The Role of Incubation Temperature in Determining Avian Phenotype: Implications for Avian Ecology, Life History Evolution, and Conservation

Sarah Elizabeth DuRant

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Fisheries and Wildlife

William A. Hopkins, Chair
Dana M. Hawley
Gary R. Hepp
Ignacio T. Moore
Jeffrey R. Walters

July 21st, 2011
Blacksburg, Virginia

Keywords: Maternal effects, bioenergetics, stress endocrinology, eco-immunology

Abstract

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The early developmental environment has a profound influence on an individual's life history trajectory and parents have tremendous influence over this environment. Despite the wealth of research demonstrating that incubation temperature affects a suite of traits important to fitness in reptiles, we are only now discovering that nest temperatures are a defining component of the avian developmental environment. Aspects of the nest environment may be an important and overlooked maternal effect in birds as nearly all birds physically incubate their eggs, thus providing a clear link between parental behavior and the developmental environment of the avian embryo. My research used an interdisciplinary approach, uniting concepts from life history theory, bioenergetics. immunology, and physiological ecology to investigate the importance of incubation temperature to avian phenotype. I found that incubation temperature affects a suite of traits important for future development, survival and reproduction in a species of birds. Using a population of wood ducks (*Aix sponsa*) that has been the subject of long term studies I investigated the effects of incubation temperature on embryonic developmental patterns and energy expenditure, and body size and condition, stress endocrinology, thermoregulatory performance, and immunocompetence in hatchling wood ducks. In all experiments freshly laid wood duck eggs were collected from nest boxes located in SC, transported to Virginia Tech and incubated at one of three temperatures (35.0, 35.9, 37.0˚C) that fell within the range of naturally-incubated wood duck nest temperatures. I found that less than 1˚C differences in incubation temperature affected duckling growth and body condition, stress endocrinology, immune responses, and energy expended to thermoregulate. Many of these effects persisted days to weeks after hatching. In most cases, ducklings that hatched from eggs incubated at lowest temperature performed poorer than ducklings that hatched from eggs incubated at the higher temperatures. Incubation temperature also affected wood duck embryonic developmental trajectories and energy expended during incubation with embryos from the low incubation temperature expended more energy and developing slower than ducklings incubated at the higher temperatures. Embryonic energy expenditure could contribute to effects observed on hatchling phenotype. Because I demonstrate that incubation temperature affects hatchling phenotypic quality, the variability upon which natural selection acts, my findings have implications for avian ecology, life history evolution and conservation.

Funding

Primary funding for this research was provided by National Science Foundation Grant IOB-0615361 and a National Science Foundation Doctoral Dissertation Improvement Grant. Additional funding was provided by a Sigma Xi Grants-in-aid of-Research (GIAR) grant, Society of Integrative and Comparative Biology GIAR, a research grant from the Virginia Tech Graduate School, and small scholarships from the Department of Fish and Wildlife Conservation at Virginia Tech.

Acknowledgements

What's the old adage, "it takes a community to raise a child?" Well, it definitely takes a community to produce a doctoral student. I never could have completed my dissertation without the help of many people. Some folks kept me sane, some silly, some happy, some intellectually stimulated, some grounded, and some folks just held my hand whenever I needed it.

Among those in my community include my friends and family. You discover who truly loves you when you embark on a process as consuming as a doctoral program. I cherish the friends from my past for believing in me even if they didn't understand what I was doing, and my current friends because they could relate. Many friends and family helped keep me in touch with the outside world (um-um Katie, Abby, Ryan, Mel, Barbara), who's hot, who's not, what's new, what I need to throw out of my closet stat, and how to speak without the use of scientific jargon. I thank my family because they are my family, and nothing in the world is quite like family. Family let's you neglect and abuse them, and they still invite you home for Christmas and on the annual beach vacation. I would sincerely like to thank my grandmother for calling me every week and being (or acting) genuinely interested in my research. I wish she could be here for the grand finale. I also thank my parents for giving me the freedom and confidence to pursue any path I saw fit.

I would also like to thank my lab mates and fellow graduate students for going through the process with me. It's not always easy to receive constructive criticism and nobody understands that quite like your peers. My peers were also very humble in giving advice and teaching me new techniques, analyses, and editing my writing (thank you JD for reviewing almost everything I ever wrote during my graduate career). I thank the extended Winkel lab for providing me with a different perspective on the biological sciences (what's a plasmid again?) and for hosting the best game nights, parties, and downtown outings. Many thanks to all of the volunteers and technicians that gave me incredible logistical support (including Jeffrey, hands-off Thera).

In addition to my peers, the undergraduate students I had the pleasure to mentor were an unexpected and integral part of my community. Teaching novice students how to conduct an experiment and why it matters was one of the most rewarding experiences of my graduate career. Nothing gets me quite like seeing that spark in a student's eye. Amanda Wilson, Brittney Hopkins, and Chad Stachowiak taught me more than they probably will ever realize.

Of course, I never would have completed my dissertation without the help of my committee. Each member of my committee brought a unique skill and personality to the table that enhanced my doctoral project immensely. I appreciate all of the time and effort they put into helping me design experiments, teaching me lab techniques, fine-tuning my writing, or helping me think outside of the box. I feel fortunate to have had a committee that whole-heartedly supported both my project and me.

Perhaps the most influential member of my community was my advisor, Sir William of Hopkins. Sir William took me under his wing over 8 years ago and has been committed to my professional (and often times personal) development ever since. Everything I know about science I have built on the foundation he provided me with. I will never be able to put into words what his mentorship has meant to me and how it has shaped not just the scientist, but the person that I have become. I value his advice and guidance more than anyone I have ever met.

The last members of my community that I would like to thank are the ducks. I have a special place in my heart now for ducklings. They provided endless entertainment and brought

out the ridiculous maternal instinct in me. I appreciate the sacrifice they made, and I truly hope that my research broadens our understanding of avian ecology and life history, ultimately aiding in their conservation.

Attribution

I co-authored Chapter 1 with my advisor, William A. Hopkins, committee members Gary Hepp, and Ignacio Moore, and an undergraduate technician Brittney Hopkins. Brittney Hopkins aided in data collection and reviewed the chapter. All other co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

Chapter 2 was co-authored with my advisor, committee members Gary Hepp and Dana Hawley. All co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

Chapter 3 was co-authored with my advisor, committee member Gary Hepp, and an undergraduate technician Amanda Wilson. Amanda aided in data collection and reviewed the chapter. All other co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

Chapter 4 was co-authored with my advisor and committee member Gary Hepp. Both co-authors co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

The conclusions chapter was co-authored with my advisor and committee members Gary Hepp and Jeff Walters. All co-authors provided input on the framework of the paper and insightful comments on conceptual and technical aspects of the writing.

Table of Contents

List of Tables

Introduction

Chapter 2: Incubation Temperature Affects Multiple Measures of Immunocompetence in Young Wood Ducks (*Aix Sponsa*).

Conclusions: Incubation temperatures role in determining avian phenotype: implications for avian ecology, life history evolution, and conservation.

List of Figures

Introduction.

Chapter 1: Slight differences in incubation temperature affect early growth and stress endocrinology of wood duck (*Aix sponsa*) ducklings.

Chapter 2: Incubation Temperature Affects Multiple Measures of Immunocompetence in Young Wood Ducks (*Aix Sponsa*).

Introduction

Parents have enormous non-genomic influences on the health and development of their offspring (Mousseau and Fox 1998; Badyaev and Uller 2009). Parental behavior and physiology can influence offspring development and play a critical role in determining offspring phenotype (Mousseau and Fox 1998; Badyaev and Uller 2009). These non-genomic contributions, referred to broadly as parental effects, can sometimes persist across generations, and can ultimately shape the life history trajectory of offspring by influencing growth rates, age at maturity, survival, and reproduction (Lindstrom 1999; Metcalfe and Monaghan 2001; Badyeav and Uller 2009). Non-genomic contributions may also serve as a key link between changing environmental conditions, such as climate, and resulting phenotypic change (Mousseau and Fox 1998; Hayward and Wingfield 2004).

Because scientists have long appreciated the importance of parental health during reproduction and the adaptive interplay between parental and offspring health (Trivers and Willard 1973), parental effects have been widely studied in a variety of organisms, including birds. Important parental effects in birds include mate choice, nest-site selection, nutrient and hormone allocation to the egg, incubation behavior, and feeding behavior (Price 1998; Schwabl 1996). These parental effects influence a variety of offspring characteristics that are important to their success, including time to hatching, size at hatching, hatchling growth rate, begging behavior, immunocompetence, and secondary sexual characteristics (Schwabl 1996; Price 1998; Saino et al. 2005).

In birds, parental incubation determines much of the early developmental environment, influencing both the humidity and temperature under which the eggs develop (Deeming 2002). Incubation behavior by avian parents consists of on-off bouts which largely keep eggs within a

narrow temperature range. This is also a demanding time for the parents because they must tightly control the microclimate of the nest (Webb 1987; Deeming 2002a), while maintaining their own body condition (White and Kinney 1974; Carey 1980; Deeming 2002b). Optimal incubation temperatures become compromised when parents spend time away from the nest leading to greater predation risks and lower hatching success of eggs (Reid et al. 2002). Nest temperatures are not just dictated by incubation behavior, but can also be affected by nest structure, insulation, nest site and climate (Deeming 2002). Nonetheless, the incubation period, via variation in nest microclimate, creates conditions for the phenotype of avian young to be shaped by the parents' environment, behavior and physiology.

Despite knowing that temperatures of naturally-incubated nests vary within avian species, we know very little about the consequences of variation in incubation temperature for avian phenotype. What we do know about incubation temperature and its effect on avian phenotype comes primarily from studies on domesticated species. In these studies, incubation temperature is typically manipulated during a portion of the incubation period, and has been shown to affect avian thermoregulation, post-hatch growth, metabolism, feed conversion efficiency and hatchling morphology (Hill, 2001; Hulet et al. 2000; Lourens and can Middelkoop, 2000; Lourens, 2001; Nichelmann and Tzchentke, 2003). However, these studies do not examine effects of temperature on phenotype from an ecological or evolutionary standpoint. For example, how do temperature manipulations correspond with natural variations in incubation temperature?

In reptiles, we know much more about the implications of naturally-occurring variations in nest temperature on offspring phenotype than we do in birds. Incubation temperature has been shown to influence traits important to population dynamics and individual fitness, including

offspring sex ratios, body size, body morphology, growth rate, locomotor performance, behavior, and survival (Bull, 1980; Joanen et al., 1987; Burger, 1990; Brooks et al., 1991; Van Damme et al., 1992; Shine et al., 1997; Angilletta, 2000; Booth et al., 2000; Nelson et al., 2004). Importantly, at least one study has shown that incubation-induced phenotypic effects are the same in natural nests as they are in the laboratory (Shine et al. 1997).

The few studies that have examined the effects of biologically-relevant differences in incubation temperature on birds demonstrate that temperature is an integral component of avian development. One such study explored the effects of incubation temperature on incubation duration and energy expenditure of the eggs of brush turkeys (Booth 1987). The authors found that decreasing incubation temperature 6ºC increased the length of the incubation period by 15-25 days and subsequently increased total energy expenditure of the embryos during incubation by 74%. The increase in energy expenditure of embryos suggests that hatchlings from eggs incubated at lower temperatures had smaller remaining energy reserves at hatching. While decreased temperatures lower embryonic metabolic rates, total energy expenditure during incubation is greater because lower temperatures extend the duration of incubation (Vleck and Hoyt 1991; Vleck and Vleck 1996). Another study observed sex-specific mortality of embryos at different temperatures, with the lower temperature producing more males and the higher temperature producing females (Goth and Booth 2005).

Recently, Hepp et al (2006) investigated the effects of incubation temperature on incubation duration and offspring phenotype in wood ducks (*Aix sponsa*). Previous work by the authors indicated that incubation temperature of naturally incubated wood duck nests varied among females and was inversely related to incubation duration (Fig 1). Based on these results, the authors artificially incubated wood duck eggs at three temperatures (34.6, 36.0, and 37.6ºC)

that encompassed the range of temperatures of naturally incubated wood duck nests (range 34.8-37.2ºC, Hepp et al. 2006; range 34.9-37.5ºC, Manlove and Hepp 2000; 34.9-37.7ºC, Folk 2001). They found that incubation at the lower temperatures significantly increased incubation duration, with eggs incubated at 34.6ºC hatching almost 10 days later than eggs incubated at 37.6ºC. Proximate analysis of ducklings revealed that ducklings from eggs incubated at the lower temperatures had significantly lower wet and dry mass, lower protein content, and higher ash content than ducklings incubated at the higher temperatures (Table 1). In addition, incubation temperature influenced protein use during incubation and body composition of the ducklings. Ducklings that hatched from eggs incubated at 34.6ºC depleted ~33% of egg protein whereas ducklings that hatched from eggs incubated at 37.6ºC depleted ~24% of egg protein.

Working in collaboration with Auburn University, my research explores the effects of incubation temperature on hatchling phenotype, focusing on important physiological processes for development and survival in the initial days and weeks after hatching. Specifically, I investigated the influence of incubation temperature on a duckling's ability to thermoregulate, the energetic cost of thermoregulating, stress physiology of young ducklings, and duckling immunocompetence (i.e., bacterial killing ability of the blood, cell-mediated immunity, and antibody production in response to exposure to a novel antigen). I also investigated a possible mechanism underlying any observed effects on incubation temperature on hatchling phenotype by measuring embryonic metabolism and examining developmental patterns. My research provides unprecedented insight into the link between the avian incubation environment and offspring phenotype.

Objectives

My research used an integrative approach to explore the influence of incubation temperature, an important component of an individual's early developmental environment, on traits important to hatchling survival and development in wood ducks (*Aix sponsa*). Specifically, I investigated:

1) The influence of incubation temperature on the hypothalamo-pituitary adrenal axis in ducklings.

2) The influence of incubation temperature on immunocompetence in ducklings.

3) The influence of incubation temperature on the ability of ducklings to thermoregulate.

4) The influence of incubation temperature on embryonic metabolism and developmental patterns.

General Methods

Study Species. The wood duck (*Aix sponsa*) is a widely-distributed dabbling duck whose breeding range includes a diversity of aquatic habitats extending throughout much of the eastern half of North America and along the west coast from southern California to British Columbia (Hepp and Bellrose, 1995). Several natural history attributes of the Wood Duck make them well-suited for testing the effects of incubation temperature on avian phenotype. Wood Ducks nest in cavities and readily use nest boxes which facilitate locating nests and collecting eggs (Hepp et al. 1987b). The egg-laying season is fairly long, particularly in South Carolina where nesting begins in February and continues until mid-July, and average clutch size is 12 eggs (Bellrose and Holm, 1994). In addition, females can produce multiple broods in a breeding season, thus giving

us access to many eggs. Finally, long-term data exists on the population of Wood Ducks we work with and much of their breeding ecology is well-studied. Minimum, maximum, and average temperatures of naturally-incubated wood duck nests at our study site are 34.98, 38.70, and 36.79°C, respectively (Hepp unpublished data).

Wood duck reproductive ecology also makes studies of variation in incubation temperature on offspring phenotype relevant for this species. Wood ducks are intermittent incubators and only the female cares for the eggs (Bellrose and Holm 1994). Wood ducks form pair bonds in the fall and males guard females from predators and other males allowing her to meet the nutritional demands of egg production and incubation (Bellrose and Holm 1994). However, once egg-laying is complete females begin incubating eggs and males offer no aid to the female during incubation (e.g., feeding the female, guarding the nest; Bellrose and Holm 1994), with pair bonds completely dissolving late in incubation. Thus, females typically leave the nests twice a day to forage (~1.5 hrs in the morning and early evening), which creates fluctuations in nest temperatures (Manlove and Hepp 2000). In a population of wood ducks in South Carolina, female quality appears to dictate when she will nest with better quality females nesting early in the year when females must maintain higher incubation constancy, and poorer quality females nesting later in the season when ambient temperatures are warmer (Hepp and Kennamer 2011). Although the energetic demands of incubating a clutch early in the year are greater (Hepp and Kennamer 2011), nest predation is greater later in the season (Bellrose and Holm 1994).

Study site. We collected eggs from nest boxes on Par Pond (n = 84) and L-Lake (n = 30) at the Department of Energy's Savannah River Site in west-central South Carolina. These nest boxes

have been available to wood ducks for ~20 years and have been monitored every year by staff and graduate students from the Savannah River Ecology Laboratory. Each year > 100 nests are initiated in these nest boxes. There are at least 100 breeding females using the sites and > 2500 eggs are laid annually (R. Kennamer, pers. comm). Radiation exposure on Par Pond and surrounding areas does not pose a problem to female wood ducks and their eggs. Radiation doses are > 2 orders of magnitude below dose levels known to cause adverse effects in terrestrial animals (Kennamer et al. 1993, 1995).

Collecting and Incubating Eggs, Manipulating Incubation Period. We collected fresh, unincubated eggs from nest boxes each day and stored them at 20ºC and 55-60% humidity, until they were placed in egg incubators at Virginia Tech. Avian embryos do not develop below 24-27ºC (White and Kinney 1974) and holding duck eggs for < 5 days at low temperatures before incubation does not affect hatchability (Arnold et al. 1987). Wooden eggs were used to replace wood duck eggs so that females do not abandon nests (Hepp et al. 1987a).

We artificially incubated eggs in Grumbach incubators (model BSS 160) at 3 temperatures (35.0, 35.9, and 37.0 º C), so that three incubation durations were created (37, 35, and 31 days, respectively). Incubators were programmed to allow two cool-down periods each day (~3°C reduction in mean temperature for 75 min at 0815 and 1830) to simulate natural daily feeding recesses taken by mothers during incubation (Manlove and Hepp, 2000). Furthermore, periodic chilling of the eggs may increase hatchability (Landauer 1967). To avoid pseudoreplication, and thus female/clutch effects on any variables we measured, we only included a single duckling per clutch per treatment in each of our experiments.

All laboratory experiments were conducted on the Virginia Polytechnic Institute and State University campus in labs located in Latham Hall.

