burning ovens with particular attention given to maintaining incubation temperature.
Much of the 20th century research investigating the relationship between temperature and avian development largely focused on determining the optimum incubation temperatures to achieve the highest hatchability of chicken eggs (Alsop, 1919; Romanoff, 1960; Kosin, 1964; Landauer, 1967; Decuypere and Michels, 1992; French, 1997; Conway and Martin, 2000; Christensen et al., 2003). Incubation temperature is generally thought to be highly predictive of embryonic growth efficiency (Henderson and Brody, 1927; Romanoff, 1936) and most ideal for incubating avian species within the range of 35–38 °C (Romanoff, 1960; Kosin, 1964; Landauer, 1967; Webb, 1987; Decuypere and Michels, 1992). Within this ideal range, an increase in incubation temperature results in decreased incubation time, increased growth of embryos, and increased water loss (Henderson, 1971; Wilson, 1991a; Decuypere and Michels, 1992). Decreases in incubation temperature within the range of 35–38 °C reduced water loss but increased incubation time (Romanoff, 1936; Romanoff, 1960; Wilson, 1991b).

Although there are interspecific differences in incubation temperature (Drent, 1975; Hoyt, 1980; Wilson, 1991b; French, 1997), even slight deviations from optimum temperature within a species can disrupt embryonic development and affect hatchability of eggs (Romanoff, 1960; Dawson, 1984; Wilson, 1991b; Gill, 1999; Chapter 3). An internal egg temperature >41 °C resulted in developmental abnormalities and reduced hatchability in the chicken (Alsop, 1919; Romanoff et al., 1938). Indeed, most avian embryos exhibit
developmental abnormalities when briefly exposed to 39 °C, just slightly out of the optimum range for incubation (for interspecific reviews see Webb, 1987; Decuypere and Michels, 1992; French, 1997; Christensen, 2001).

**Fluctuating Temperatures**

The aforementioned studies utilized constant temperatures during incubation to model the relationship between temperature and avian development. However, constant temperatures do not fully represent the thermal exposure that most eggs of precocial avian species encounter prior to incubation (Chapter 4). Many precocial birds which lay multiple eggs in a clutch (primarily Anseriformes and Galliformes such as ducks, *Anas platyrhynchos*, and northern bobwhites, *Colinus virginianus*), deposit eggs in their nest at a rate of one egg per day, and do not begin incubation until the penultimate or ultimate egg is laid (see Tazawa and Whittow, 2000 for review). Thus, there is an extended pre-incubation period—the focal period of this study—where eggs that are laid first are unattended and stored for the entire pre-incubation period without the thermal protection provided by an incubating parent. Egg temperatures during pre-incubation follow the diurnal thermal fluctuations of the environment (Guthery et al., 2004). The peak temperature of the diurnal fluctuations, for species like northern bobwhites, is often greater during drought years than non-drought years; in fact, potentially lethal temperatures (Chapter 3) have been experienced in ground-nests during drought conditions (Decuypere and Michels, 1992; Gill, 1999; Guthery et al., 2004).
Environmental temperature perturbations (e.g., temperature fluctuations) may have different effects on the ontogeny of vertebrates depending on the timing of exposure (Romanoff, 1949; Romanoff, 1960; West-Eberhard, 1989; Burggren, 1998; Christensen, 2001; Tzschentke, 2008). Romanoff (1949; 1960) reviewed several studies evaluating thermal tolerance of chicken embryos and suggested that they were more susceptible to thermal stress at different stages of incubation. Similarly, Christensen (2001) and Tzschentke (2008) both demonstrated that the effects of increased incubation temperature were time dependent.

Time or age dependent results suggest that critical periods of development exist (Romanoff, 1949; West-Eberhard, 1989; Burggren, 1998; Dzialowski et al., 2002) in which an embryo is most susceptible to experiencing developmental change (morphological or physiological) or death due to exposure to environmental stressors (e.g., hyperthermal conditions). Development—as referred to in this study—is phenotypic change of an individual from zygote to hatching and occurs in response to the interaction of the genome and environment (West-Eberhard, 1989; Newman and Muller, 2006). Critical periods of development are the result of periods of phenotypic readiness to respond to environmental inputs (West-Eberhard, 1989).
**Model Species for Thermal Tolerance: Northern Bobwhite**

Previous studies utilized constant temperatures to examine the age-specific developmental response to hyperthermia during avian incubation. In the present study, I tested for critical periods of responsiveness in the developing northern bobwhite during pre-incubation, using fluctuating temperatures as an environmental input.

The northern bobwhite was used as a model species for this study since it is a ground-nesting gallinaceous game-bird that exhibits an extended pre-incubation period. Bobwhite hens lay one egg per day for approximately 12–14 d (Stoddard, 1931; Lehmann, 1984; Rosebererry and Klimstra, 1984), during which deposited eggs remain stored in the nest, largely unattended, until the hen begins incubation ≤7 d after laying is complete (Stoddard, 1931). During the pre-incubation period, bobwhite eggs are potentially exposed to extremely high, fluctuating temperatures without the thermal buffer of the incubating parent, especially during drought years.

In the southern portion of the bobwhites range population numbers fluctuate with climate; increasing or remaining constant in non-drought years, and decreasing in drought years (Bridges et al., 2001; Guthery et al., 2001; Reyna, 2008). In non-drought years, internal temperatures of non-incubated bobwhite eggs followed ambient temperature fluctuations and often exceeded 40 °C during peak thermal intensity (Guthery et al., 2004). Bobwhite nest temperatures in simulated drought conditions regularly peaked at 45 °C and were recorded as
high as 60 °C (Guthery et al., 2000a). By evaluating the developmental response to hyperthermia during early, middle, and late stages of pre-incubation, insight may be gained into whether avian embryos are susceptible to chronic and acute doses of hyperthermic fluctuations, as experienced in the nest, and whether the response is stage dependent.

**Oxygen Consumption: Indicator of Development**

Embryonic development is often monitored by measuring oxygen consumption, an index of metabolism (Romanoff, 1940; Vleck et al., 1980). Many factors influence oxygen consumption (\(\dot{V}O_2\)), most notably body mass, oxygen availability, and environmental temperature (e.g., Dejours, 1981). In precocial birds, the rate of whole animal oxygen consumption (in ovo) typically increases until approximately 80% of incubation time (Visschedijk, 1968; Vleck et al., 1980). Thereafter, the rate slows (gradually increasing, remaining constant, or decreasing) until the embryo internally pips into the air cell (Vleck et al., 1980) when the rate increases, followed by another increase at the time the chick pips the shell and hatches (Visschedijk, 1968). This study will further investigate whether the timing of the initial peak in \(\dot{V}O_2\), pipping (internally or externally), or hatching is variable due to the timing of exposure to fluctuating temperatures during the pre-incubation period.

**Hatching Synchrony**

Hatching is a life changing developmental event (Warkentin, 2007) that typically occurs either asynchronously (eggs hatch sequentially over an extended
period of time) or synchronously (all eggs hatch within a 24 hr period) mostly depending on the commencement time of incubation (Vleck et al., 1980; Vince et al., 1984). Northern bobwhites, for example, do not begin incubation until the last egg is laid (Stoddard, 1931), meaning all eggs receive equal amounts of incubation, resulting in synchronous hatching. This type of hatching seems beneficial in that it allows the incubating parent to quickly transition behavior from incubating sessile eggs to brooding motile hatchlings (Vleck et al., 1980; Warkentin, 2007).

The nature and magnitude of ambient temperature (Chapter 4) during pre-incubation could influence hatching synchrony in wild species (Twomey, 1936). In temperate climates, cold torpor suspends development of earlier laid eggs so that once incubation begins, development and hatching occur synchronously (Drent, 1975; Ewert, 1992). Few studies have investigated hatching synchrony in warmer climates where nest temperatures fluctuate during pre-incubation (chapter 4) and often exceed physiological zero (the temperature above which development begins). In warmer climates, earlier-laid eggs presumably develop prior to incubation (Chapter 4) resulting in asynchronous development at the onset of incubation if the quantity of heating degree-hours is not equal among eggs. In this case, eggs might hatch asynchronously (Stoleson and Beissinger, 1999) unless a synchronizing mechanism takes place (Vince, 1964; Vince, 1968; Vince et al., 1984; Stoleson and Beissinger, 1999).
Although hatching synchrony is primarily a function of the interaction of pre-incubation temperature and the timing of incubation commencement, it has been observed in the common rhea, *Rhea americana*, which begins incubation prior to the last egg being laid and still achieves hatching synchrony (Vleck et al., 1985). Additionally, hatching synchrony has been observed in the laboratory among northern bobwhite eggs that differed in developmental stages by 2 d, (Vince et al., 1984), but not by 12-14 d, the duration of pre-incubation for the northern bobwhite in nature.

For hatching synchrony to occur, eggs must remain in contact with each other, especially in the last 20% of incubation (Vince, 1964) when hatching begins with pipping (Visschedijk, 1968). Regardless of relatedness or order of laying, bobwhite eggs of the same age hatch synchronously if touching (Pani et al., 1969) and, when isolated, their time of hatch is spread over a longer period (Vince, 1968). Vince (1968) found that bobwhite eggs accelerated hatching when placed in contact with more-advanced eggs (24 h advanced in development) and delayed hatching when placed in contact with more-retarded eggs (24 h retarded in development), suggesting that both an acceleration and delay of hatching time occur between earlier laid and later laid eggs in the wild.

The mechanism for synchronization involves both audible sounds and low frequencies emitted from the eggs beginning as early as 6 d before and continuing until hatching (Freeman and Vince, 1974). Earlier in this period (3–6 d before hatch), the sounds are produced by small, irregular movements occurring
throughout the incubation period (Hamburger, 1968; Freeman and Vince, 1974). Approximately 1–3 days before hatching (<2 d for chicken; 2–3 d for bobwhite), sharp and short higher frequencies are produced from beak clapping, followed by low frequency pulses introduced at the onset of pulmonary respiration (Freeman and Vince, 1974). After regular breathing has been established (about 10-15 hours before hatching), short, intermittent click-like signals of higher amplitude occur and \( \dot{V}o_2 \) increases. These clicks, associated with increased \( \dot{V}o_2 \), are thought to be the primary accelerating stimuli in bobwhites that promote hatching synchrony (Vince, 1968; Freeman and Vince, 1974; Vleck et al., 1980; Martin, 1999). This study also investigates if exposure to fluctuating, hyperthermal treatments during different periods within pre-incubation compromises hatching synchrony.

**Objective and Hypothesis**

This study tested for critical periods of development during the pre-incubation period—to my knowledge the first to do so—to determine whether a time or stage dependent response to hyperthermic temperatures exists by exposing fertilized bobwhite eggs to chronic and acute doses of high fluctuating heat during 3 different periods of the pre-incubation period. I hypothesized that equal heating degree-hours given in different periods of the pre-incubation period will induce differential rates of development and effect hatchability and hatching synchrony.
Materials and Methods

Fertilized northern bobwhite eggs were collected from wild-type breeding pairs at Lake Cumberland Game Bird Farm (Mill Springs, KY, USA). Eggs were packaged and shipped to the University of North Texas (Denton, USA) on the day of collection. Eggs arrived on-site within 2–3 business days with a written record of the date and time of egg collection. Upon arrival, bobwhite eggs were randomly divided into 5 groups of 15 eggs, placed on plastic egg trays blunt end up, labeled with an indelible marker as either control or treatment and assigned a number (e.g., C-1 = Control group, egg #1). Each egg was weighed to the nearest 0.01 g with a digital scale (Ohaus Explorer Pro, Pinebrook, NJ, USA).

Lake Cumberland Game Bird Farm was approved for egg production by the United States Department of Agriculture (USDA) and certified by the USDA National Poultry Improvement Plan. This research was approved by the University of North Texas Institutional Animal Use Care Committee, protocol # 0808.

Control Incubation

Control eggs went directly into the control incubator, a G.Q.F. 1502 Sportsman incubator (G.Q.F. Manufacturing Co., Savannah, GA, USA) and did not receive any pre-incubation treatment. The temperature of the control incubator was 37.5 ± 0.5 °C with a relative humidity (RH) of 60%. Eggs were turned automatically every 4 h for the first 19 days of the 23-d incubation (Romanoff, 1960). On day 20, eggs were weighed to the nearest 0.01 g and
placed in the hatching chamber of the same incubator where they remained in physical contact (Vince et al., 1984) and were no longer turned (after Romanoff, 1960). Hatching was determined when eggs were star-pipped, defined as an externally pipped egg where the embryo created a small hole (approximately 3 mm²) in the shell to initiate hatching (MacCluskie et al., 1997). This definition of hatching was used to compensate for any artificial hatching difficulties created by fluctuations in microclimate within the incubator. Upon star-pipping, the duration of incubation and percentage of eggs hatched was recorded. The age at the time of death was recorded for eggs that did not hatch.