Duckling Husbandry. After hatching, all ducklings were exposed to the same housing conditions. We housed ducklings communally in 46 x 32 x 24.5cm bins (n = 2 ducklings/bin) arranged in a rack system in a temperature controlled environmental chamber held at 30ºC. Ducklings had constant access to food (ground-up Dumor Chick Starter/Grower 20%) and water and were warmed by a 30 watt light bulb suspended 32.5cm above the bottom of the bin and held on a 12L: 12D photoperiod. At the end of experimental procedures ducklings were euthanized using CO_2.

All experimental and husbandry procedures followed IACUC guidelines and were VT IACUC approved.

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Table. Intro.1. Least squares means (±SE) of body mass (g) and body components (g) of wood duck ducklings incubated at different temperatures. Means within rows followed by different letters are significantly different ($P < 0.05$).

	Incubation temperature			
Variable	Low[†]	Mid	High	P[††]
Incubation duration	37	33	29	
Wet duckling	26.68a (0.21)	27.35b (0.16)	27.62b (0.17)	0.003
Dry duckling	8.32a (0.10)	8.72b (0.08)	8.96c (0.09)	0.0001
Protein	4.00a (0.09)	4.48b (0.08)	4.69c (0.08)	0.0001
Lipid	3.37 (0.10)	3.45 (0.08)	3.47 (0.09)	0.42
Ash	0.85a (0.01)	0.81b (0.01)	0.79b (0.01)	0.0004

[†] Low: 34.5º C; Mid: 36.0º C; High: 37.5º C

[††] Mixed model ANCOVA, wet duckling mass as covariate

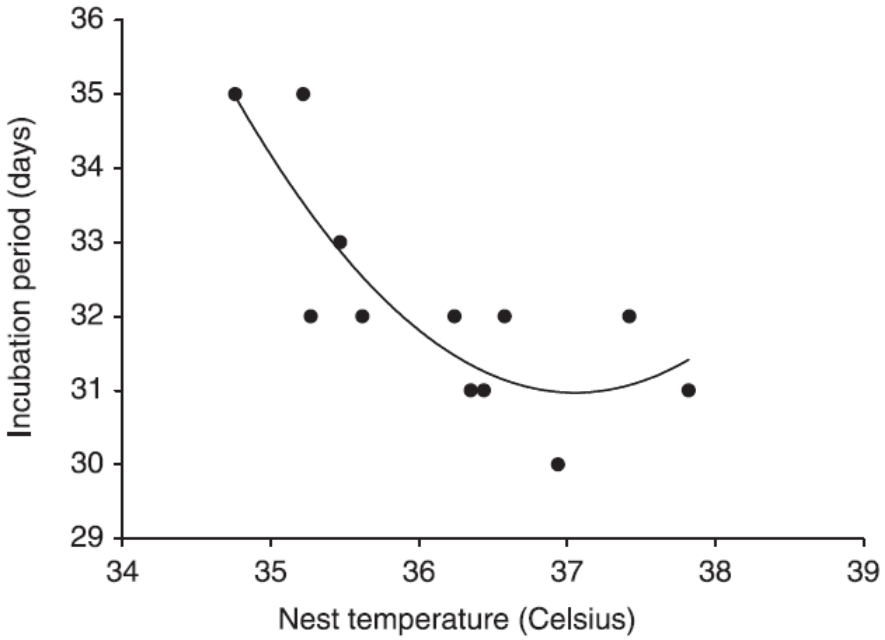

Figure. Intro.1. Relationship between average nest temperature and incubation period for wood ducks.

Chapter 1: Slight differences in incubation temperature affect early growth and stress endocrinology of wood duck (*Aix sponsa*) ducklings.

Co-authors: William A. Hopkins, Ignacio Moore, Gary Hepp, Brittney Hopkins

*Formatted for the Journal of Experimental Biology.

Abstract

Early developmental experiences, such as incubation conditions, can have important consequences for post-hatching fitness in birds. Although the effects of incubation temperature on phenotype of avian hatchlings are poorly understood, recent research suggests that subtle changes in incubation conditions can influence hatchling characteristics including body size and condition. We designed an experiment to explore the effects of incubation temperature on hatching success, survival to 9-d post hatch, growth, and the hypothalamo-pituitary-adrenal (HPA) axis in Wood Ducks (*Aix sponsa*). Wood Duck eggs were collected from nest boxes and experimentally incubated at three temperatures (35.0, 35.9, and 37.0ºC), each falling within the range of temperatures of naturally-incubated wood duck nests. Survival and growth were monitored in ducklings fed *ad libitum* for 9-d post hatch. In addition, baseline and stress-induced plasma corticosterone concentrations were measured in 2- and 9-d old ducklings. Hatching success and survival to nine days was greatest in ducks incubated at the intermediate temperature. Ducklings incubated at 35.9 and 37.0ºC had 43% higher growth rates than ducklings incubated at 35.0ºC. In addition, ducklings incubated at 35.0ºC had higher baseline (17-50%) and stress-induced (32-84%) corticosterone concentrations than ducklings incubated at 35.9 and 37.0ºC at 2- and 9-d post hatch. We also found a significant negative correlation between body size and plasma corticosterone concentrations (baseline and stress-induced) in 9-d

old ducklings. To our knowledge, this is the first study to demonstrate that thermal conditions experienced during embryonic development can influence the HPA axis of young birds. Our results illustrate that subtle changes (< 1.0ºC) in the incubation environment can have important consequences for physiological traits important to fitness.

Keywords: maternal effects, corticosterone, incubation

Introduction

Maternal effects are maternal traits that influence offspring phenotype via non-genetic pathways (Mousseau and Fox, 1998). In many cases maternal effects can have profound influences on offspring, sometimes rivaling the effects of genomic contributions to offspring fitness (Bernardo 1996; Price 1998). In fact, recent reviews have highlighted the importance of such early developmental experiences because of the influence they can have on subsequent life history decisions that ultimately influence survival and reproductive success (Lindstrom 1999; Metcalfe and Monaghan 2001). Maternal effects (eg., mate choice, nest-site selection, incubation behavior, nutrient allocation to embryo, etc) have been widely studied in a variety of organisms, including birds, and have been shown to affect a multitude of offspring characteristics that are important to their success. Such effects include time to hatching, size at hatching, hatchling growth rate, begging behavior, immunocompetence, and secondary sexual characteristics (Schwabl 1996; Price 1998; Saino et al. 2005).

In birds, an important maternal effect is incubation behavior, a critical component of avian reproduction. Incubation influences both the current reproductive success and future fitness of the parent (Gloutney et al., 1996; Williams, 1996; Tinbergen and Williams, 2002;

Heaney and Monaghan, 1996; Bryan and Bryant, 1999; Reid et al., 2000; Visser and Lessels, 2001). Egg incubation is a demanding period because parents are confronted with the conflicting demands of simultaneously maintaining their own body condition (White and Kinney, 1974; Carey, 1980; Deeming, 2002) and the environmental conditions of the nest (Webb, 1987; Deeming, 2002). The demands of incubation can be particularly severe in species that display uniparental incubation, particularly when the incubating parent receives no aid in incubating the eggs or procuring its own food. Time spent foraging away from the nest can decrease the temperature at which eggs are incubated, thus slowing their development and subsequently increasing the duration of incubation (Lyon and Montgomerie, 1985; Deeming and Ferguson, 1991; Zicus et al., 1995; Neuchterlein and Buitron, 2002; Martin et al., 2007). Extended incubation duration can incur risks, including increased susceptibility to egg predation (Reid et al., 2002; Tombre Erikstad, 1996).

In addition to influencing the probability of egg predation, changes in the incubation environment also can influence offspring phenotype. In reptiles, incubation temperature has been shown to influence traits important to population dynamics and individual fitness, including offspring sex ratios, body size, body morphology, growth rate, locomotor performance, behavior, and survival (Bull, 1980; Joanen et al., 1987; Burger, 1990; Brooks et al., 1991; Van Damme et al., 1992; Shine et al., 1997; Angilletta, 2000; Booth et al., 2000; Nelson et al., 2004). In contrast, the effect of incubation conditions on hatchling fitness in birds is poorly-understood. However, recent research suggests that subtle changes in incubation conditions can have serious effects on hatchling characteristics. Hepp et al. (2006) investigated the effects of incubation temperature on the duration of embryonic development and offspring phenotype in wood ducks (*Aix sponsa*) by artificially incubating eggs at three temperatures (34.6, 36.0, and 37.6ºC) within

the range of temperatures found in naturally incubated nests (range 34.8-37.7ºC; Manlove and Hepp, 2000; Folk, 2001; Hepp et al., 2006). They found that incubation at lower temperatures significantly extended embryonic development, with eggs incubated at 34.6ºC hatching nearly 10 days later than eggs incubated at 37.6ºC. Further, proximate analysis revealed that ducklings from eggs incubated at lower temperatures had significantly lower wet and dry mass, lower protein content, and higher ash content than ducklings incubated at the higher temperatures (Hepp et al., 2006).

Recent research suggests that conditions experienced by avian embryos during incubation can also influence a hatchling's stress physiology. Two studies observed significant differences in stress-induced corticosterone (the primary stress hormone in birds) concentrations between groups of hatchlings that experienced different incubation conditions, suggesting that these effects were mediated through the behavior or physiology of the parents (Walker et al., 2005; Cyr et al., 2007). Walker et al. (2005) found that newly hatched Magellanic Penguin (*Spheniscus magellanicus*) chicks from frequently disturbed (tourist visited) areas had similar baseline corticosterone concentrations as chicks in unvisited areas. However, chicks from disturbed areas expressed significantly greater stress-induced free corticosterone levels after capture compared to chicks from undisturbed areas. Although the authors attributed these effects to cues relayed to the offspring from the parent during incubation, the possibility exists that these effects were attributable to events that occurred prior to egg-laying (e.g., steroid deposition to the yolk). A recent study on European Starlings (*Sturnus vulgaris*) provides even more compelling evidence of the influence of incubation conditions on the HPA axis (Cyr et al., 2007). Adult Starlings exposed to a stressor while they were incubating eggs produced chicks that, at 18 days post hatch, displayed higher levels of stress-induced free corticosterone than chicks produced by

undisturbed parents. In that study, females were exposed to a chronic stress protocol (CSP; a rotation of 4 stressors per day, spaced 1-2 h apart) for eight days while they were incubating eggs. Females left the nest when stressors were present and typically did not return until the stressor was removed (30 min later). Because the CSP was present only during incubation, these data suggest that the effects of the CSP on the chicks were attributable to either 1) alterations in the female's behavior or physiology during incubation (e.g., reduced time on nests, change in maternal body temperature or heart rate) or 2) from changes in parental care post-incubation.

In this study, we used Wood Ducks (*Aix sponsa*) to investigate whether variation in incubation temperature alone can affect important post-hatching characteristics of hatchlings. Specifically, we evaluated whether slight differences in incubation temperature, which is largely dependent on female behavior, would influence early survival, growth, body condition, and the HPA axis of ducklings. We randomly allocated eggs from natural nests to one of three incubation temperatures (35.0, 35.9, and 37.0 C). We monitored survival and growth to 9 d post hatch and measured baseline and stress-induced corticosterone in ducklings at 2 and 9 d post hatch. Based on the aforementioned studies (Hepp et al. 2006; Walker et al. 2006; Cyr et al. 2007), we predicted that ducklings incubated at the lowest temperature would have lower survival and growth, and higher corticosterone concentrations than ducklings incubated at the higher temperatures.

Methods

Study Species. The wood duck (*Aix sponsa*) is a widely-distributed dabbling duck whose breeding range extends throughout much of the eastern half of North America and along the west

coast from southern California to British Columbia (Hepp and Bellrose, 1995). Wood ducks are relatively small-bodied (~ 650-700 g) and occupy a great diversity of aquatic habitats, including freshwater marshes, wooded swamps, beaver ponds, and bottomland habitats along major tributaries. Female wood ducks nest in tree cavities, but will also use artificial nest boxes, a factor that facilitates locating nests and capturing females (Hepp et al., 1987b). Although they are socially monogamous and begin to form pair bonds in autumn and winter (Armbruster, 1982; Hepp and Bellrose, 1995), only the female incubates the eggs and cares for the young (Fredrickson, 1990). During this time she receives no aid from the male. Females lay one egg per day and the average clutch size is 12 eggs (Bellrose and Holm, 1994). Night incubation begins when ~75% of the clutch is laid, and 24 h incubation doesn't begin until egg-laying is complete (Hepp and Bellrose, 1995). Minimum, maximum, and average temperatures of naturally-incubated wood duck nests at our study site were 34.98, 38.70, and 36.79°C, respectively (unpublished data). In our study area, wood ducks initiate nesting in mid-late February and continue nesting until mid-July. Females can produce multiple broods in a breeding season.

Study site. Eggs were collected from aquatic habitats located on the Department of Energy's Savannah River Site (SRS) in west-central South Carolina. Nest boxes are located throughout the SRS on two large reservoirs (n = 120) and 10 isolated wetlands (n = 81). These nest boxes have been available to wood ducks for > 10 years and have been monitored every year by staff and graduate students from the Savannah River Ecology Laboratory. Each year > 100 nests are initiated in these nest boxes. There are at least 100 breeding females using the sites and > 2500 eggs are laid annually (R. Kennamer, pers. comm.).

Egg Collection and Incubation. To collect fresh, non-incubated eggs, nest boxes were checked every four days during the breeding season until nests were initiated. Active nests were visited daily, and new eggs were collected, individually marked, and stored at 20ºC and 55-60% humidity until they were placed in incubators. Avian embryos do not develop when maintained below 24-27ºC (White and Kinney, 1974) and holding duck eggs for < 5 days at low temperatures before incubation does not affect hatchability (Arnold et al., 1987). Wooden eggs were used to replace wood duck eggs so that females would continue to lay eggs and would not abandon nests (Hepp et al., 1987a).

After four days of collection, eggs were transported to Virginia Tech and artificially incubated in Grumbach incubators (model BSS 160) at one of three temperatures (35.0, 35.9, and 37.0 ºC), which produced three incubation durations (31 ± 0.27, 35 ± 0.15, and 37 ± 0.19 days, respectively). Although experimental mean temperatures were achieved, incubators were not maintained at constant temperatures. Instead, incubators were programmed to allow two cool-down periods each day (~3ºC reduction in mean temperature for 75 min at 0815 and 1830) to simulate natural daily feeding recesses taken by mothers during incubation (Hepp and Bellrose ,1995, Manlove and Hepp, 2000). Such periodic chilling of the eggs generally results in increased hatchability (Landauer, 1967). Throughout incubation we candled eggs every 7-10 days to check for embryonic mortality. Stage at death was determined using age criteria of excised wood duck embryos from Bellrose and Holm (1994).

Duckling Husbandry. After hatching, all ducklings were housed under identical conditions. Ducklings were maintained communally in 46 x 32 x 24.5 cm plastic cages (2-3 ducklings/ cage)

arranged in a rack system in a temperature controlled environmental chamber held at 28ºC. Ducklings were allowed constant access to food (ground-up Dumor Chick Starter/Grower 20%) and water. Ducklings were warmed by a 50 watt infrared light bulb suspended 32.5cm above the bottom of the cage (creating a thermal gradient) and held on a 14L: 10D photoperiod. Each morning all cages, food, and water dishes were cleaned. At the end of the experiment, ducklings were euthanized via asphyxiation with carbon dioxide followed by cervical dislocation in accordance with IACUC standards.

Duckling Growth and Survival. We monitored duckling growth by weighing ducklings every morning during cage cleaning. We calculated daily growth rate of ducklings by subtracting their hatch mass (day 0) from their mass at 9 days post hatch and dividing by 10 (the total number of days elapsed). We checked for duckling mortality each morning and evening.

Stress Hormone Protocol. To determine whether incubation temperature influences the HPA axis, baseline and stress-induced corticosterone concentrations were measured in 2 and 9 day old ducklings from each of the three incubation temperature treatments (35.0, 35.9, and 37.0ºC). We used a repeated measures design, therefore all blood samples were taken from the same individuals at both ages. Ducklings were bled via the femoral vein immediately after removal from their cage (< 3 minutes) then held in a cloth bag. Ducklings were bled again at 15 minutes post removal from their cage. To control for circadian influences on plasma corticosterone levels, all samples were collected between 1250 and 1545 hours. Blood was collected in heparinized capillary tubes and stored on ice for no longer than 1.5 hours before separating plasma via centrifugation. Plasma samples were then stored at -80˚C.

Radioimmunoassay. Plasma corticosterone was assayed by radioimmunoassay (RIA) techniques following extraction based on the methods of Wingfield et al. (1992). To determine sample extraction efficiency we equilibrated each sample overnight with 2000cpm of tritiated steroid. Each sample was extracted with 4 mL of dichloromethane, dried using nitrogen gas and resuspended in 600 µL of phosphate buffered saline. Individual extraction efficiency was determined from 100µl of the sample (mean recoveries were 86.5% and 82.7% for assay I and assay II, respectively). Two hundred µl of the sample was allocated to each of two duplicates for the assay. Then, duplicate samples were compared to a standard curve, which contained known amounts of corticosterone, run with each assay. Inter- and intra-assay variation were 17 and 9.6 %, respectively, as determined by running standards in each assay.

Statistical Analyses. All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and statistical significance was recognized at $\alpha = 0.05$. In the few instances where multiple individuals from a single clutch were in the same treatment group, we randomly selected one of the individuals to be used in analyses to avoid pseudo-replication. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively.

Hatching success and post-hatch survival were binary data and therefore were analyzed using a Chi Square test and Fisher's exact test (SAS proc freq), respectively. Because the hatching success and post hatch survival data set included individuals from the same clutch, we initially included clutch as a random effect. Clutch had no significant effect on hatching success of fertilized eggs or post-hatch survival and was eventually dropped from the model because

models excluding this variable were a better fit to the data set based on AIC values. We determined the effects of incubation temperature on the duration of incubation using a one-way analysis of variance (ANOVA; SAS proc glm).