**Experimental Design**

The 12-d pre-incubation period (PI) was divided into thirds: PI days 1–4, PI days 5–8, and PI days 9–12. During each third of pre-incubation, treatment eggs were exposed to either Low Fluctuating (LF) or High Fluctuating (HF) thermal regimes (Table 5.11).

Table 5.1. Schedule for diurnally fluctuating thermal treatments during a 12-d pre-incubation period.

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>Fluctuating Thermal Regime</th>
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<tbody>
<tr>
<td></td>
<td>Low(LF)</td>
</tr>
<tr>
<td>0000–0759</td>
<td>25°C</td>
</tr>
<tr>
<td>0800–1059</td>
<td>30°C</td>
</tr>
<tr>
<td>1100–1359</td>
<td>35°C</td>
</tr>
<tr>
<td>1400–1659</td>
<td>40°C</td>
</tr>
<tr>
<td>1700–2359</td>
<td>25°C</td>
</tr>
</tbody>
</table>
Treatment groups were placed in either the HF or LF thermal chamber (G.Q.F. 1583 Hova-Bator with circulated air) according to the pre-incubation schedule (Table 5.2) which was based on the timing, or absence, of exposure to a HF thermal regime during pre-incubation: chronic low fluctuating (LF), chronic high fluctuating (HF), Hyperthermic I (H1; HF during PI days 1–4), Hyperthermic II (H2; HF during PI days 5–8), or Hyperthermic III (H3; HF during PI days 9–12).

Table 5.2. Pre-incubation schedule used to expose northern bobwhite eggs to fluctuating thermal regimes.

<table>
<thead>
<tr>
<th>Group</th>
<th>PI days 1–4</th>
<th>PI days 5–8</th>
<th>PI days 9–12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Fluctuating (LF)</td>
<td>25–40°C</td>
<td>25–40°C</td>
<td>25–40°C</td>
</tr>
<tr>
<td>Hyperthermic I (H1)</td>
<td>30–45°C</td>
<td>25–40°C</td>
<td>25–40°C</td>
</tr>
<tr>
<td>Hyperthermic II (H2)</td>
<td>25–40°C</td>
<td>30–45°C</td>
<td>25–40°C</td>
</tr>
<tr>
<td>Hyperthermic III (H3)</td>
<td>25–40°C</td>
<td>25–40°C</td>
<td>30–45°C</td>
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<tr>
<td>High Fluctuating (HF)</td>
<td>30–45°C</td>
<td>30–45°C</td>
<td>30–45°C</td>
</tr>
</tbody>
</table>

Fluctuating chambers were kept at base temperatures, 25 °C (LF) and 30 °C (HF), from 12:00 (midnight) to 08:00 and were increased 5 °C at 08:00, 11:00, and 14:00. At 17:00, chamber temperatures were returned to their base temperature. These diurnal fluctuations were carried out each day of the 12-d pre-incubation period. Eggs were not turned during this period, as they would not be turned in nature.
The peak temperatures of the low and high groups were selected based on nesting studies showing temperatures peaked ≥40 °C in non-drought years and ≥45 °C in simulated drought years (Guthery et al., 2000a; Guthery et al., 2004). The nature of each treatment was such that the LF treatment exposed eggs to 92.4 heating degree-hours per day, and the HF treatment exposed eggs to 212.4 heating degree-hours per day (chapter 4). A heating degree-hour was defined as 1 °C above physiological zero (25 °C for northern bobwhites; Miller and Wilson, 1975) for 1 hour, such that 26 °C for 1 h = 1 heating degree-hour, and 27 °C for 1 h = 2 heating degree-hours.

Pre-incubation Development

To investigate the possibility of differential development among treatments during pre-incubation, eggs were removed from the pre-incubation treatment the morning of the 13th day and weighed to the nearest 0.01 g to determine the amount of water loss during pre-incubation. Embryos were subsequently separated from the egg (yolk-free), weighed to the nearest 0.01 g, aged and staged according to morphological development markers (Hamburger and Hamilton, 1951; Hendrickx and Hanzlik, 1965; Appendix).

Treatment Incubation

Following pre-incubation, treatment groups were removed from the thermal chamber, weighed to the nearest 0.01 g, and immediately placed into the control incubator (37.5 °C; RH = 60%). Eggs were turned automatically every 4 h for the first 19 days of the 23-day incubation (Romanoff, 1960). On day 20,
treatment groups were placed in separate hatching chambers where they were kept in physical contact with one another (Vince et al., 1984) and were no longer turned (Romanoff, 1960). Upon star-pipping, the duration of incubation, degree of hatching synchrony, and percentage of eggs hatched was recorded. The age at the time of death was recorded for eggs that did not hatch.

**Oxygen Consumption**

On incubation days 10, 15, 17–23, and on 1-d post hatch, \( \dot{V}O_2 \) was recorded via flow-through respirometry (see chapter 4 for description of methodology) as an indicator of development and timing of physiological process (e.g., onset of air breathing). Northern bobwhite eggs (N=6) were placed in 6 of the 7 individual metabolic chambers (Figure 5.1 A) which were placed into a modified incubator (Figure 5.1 B; 37.5° ± 0.5°C), as part of the flow-through respirometry system (Figure 5.2).
Figure 5.1. Metabolic chambers (modified Gerber® baby food jars) containing northern bobwhite eggs (A) were placed into a custom Plexiglas® incubator (B) as part of a flow-through respirometry system.
Figure 5.2. Diagram of a flow-through respirometry system used for recording oxygen consumption of northern bobwhite embryos (in ovo). Air was pumped into 7 metabolic chambers simultaneously, and through a chamber of drierite and soda lime (DS) to remove water and CO₂. Oxygen consumption was recorded via the O₂ analyzer as the difference between the blank chamber (chamber 1) and each chamber containing an egg (chambers 2–7).
**Statistical Analyses**

All data were tested with a Shapiro–Wilks normality test (Zar, 1999) and Hartley's $F_{\text{max}}$ test (Zar, 1999) before specific statistical analyses are performed. An independent t-test (Zar, 1999) was used to compare fluctuating to constant temperature groups. A one-way ANOVA (Zar, 1999) was used to identify the relationship between treatment and control groups for all experiments. A Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to compare survival rates and time to mortality among groups. Significance between groups was determined with a Student–Newman–Keuls (SNK) multiple range post hoc test (Zar, 1999). All statistical tests were conducted using SigmaStat 3.5 software (Systat Software Inc. San Jose, CA), or by hand (Zar, 1999; Beitinger, 2006). Statistical decisions were made with a 0.05 level of probability.

**Results**

**Pre-incubation Development**

Eggs exposed to chronic high-fluctuating temperature (HF) received the most heating degree-hours during pre-incubation (2,548.8) and developed the most, which advanced them to developmental stage $13.7 \pm 0.2$, equivalent of 2.2 d of incubation at a constant 37.5 °C (Hamburger and Hamilton, 1951; Hendrickx and Hanzlik, 1965; c.f. Appendix). Eggs exposed to chronic low-fluctuating temperature (LF) received 1,108.8 heating degree-hours during pre-incubation which advanced them to the equivalent of 0.5 d of incubation (developmental stage $2.4 \pm 0.2$; Figure 5.3). However, eggs in the acute dose groups (H1, H2,
and H3) displayed differential development during pre-incubation even though they each received 1,588.8 heating degree-hours (Figure 5.3). Eggs in the H1 and H2 groups were more responsive to the hyperthermic conditions, advancing to stages 6.5 ± 1.1 (equivalent to 1.1 incubation days) and 5.7 ± 1.0 (equivalent to 1.0 d of incubation). H3 eggs, receiving hyperthermic treatment in the last 3rd of pre-incubation, were less responsive to thermal stress and only advanced to stage 2.6 ± 0.1, the equivalent of 0.5 d of incubation at a constant 37.5 °C (Figure 5.3).
Figure 5.3. Mean developmental stages of northern bobwhite embryos after exposure to thermal regimes during a 12-d pre-incubation period. Numbers above bars indicate equivalent days of incubation. Letters indicate statistical groupings. Data are presented as mean ± S.E., N=23 eggs per regime.
**Water Loss**

The difference in mean percent fresh egg weight loss during pre-incubation among treatment groups was not significantly different (P=0.22, ANOVA). LF eggs lost 4.9% ± 0.1 of fresh egg mass during pre-incubation. H2 eggs lost 4.8% ± 0.3, H3 eggs lost an average of 4.5% ± 0.3, H1 eggs lost 4.2% ± 0.3, and HF eggs lost 4.4% ± 0.1 (Figure 5.4).

During incubation, LF eggs lost the highest percentage of fresh egg mass (13.4% ± 0.6; P<0.001, ANOVA). Control and HF eggs lost statistically the same percentage of fresh egg mass, 11.5% ± 0.2 and 11.1% ± 1.0 respectively. H1, H2 and H3 egg groups lost statistically similar percentages of fresh egg mass, 8.5% ± 0.2, 8.4% ± 0.2, and 8.4% ± 0.3 respectively (Figure 5.4).

Combining the pre-incubation and incubation periods, HF and LF eggs lost a greater percentage of fresh egg mass than H1, H2, H3, and control eggs (Figure 5.4; P<0.001, ANOVA). The total percentage of fresh egg mass was highest in LF eggs (18.3% ± 0.7; P<0.001). HF eggs lost a total of 14.9% ± 1.4, which was statistically the same as H1 (12.7% ± 0.4), H2 (13.2% ± 0.5), and H3 (12.9% ± 0.4) groups (Figure 5.4)
Figure 5.4. Mean percentage of fresh egg weight loss for northern bobwhite eggs exposed to chronic and acute fluctuating thermal regimes during a 12-d pre-incubation period. N=45 eggs per regime. Letters indicate statistical groupings with a 0.05 level of significance. Time periods are identified with lower-case, italicized, and capital lettering.
Oxygen Consumption

Oxygen consumption was similar in overall pattern throughout incubation between chronic (HF and LF) and acute exposure eggs (H1, H2, and H3; Figure 5.5). However, the absolute values differed among groups where \( \dot{V}O_2 \) of HF eggs was higher than all other groups on day 10 (P<0.001, ANOVA). On day 15, \( \dot{V}O_2 \) was statistically divided into 3 groups where HF and control were equal and higher than LF eggs whose \( \dot{V}O_2 \) was higher than the acute groups (H1, H2, and H3; statistically equal). On day 18, LF eggs consumed more oxygen on average than all other groups (P=0.01). For all other time periods, oxygen consumption among groups was not statistically significant (Figure 5.5).

Stage at Mortality

Mortality occurred in all groups prior to hatching. The survival rate and stage of development at the time of death was significantly different between groups (Figure 5.6; Log rank survival analysis, P<0.001). A Holm-Sidak Pairwise MCP statistically grouped the low fluctuating group with a mean survival time of 22.1 d ± 0.07 with the control group which had a survival time of 22.7 d ± 1.3 (P=0.05). Although different from control, survival time of H1 (18.4 ± 1.5), H2 (16.1 ± 2.1), and H3 (17.1 ± 1.8) were not statistically different (P=0.98), and were grouped with the low fluctuating group (P=0.05). Survival of HF (12.2 ± 1.9) embryos was statistically different from all other groups (P=0.02; Figure 5.6).
Figure 5.5. Oxygen consumption (mean ± S.E.) of northern bobwhite embryos as a function of development and pre-incubation thermal regimes. Statistically similar means are grouped within boxes.
Figure 5.6. Kaplan-Meier survival curves showing survival rate and time to mortality of northern bobwhite embryos exposed to thermal regimes during a 12-d pre-incubation. Stair-step declines show time of mortality. N=45 eggs per regime. Letters indicate statistical groupings.
Hatching Assessment

Mean hatch rates (± S.E.) for control eggs (80.7% ± 3.1) were significantly different from all treatment groups (Figure 5.7; ANOVA, P<0.001). Hatching success between the HF (5.9% ± 2.0) and LF treatment (53.4% ± 6.3) was significantly different (P<0.001). Mean hatch rates were not significantly different among the groups receiving acute doses of high heat (P=0.48): H1 (21.7% ± 4.4), H2 (32.5% ± 8.9), and H3 (22.9% ± 5.7).