To ensure that egg mass did not differ among treatments we conducted a one-way ANOVA (SAS proc glm). In addition, we tested for differences in duckling mass at hatching among treatments using a one-way ANCOVA with egg mass as the covariate. Differences in growth rates among incubation temperature treatments were assessed using a one-way ANOVA (SAS proc glm). Only individuals that survived to nine days post hatch were included in the analysis and all growth data met assumptions of normality and homoscedasticity. Growth trajectories were compared among treatments using a repeated measures ANOVA (SAS proc glm). The model included incubation temperature, age and their interaction as main effects and only included individuals that survived to nine days post hatch. Not all ducklings were weighed on day 1 and day 2, therefore these days were omitted from the model. Differences between treatments in body condition of 9 d old ducklings were evaluated using an analysis of covariance (SAS proc glm) with incubation temperature as the independent variable, mass as the dependent variable, and tarsus length as the covariate.

Effects of incubation temperature on the HPA axis were examined using a mixed-model ANOVA (SAS proc mixed), including only individuals for which we had baseline and stress-induced data points at both ages. All data met assumptions of normality, but plasma corticosterone concentrations were $\log_{10}$-transformed to better fit assumptions of homoscedasticity. Incubation temperature, age, stress (baseline v stress-induced), and all interactions between these variables were included as main effects in the initial model. Non-significant interactions were dropped from subsequent iterations of the model. Because plasma

samples were collected from the same individuals at both time points (baseline and stress-induced) and ages, individual was included in the statistical model as a random effect to account for non-independence of plasma corticosterone concentrations. Differences in the magnitude of the stress response among treatment groups were also evaluated by comparing the factorial increase in plasma corticosterone concentration across treatments and age groups using a repeated-measures ANOVA (SAS proc glm). Finally, we used Pearson correlation coefficients to examine relationships between body mass and body condition, versus $\log_{10}$-transformed plasma corticosterone concentrations, both baseline and stress-induced. In these analyses, the residuals of a regression of mass on tarsus length were used as our measure of body condition.

Results

There was a trend towards higher hatching success of eggs incubated at the medium temperature ($p = 0.073$; Table 1). Eggs incubated at this temperature had 51 and 47% higher hatching success than the eggs incubated at the low and high temperatures, respectively. A similar trend was seen when examining the effects of incubation temperature on post-hatching survival to nine days ($p = 0.075$; Table 1). Post hatching survival for ducklings from the medium incubation temperature was 32 and 22% greater than for ducklings that hatched from eggs incubated at the low and high temperatures, respectively. All of the duckling mortality in the high and intermediate temperature groups occurred within 3 days of hatching, whereas 71% of ducklings that hatched from eggs incubated at the lowest temperature died > 4 days post hatch. Incubation temperature significantly affected the duration of incubation ($F_{2,72} = 171.56$; $p < 0.001$; Table 1), with higher temperatures inducing more rapid development. Post hoc

comparisons (Tukey HSD) revealed that all the treatments differed significantly different from one another in duration of incubation.

There were no differences in egg mass among treatments ($p = 0.1091$; Table 1). Egg mass correlated strongly with duckling mass at hatching ($F_{1,67} = 159.30$; $p < 0.001$), but duckling mass at hatching did not differ among treatment groups ($p = 0.943$; Table 1). Ducklings from all incubation temperatures had positive growth rates and gained 23-33 g from hatching to nine days post hatch. However, ducklings from the medium and high incubation temperatures both had 43% higher growth rates than ducklings from the low treatment group ($F_{2,47} = 5.23$; $p = 0.0089$; Table 1). Changes in body mass were not apparent until 5 days post hatch (incubation temperature X age: $F_{14,47} = 3.90$; $p = 0.0075$; Fig. 1). By nine days post-hatch, ducklings that hatched from eggs incubated at the high and intermediate temperatures were 17% larger than ducklings that hatched from eggs incubated at the lowest temperature.

Tarsus length correlated strongly with body mass ($F_{1,47} = 66.21$; $p <0.001$) and there was a significant effect of incubation temperature on body condition at 9 d post hatch ($F_{2,47} = 4.25$; $p = 0.020$; Fig. 2). There was no temperature by tarsus interaction ($p = 0.728$). Post hoc comparisons (Tukey HSD) revealed that ducklings that hatched from eggs incubated at the medium and high temperature treatments were heavier in relation to tarsus length (i.e., had higher body condition) than ducklings that hatched from eggs incubated at the lowest temperature. For visual purposes these data are presented as mean (±SE) residuals of mass on tarsus (Fig. 2).

Capture and restraint resulted in a 140-262% increase in plasma corticosterone in two and nine day old ducklings from all incubation temperatures (stress: $F_{1,140} = 105.13$ $p < 0.0001$; Fig. 3). There also was a significant effect of age on baseline and stress-induced plasma

corticosterone concentrations (age: $F_{1,140} = 24.99$; $p < 0.0001$). Specifically, both baseline and stress-induced plasma corticosterone concentrations were lower at nine days post hatch than at two days post hatch. Moreover, incubation temperature had a significant effect on the HPA axis of both two and nine-day old ducklings ($F_{2,140} = 4.98$; $p = 0.0082$). Ducklings from the lowest incubation temperature treatment had higher baseline (17-50%) and stress-induced (32-84%) corticosterone concentrations than ducklings that hatched from eggs incubated at the intermediate and high temperatures at both 2- and 9-d post hatch. Ducklings from the intermediate incubation temperature had the lowest plasma corticosterone concentrations (baseline and stress-induced) at day 2. However, at nine days post-hatching, ducklings from the high incubation temperature had similar corticosterone profiles as ducklings from the intermediate temperature, whereas ducklings from the low incubation temperature continued to exhibit higher levels of both baseline and stress-induced plasma corticosterone concentrations. There was no effect of age, incubation temperature, or their interaction on the factorial increase in plasma corticosterone concentrations ($p > 0.179$ in all cases).

We also found a significant negative correlation between body mass and plasma corticosterone concentrations (baseline: $r = -0.284$, $p = 0.048$; stress-induced: r = -.294, $p = 0.040$) in 9-d old ducklings (Fig. 4A, B). A similar trend existed between tarsus length and plasma corticosterone (baseline: $r = -0.308$, $p = 0.032$; stress-induced: $r = -0.248$, $p = 0.085$) in ducklings that were 9 d post hatch (data not shown). There was no relationship between body mass or tarsus length and plasma corticosterone concentrations in ducklings that were 2 d post hatch (in all cases $p > 0.37$; data not shown) and there was no relationship between body condition and plasma corticosterone concentration in either age group (in all cases $p > 0.20$; data not shown).

Discussion

We found that small differences in incubation temperature, within the temperature range of naturally-incubated nests, can affect a suite of important post-hatching characteristics in Wood Ducks. Ducklings that hatched from eggs incubated at the lowest temperature had lower survival, slower growth, lower body condition, and higher baseline and stress-induced corticosterone than ducklings incubated at the higher temperatures. To our knowledge, this is the first study in a wild bird to demonstrate that slight variations in the incubation environment can affect the growth, development and physiology of the hatchlings.

Although ducklings from all treatment groups were similar in size at hatching, by nine days after hatching ducklings from the lowest incubation temperature were 15% smaller and were in poorer body condition than ducklings incubated at higher temperatures. The lack of treatment effect on hatchling mass in our study was surprising as there was a clear effect of incubation temperature on hatchling mass in Hepp et al. (2006). Body size is important for survival in young birds, as smaller body size can decrease their ability to recover from mass loss and their ability to compete with conspecifics during rapid growth phases (Arroyo, 2002), and could increase their susceptibility to gape-limited predators (e.g., fish). Additionally, ducklings of larger body size have reduced mass-specific energetic demands when maintaining homeothermy. In many animals, juvenile body size also is an important contributor to future survival and reproduction (Semlitsch et al., 1988; Ringsby et al., 1998; Van der Jeugd and Larsson, 1998; Monros et al., 2002; Altwegg and Reyer, 2003). For example, larger offspring are often more likely to survive strenuous events (e.g., over-wintering, migration; Milner et al., 1999; Bodie and Semlitsch, 2000; Munch et al., 2003) and reach maturity more quickly than offspring of smaller body size (Dawson and Clark, 2000). Therefore, it is possible that eggs

incubated at lower temperatures produced offspring that are less likely to survive their first year or may begin breeding later than ducklings that hatched from eggs incubated at the higher temperatures. However, future studies are needed to evaluate these hypotheses.

We also found that incubation temperature strongly influenced the HPA-axis in ducklings at both 2 and 9 d post-hatching. At 2 d post-hatching, ducklings incubated at the lowest temperature had higher baseline and stress-induced corticosterone concentrations than ducklings incubated at the medium and high temperature, respectively. Although all of the ducklings exhibited a decrease in baseline plasma corticosterone concentrations from day 2 to day 9, ducklings from the lowest temperature continued to express both higher baseline and stress-induced plasma corticosterone concentrations than ducklings incubated at the higher temperatures. The ontogenetic shift in plasma corticosterone concentrations we saw in Wood Duck ducklings is consistent with patterns of plasma corticosterone in Mallard (*Anas platyrhynchos*) ducklings; Mallards had higher baseline and stress-induced corticosterone 1 day after hatching than at 7 days after hatching (Holmes et al., 1989). Interestingly, Wood Duck ducklings in this study did not appear to undergo a refractory period, a brief period of unresponsiveness to stressors that occurs shortly after hatching/birth, thought to protect young from the detrimental effects of elevated glucocorticoids (reviewed in Sapolsky and Meaney 1986).

Our finding that incubation temperature influences the HPA axis of hatchling ducks has many implications for their future development. Corticosterone is an extremely important hormone because it affects a multitude of physiological systems in vertebrates (e.g., reproductive physiology, immune function, cardiovascular function, metabolism; Shreck, 1993; Dhabar and McEwen, 1999; Moore and Jessop, 2002; van den Buuse et al., 2002) and depending on the

context, it may have stimulatory, inhibitory, or permissive effects (Sapolsky, Romero, and Munck, 2000). There is evidence among vertebrates that high levels of baseline corticosterone early in ontogeny can have negative effects on growth (Morici et al., 1997; Spencer et al., 2003), immune function (Morici et al., 1997), neural development (Caldjii et al., 2001) and cognitive function (Kitaysky et al., 2003, 2006). Although the underlying cause of differences in corticosterone concentrations among treatments in our study remains unclear, one possibility is that ducklings incubated at the lowest temperature are developmentally-delayed compared to ducklings from eggs incubated at the higher temperatures. If this is the case, then we would expect ducklings from the lowest incubation temperature to eventually express plasma corticosterone profiles that are similar to ducklings incubated at the higher temperatures. However, longer term studies that monitor baseline and stress-induced corticosterone concentrations throughout adolescence would be needed to directly address this hypothesis.

An interesting finding was that baseline and stress-induced plasma corticosterone concentrations at nine days of age were negatively correlated with body mass and tarsus length. Many studies have found negative correlations between aspects of body size (e.g., body mass, body condition) and baseline and/or stress-induced corticosterone (Schoech et al., 1997; Kitaysky et al., 1999; Moore et al., 2000; Romero and Wikelski, 2001; Perfito et al., 2002). Our failure to detect a significant correlation between morphology and corticosterone concentrations in ducklings at 2 d post-hatching may reflect the small range of body sizes at this age. Although, we cannot determine whether corticosterone is directly influencing body size or vice versa, previous studies have shown that exposure of young animals to exogenous corticosterone can inhibit growth (Beckett et al., 1996; Morici et al., 1997; Spencer et al., 2003; Kilic et al., 2008; Wada and Breuner, 2008). Such findings suggest that high baseline corticosterone

concentrations of ducklings incubated at the low temperature may have contributed to the slower growth and reduced body sizes we observed in this treatment group. Again, however, 9 day old ducklings incubated at the lowest temperature may be developmentally younger than ducklings incubated at higher temperatures. Retarded development might account for both their smaller body size and higher plasma corticosterone concentrations compared to ducklings incubated at the higher temperatures. This may also explain why we did not detect a relationship between corticosterone and body condition. Body size and corticosterone may be tightly linked with developmental stage, whereas body condition may not.

Biologists have long appreciated the importance of maternal condition during reproduction and the adaptive interplay between maternal and offspring health (Trivers and Willard, 1973). Indeed, maternal effects are known to have profound effects on offspring, often rivaling the effects of genomic contributions to offspring fitness (Bernardo, 1996; Price, 1998). Several recent reviews have highlighted the importance of such early developmental experiences because of the effects they can have on subsequent life history decisions that ultimately affect survival and reproductive success (Lindstrom, 1999; Metcalfe and Monaghan, 2001). Only recently, however, have we begun to appreciate the role that female incubation behavior in birds might play in shaping the phenotype of her offspring (Hepp et al., 2006; Walker et al., 2005; Cyr et al., 2007). Female behavior plays a large role in determining the environment of the nest, because her attendance positively affects nest temperatures (Martin et al. 2007). Our study clearly demonstrates that even small changes in nest temperature caused by variation in incubation behavior can have dramatic effects on the physiology of hatchlings. If these sublethal effects translate into differences in survival or recruitment then there may be strong selection on females to maintain a narrow range of nest temperatures. Furthermore, suboptimal foraging

conditions or frequent disturbance may reduce female reproductive success by forcing females to allocate time away from incubation.

Acknowledgements

Data presented in this chapter was reproduced with permission from The Journal of Experimental Biology in reference to manuscript: DuRant, S.E., G.R. Hepp, I.T. Moore, B.C. Hopkins*, W.A. Hopkins. 2010. Slight differences in incubation temperature affect early growth and stress endocrinology in wood duck (*Aix sponsa*) ducklings. *Journal of Experimental Biology,* 213: 45-51. DOI: 10.1242/jeb.o34488.

We would like to thank Anne McNabb, Paul Siegel, Bobby Kennamer, John Burke, Haruka Wada, Christine Bergeron, and Sarah Budischak for technical assistance and John Cohen for statistical assistance. We would also like to thank Jason Scott, Emily Butler, J.D. Willson, Matt Carney, Bobby Kennamer, and Benton Gann for their help in the field. J.D. Willson, Brian Todd, Jeff Walters, and Dana Hawley reviewed earlier drafts of the manuscript. Primary funding for this project was provided by the National Science Foundation grant IOB-0615361 to WAH and GRH. A Virginia Tech Graduate Student Assembly Graduate Research and Development Program Award to SED also helped support this research.

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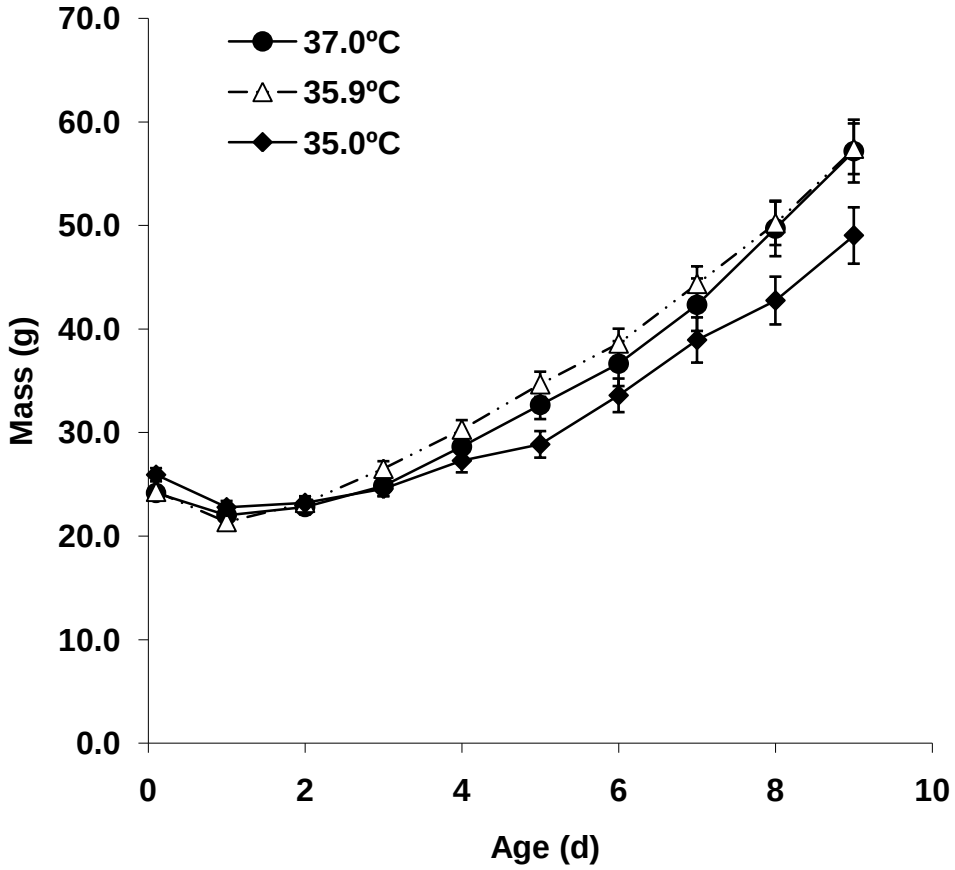

Figure 1.1. Patterns of growth among Wood Duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0ºC). Only ducklings that survived to 9 d post hatch are included. Error bars are ± 1 standard error of the mean.

35.0ºC: N = 12; 35.9ºC: N = 27; 37.0ºC: N = 15.

An * denotes when significant treatment effects were detected in post hoc analyses of the overall statistical model.