Figure 5.7. Mean hatching success of northern bobwhite eggs exposed to thermal regimes during pre-incubation. Mean (± S.E.) shown, N= 9 incubation trials, letters indicate statistical groupings.
The mean time to internal pipping (i.e., the onset of pulmonary respiration) was affected by pre-incubation treatment with a significant difference between control and treatment groups and within treatment groups (Figure 5.8; ANOVA, P<0.001). The mean time to internally pip for HF eggs was 18.3 d ± 0.2, significantly earlier (P=0.007) than H1 eggs (19.8 d ± 0.2), H2 eggs (19.3 d ± 0.1), and H3 eggs (19.3 d ± 0.3) which were not statistically different (Figure 5.8; P=0.06). The mean time to internally pip for LF eggs was 20.08 d ± 0.21, which was not significantly different from control eggs (20.2 d ± 0.1; P=0.64, Figure 5.8).

The mean (± S.E.) time to external pipping was significantly different between control and treatment groups and within treatment groups (Figure 5.8; ANOVA, P<0.001). Control eggs externally pipped at 21.5 d ± 0.1, statistically equal to LF eggs (21.6 d ± 0.1), and H1 eggs (21.0 d ± 0.3). H2 eggs had a mean time to external pip of 20.6 d ± 0.1 which was not statistically different than H3 eggs (20.5 d ± 0.2) or HF eggs (20.1 d ± 0.3; Figure 5.8).

The mean (± S.E.) time to hatch was significantly different between control and treatment groups and within treatment groups (Figure 5.8; ANOVA, P<0.001). The mean (± S.E.) time to hatch for HF eggs was 21.0 d ± 0.0, which was not different than H1 eggs (21.6 d ± 0.3), H2 eggs (22.4 d ± 0.2), and H3 eggs (22.0 d ± 0.2). Control eggs hatched at 23.0 d ± 0.0, which was not statistically different from LF eggs (23.2 d ± 0.1) or H1 eggs (Figure 5.8).
Figure 5.8. Time to internal and external pipping, and hatching for northern bobwhite eggs exposed to thermal treatments during a 12-d pre-incubation period. Data are presented as meant (± S.E.). Letters indicate statistical groupings, sample size in parentheses.
Mean (± S.E.) duration between internal pip and external pip was significantly different among thermal groups (Figure 5.9; P<0.001, ANOVA). HF eggs took 1.8 d ± 0.1 to pip externally after internally pipping, significantly longer than any other group (P<0.001, Figure 5.9). The mean time for LF eggs to externally pip was 1.6 d ± 0.0, which was longer than H1 (1.2 d ± 0.1), H2 (1.3 d ± 0.0), and H3 (1.2 d ± 0.0) treatments, but statistically the same as control (1.4 d ± 0.0).

Mean (± S.E.) duration between external pip and hatch was significantly different among thermal groups too (Figure 5.9; P<0.001, ANOVA). HF eggs (0.9 d ± 0.2) and H1 eggs (0.5 d ± 0.0) exhibited the shortest duration between external pip and hatch (P<0.05). H2 eggs (1.9 d ± 0.0) took longer than control (1.5 d ± 0.1), but was not statistically different than H3 (1.5 d ± 0.0) and LF egg (1.6 d ± 0.0).

Finally, the duration between internal pip and hatching was significantly different among groups experiencing different treatments during pre-incubation (Figure 5.9; P<0.001). LF (3.2 d ± 0.0) and H2 (3.1 d ± 0.1) eggs took the longest period of time to proceed from internally pipped to hatch (P<0.001), followed by control (2.8 d ± 0.1), H3 (2.7 d ± 0.0), and HF (2.7 d ± 0.1). H1 took the least amount of time to execute hatching, taking 1.7 d ± 0.1.
Figure 5.9. Duration of hatching events of northern bobwhite eggs exposed to pre-incubation thermal regimes. Data are presented as mean (± S.E.). Letters indicate statistical groupings.
Hatching Synchrony

Eggs of the high-fluctuating (HF), low-fluctuating (LF), and control groups hatched synchronously within groups, i.e., they hatched within a 24-hour period. The acute exposure groups (H1, H2, and H3) received the same amount of heating degree-hours during pre-incubation; however, H3 is the only group that consistently exhibited hatching synchrony. H1 eggs regularly hatched within the range of 20–24 days, and H2 eggs repeatedly hatched across the range of 21–24 d.

Hatchling Morphology

Mean hatchling wet mass was statistically different among treatment groups experiencing different pre-incubation treatments (P=0.009, ANOVA). Control (7.04 g ± 0.17) and LF (7.05 g ± 0.12) hatchlings were the heaviest and not statistically different. Acute exposure groups had significantly lower mean wet mass than control and LF groups: H1=6.26 g ± 0.20, H2=6.11 g ± 0.24, and H3=6.40 g ± 0.29. HF hatchlings (6.65 g ± 0.17) had lower masses than control and LF groups but were not statistically different than acute exposure hatchlings. Hatchling 3rd toe length and beak length were not statistically different among treatment groups (P=0.14 and P=0.30 respectively).
Discussion

*Pre-incubation development*

Expanding on the findings of chapter 4, this study shows that embryos exhibit differential development during pre-incubation when exposed to chronic and acute doses of fluctuating temperatures above physiological zero. I expected that more development would occur with more heating degree-hours and less development with fewer heating degree-hours as was the case in chapter 4. However, this only happened in the chronic exposure groups (Figure 5.3). Eggs exposed to chronic high-fluctuating temperature (HF) received more heating degree-hours during pre-incubation and accordingly developed more than eggs exposed to chronic low-fluctuating temperature (LF; Figure 5.3). However, eggs in the acute dose groups (H1, H2, and H3) displayed differential development during pre-incubation even though they received equal quantities of heating degree-hours (Figure 5.3). Eggs in the H1 and H2 groups were more responsive to the hyperthermic conditions, advancing to the equivalent of 1.0 d of incubation. H3 eggs, receiving hyperthermic treatment in the last 3rd of pre-incubation, were less responsive to thermal stress and only advanced to the equivalent of 0.5 d of incubation (Figure 5.3). This shows that phenotypic responsiveness to acute doses (4 d) of hyperthermic stress is more pronounced during the first 2/3 of pre-incubation (i.e., in the earliest stages of development), and that differential developmental responses occur with equal quantities of heating degree-hours.
Interestingly, control eggs (constant 37.5 °C) required much less heating degree-hours than fluctuating temperature groups to advance to the same stage of development. This further illuminates that the nature of thermal exposure (i.e., constant versus fluctuating), duration (i.e., chronic versus acute), and timing (e.g., different thirds of pre-incubation) of thermal exposure impact development in addition to magnitude.