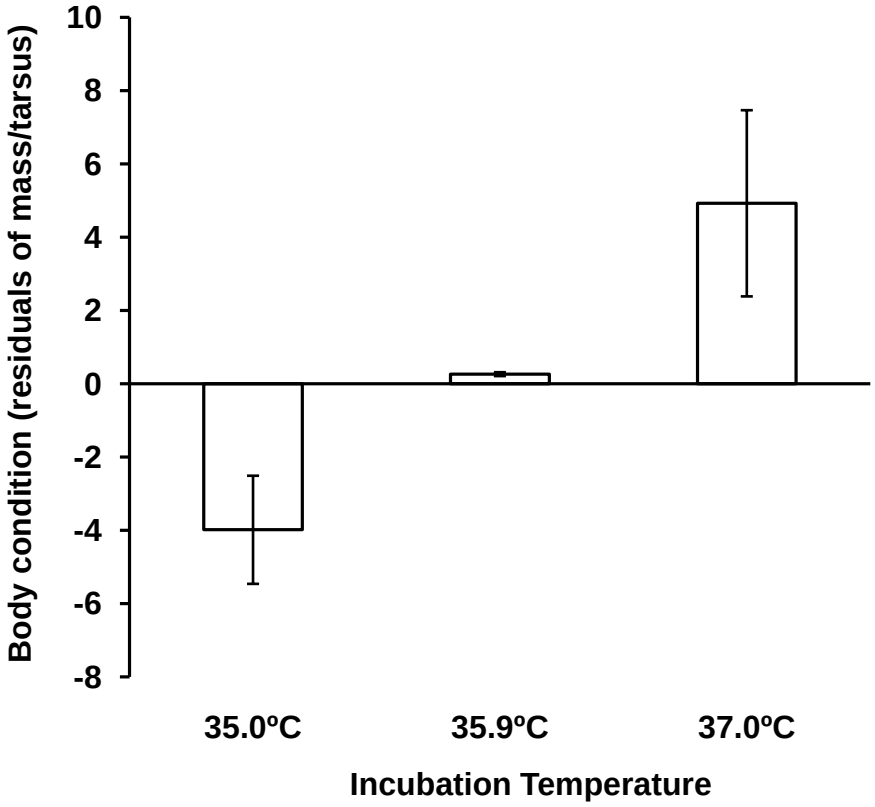

Figure 1.2. Body condition (residuals of body mass/tarsus length) of 9 d post hatch Wood Duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0ºC). 35.0ºC: N = 15; 35.9ºC: N = 26; 37.0ºC N = 10. Differences in body size among treatments were examined using ANCOVA with mass as the independent variable and tarsus as the covariate.

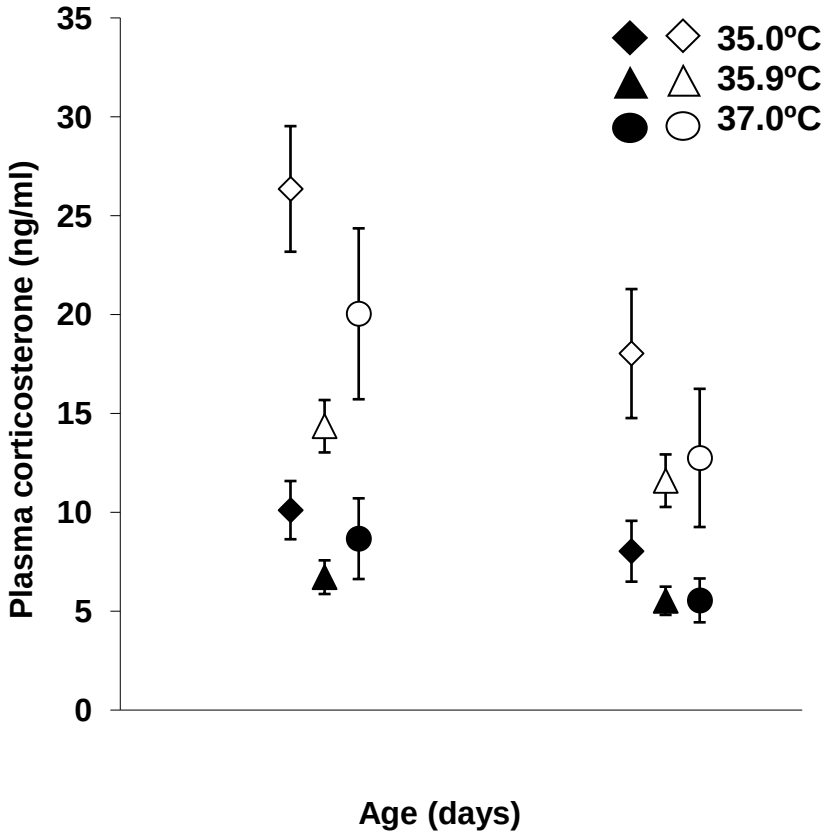

Figure 1.3. Plasma corticosterone concentrations (ng/ml) of Wood Duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0ºC). Closed symbols represent baseline plasma corticosterone concentrations and open symbols represent stress-induced corticosterone concentrations. The same individuals were bled at each data point. Error bars are ± 1 standard error of the mean. 35.0ºC: N = 14; 35.9ºC: N = 25; 37.0ºC: N = 10.

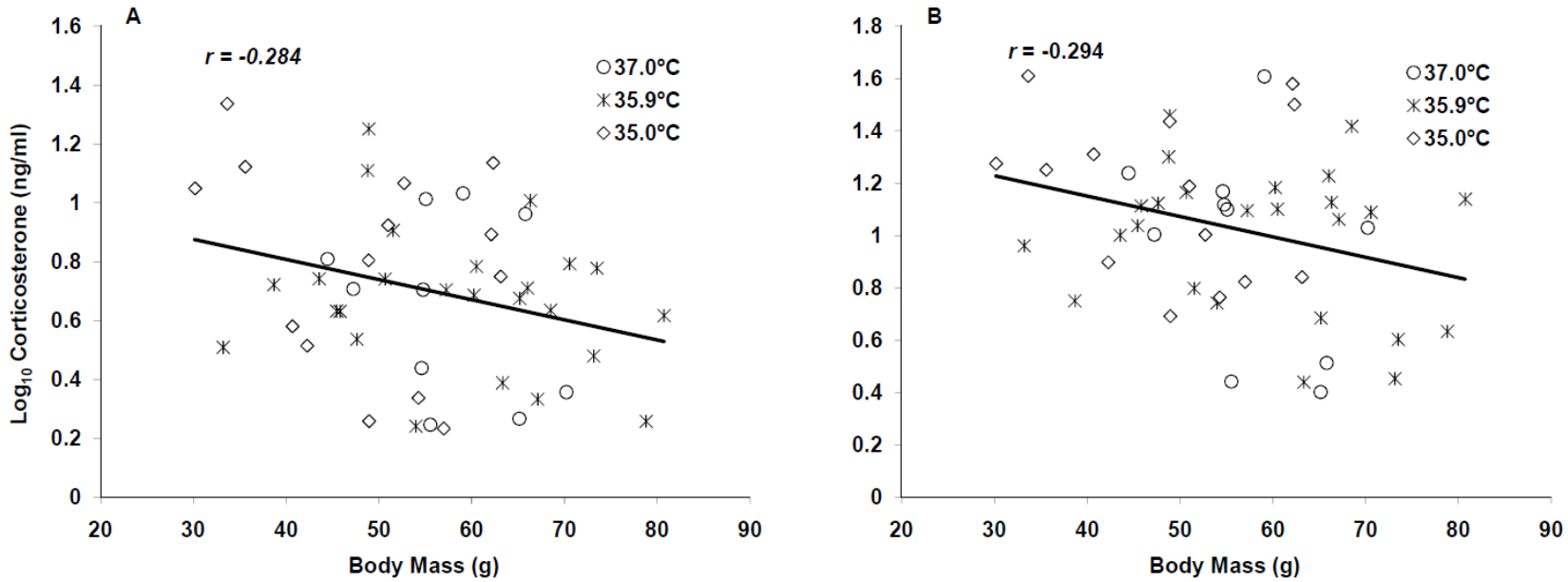

Figure 1.4. Relationships between corticosterone concentrations (A: Baseline; B: Stress-induced) versus body mass in 9 d post hatch Wood Duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0ºC). 35.0ºC: N = 14; 35.9ºC: N = 25; 37.0ºC N = 10.

Chapter 2: Incubation Temperature Affects Multiple Measures of Immunocompetence in Young Wood Ducks (*Aix Sponsa*)

Co-authors: William A. Hopkins, Dana Hawley, Gary Hepp

* Formatted for Functional Ecology

Abstract

In birds, incubation behaviour is a critical parental effect as it influences the early developmental environment and can consequently have lifelong consequences for offspring phenotype. Recent studies that manipulated incubation temperature have noted profound effects on hatchling body composition, condition, and growth suggesting that incubation temperature also could affect energetically-costly physiological processes of young birds that are important to survival (e.g., immune responses). To control for other environmental factors that could influence avian development, we artificially incubated wood duck *(Aix sponsa*) eggs at three biologically-relevant temperatures. Following experimental incubation, we used three immunoassays to measure innate and acquired immune responses of ducklings. Incubation temperature affected responses requiring acquired immunity but not responses dependent solely on innate immunity. These effects may have been mediated via differences in duckling resource availability (e.g., energy, protein stores) as we also detected differences in duckling growth and body condition. Our results show that incubation temperatures can be an important driver of phenotypic variation in avian populations.

Key Words

Maternal effects, acquired immunity, innate immunity, PHA, SRBC, BKA, hematology

Introduction

Parents have enormous non-genomic influences on the health and development of their offspring (Mousseau & Fox 1998; Badyaev & Uller 2009). Parental behaviour or physiology can influence offspring development and play a critical role in determining offspring phenotype (Mousseau & Fox 1998; Badyaev & Uller 2009). These non-genomic contributions, referred to broadly as parental effects, can ultimately shape the life history trajectory of the offspring by influencing growth rates, age at maturity, survival, and reproduction (Lindstrom 1999; Metcalfe & Monaghan 2001; Badyaev & Uller 2009). Non-genomic contributions may also serve as a key link between changing environmental conditions, such as climate, and resulting phenotypic change (Mousseau & Fox 1998; Hayward & Wingfield 2004).

In birds, parental incubation determines much of the early developmental environment, influencing both the humidity and temperature under which the eggs develop (Deeming 2002). Incubation behaviour by avian parents consists of on-off bouts which largely keep eggs within a narrow temperature range (Deeming 2002), but optimal incubation temperatures become compromised when parents spend time away from the nest leading to greater predation risks and lower hatching success of eggs (Reid, Monaghan & Nager 2002). Although we know that temperatures of naturally-incubated nests vary within avian species, we are just beginning to appreciate the consequences of variation in incubation temperature for avian phenotype. For instance, in wood ducks (*Aix sponsa*, Linneaus), slight differences (< 1 °C) in average incubation temperature influence duckling body composition, growth, body condition, locomotor performance and plasma stress hormone concentrations (Hepp, Kennamer & Johnson 2006; DuRant et al. 2010; Hopkins et al. In review). The results of these studies suggest that incubation

temperature also might influence energetically demanding physiological processes, such as mounting an immune response.

Maintaining and using the vertebrate immune system can be quite costly (Lochmiller & Deerenberg 2000; Martin, Scheuerlein & Wikelski 2003). For example, animals exposed to benign immune challenges, designed to up-regulate immune function without any associated pathology, exhibit decreased growth (Uller, Isaksson, & Olsson 2006), survival (Hanssen et al. 2004), and reproduction (Uller et al. 2006; Ilmomen, Taarna & Hasselquist 2004). In addition, immune responses require significant amounts of protein (Beisel 1977), much of which is derived from the breakdown of skeletal muscle.In this study we sought to determine whether incubation temperature influences the immune system of hatchling wood ducks. Because previous studies indicated that ducklings incubated at lower temperatures have lower protein content, are in poorer body condition, and grow more slowly than ducklings incubated at the higher temperatures (Hepp et al. 2006; DuRant et al. 2010), we predicted that ducklings incubated at the lowest temperature would have lower immune responses than ducklings at higher temperatures. In order to pinpoint temperature as the key environmental factor affecting duckling immunity we incubated wood duck eggs in the laboratory at three biologically-relevant temperatures.

Methods

Egg Collection and Incubation. We collected wood duck eggs from nest boxes (n = 201 nest boxes) located in west-central South Carolina and stored eggs at 20°C and 55-60% humidity prior to transport. Every four days we transported eggs to Virginia Tech, where they were artificially incubated in Grumbach incubators (model BSS 160) at one of three temperatures

(35.0, 35.9, and 37.0 °C) at 60-65% humidity. Our incubation temperatures were chosen because they fall within the range of naturally-incubated wood duck nests (Hepp et al. 2006) Incubators were programmed to allow two cool-down periods each day (~3°C reduction in mean temperature for 75 min at 0815 and 1830) to simulate natural daily feeding recesses taken by mothers during incubation (Manlove & Hepp 2000).

Duckling Husbandry. After hatching we maintained ducklings communally in 46 x 32 x 24.5 cm plastic cages (2-3 ducklings/ cage) in a temperature controlled environmental chamber (28°C, 14L:10D photoperiod). A 50 watt infrared light bulb suspended 32.5 cm above the bottom of each cage (creating a thermal gradient) provided additional warmth to ducklings. Ducklings were allowed constant access to food (Dumor Chick Starter/Grower 20%) and water. All experimental procedures were conducted in accordance with approved Virginia Tech IACUC protocols.

Duckling Growth and Body Condition. We monitored growth daily by weighing ducklings and measuring tarsus length (tarsometatarsus). We calculated body condition by regressing body mass against tarsus length and used the residuals as an individual's body condition. The residuals from these analyses were used in all statistical comparisons of body condition.

Challenging the Immune system. We measured duckling immunocompetence using hematology and three commonly-used immunoassays which provided estimates of innate, pro-inflammatory immune responsiveness (requires aspects of both innate and acquired immunity; Vinkler, Bainova & Albrecht 2010), and humoral immunity (a branch of acquired immunity). We measured innate immunity in all ducklings at 1 and 6 dph using a bacteria killing activity (BKA)

assay which quantifies the proportion of bacteria killed when exposed to duckling blood (Liebl & Martin 2009). We also quantified innate immunity of 6 dph ducklings by making blood smears to later asses for quantity of heterophils, lymphocytes, eosinophils, basophils, and monocytes (Clark, Boardman, & Raidal 2009). We measured pro-inflammatory immune responsiveness in half of these ducklings at 6 dph using phytohemattagluttinin (PHA) and humoral immunity in the remaining half of the ducklings at 6 dph using sheep red blood cells (SRBC). All blood sample collection and injections with novel antigens occurred between 1200 and 1600 h during March-August 2009. To avoid pseudoreplication and potential bias attributed to parentaleffects on immune parameters, we only measured immune function of one duckling per clutch per incubation temperature treatment per immune assay.

The first assay, bacteria killing activity (BKA) assay, examines the capacity of the blood to rapidly thwart potential pathogens and is used as a measure of innate immunity. We collected 70 µL of whole blood from the femoral artery of 1 and 6 dph ducklings that hatched from eggs incubated at the three temperatures. Blood was immediately placed in a -80°C ultracold until samples were assayed in February 2010. Assay methods followed those outlined in Liebl and Martin (2009). Briefly, we added $10^{\wedge 5}$ *E. coli* suspended in PBS to 1:20 dilution of duckling whole blood. We added CO_2 independent media to all samples and then incubated them for 1 hr at 37°C to allow bacteria-killing to occur. After the incubation period we added tryptic soy broth (TSB) to all samples and prepared positive controls. Positive controls contained $10^{\wedge 5}$ *E. coli* solution and TSB. All samples and controls were then incubated at 37°C for 8 hrs to allow bacteria growth. At the end of the 8 hr incubation we measured sample absorbance using a nandrop. We determined the proportion of bacteria killed by comparing sample absorbance against control absorbance. All controls and samples were assayed in triplicate.

We made slides by smearing a drop of the blood collected for BKA on a glass slide. Slides were air dried in slide boxes and stored until they could be stained and observed for immune cell presence. We stained slides using a wright geisma stain. To determine presence of immune cells 100 WBCs were counted from each bird using a "battlement track" along the monolayer of cells.

At six dph we exposed 46 ducklings to phytohemataglutanin (PHA), a plant lectin, to measure cutaneous immune responsiveness. PHA is a plant lectin that stimulates both innate and cell-mediated immunity and results in the migration of T-cells and B cells to the injection site (Martin et al. 2006; Vinkler et al. 2010). We injected 30µl PHA (2 mg/mL PHA dissolved in PBS) into the left proximal foot web of ducklings and then measured the amount of swelling, induced by the migration of T-cells and B cells to the injection site (Martin et al. 2006; Vinkler et al. 2010). Prior to injection we measured foot web thickness of both the right and left foot using a micrometer (Mitutoyo No. 7309). We measured swelling in response to the injection at 6 and 24h after injection and every 24 h thereafter until swelling subsided (up to 14 d). Based on our data, peak swelling occurred 24 h after injection, therefore this time point was used in statistical analyses. We calculated swelling response as the difference between left foot web thickness and right foot web thickness. Fold increase in swelling was $\log_{10}$-transformed prior to statistical analyses.

To assess humoral immunity we injected 6 dph ducklings (n = 59) intraperioteneally with 200 µl of 10 % SRBC. Immediately prior to injections we collected 70µl of blood from the femoral artery to determine baseline antibody levels, then collected blood again 6 d after injection to determine antibody responses. Preliminary data revealed that peak antibody production to SRBC injection in our ducklings occurred 6 d after injection. After we collected

blood samples, we centrifuged them, removed plasma and stored samples at -80°C until we analyzed plasma for antibody production in August 2010. We used a standard haemagglutination (HA) test to quantify the antibody concentration following methods described in Hanssen et al. 2004 and Hay and Hudson 1989.

Data Analysis. All statistical analyses were performed in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normality and homoscedasticity. We used raw data values in statistical analyses except in our analysis of swelling in response to PHA injection and heterophil to lymphocyte ratios. Both fold increase in swelling and heterophil/lymphocyte ratios were $\log_{10}$-transformed prior to statistical analyses. Body mass and body condition were initially included as covariates in all models examining effects on the immune system Body condition was retained in the SRBC model, but both body condition and body mass were dropped from final versions of all other models due to insignificance (body mass: all $p \geq 0.65$; body condition: all $p \geq 0.29$). To avoid pseudoreplication and potential bias attributed to parental effects on immune parameters, we only measured immune function of one duckling per clutch per incubation temperature treatment per immune assay.