Water Loss

Water loss in incubated eggs is necessary and inevitable (Romanoff, 1949; Rahn and Ar, 1974), however, excessive water loss in eggs (>20% of fresh egg mass) causes the embryo to incur hatching problems and desiccate (Tullett and Burton, 1982; Meir et al., 1984). The highest percentage of fresh egg weight loss occurred in LF eggs (18.3% ± 0.7) even though H1, H2, and H3 eggs experienced more heating degree-hours (Figure 5.4). Even with losing the most water, LF eggs yielded the highest hatch rates 53.4% ± 6.4 among treatment groups, which suggests that water loss does not have a linear relationship with ambient temperature and is not a primary factor causing mortality in eggs of other groups that experienced more heating degree-hours.

Oxygen Consumption

The nature of the thermal regime had important effects on oxygen consumption. Oxygen consumption was different between chronic and acute thermal regimes during 3 different days of incubation (Figure 5.5). Although the initial $\bar{V}O_2$ (day 10) and final $\bar{V}O_2$ reading (1-day post hatch) were the same, these
results indicate that acute hyperthermic exposure alters \( \dot{V}O_2 \) in some instances. The pattern of \( \dot{V}O_2 \) observed was typical of precocial species (Visschedijk, 1968) and consistent with reports of developing northern bobwhites (Williams and Swift, 1988) and Coturnix quail, *Coturnix coturnix*; a precocial bird with similar sized eggs (Vleck et al., 1980).

*Hatching Assessment*

Hatching is a developmental event (Warkentin, 2007) thus, any changes in development could affect the occurrence of hatching events (internal pip, external pip, and hatching), or hatching success.

The time to internal pip is noteworthy because it marks the onset of pulmonary respiration for the resident embryo. In this study, HF eggs internally pipped \( \geq 1 \) d earlier than all other treatment groups and consistently 2 d earlier than LF and control eggs (Figure 5.8). This indicates that chronic or acute exposure to hyperthermic fluctuations induces an early onset of air breathing in northern bobwhite embryos (*in ovo*).

The time to external pip is probably not as important as the duration between internal and external pip—i.e., the time in the internally pip state (Figure 5.9), because of the respiration and acid-base balance in embryos. As development progresses, shell conductance remains relatively constant but embryonic \( \dot{V}O_2 \) and \( CO_2 \) production increases. As a result, the air cell’s oxygen tension (\( PO_2 \)) decreases and carbon dioxide tension (\( PCO_2 \)) increases. Therefore, at the time of internal pip the air cell is hypoxic with \( PO_2 \) levels...
approximately 90 mmHg in chickens (Dawes, 1975; Tazawa, 1980; Tazawa and Whittow, 2000). Thus, for the span of time between internal and external pip the embryo is subjected to hypoxic conditions. Two different studies demonstrated that exposure to hypoxia induced changes in body mass, metabolic rate, and hematology in chicken embryos (Stock and Metcalfe, 1987; Dzialowski et al., 2002). In the present study, the duration between internal and external pip was longest in HF eggs (1.8 d ± 0.1) and shortest in acute dose groups ranging from 1.2 d ± 0.0 (H3) –1.3 d ± 0.0 (H2). The exposure to hypoxia did not appear to be the primary factor causing lower hatchling mass in treatment groups. All groups had similar metabolic rates at hatch and hematology was not measured. Future studies could measure the PO$_2$ of air cells in bobwhite eggs during internal pip and examine the effects of hypoxia on northern bobwhite development.

An avian embryo and its inner-egg milieu can dry quickly once the eggshell and associated membranes have been externally pipped. This is certainly more important for eggs exposed to high temperatures where drying occurs at a much faster rate (Bennett and Dawson, 1979). Perhaps as an adaptation to thermally stressful environments, HF and H1 eggs exhibited the shortest period (≤1 d) of time between externally pipping and hatching.

Lastly, the time to hatch was also different among treatment groups. HF eggs consistently hatched on incubation 21, 2-days earlier than all other groups. Although this could be thought of as being a result of the 2.2 d of pre-incubation development, it seems just as related to the timing of hyperthermic fluctuations.
Although LF eggs had 0.5 d of development during incubation, they routinely hatched 0.5 d after control (Figure 5.8). H2 and H3 eggs only varied in developmental days by 0.5 d, meaning they had 0.5–1 d of pre-incubation development and hatched 0.5–1 d early. More closely in pattern to HF eggs, H1 eggs also hatched earlier than control, albeit asynchronously, which suggests that hyperthermic exposure in the earliest stages of the embryo caused more hatching plasticity than groups receiving equal amounts of heat during other periods of pre-incubation. Groups receiving any duration of hyperthermic fluctuations exhibited a reduced incubation period which is perhaps an adaptation to the thermally stressful environments that northern bobwhites inhabit.

**Hatching Outcome**

Hatching success and hatchling mass suffered in groups receiving chronic and acute hyperthermic thermal stress. HF and H1 eggs had the lowest hatch rates and all groups exposed to hyperthermic temperature fluctuations had reduced hatchling mass as compared to groups that were not exposed, demonstrating that thermal stress during pre-incubation has subsequent effects on hatch rates and hatchlings.

Hatching synchrony occurred in groups with chronic exposure to fluctuation thermal regimes (LF and HF) but only occurred in H3 eggs among the acute exposure groups. Asynchronous hatching was exhibited by H1 and H2
groups showing that hyperthermic exposure during the earliest stages, in acute doses, results in hatching plasticity and altered hatch timing.

**Critical Periods of Development**

The study is the first to demonstrate critical periods of development in response to hyperthermic temperature fluctuations during the pre-incubation period. The earliest stages of development appear to be the most critical for the northern bobwhite embryos as witnessed first by differential development during pre-incubation. H1 and H2 eggs developed significantly more than H3 eggs even though all 3 groups received identical heating degree-hours during pre-incubation. H1 eggs proceeded through the hatching sequence (i.e., from internal pip to hatch) significantly faster than H2 and H3 eggs, and also had the shortest duration from external pip to hatching among the 3 acute exposure groups. H1 and H2 eggs also lost the ability to hatch synchronously. Finally, H1 eggs had a significantly lower hatch rate and H2 had a significantly lower hatchling mass than other acute dose groups.