Results

Hatching success and post-hatch survival were binary, therefore we analyzed these variables using a Chi Square test and Fisher's exact test (SAS Proc Freq), respectively. We initially included clutch (i.e., nest of origin) as a random effect in these analyses, but subsequently removed clutch from models because models excluding this variable were a better

fit to the data set. There was no effect of incubation temperature on hatching success (35.0°C: 61 %, 35.9°C: 65 % and 37.0 °C: 57 %) or post hatch survival (35.0°C: 80 %, 35.9°C: 82 % and 37.0 °C: 86 %), in both cases $p \geq 0.54$.

Incubation temperature significantly affected the duration of incubation (ANOVA, $F_{2,129} = 160.89$; $p < 0.001$; all post-hoc pairwise comparisons $p < 0.05$), with higher temperatures inducing more rapid development (35.0°C: 32.1 ± 0.18 days; 35.9°C: 34.4 ± 0.18 days; 37.0 °C: 37.2 ± 0.24 days). To examine the effects of incubation temperature on hatchling mass we used an ANCOVA (SAS Proc GLM) with fresh egg mass as the covariate. There was a strong positive relationship between hatchling mass and egg mass ($F_{1,92} = 219.22$; $p < 0.001$), however there was no effect of incubation temperature on hatchling mass ($p = 0.737$).

We found a significant influence of incubation temperature on both duckling growth (Repeated measures ANOVA: temperature X time: $F_{30,1380} = 3.54$; $p = 0.016$; Figure 1A) and duckling body condition (Repeated measures ANOVA: temperature X time: $F_{8,138} = 2.75$; $p = 0.008$; Figure 1B). In our analysis of body condition we compared duckling body condition at five ages: hatching, 2, 6, 10, and 20 dph. Ducklings were similar in mass and body condition during the first few days post-hatching; however as ducklings aged, individuals from the lowest incubation temperature exhibited slower growth and poorer body condition compared to ducklings from the higher incubation temperature treatments. By 20 dph, ducklings from the lowest incubation temperature weighed 7-8% less and were in much poorer body condition than ducklings from the medium and high incubation temperatures.

The proportion of bacteria killed by duckling blood was not affected by incubation temperature (temperature: $p = 0.290$; temperature X age: $p = 0.866$; Figure 2A). Ducklings from all incubation temperatures had similar BKA at both 1 and 6 dph (repeated measures ANOVA;

age: $p = 0.910$). Linear regression revealed a significant correlation between when an individual was sampled and it's BKA on both day 1 and day 6 such that samples that spent a longer time in the freezer had lower killing ability (Fig. 2.3; Day 1: $R^2 = 0.107$; $p < 0.001$; $R^2 = 0.117$; $p < 0.001$). Individuals also had similar BKA on day one as they did on day 6 (Fig. 2.4; $R^2 = 0.177$; $p < 0.001$).

We also did not detect a significant effect of incubation temperature on duckling cells associated with innate immunity, nor lymphocytes, cells associated with acquired immunity ($F_{8, 168} = 1.28$; $p = 0.250$; table 1). These data were analyzed using a MANOVA (SAS proc glm). Investigation of individual ANOVA's for each cell type also revealed that no cell type differed among incubation temperatures (in all cases $p > 0.173$). We also did not detect a significant difference in $\log_{10}$transformed heterophil/lymphocyte ratios among incubation treatments (ANOVA, SAS proc glm; $p = 0.315$).

Ducklings from each incubation temperature treatment exhibited a pronounced swelling response after injection with PHA (Figure 2B). Contrary to duckling bactericidal ability, swelling responses differed significantly among incubation temperature treatments (ANOVA; temperature: $F_{2,43} = 3.57$; $p = 0.037$). Peak swelling was lower in ducklings incubated at the lowest temperature compared to ducklings incubated at the higher temperatures. Ducklings from all incubation temperatures produced antibodies in response to SRBC injection (Figure 2C). Similar to our PHA results, ducklings incubated at the lowest temperature had lower antibody responses to SRBC injection than ducklings incubated at the higher temperatures (ANCOVA; temperature: $F_{2,54} = 5.03$; $p = 0.001$). In addition, there was a trend towards ducklings in better body condition producing more antibodies (body condition: $F_{1,54} = 3.35$; $p = 0.073$).

Discussion

To our knowledge, this is the first study to definitively demonstrate in a wild bird that incubation temperature affects the immune system, a requirement for defending against pathogens. We found that ducklings incubated at the lowest temperature had less robust acquired immune responses than ducklings incubated at higher temperatures. Effects on the immune system early in life may be particularly important for precocial species which begin interacting with and foraging in their environment shortly after hatching. In addition, we demonstrated that incubation temperature influences both duckling growth and body condition, with low temperatures producing ducklings that grow more slowly and exhibit poorer body condition than higher temperatures. As these effects persisted until at least 20 dph, our results suggest that effects of incubation temperature on body size and mass may persist, or even amplify, throughout juvenile development. Body size is important to over winter survival and age at first breeding events (Ringsby, Saether, & Solberg 1998; Hepp, Kennamer & Harvet 1989), therefore differences in condition and growth trajectories have implications for lifetime reproductive success.

Contrary to our predictions, we did not detect an effect of incubation temperature on microbicidal capacity of duckling blood, a measure of innate immunocompetence, nor among the quantity of immune cells present in duckling blood that are associated with innate immunity (e.g., heterophils, basophils, eosinophils, and monocytes; Clark, Boardman, & Raidal 2009). Our findings differ from a study that experimentally-manipulated temperatures of tree swallow nest boxes (Ardia, Perez & Clotfelter 2010). Nestling tree swallows (*Tachycineta bicolor*,Vieillot), an altricial species, from cooled nests had lower killing ability than control nestlings. The discrepancy between our study and Ardia et al. (2010) could be explained by the profound

differences in life history and early development of these two species. Alternatively, differences could be attributable to female tree swallow behaviour, as female tree swallows from experimentally-cooled nest boxes exhibited differing incubation patterns than control females. For instance, the differences in female tree swallow behaviour affected how frequently eggs underwent cooling and rewarming and could have produced additional differences in incubation constancy or humidity which also may have influenced hatchling innate immunity. In contrast, the only incubation condition manipulated in our study was temperature.

In contrast to microbicidal activity we found that incubation conditions did influence both immune responses that require acquired immune function. Although all ducklings exhibited a swelling response after injection with PHA, an immune challenge that stimulates both innate and acquired immunity (Martin et al. 2007; Vinkler et al. 2010), ducklings from the low incubation temperature had 19—21% lower swelling than ducklings from the higher incubation temperatures. Similarly, all ducklings produced antibodies in response to SRBC injection, but ducklings from the lowest incubation temperature produced 38 and 32 % fewer antibodies than ducklings incubated at the medium and high temperatures, respectively. These findings are consistent with an across species comparison of response to PHA injection and length of incubation (Palacios & Martin 2006). Less robust acquired immune responses could indicate that ducklings from the lowest incubation temperature were developmentally-delayed relative to ducklings incubated at the higher temperatures, lending support to the hypothesis that retarded development occurs in embryos incubated at suboptimal temperatures (Martin et al. 2007). We did not detect a difference in blood lymphocyte levels, important adaptive immune cells (Clark, Boardman, & Raidal 2009), among incubation treatments. However, this is probably because blood samples were taken before ducklings were exposed to an antigen, and represent normal

circulating levels of lymphocytes, and not the duckling's ability to upregulate lymphocytes or lymphocyte ability to produce antibodies, etc (Lattimer et al. 2003).

The differences in effect of incubation temperature on innate immunity (BKA and innate immune cell levels in the blood) versus responses to SRBC and PHA may result from the resource dependence of acquired immune responses. For instance, previous studies have shown that measures of innate immunity, (e.g., microbicidal capacity) are less-likely to correlate with measures of condition than measures of acquired immune function (e.g., PHA and SRBC; Forsman et al. 2010; Palacios et al. 2009). It has been proposed that this discrepancy may arise because innate immunity relies on factors already present in the blood before an animal is exposed to an antigen, whereas a response involving acquired immunity is more resource-intensive, involving up-regulation of immune cells after an antigen is detected. Ducklings that hatch from eggs incubated at the lowest temperature have lower protein content, are in poorer body condition and exhibit slower growth than ducklings from higher incubation temperatures suggesting that they have fewer resources available for mounting an acquired immune response. This could explain why we saw effects of incubation temperature on SRBC responses, an acquired component, and to a less extent, PHA, which is thought to induce both innate and acquired components (Vinkler et al. 2010), but not BKA. Indeed, there was a trend towards greater SRBC antibody production in ducklings in better body condition. Thus, our results suggest that that the effects of incubation temperature on the immune system may be mediated through indirect pathways (e.g., resource and energy availability). Alternatively, our results could stem from direct effects of incubation conditions on the immune system. Such hypotheses have been proposed to explain a negative relationship between malaria prevalence and incubation period across 36 altricial avian species observed by Ricklefs (1992). However, our

within species comparison shows the opposite pattern of what would be predicted by the Ricklefs hypothesis.

The importance of maternal health to offspring phenotype is well-documented (Mousseau & Fox 1998), however only recently have we begun to appreciate the role incubation conditions, which are largely determined by parental incubation behaviour, play in determining avian offspring phenotype. Here we demonstrate that slight differences in incubation temperature produce variability in duckling immunocompetence. If less robust immune responses translate into greater disease susceptibility, then incubation conditions could influence disease dynamics in avian populations, especially if differences in immunocomptence persist into adulthood. For instance, there may be a greater number of disease susceptible individuals when parents are nesting in sub-optimal habitats (e.g., areas of high disturbance or low resource availability) or sub-optimal environmental conditions (e.g., during droughts) and are less capable of maintaining nest temperatures due to increased time spent away from their nest foraging, etc. Furthermore, as immune function, growth and body condition have important implications for survival there may be strong selection pressure on parents to maintain optimal nest temperatures. Perhaps most importantly, our findings suggest that incubation conditions play an important role in providing phenotypic variation, which provides the variability upon which natural selection acts, within avian populations.

Acknowledgements

Many thanks to B. Hopkins who provided invaluable laboratory assistance. Also thank you to J. Fallon for staining and analyzing slides. We would also like to thank A. Wilson, J. McPherson, J. Walls C. Earle, C. Gaston, A. McNabb, P.Siegel, B. Kennamer, J. Burke, H. Wada, M. Martin,

A. Liebl, L. Kirkpatrick, W. Slade, P. Bowerman and M. Hepner for field and laboratory assistance and J. Cohen for statistical assistance. J.D. Willson, J. Walters, and I. Moore reviewed earlier drafts of the manuscript. Funding for this research was supported by National Science Foundation (NSF) grant IOB-0615361 (GRH and WAH) and small grants from the Sigma Xi, Society of Integrative and Comparative Biology, and Virginia Tech Graduate Research and Development program awarded to SED. IACUC approved proposal #08-067-FIW; Collection Permits:South Carolina Department of Natural Resources G-08-07; US Fish and Wildlife Service MB748024-0.

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Table 2.1. Immune cell levels in blood of 6dph ducklings incubated at three different temperatures as determined by 100 white blood cell counts of blood smears. Data were analyzed using both MANOVA (including all but N/L ratios) and 6 separate ANOVA's. *P*-values were derived from individual ANOVA's. The *p-value* for the overall MANOVA was (0.250)

	Incubation temperature			
Cell type	35.0˚C Mean (1SE)	35.9˚C Mean (1SE)	37.0˚C Mean (1SE)	*p*
Eosinophils	2.73 (0.30)	3.33 (0.31)	2.62 (0.30)	0.186
Basophils	1.88 (0.26)	1.50 (0.25)	1.90 (0.33)	0.548
heterophils	47.0 (2.2)	45.8 (1.6)	43.1 (2.1)	0.263
Lymphocytes	45.7 (2.2)	47.3 (1.6)	50.2 (2.0)	0.173
Monocytes	2.73 (0.34)	2.06 (0.32)	2.37 (0.32)	0.350
Heterophil/lymphocyte ratio	1.16 (0.11)	1.07 (0.10)	1.00 (0.11)	0.315

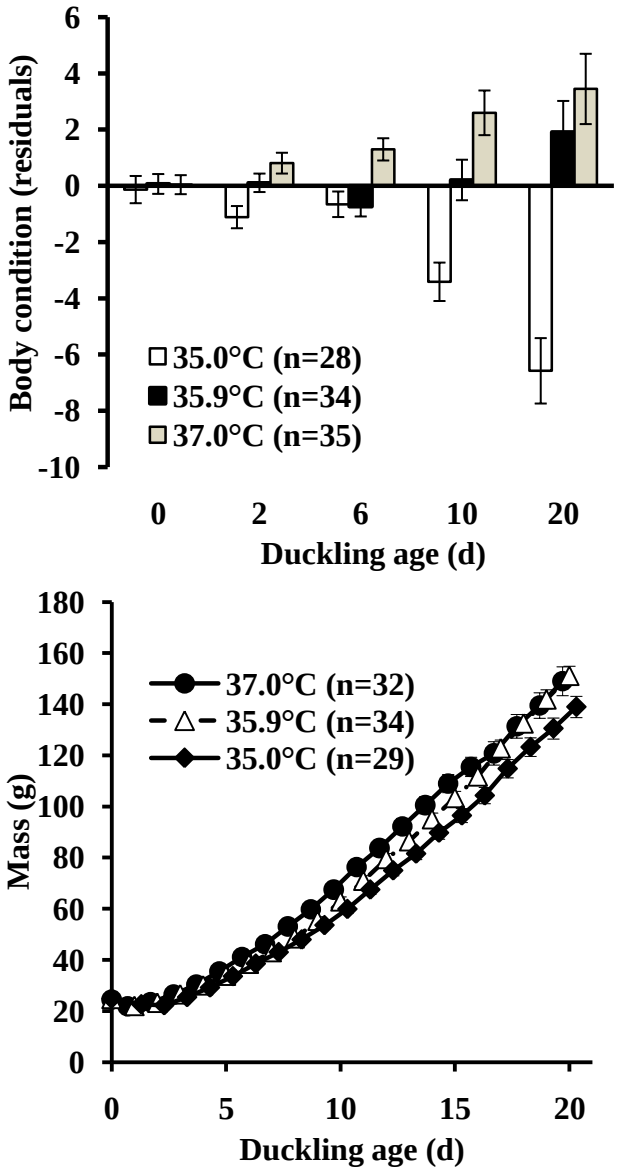

Figure 2.1. Duckling growth (A) and body condition (B) of wood duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Body condition is represented as the average residual of a regression of body mass versus tarsus length. Error bars are ± 1 standard error of the mean.

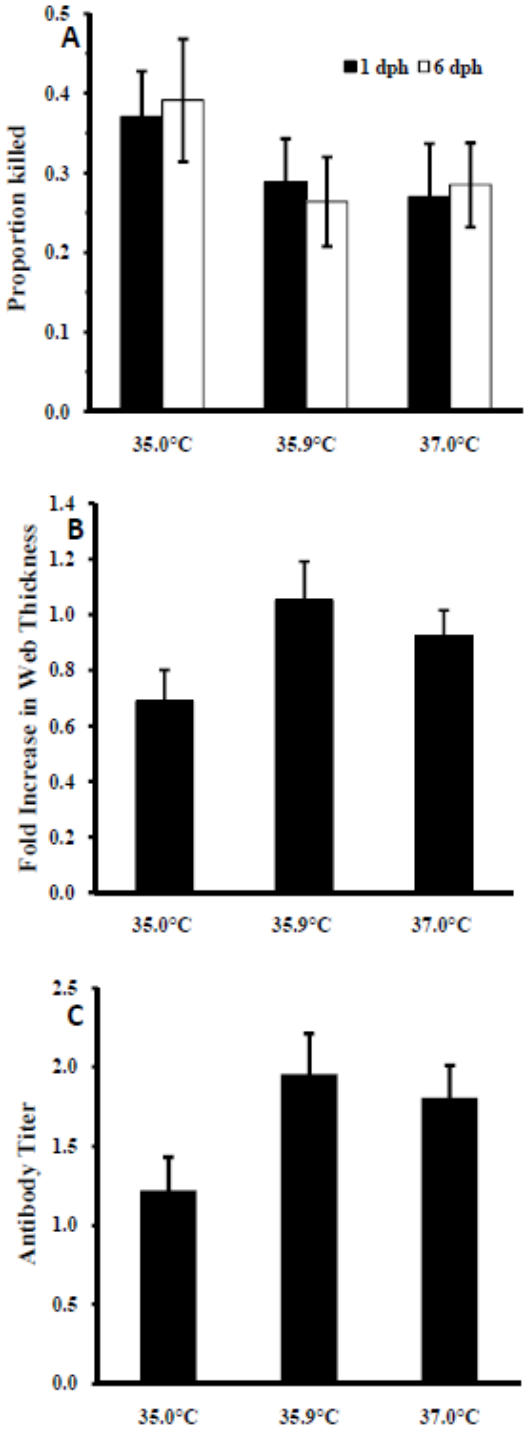

Figure 2.2. Immune responses of wood duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Error bars are ± 1 standard error

of the mean. A) Bacteria killing assay (BKA) – Proportion of *E. coli* killed by duckling blood collected when ducklings were 1 and 6 dph. 35.0°C: N = 31; 35.9°C: N = 39; 37.0°C: N = 33 B) Fold increase in foot web thickness of ducklings 24 h after exposure to a novel antigen, phytohemagglutinin (PHA). Ducklings were injected with PHA at 6 days post hatching (dph), 35.0°C: N = 14; 35.9°C: N = 16; 37.0°C: N = 16. C). Antibody titers of ducklings exposed to a novel antigen, sheep red blood cells (SRBC). Ducklings were injected with SRBC at 6 dph and antibody production in response to injection was measured at 12 dph. Error bars are ± 1 standard error of the mean, 35.0°C: N = 18; 35.9°C: N = 22; 37.0°C: N = 18.

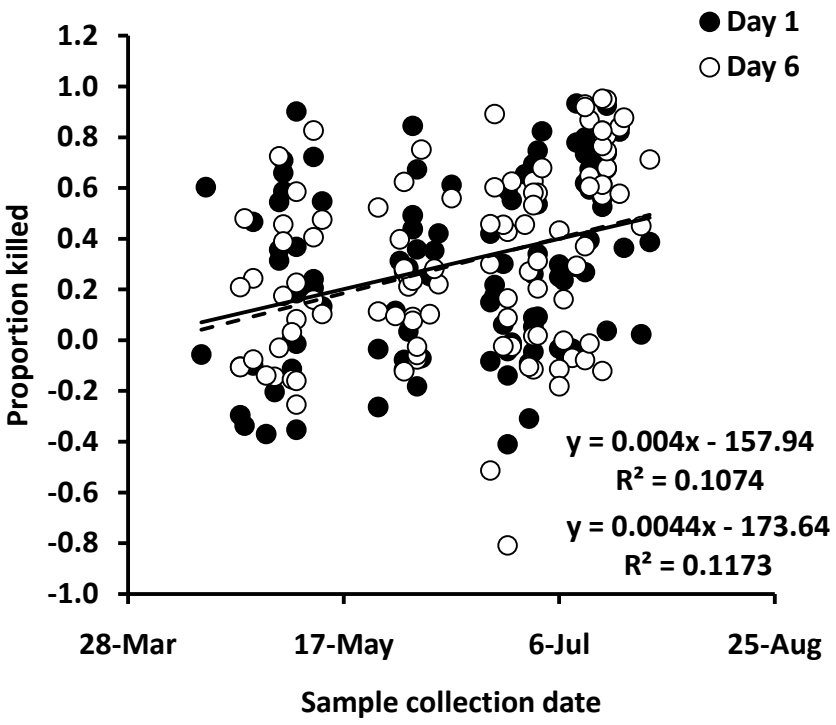

Figure 2.3. The relationship between sample collection date and the proportion of bacteria killed in 1 day (closed circles) and 6 day (closed circles) old ducklings.

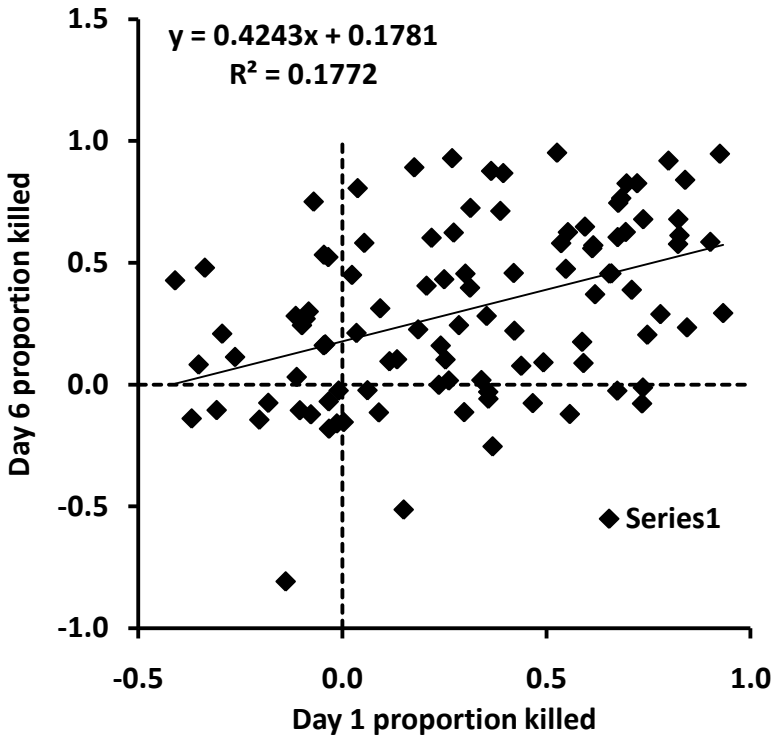

Figure 2.4. The relationship between an individual's bacteria killing ability on day one versus day six in wood duck (*Aix sponsa*) ducklings.

Chapter 3: Incubation temperature determines the early thermoregulatory ability of a precocial bird.

Co-authors: William A. Hopkins, Amanda Wilson, Gary Hepp

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Abstract

The developmental environment plays a key role in determining offspring phenotype, and the parents' behavior and physiology often dictates developmental conditions. Despite the plethora of studies documenting the importance of incubation temperature on offspring phenotype in reptiles, very few studies have examined such relationships in birds. Because nearly all birds physically incubate their eggs, altering the nest environment may be an important but previously-overlooked way parents can influence their offspring's phenotype. Here, we tested the hypothesis that incubation temperature would affect thermoregulation in wood duck (*Aix sponsa*) hatchlings. We show that a reduction of less than 1°C in incubation temperature affects the metabolic costs of thermoregulation in offspring of a non-domesticated bird, resulting in 27-40 % greater increases in oxygen consumption of ducklings incubated at the lowest temperature relative to ducklings incubated at higher temperatures. Because we demonstrate that incubation temperature affects hatchling phenotypic quality, our findings support newly proposed frameworks that highlight the importance of incubation temperature to the evolution of clutch size in birds.

Key Words *Aix sponsa*, bioenergetics, endothermy, incubation temperature, maternal effects, wood duck

Introduction

The behavior and physiology of parents can have tremendous influence on the early developmental environment of their young, which helps to shape their offspring's phenotype via non-genomic contributions (Badyaev & Uller 2009). Because birds physically incubate their eggs, brooding behavior provides a clear connection between avian parental behavior and incubation conditions experienced by the embryos. Physical contact with the eggs influences important aspects of embryonic microclimate including nest temperature and humidity (Deeming 2002; Martin et al. 2007). Recent research in non-domesticated birds demonstrates that incubation conditions can affect a suite of phenotypic traits in hatchling birds, many of which have implications for future development and survival (Cyr et al. 2007; Ardia, Perez & Clotfelter 2010)., In fact, a series of recent laboratory studies pinpoint incubation temperature as a major contributor to such phenotypic variation. These studies demonstrate that small differences in incubation temperature, reflecting variation in temperature found in naturally incubated nests, drastically affects hatchling immunocompetence (DuRant et al. In Review a), locomotor performance (Hopkins et al. In press), stress endocrinology (DuRant et al. 2010), growth, and body condition (DuRant et al. 2010).

For precocial species, perhaps one of the most important traits influencing survival of hatchlings is the early development of thermoregulatory ability. Among some waterfowl, mortality of hatchlings due to hypothermia is estimated to be as high as 25% (Korschgen et al. 1996). As hatchling birds transition to homeothermy, they rely on energy and protein stores and frequent brooding to survive cold periods. Development of homeothermy requires maturation of integument and increased protein in skeletal muscles (Visser1998). A previous study in wood

ducks (*Aix sponsa*; fig. 1) revealed that ducklings, at the pipping stage, from eggs incubated at lower temperatures had lower protein content than ducklings incubated at higher temperatures (Hepp, Kennamer & Johnson 2006). In addition, ducklings incubated at lower temperatures expend more energy during incubation than ducklings from higher incubation temperatures (DuRant et al. In Review b). The findings of those studies suggest that ducklings incubated at lower temperatures may have fewer energy reserves remaining after hatching to meet the demands of thermoregulatory challenges than ducklings from higher incubation temperatures. Since wood ducks begin nesting in late winter, hatchlings are frequently exposed to low temperatures (at times below freezing) that pose substantial physiological challenges for ducklings.

In this study, we tested the hypothesis that incubation temperature affects thermoregulation in hatchling wood ducks, and that ducklings hatched from eggs incubated at lower temperatures would be less effective and efficient at maintaining their body temperatures. In order to eliminate other environmental factors that affect avian development, we incubated wood duck eggs in the laboratory at three temperatures (35.0, 35.9, and 37.0°C) that fall within the range of temperatures of naturally-incubated nests (Hepp, Kennamer & Johnson 2006). Twenty-four hours after hatching we tested ducklings in one of two thermal challenge experiments, following the methods of Rhymer (1988). In the first experiment, we measured the change in a ducklings' body temperature after being exposed to a 1 hr thermal challenge at 5, 10, 15, 20, or 36°C (controls). In the second experiment, we estimated energy expenditure of ducklings for 1 hr at 36°C then again during a 1hr thermal challenge at 15°C. We indirectly measured energy expenditure by monitoring duckling oxygen consumption (Dorcas, Hopkins &

Roe 2004; Hopkins et al. 2004) which can be converted into energy equivalents assuming 1L O_2 = 19.6 kJ (Vleck, Vleck & Hoyt 1980).

Methods

Egg Collection and Incubation. Every four days until egg laying was initiated, we checked wood duck nest boxes (n = 201) located on two large reservoirs and several isolated wetlands in west-central South Carolina. We visited active nests daily, and collected and marked any new eggs. Eggs were stored at 20°C and 55-60% humidity. After four days of collection, we transported eggs to Virginia Tech and artificially incubated them in Grumbach incubators [model BSS 160) at one of three temperatures (35.0, 35.9, and 37.0 °C) at 60-65% humidity, which produced three incubation durations [37.2 ± 0.24, 34.4 ± 0.18, and 32.1 ± 0.18 days, respectively). Incubators were not maintained at constant temperatures, but were programmed for two cool-down periods each day (~3°C reduction in mean temperature for 75 min at 0815 and 1830 hr) to simulate natural daily feeding recesses taken by mothers during incubation (Manlove & Hepp 2000). All experimental procedures were conducted in accordance with approved Virginia Tech IACUC protocols.

Duckling Hatching and Duckling Husbandry. We monitored hatching success of eggs incubated at the three temperatures by checking for newly hatched ducklings 4 times a day between 0800 and 2000 hr. Upon hatching, we measured duckling mass and tarsus length and calculated body condition as the residual of mass regressed against tarsus. We maintained ducklings communally in 46 x 32 x 24.5 cm plastic cages (2-3 ducklings/cage) in a temperature-controlled environmental chamber (28°C, 14L:10D photoperiod). A 50 watt infrared light bulb

suspended above each cage provided additional warmth to ducklings. Ducklings were allowed constant access to water.

Thermoregulation and Bioenergetics of Ducklings. We examined the thermoregulatory capacity and energetics of ducklings by conducting cold-challenge experiments similar to those described by Rhymer (1988). In the first experiment, unfed one-day old ducklings incubated at three experimental temperatures (35.0, 35.9, and 37.0°C) were placed in individual 1-L glass chambers within an environmental chamber and allowed to acclimate for one hour at 36°C. Next, we measured body temperature of each bird using a cloacal thermometer (Scultheis T6000), then dropped temperatures of the environmental chambers to 5°C, 10°C, 15°C, 20°C, or left the temperature at 36°C (these ducklings served as controls) for 1 hr (n = 11-33 ducklings/per incubation temperature/per challenge temperature). After 1 hr at the ducklings' thermal challenge temperature, we again measured duckling body temperature. We used the percent change in a ducklings' body temperature from before to after the thermal challenge in statistical analyses. All of our challenge temperatures were within the range of temperatures experienced by ducklings in the field in South Carolina, USA.

Our second challenge experiment examined the metabolic costs associated with maintaining homeothermy. One-day old ducklings from eggs incubated at the three experimental temperatures (35.0, 35.9, and 37.0°C; n = 10-12 ducklings/per incubation temperature) were placed in an environmental chamber at 36.0°C (approximating thermoneutrality). After allowing ducklings to settle for 1 hr, we measured duckling respiration every 12 min for 1 hr using open flow respirometry (MicroOxymax, Columbus Instruments, Columbus, OH). Details on respirometry techniques are provided in Dorcas et al. (2004) and Hopkins et al. (2004).. We then

measured each duckling's body temperature using a cloacal thermometer in less than two minutes to minimize disturbance and potential influences on metabolic rate. Next, we dropped the environmental chamber to 15°C over a 15 min period and monitored respiration for an additional 1 hr. At the end of the 1 hr thermal challenge, we again measured duckling body temperature. We obtained 5 respiration measurements for our estimate of pre-trial respiration (or resting metabolic rate; RMR) and 5 measurements for our estimate of respiration during the thermal challenge. We calculated the integral of the respiration curves at thermoneutral (36°C) and 15°C to determine the volume of oxygen consumed before the thermal challenge and during the thermal challenge. Integrals were compared to determine the relative cost of homeothermy.

To avoid pseudoreplicationand account for parental effects on duckling thermoregulation and respiration, we only used one duckling per clutch per incubation temperature x thermal temperature treatment in both experiment 1 and 2.

Statistical Analyses. All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Unless otherwise noted, raw data were used in statistical analyses.

Results

The length of the incubation period differed significantly among incubation temperatures, with lower temperatures slowing developmental rates (ANOVA; SAS Proc GLM; $F_{2,342} = 359.2$; $p < 0.001$; see methods for incubation durations). Post hoc comparisons (Tukey HSD) revealed

that all incubation temperatures produced incubation periods that differed from one another. Using a Chi Square test (SAS proc freq) we determined that hatching success did not differ among incubation temperatures (p = 0.189; 35.0°C: 63%, 35.9°C = 71%, and 37.0°C = 70%).

To test for the effects of incubation temperature on duckling thermoregulatory ability, we used an ANCOVA (SAS Proc Mixed). Models were performed with both hatchling mass and body condition as covariates, but because hatchling mass had a stronger influence on changes in body temperature (see below) we used mass as the covariate in our final model. As the challenge temperature decreased, duckling body temperature also decreased (thermal challenge temperature: $F_{4,278}$ = 22.26; p < 0.001; Fig. 1). However, there was no effect of incubation temperature on a duckling's ability to maintain its body temperature (incubation temperature: p = 0.930; incubation temperature X thermal challenge temperature: p = 0.499). Post hoc comparisons (Tukey HSD) revealed that the percent change in body temperature of ducklings held at 36°C was significantly lower than that for all other thermal challenge temperatures. In addition, ducklings thermally challenged at 5°C had a 29-56% greater reduction in body temperature compared to ducklings challenged at 20°C. When controlling for incubation temperature, smaller ducklings were less effective at maintaining their body temperature than larger ducklings (hatchling mass [covariate]: $F_{1,278}$ = 6.45; p = 0.012). We detected a similar trend with body condition; ducklings in poorer body condition exhibited the greatest decreases in body temperature (body condition: $F_{1,278}$ = 2.91; p = 0.089).

We also tested for differences among incubation temperature treatments in hatchling mass and body condition of ducklings used in experiment 1 using ANCOVA (SAS Proc GLM) with egg mass included as the covariate. In addition, body condition of ducklings used in experiment 1 differed significantly among incubation temperatures ($F_{2,290}$ = 6.09; p = 0.003),

with the low incubation treatment producing ducklings in the poorest body condition. There was a similar trend with hatchling mass ($F_{2,291}$ = 2.37; p = 0.095); the lowest incubation temperature produced less heavy ducklings than the higher incubation temperatures. Egg mass also significantly affected hatchling mass and hatchling body condition (hatchling mass: $F_{1,290}$ = 303.05; $p < 0.001$; body condition: $F_{1,290}$ = 526.71; $p < 0.001$) where larger eggs produced heavier ducklings in better body condition.

Using an ANCOVA (SAS Proc Mixed) with $\log_{10}$-transformed oxygen values we determined that although ducklings from all incubation temperatures exhibited similar changes in body temperature when confronted with a thermal challenge, ducklings that hatched from eggs incubated at the lowest temperature had a greater increase in oxygen consumption during a thermal challenge than ducklings incubated at the higher temperatures (incubation temperature X time: $F_{2,31}$ = 5.60; p = 0.008; Figs. 2 and 3). In addition, ducklings in poorer body condition consumed more oxygen than those in better body condition (body condition [covariate]: $F_{1,30}$ = 11.66; p = 0.002). We detected a similar effect of hatchling mass (hatchling mass: $F_{1,30}$ = 6.74; p = 0.015) on oxygen consumption.

Again, we tested for differences among incubation temperature treatments in hatchling mass and body condition of ducklings used in experiment 2 using ANCOVA (SAS Proc GLM) with egg mass included as the covariate. There was no difference among incubation temperatures in hatchling mass or body condition of ducklings in experiment 2 (p = 0.479 and 0.255, respectively). Hatchling mass and body condition were significantly affected by fresh egg mass in which larger eggs produced heavier ducklings in better body condition (hatchling mass: $F_{1,30}$ = 61.68; $p < 0.001$; body condition: $F_{1,30}$ = 40.54; $p < 0.001$).

Discussion

Our findings reveal that incubation temperature is an important determinant of avian offspring phenotype, a phenomenon that has rarely been considered by avian ecologists (Deeming 2004). Here we demonstrate for the first time, that slight differences in incubation temperature, within the range found in naturally incubated nests, can affect energy expended by non-domesticated avian hatchlings during thermoregulation. In the wild, most birds likely develop in environments with limited resource availability, and thus, may not be able to compensate for large increases in energy expenditure. Energetic constraints could ultimately have implications for traits important to survival and reproduction (e.g., immunocompetence, growth, and size at maturity), suggesting that there is selection pressure on parents to maintain optimal incubation temperatures, in some cases within a very narrow range.

Contrary to our prediction, incubation temperature did not affect a duckling's ability to maintain its body temperature during a brief thermal challenge (Fig. 1). However, thermal challenge temperature did affect changes in body temperature, with all ducklings exhibiting greater decreases in body temperature as thermal challenge severity increased. Average body temperature of all ducklings at 1 day post hatch (dph) at thermoneutral was 38.6 ± 0.05°C, which is slightly lower than other duck species at 1 dph (e.g., Mallards, Common Goldeneye, Pekin ducks, Common Merganser: 39.0—40°C; Koskimies & Lahti 1964). Similar to the young of other precocial species, hatchling wood ducks appear to be relatively resilient to changes in body temperature as their body temperature regularly dropped 2-4°C when exposed to a thermal challenge (Visser 1998). The majority of mortality occurred in ducklings (8 of 294 ducklings) whose body temperature dropped below 33°C. Resilience to changes in body temperature is

thought to allow ducklings to transition to homeothermy without constantly relying on brooding from parents or huddling with siblings (Visser 1998). Because wood ducks in South Carolina begin hatching in early March and leave the nest within 24 hr, the ability to tolerate brief reductions in body temperature would increase their chances of survival during the first days after hatching.