Collectively, these data indicate that the earliest developmental stages of the northern bobwhite embryo are more affected by hyperthermic temperature fluctuations during pre-incubation. Indeed, a critical period of development exists during the first 2/3 of the pre-incubation period during which exposure to hyperthermic temperatures results in aberrant development, hatching plasticity, and reduced hatch rates. For the first time, this study shows that the timing of thermal exposure during pre-incubation plays a role in avian development.
Future investigations should focus the regulation of proteins during temperature fluctuations and the protein composition of different treatment groups to determine the cause of the differential responses to hyperthermic temperature fluctuations during pre-incubation.
CHAPTER 6
PHYSIOLOGICAL CONCLUSIONS AND APPLICATIONS FOR NORTHERN BOBWHITE CONSERVATION

General Overview

The ultimate goal of this study was to perform research at the boundaries of wildlife biology and developmental physiology to contribute to the understanding of how global warming may impact oviparous embryos. Global climate-warming is occurring at a much higher rate than previously predicted (IPCC, 2001; Root and Schneider, 2002; IPCC, 2007). Taking into account natural cycles in global climate and the unprecedented increase in greenhouse gasses in the atmosphere (Root and Schneider, 2002), scientists predict an increase in global average temperature of 5 °C ± 1 °C in the United States by the period 2071–2100 relative to the period 1961–1990 (Root and Schneider, 2002; IPCC, 2007).

Concurrent with higher mean temperatures, scientists forecast variability in daily temperatures (Easterling et al., 1997; IPCC, 2001) and expect an increase in drought frequency for the southern portion of the United States (IPCC, 2007). Thus, additional research goals were to analyze how diurnally fluctuating temperatures affect embryonic development differently than mean
temperatures, and to determine if simulated drought conditions impact development. Northern bobwhite embryos (*in ovo*) were used as model organisms for this research. Northern bobwhite population numbers are declining across their range (Brennan, 1991) to the extent that they were recently placed on the IUCN’s red list as a “near-threatened” species (IUCN, 2008). Bobwhite populations fluctuate with local and regional changes in climate (Bridges et al., 2001; Reyna, 2008); increasing in rainy years, and decreasing in drought years. Known as the most crucial period of a bird’s life-cycle (Romanoff, 1960), the embryonic stage of the northern bobwhite’s life-cycle presents itself as a limiting factor in production with the predicted increase in drought frequency as global warming continues. As a species that is often exposed to hyperthermic conditions during development and is declining concurrent with climate warming, the northern bobwhite served as a natural animal model to investigate how developing vertebrates respond to climate warming and to examine how northern bobwhite embryos respond to acute and chronic doses of simulated drought temperatures.

The focal period of this study was the pre-incubation period—i.e., the period of time when eggs of precocial species (e.g., northern bobwhite) are stored in the nest without the protection of an incubating parent. During this period, eggs are exposed to ambient temperatures which can reach potentially lethal levels in semi-arid habitats. This study began by determining the maximum duration of egg storage in the nest or laboratory (Chapter 2). Then,
the upper lethal temperature for northern bobwhite embryos was discovered (Chapter 3). The study progressed by determining how diurnally-fluctuating and constant temperatures differentially affected development (Chapter 4). Subsequently, investigations were made to resolve whether critical periods of development existed during pre-incubation (Chapter 5).

Objectives and Physiological Conclusions

In the first study (Chapter 2), the objectives were to determine the effects of prolonged egg storage on the pH of egg albumen and yolk, and subsequent hatchability of northern bobwhite eggs. During this study, hatching success of northern bobwhite eggs stored at 20–22 °C declined significantly after 14 days, indicating that clutch size and duration of pre-incubation might be a function of egg viability—i.e., the benefit of laying additional eggs is offset by loss of egg viability. The pH of egg albumen, a common indicator of egg quality, reached 9.96 ± 0.04 after 14 days of storage, a high value that does not appear in other poultry studies. The observed increased albumen pH could be an adaptation to bacteria penetration of the wet cuticle post oviposition. As observed in this study, the albumen pH increases over time to a value exceeding optimum conditions for bacteria growth, 6.5–7.5 (Tortora et al., 2009). The pH gradient between albumen and yolk is then established allowing optimum growth under incubation conditions (i.e., higher temperatures). However, after 14 days, degradation of the albumen and yolk occur which is detrimental to embryonic development and subsequent hatching.
In the second study (Chapter 3), the objective was to determine the thermal properties and lethal temperature doses of freshly laid northern bobwhite eggs. The study revealed that pre-incubated bobwhite eggs exhibit a remarkable tolerance to brief hyperthermic exposures, surviving temperatures up to 50 °C for 1 h, 49 °C for 3 h and 46 °C for 6 h. This may be an adaptation to the naturally occurring temperature extremes that can occur in their naturally hot, semi-arid environment while awaiting incubation (Guthery et al., 2001; Guthery et al., 2004; Hernandez and Peterson, 2007).

In the third study (Chapter 4), the objectives were to determine: 1) if avian embryos develop differently when exposed to diurnally fluctuating temperatures or a constant temperature equal to the mean of the fluctuating regime, during pre-incubation, and 2) if development or hatching is altered as a result of increased heat loads equal to simulated drought conditions and conditions predicted as a result of global warming. The results suggested that fluctuating temperatures act differently on developing organisms than do constant temperatures of equal mean and heating degree-hours. The impact of these differences is increased as heat loads are increased—i.e., more variation in development resulted from higher heating degree-hours. Further, within fluctuating regimes, higher fluctuating heat loads caused an increase in the rate of development during pre-incubation, a decrease in rate of development during incubation and had more of a negative effect on hatching. These findings indicate that observed reductions in the percentage of northern bobwhite juveniles during
droughty years (Bridges et al., 2001; Reyna, 2008) could be caused by increased heat loads acting upon embryos (in ovo) during the 12-d pre-incubation period. Additionally, these results suggest that the nature (i.e., fluctuating or constant) and magnitude of diel temperatures should be considered when evaluating the biological response to predicted temperatures of global warming.

Lastly, the fifth study (Chapter 5) tested for critical periods of development during the pre-incubation period—to my knowledge the first to do so—to determine whether a time or stage dependent response to cyclic hyperthermic temperatures existed by exposing fertilized bobwhite eggs to chronic and acute doses of high-fluctuating heat during 3 different periods of the pre-incubation period. Collectively, the data indicated that the earliest stages of the northern bobwhite embryo were more affected by cyclical hyperthermic temperatures during pre-incubation. Indeed, a critical period of development exists during the first 2/3 of the pre-incubation period during which exposure to hyperthermic temperatures results in aberrant development, hatching plasticity, and reduced hatch rates. For the first time, this study shows that the timing of thermal exposure during pre-incubation plays an important role in avian development.
Wildlife Biology and Conservation Applications

Elucidation of the thermal susceptibility of developing northern bobwhites may reveal how oviparous vertebrates in the embryonic stage respond to thermal stress in changing environments. Such information will undoubtedly prove useful to researchers, managers, and policy makers when determining conservation efforts to sustain populations in response to seasonal droughts, species introductions to new habitats (a conservation tool), or projected global warming conditions. Rather than using the mean daily-temperature in decision making, focus would better be given to evaluating the nature and magnitude of temperatures and how those regimes may affect developing species spatially and temporally. Further, when introducing or re-introducing ground nesting avian species, like northern bobwhites, to new habitats that potentially pose a thermal risk (Parmesan and Yohe, 2003; Root et al., 2003), consideration must be given to the embryonic stage of the avian life-cycle as a critical period that may limit the species range in space and time.

While it was not my primary intention to set forth northern bobwhite management goals, portions of my findings certainly apply to those who manage quail and quail habitats as a profession; either for conservation purposes or to sustain huntable populations for sport. Collectively, these studies show that northern bobwhite hatch rates could be severely reduced during drought as a result of acute or chronic doses of hyperthermic temperatures. As habitat managers, the general response may be to create cooler nesting microclimates
by increasing the presence of herbaceous and woody cover within recommended limits. Consideration might also be given to increasing the usable space and thermal refugia of nesting hens and their clutches.

In the American classic rock and roll song *Free Bird* by Lynyrd Skynyrd, band members sing, “and this bird you cannot change”. The same goes for the northern bobwhite, the bird cannot be changed to adapt to the habitat, but the habitat and human interaction can be changed to fit the bird. In addition to the habitat prescriptions above, harvest limits and hunting season lengths could be reduced during drought years to reduce the impact on surviving birds (Peterson, 2001). While some suggest that quail hunting is self regulating during droughty years because it’s plausibly more difficult to find birds when population are low (Peterson and Perez, 2000; Peterson, 2001), the opinion of the author differs. Quite frankly, any human induced mortality is additive to naturally occurring mortality (i.e., non-human induced) and should be limited when population numbers “bust” as witnessed during droughts (Bridges et al., 2001; Reyna, 2008).

Further evidence for harvest restrictions or season reductions may be better illustrated in how the northern bobwhite population decline is viewed. The reduction in bobwhite population numbers is a complex issue, likely with causal mechanisms that vary spatially and temporally. However, in the southern portions of the habitats range, bobwhite population numbers correlate to drought conditions (Bridges et al., 2001; Reyna, 2008) which may be a function of the
thermal susceptibility of the northern bobwhite embryo as detailed in this study. The population decline of northern bobwhites is typically viewed as a regression of quail observed along monitoring transects with concurrent years (Figure 6.1A). However, if the resulting data is divided between periods of consecutive population declines (likely correlating with drought; Bridges et al. 2001) and regressed with shorter time periods, observations can be made that after consecutive drought years bobwhite populations decline to a level that cannot rebound to previous numbers without conservation measures (Figure 6.1B). Any reduction in bag limits or duration of hunting season, primarily during late hunting season (Peterson, 2001), could help sustain populations during this stressful time and further help sustain huntable populations in future years.
Figure 6.1. Northern bobwhite population numbers from the North American Breeding Bird Survey from 1967–2008 (Sauer et al., 2008). A normal regression and 95% C.I. is shown (A), and regressions of shorter time periods in B.
Future Directions

While the present study has filled voids in the literature regarding the plausible vertebrate ontogenetic response to global warming and the thermal susceptibility of northern bobwhite embryos, several new opportunities were created. Future research should be directed towards understanding the physiological mechanisms involved in embryo quiescence when exposed to temperature extremes. Investigations into the protein composition of surviving embryos compared to the deceased may provide a good start. For example, it would be beneficial to determine the presence, quantity, or type of heat shock protein known to be expressed in vertebrates (Lindquist, 1986; Lindquist and Craig, 1988; Becker and Craig, 1994). Additionally, protein profiles of fresh, developing, and adult bobwhites may prove useful when examining the thermal limits imposed by climate change or drought conditions. Wildlife studies could verify results by comparing developmental responses to simulated drought with responses in naturally occurring drought years.

Finally, this dissertation has demonstrated the rewarding power of combining wildlife biology with developmental physiology to further advance the emerging field of conservation physiology—that is, the study of physiological responses of organisms to environmental change that might cause or contribute to population declines (Wikelski and Cooke, 2006). Climate change, habitat loss, and the release of toxic chemicals are among the many factors that increasingly threaten our environment and contribute to biodiversity loss (Stevenson et al.,
As human influence on natural systems increases, it will become progressively more important to understand what factors cause stress in animals, the nature of these factors, and how animals respond morphologically, physiologically, and behaviorally. Conservation physiology is an interdisciplinary approach that goes beyond the descriptive nature of wildlife biology and ecology, by incorporating physiological tools that allow a more detailed mechanistic understanding of the factors that contribute to population declines. With the current disconnect between physiological information and wildlife and conservation management, conservation physiology is poised to bridge the gap by providing physiological assessments of conservation problems, prescriptions for animal conservation, and informed consequences of conservation decisions. This field will undoubtedly allow conservation biologists, courts, and policy makers to put together more effect conservation policy.
APPENDIX

DEVELOPMENTAL STAGES OF THE NORTHERN BOBWHITE EMBRYO
In the present study, northern bobwhite embryos (*Colinus virginianus*) were staged according to Hamburger and Hamilton (1951) for incubation days 1–4, and Hendrickx and Hanzlik (1965) for incubation days 5–23. The stages established by Hamburger and Hamilton (1951) are well known; however, due to the obscurity of Hendrickx and Hanzlik (1965), the stages of the northern bobwhites and how they relate to chicken (*Gallus gallus*) development is presented in Table 1, and the plates follow; all of which are duplicates from Hendrickx and Hanzlik (1965).
Table A.1. A comparison of incubation times and stages of development for northern bobwhite and chicken embryos (Hendrickx and Hanzlik, 1965).

<table>
<thead>
<tr>
<th>Quail</th>
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<td>41*</td>
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* Newly-hatched chick.
Figure A.1. Plate 1, Stages 24–33 of northern bobwhite quail development (Hendrickx and Hanzlik, 1965).
Figure A.2. Plate 2, Stages 34–41 of northern bobwhite quail development (Hendrickx and Hanzlik, 1965).
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