Consistent with our predictions, incubation temperature had a strong effect on energy expended by ducklings during a thermal challenge. Despite similar resting metabolic rates (RMR) among incubation treatments, ducklings incubated at the lowest temperature had 27 and 40% higher metabolic rates during the thermal trial than ducklings incubated at the medium and high temperatures, respectively (Figs. 2 and 3). Our results suggest that even though ducklings incubated at the lowest temperature maintain their body temperature as well as those incubated at higher temperatures, they work harder to do so. Although no other studies have examined the influence of biologically relevant differences in incubation temperature on avian thermoregulation, a series of studies on domesticated species (domestic poultry and Muscovy ducks) suggest that brief exposure to reduced temperature during late incubation can influence offspring thermoregulation (summarized in Nichelmann 2004). Together, our work and the poultry work indicate that the effects of temperatures experienced during incubation on avian thermoregulation warrants further study in wild species.

Previous studies have shown that body composition of ducklings differs among incubation temperatures (Hepp, Kennamer, & Johnson 2006), which may account for the lower thermoregulatory efficiency of ducklings from the lowest incubation temperature. The disparity in duckling thermoregulatory ability among incubation treatments do not appear to be driven by differences in size and surface area to volume (SA/V) ratios as there was no difference in

duckling size or body condition among incubation temperatures in this experiment. Moreover, incubation temperature affected metabolism during a thermal challenge even after body size and condition were accounted for in statistical models. Greater energy expenditure in ducklings incubated at the lowest temperature could arise from differences in insulation as ducklings incubated at lower temperatures have lower lipid content than ducklings incubated at higher temperatures (Hepp et al. unpublished data). Less insulation could increase heat loss and therefore require greater heat production to compensate for these losses. Feather density is also critical for insulation in young ducklings, and future studies should quantify whether incubation temperature affects duckling feather development. Lower thermogenic capacity may also result from less mature muscle fibers of low incubation temperature ducklings as muscle maturity plays an important role in thermoregulation (Hohtola & Visser 1998). Indeed, a previous study demonstrated that low temperature ducklings have reduced locomotor performance, a trait also affected by muscle maturity, than ducklings incubated at higher temperatures (Hopkins et al. In Press).

Thermoregulation is essential to survival of precocial avian offspring; hypothermia accounts for 8-9% of mortality of mallards (*Anas platyrynchos*) and 24-25% of canvasbacks (*Aythya valisineria*) in the first days after hatching (Talent, Jarvis & Krapu 1983; Mauser, Jarvis & Gilmer 1994; Korschgen et al. 1996). High mortality due to hypothermia is probably the case with wood ducks as well, as up to 95% of wood duck duckling mortality occurs within two weeks of hatching (Bellrose & Holm 1994). In more northerly populations, mortality is often greater in broods hatched early in the nesting season, presumably because these ducklings are exposed to more severe weather (Bellrose & Holm 1994). Because ducklings are frequently exposed to ambient temperatures below thermoneutral, ducklings that are less efficient at

thermoregulating may have reduced capacity for other energetically costly processes, such as growth and immune function, as duckling metabolic rates were 155-250% greater during the thermal challenge than at thermoneutral. It is also important to note that we detected differences in respiration rates when ducklings were exposed to a relatively mild thermal challenge (15°C), therefore the magnitude of differences in respiration when thermoregulating should be exacerbated during more severe thermal challenge temperatures. Growing larger rapidly, thus reducing the SA/V ratio, is perhaps one of the best strategies for reducing the cost of homeothermy in precocial waterfowl. However, the ducklings expending more energy to thermoregulate may not be capable of offsetting the energetic demands of thermoregulating while concomitantly allocating more resources toward early growth.

Avian ecologists have long appreciated the role incubation temperature plays in hatching success and the length of incubation. Recently, however, theory on the evolution of avian clutch size has begun to incorporate incubation temperature as an important selective factor (Cooper et al. 2005; Martin 2008). Such evolutionary frameworks focus primarily on the relationships among incubation temperature, seasonal and latitudinal variations in clutch size, egg size, and incubation period, and how these relationships subsequently influence hatching success. Our research suggests that perhaps there is an additional layer of complexity to the relationships among nest temperature, egg size, and clutch size, as incubation temperature may also act as an important source of phenotypic variation among offspring within a population. In other words, clutch size also may be constrained by the phenotypic quality of young produced at various incubation temperatures.

Acknowledgements

We would like to thank J. Walls, M. McClintock, M. Nunez , P. Siegel, R. Kennamer, C. Stachowiak, T. Lombardi, M. Fink, C. Espada, G. Zapatero, C. Whitaker, M. Williams and M. Valett for field and laboratory assistance and J. Cohen for statistical assistance. J. Congdon, J. Willson, D. Hawley, J. Walters, and I. Moore reviewed earlier drafts of the manuscript. Funding for this research was supported by National Science Foundation (NSF) grant IOB-0615361 (GRH and WAH) and small grants from the Sigma Xi, Society of Integrative and Comparative Biology, and Virginia Tech Graduate Research and Development program awarded to SED. IACUC approved proposal #08-067-FIW; Collection Permits:South Carolina Department of Natural Resources G-08-07; US Fish and Wildlife Service MB748024-0.

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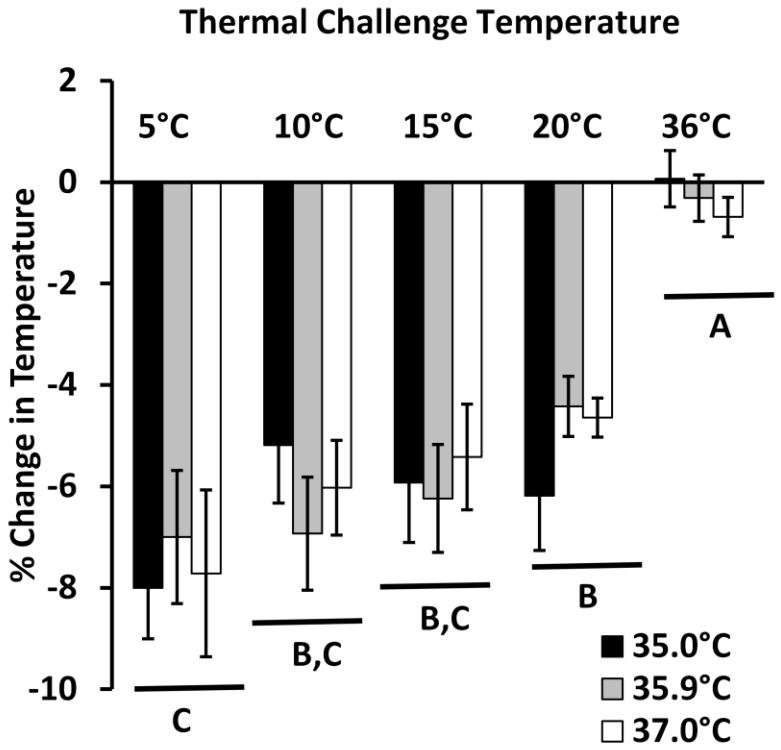

Figure 3.1. The effect of incubation temperature on thermoregulatory ability of hatchling wood ducks (Aix sponsa). Means (± 1 standard error) represent the percent change in a duckling's body temperature after being held at thermoneutral (36 °C) for 1 hr then being thermally-challenged for 1 hr at one of five temperatures (5, 10, 15, 20 or 36 °C). Ducklings hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). n = 11-33 ducklings/ thermal challenge temperature/incubation temperature.

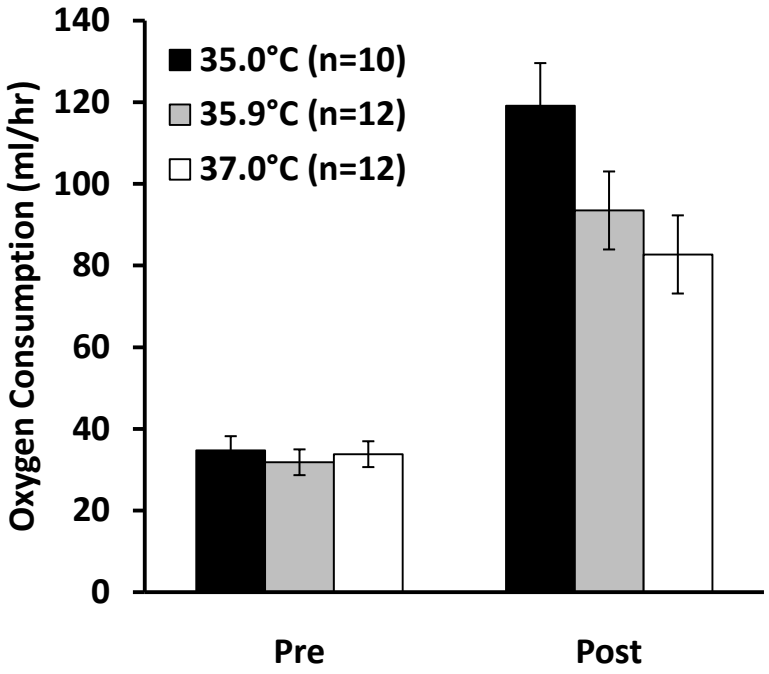

Figure 3.2. The effect of incubation temperature on oxygen consumed during a thermal challenge in 1 day post hatch wood ducks (*Aix sponsa*). Least-squares means (corrected for body condition) represent the volume of oxygen consumed (ml; integral of respiration curve) of ducklings held at thermoneutral (36 °C) for 1 hr then thermally-challenged at 15 °C for 1 hr. Ducklings hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Statistical analyses were conducted on $\log_{10}$-transformed oxygen data. Error bars are ± 1 standard error of the LSmean.

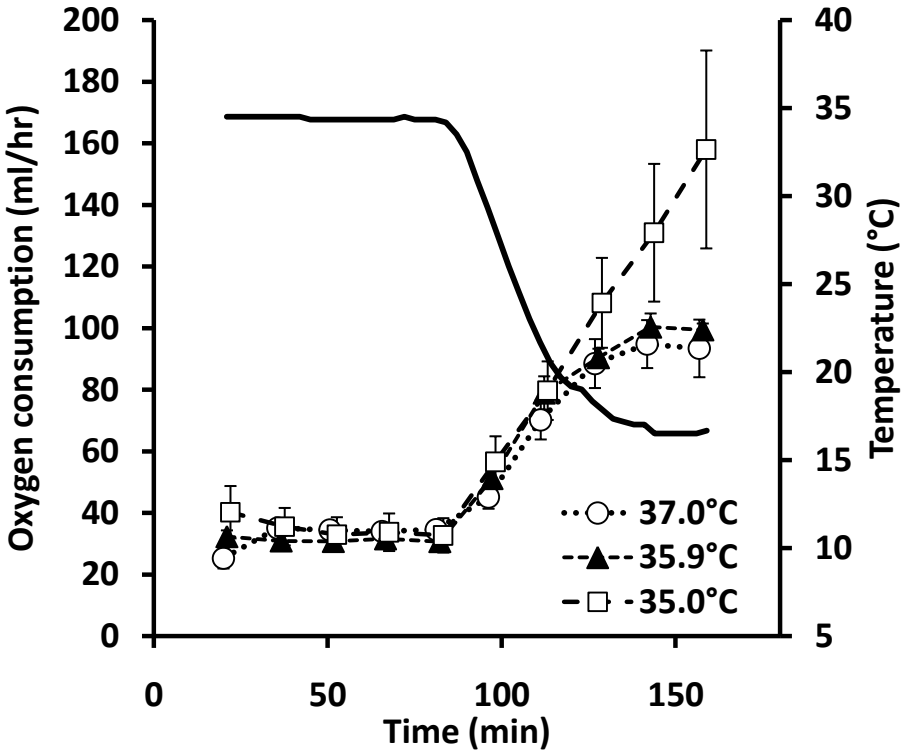

Figure 3.3. Patterns of oxygen consumption (ml/hr) of 1 day post hatch wood duck (*Aix sponsa*) ducklings while at thermoneutral (36°C) then during a 1 hr thermal challenge (15°C). Ducklings hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). The solid line represents the temperature inside individual respiration chambers. Error bars are ± 1 standard error of the mean. 35.0°C: n = 10; 35.9°C: n = 12; 37.0°C: n = 12.

Chapter 4: Embryonic developmental patterns and energy expenditure are affected by incubation temperature in wood ducks (*Aix sponsa*)

Co-authors: William A. Hopkins, Gary Hepp

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Abstract

Recent research in birds, demonstrated that incubation temperature influences a suite of traits important for hatchling development and survival. We explored a possible mechanism for the effects on hatchling quality by determining whether incubation temperature influences embryonic energy expenditure of wood ducks (*Aix sponsa*). Because avian embryos are ectothermic, we hypothesized that eggs incubated at higher temperatures would have greater energy expenditure at any given day of incubation. However, because eggs incubated at lower temperatures take longer to hatch than embryos incubated at higher temperatures, we hypothesized that the former would expend more energy during incubation. We incubated eggs at three temperatures (35.0, 35.9, and 37.0°C) that fall within the range of temperatures of naturally-incubated wood duck nests. We then measured respiration of embryos every three days during incubation, immediately after ducks externally pipped, and immediately after hatching. As predicted, embryos incubated at the highest temperature had the highest metabolic rates at most days of incubation and they exhibited faster rates of development. Yet, due to greater energy expended during the hatching process, embryos incubated at the lowest temperature expended 20-37 % more energy during incubation than embryos incubated at the higher temperatures. Slower developmental rates and greater embryonic energy expenditure of

embryos incubated at the lowest temperature could contribute to their poor physiological performance as ducklings compared to ducklings that hatch from eggs incubated at higher temperatures.

Keywords: respiration, bioenergetics, maternal effects

Introduction

Incubation conditions can have profound effects on offspring phenotype in reptiles, yet this phenomenon rarely has been explored in birds. However, recent research demonstrated that subtle changes in incubation temperature can greatly influence avian hatchling characteristics (Goth and Booth 2005; Hepp et al. 2006; Olson et al. 2008; DuRant et al. 2010; DuRant et al. In Review a,b; Hopkins et al. In Press). For example, Hepp and colleagues (2006) investigated the effects of incubation temperature on the duration of embryonic development and hatchling size and body composition in wood ducks (*Aix sponsa*, Linneaus) by artificially incubating eggs at three temperatures (34.6, 36.0, and 37.6°C) that approximated the range of temperatures found in naturally incubated nests (range 34.8-37.7°C; Manlove and Hepp, 2000; Folk and Hepp, 2003; Hepp et al., 2006). They found that incubation at lower temperatures significantly extended embryonic development, with eggs incubated at 34.6°C pipping nearly 10 days later than eggs incubated at 37.6°C. Further, proximate analysis revealed that duckling embryos at the pipping stage from eggs incubated at lower temperatures had significantly lower wet mass, dry mass, and lower protein content, than ducklings incubated at the higher temperatures (Hepp et al., 2006). Several other studies on wood ducks have revealed that incubation temperature affects duckling phenotype after hatching as well. These studies have found that ducklings from the low

incubation temperature have slower growth, reduced thermoregulatory performance, reduced immune responses, higher corticosterone concentrations, and reduced locomotor performance (DuRant et al. 2010, In Review a,b; Hopkins et al. In Press).

Evaluation of embryo bioenergetics could provide an important mechanistic basis for variation in hatchling phenotype noted in the previously mentioned studies on wood ducks.. For instance in the crocodilian, *Crocodylus johnstoni* (Krefft), eggs incubated at lower temperatures have longer incubation periods and thus, exhibit greater overall embryonic energy expenditure (Whitehead 1987). The greater energy expenditure during incubation presumably gives rise to reductions in hatchling residual yolk (Webb et al. 1987). However, in other reptilian species, total energy expended during incubation has been shown to be relatively insensitive to incubation temperature (Booth and Thompson 1991; Booth 1998; Oufiero and Angilletta 2010). Although many studies have examined the energetic cost of development in birds (Vleck and Bucher, 1998), surprisingly few studies have examined the influence of incubation temperature on avian embryonic metabolic rate and/or the total energetic cost of avian development (but see Booth, 1987; Olson et al. 2006).

In this study, we explored a possible mechanism for the above referenced effects of incubation temperature on duckling phenotype by quantifying metabolic rates of embryos throughout incubation and estimating the total energetic cost of development in wood duck embryos incubated at three temperatures that fall within the range of naturally-incubated nests (35.0, 35.9, and 37.0°C). We hypothesized that embryonic metabolic rate at any given point in development would increase with increases in incubation temperature. However, because incubation period is extended at cooler temperatures, the total energetic cost of developing at cooler temperatures would be higher than the cost experienced at higher incubation temperatures

(Angilletta et al. 2000). Thus, we predicted that embryos developing at cooler temperatures would expend more energy to support maintenance costs associated with protracted development.

Methods

Egg Collection and Incubation. We collected wood duck eggs from nest boxes (n = 201) located on two large reservoirs and several isolated wetlands on the U.S. Department of Energy's Savannah River Site in west-central South Carolina. We checked nest boxes every four days until nests were initiated, then visited active nests daily, collected and marked all new eggs, and stored them at 20°C and 55-60% humidity. After four days of collection, we transported eggs to Virginia Tech and artificially incubated them in Grumbach incubators (model BSS 160) at one of three temperatures (35.0, 35.9, and 37.0 °C), and consistent 60-65% humidity, which produced three incubation durations (37.2 ± 0.24, 34.4 ± 0.18, and 32.1 ± 0.18 d, respectively). Incubators were not maintained at constant temperatures, but were programmed to allow two cool-down periods each day (~3°C reduction in mean temperature for 75 min at 0815 and 1830) to simulate natural daily feeding recesses taken by mothers during incubation (Manlove and Hepp, 2000). Eggs were randomly assigned to an incubation temperature ensuring that lay sequence and the number of days eggs were held were evenly distributed across treatments. All experimental procedures were conducted in accordance with IACUC standards (protocol # 10-015FIW).

Energetic Measurements. We determined embryonic metabolic rates throughout development and the total energetic cost of development in wood duck embryos incubated at different

temperatures using closed- and open-flow respirometry (Microoxymax, Columbus Instruments, Columbus OH). The instrument is computer-controlled, allows monitoring of multiple (n = 10) independent respiratory chambers simultaneously, and is interfaced with a large environmental chamber that allows control of temperature. Respirometry procedures generally follow those outlined in recent publications (Dorcas et al. 2004, Hopkins et al. 2004, DuRant et al. 2008). To avoid pseudoreplication, and thus female effects on embryonic respiration, we only measured metabolic rates of one egg per clutch per incubation temperature treatment at each time point.

We measured respiration of embryos at days 1, 4, 10, 13, 16, 19, 22, 25, 28, 31, and 34 of incubation and at pipping (n = 7-9 embryos/day of incubation/incubation temperature). We also measured respiration rates of hatchling wood ducks within the first 2-20 hrs after hatching (35.0 °C n = 21, 35.9 °C: n = 17; 37.0°C: n = 11). At each sampling interval we placed eggs in individual respiratory chambers (either 500mL or 1L mason jars depending on embryonic stage) which contained a small platform to secure the egg. Because air is dried prior to sampling by the respirometer, a moist paper towel was placed in the chamber to maintain high chamber humidity during the measurement period. Respiratory chambers were placed inside the environmental chamber set at the assigned incubation temperature and kept in complete darkness. We measured oxygen consumption rate (ml/hr; VO_2) of embryos every 1.7 hrs for 12 hrs, resulting in 8—12 measurements of VO_2 per embryo collected over 3-minute sampling intervals. Following the 12 hr respirometry trial, we returned eggs to their respective incubator. We measured respiration of each individual embryo at only one sampling interval. Because respiration rates increased dramatically over the period of incubation we used both open and closed flow respirometry methods to determine respiration rates of embryos depending on their

developmental stage. We used closed flow respirometry on embryos at up to day 13 of incubation and open flow respirometry at all later sampling intervals.

We express respiratory rates as a function of day of incubation and percentage of total incubation period (Angilletta et al. 2000). To quantify the total energetic cost of embryonic development, we calculated the integral of VO_2 to external pipping, from external pipping to hatching, and during the entire incubation period. Then we converted total respiration values to energy equivalents assuming 1L O_2 = 19.6kJ (Vleck et al. 1980; and similar to procedures outlined in Angilletta et al. 2000 and Hopkins et al. 2004). Because we did not use a repeated measures design, we used a randomized sampling of data to allow statistical comparisons among the three incubation treatments. To calculate integrals we randomly chose respiration profiles of 1 embryo/hatchling at each sampling point per incubation temperature and generated a time sequence and resulting integral for that curve. We repeated this procedure 7 times for each incubation temperature. Each egg was only used once in developmental simulations.

Statistical analyses. All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Unless otherwise noted, raw data values were used in statistical analyses.

To confirm that fresh egg mass (pre-incubation) did not differ among incubation temperatures we used an ANOVA. Incubation temperature was the main effect in the model. We also tested for differences in egg mass at each respiration time point to confirm that egg mass did not differ among treatments at any given day during incubation (ANOVA, SAS Proc GLM).

The model included incubation temperature, day of incubation, and their interaction as independent variables. In addition, we tested for differences in mass of hatchlings among incubation temperatures used in the respiratory trial using an ANOVA.

To determine whether respiration profiles differed throughout incubation we used an analysis of covariance (ANCOVA; SAS proc mixed). In the model, incubation temperature, day of incubation, and their interaction were included as independent variables and egg/duckling mass was included as a covariate. We $\log_{10}$-transformed VO_2 to better meet model assumptions. For qualitative purposes we also present VO_2 as a function of percentage of the total incubation period.

We tested for effects of incubation temperature on the total energetic cost of embryonic development (the integral of VO_2 during incubation), energy expended up to external pipping, and energy expended during the hatching process using a MANOVA. In the model, we included incubation temperature as the main effect. We were unable to include mass as a covariate in the model because 11-13 unique eggs and 1 hatchling were used for each simulation that produced integrals. To account for any influence of mass we also ran a MANOVA on integrals derived using mass corrected VO_2 values (VO_2/embryo or hatchling mass). Analyses using mass-corrected estimates of total energetic cost (not presented) yielded similar results as analyses using raw (not mass-corrected) VO_2 values.

Results

There was no difference in fresh egg mass among incubation temperatures (Egg mass: $F_{2,297} = 0.51$; $p = 0.600$). There was also no difference in egg mass among incubation temperatures at any day during incubation (Egg mass: incubation temperature: $p = 0.445$;

incubation temperature x days of incubation: p 0.329). However, egg mass did vary with the number of days incubated ($p = 0.030$), but not with any distinct pattern (e.g., decreasing or increasing slowly over time) since a different group of eggs was represented at each sampling day with each day representing a broad range of egg masses. There was no difference in mass of hatchlings used in the respiration trial (Hatchling mass: incubation temperature: $p = 0.143$).

Oxygen consumption rates (ml/hr) differed among incubation temperatures throughout the incubation period (incubation temperature X day of incubation: $F_{21,263} = 2.68$; $p < 0.001$; Figure 1). Embryos from the high incubation temperature treatment had slightly higher respiration rates at most days of incubation and hatched approximately 2-5 days sooner than embryos incubated at the medium and low temperatures (incubation periods: 35.0˚C: 37.2 ± 0.24, 35.9˚C: 34.4 ± 0.18, and 37.0˚C: 32.1 ± 0.18 d). Similarly, embryos incubated at the medium temperature had slightly higher respiration rates at most days of incubation than low temperature embryos. Mass also significantly influenced respiration rates (Mass: $F_{1,263} = 8.04$; $p = 0.005$), with larger eggs consuming more oxygen than smaller eggs.

Respiration curves plotted as a percentage of incubation period also appeared to differ among incubation temperatures (Figure 2). Embryos incubated at the high temperature exhibited more rapid increases in metabolic rate than embryos incubated at the lower temperatures. For instance, embryos incubated at the highest temperature had 11-45 % higher metabolic rates than embryos incubated at the medium and low temperature when embryos were ~70-80 % incubated.

We also detected a significant effect of incubation temperature on total energy expended during incubation (Wilks' Lambda = 0.22; $F_{4,34} = 9.65$; $p < 0.001$; Figure 3), in which embryos incubated at the lowest temperature expended 37 and 20 % more energy than ducklings incubated at the medium and high temperature, respectively. Post hoc comparisons of individual

ANOVAs revealed that the disparity in total embryonic energy expended during development ($F_{2,18}$ = 17.94; p < 0.001) arose from greater energy expenditure during the hatching process ($F_{2,18}$ = 31.45; p < 0.001) and not from differences in energy expended prior to pipping (p = 0.869). During the hatching process, embryos incubated at the lowest temperature expended 173 and 73 % more energy than embryos incubated at the medium and high temperature, respectively.

Discussion

Our study demonstrated that the total energy expended by wood duck embryos during the incubation period differs depending on incubation temperature. At most time points, embryos incubated at the highest temperature had higher oxygen consumption rates than embryos incubated at the lower temperatures. However, because embryos incubated at the two higher temperatures had shorter incubation periods than embryos incubated at the lowest temperature, they expended significantly less total energy during incubation. Our results are similar to a study on the effects of incubation temperature on embryonic metabolism in mallee fowl (*Leipoa ocellatas*, Gould), in that mallee fowl eggs incubated at the lowest temperature took longer to develop and expended more energy during incubation than eggs incubated at higher temperatures (Booth 1987). Contrary to much of the reptile literature, total energy expenditure of wood duck and mallee fowl embryos appear to be sensitive to incubation temperature (Booth and Thompson 1991; Booth 1998; Oufiero and Angilletta 2010; but see Whitehead 1987; Angilletta et al. 2000).

Oxygen consumption rates of wood duck embryos and hatchlings at all incubation temperatures were similar to those of other similar-sized waterfowl embryos/hatchlings (Koskimies and Lahti 1964; Hoyt et al. 1979; Hoyt and Rahn 1980; Figures 1 and 2), but the

patterns of oxygen consumption appear to differ among incubation temperatures. In the high incubation temperature group, embryonic metabolism increased exponentially during the first 80% of development, followed by a plateau in metabolic rates just prior to pipping (Figures 1 and 2) This plateau in metabolism is typical of precocial embryos (Hoyt et al. 1979; Hoyt and Rahn 1980) and is thought to occur because embryos transition from rapid and energetically-costly tissue development to maturation of physiological systems important for precocial behavior (Vleck et al. 1980). The plateau phase was less evident at the medium and low temperatures; however, the detectability of the plateau phase at the medium and low incubation temperatures may have been obscured by our sampling interval, every three days during incubation. Alternatively, the differences in metabolic profiles may relate to differences in the timing of development and maintenance of expensive physiological systems (e.g., nervous system). A study on zebra finches (*Taeniopygia guttata*, Vieillot) incubated at varying temperatures demonstrated that incubation temperature affects developmental rates of embryos, with the slowest development occurring in embryos incubated at the lowest temperature (Olson et al. 2006). If development of expensive systems is delayed in the medium and low temperature treatments then these embryos may continue to both develop and mature new tissues until just before pipping. Conversely, embryos incubated at the highest temperature may develop these systems more rapidly and thus undergo only maturation during the days immediately prior to pipping.

Another line of evidence that incubation temperature may be affecting the relative timing of developmental processes and not just protracting development comes from metabolic profiles of embryos plotted as a function of the percentage of incubation period (Figure 2). Because embryonic oxygen consumption among the three incubation temperatures do not overlap, we

observed clear differences in metabolic rate when embryos were 70-90 % incubated, it suggests that embryos are on different developmental trajectories. This finding contrasts with a study on lizards (Angiletta et al. 2000), wherein metabolic rates of embryos as a function of percentage of incubation period appeared to be temperature-independent and total energy consumed was solely a product of the length of incubation. The authors postulated that lizard embryos were either temperature insensitive or temperature acclimation occurred, such that at any given developmental stage embryos incubated at cool temperatures up-regulate metabolism and embryos incubated at warm temperatures down-regulate metabolism, resulting in no difference in stage-specific metabolic rates.

The differences among treatments in total energy expended during incubation in our study was attributable to greater energy expenditure of low temperature embryos during the hatching process, not energy expended prior to external pipping (Figure 3). Embryos incubated at the low temperature took 1.2—1.9 d longer to hatch once pipping occurred than embryos from the higher temperatures, which likely explains the difference in energy expended during hatching. The longer time needed for hatching in embryos incubated at the lowest temperature could result from differences in thyroid hormones and/or glucocorticoid (e.g., corticosterone) concentrations. These hormones increase in the late stages of incubation in precocial embryos and are responsible for tissue maturation and preparing organs (e.g., gut and lungs) for post-hatch life (McNabb et al. 1998; Wada 2008). Our previous study on wood ducks supports this hypothesis as we demonstrated that corticosterone concentrations differ at 2 days post hatch (dph) in ducklings incubated at the same three temperatures used in the current study (DuRant et al. 2010). It is surprising that while the time to pipping was 3—4.5 d shorter for embryos in the high incubation temperature treatment, energy expenditure up to pipping was nearly identical in

embryos from all incubation temperatures. Again, this may result from differences in the timing of key developmental stages; expensive physiological systems may develop earlier at the highest temperature, and once developed, incur higher maintenance costs (Vleck and Vleck 1987).

During incubation, wood duck embryos consumed between 25-35 % of the energy content of a freshly-laid wood duck egg (Figure 5). Average energy content of eggs used in this study was 334.7 ± 2.2 kJ, assuming 8.37 kJ/g of fresh egg mass (Hepp et al. 1987). Regression equations relating egg energy content to the energetic cost of development up to hatching for precocial birds (Vleck and Vleck 1987), predict that wood ducks would expend between 112-123 kJ during development, these regression models do not incorporate variations in incubation temperature. Our estimates among the three incubation temperatures encapsulated this range (Range: 90-120 kJ; Figure 4). Supposing that energy content of ducklings is equivalent to the energy content of an egg minus the energy expended during incubation (Vleck and Bucher 1998), then energy content of ducklings from the low, medium, and high incubation temperature treatment is 214, 251, and 240 kJ, respectively. Previous studies in wood ducks have found that low incubation temperatures reduce duckling protein (Hepp et al 2006) and lipid content (Hepp unpub data) by 9-12 % and 20-22%, respectively, which could account for the differences in energy content. A study on crocodiles noted that incubation temperature affected the size of yolk reserves, but not hatchling wet mass, with lower incubation temperatures increasing incubation periods and subsequently decreasing yolk reserves (Webb et al. 1987). Olson et al. (2006) noted that at 12 d post incubation zebra finch embryos regularly cooled to 20˚C had both lower mass and smaller yolk reserves than embryos constantly maintained at 37.5˚C. Currently, we cannot determine whether the disparities in duckling composition and energy content are attributable to differences in hatchling tissues or the size/composition of yolk reserves.

Finally, our results suggest that the effects of incubation temperature on embryonic energy expenditure and developmental trajectories may be contributing mechanisms for effects of incubation temperature on avian offspring phenotype. Our previous studies, using the same incubation temperature treatments as this study, have found that ducklings incubated at the lowest temperature have reduced physiological performance relative to ducklings incubated at the higher temperatures (Hepp et al. 2006; DuRant et al. 2010; Hopkins et al. In press; DuRant et al. In review a,b). Because low temperature ducklings expend significantly more energy during development than the higher temperature ducklings, they hatch with reduced energy reserves, subsequently inhibiting their ability to grow, develop, and fuel expensive physiological processes, such as thermoregulation and mounting an immune response. Furthermore, as incubation temperature appears to affect the relative timing of developmental events, ducklings incubated at the lowest temperature may also be developmentally delayed relative to ducklings incubated at higher temperatures. Developmental delays could also contribute to the previously observed differences in duckling physiological performance.

Acknowledgements

We thank Brittney Hopkins, Amanda Wilson, Jake McPherson, Johnathon Walls, Bobby Kennamer, Paul Siegel, Thera Lombardi, Maureen McClintock, Marilena Nunez, Corey Earle, and Caleb Gaston for field and laboratory assistance and Jonathon Cohen for statistical assistance. J.D. Willson, Dana Hawley, Jeff Walters, and Ignacio Moore reviewed earlier drafts of the manuscript. Primary funding for this project was provided by the National Science Foundation grant IOB-0615361 to WAH and GRH. A Virginia Tech Graduate Student Assembly Graduate Research and Development Program Award, a Sigma Xi Grants-in-aid of

Research (GIAR), and a Society of Integrative and Comparative (SICB) GIAR to SED also helped support this research.

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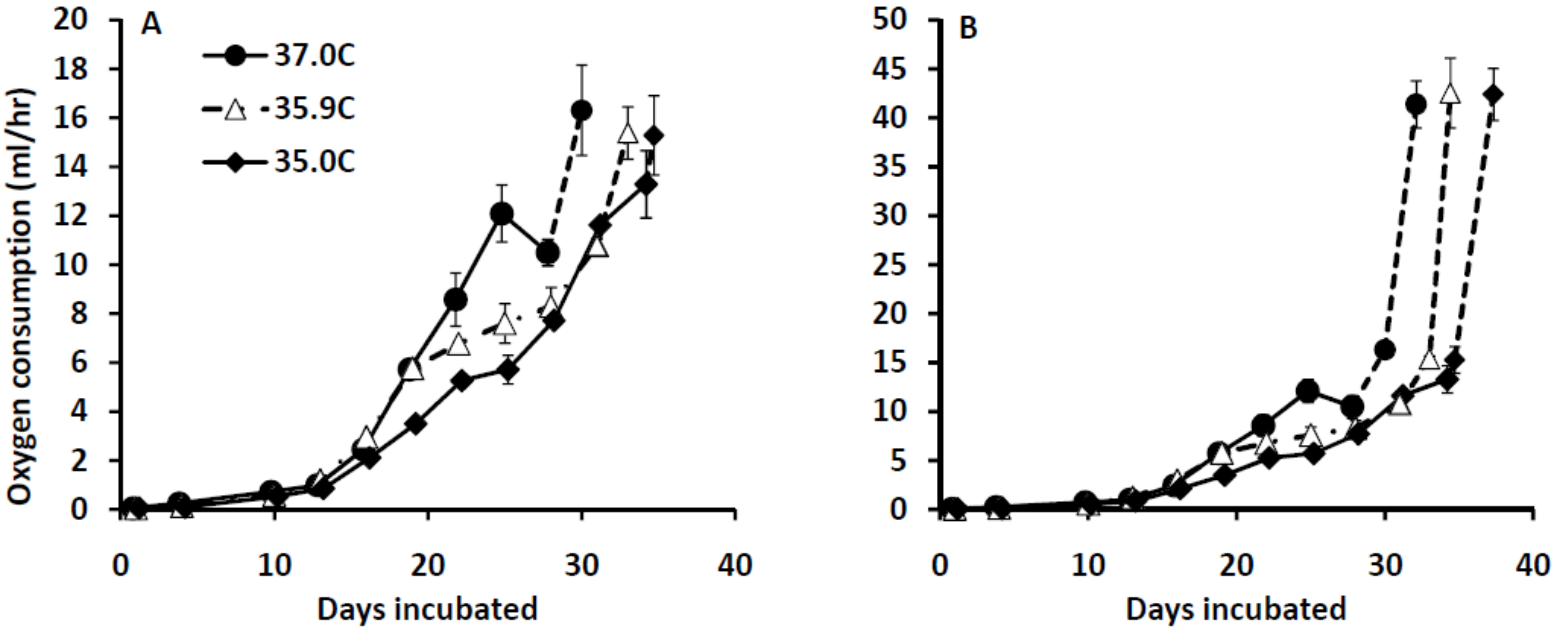

Figure 4.1. Oxygen consumption rates (ml/hr) of wood duck (*Aix sponsa*) embryos during incubation to external pipping (A) and to hatching (B). Embryos were incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Dotted lines connect respiration points associated with pipped eggs and ducklings after hatching. Error bars represent ± 1 SE of the mean. Statistical analyses were performed on Log_{10}-transformed O_2 values.

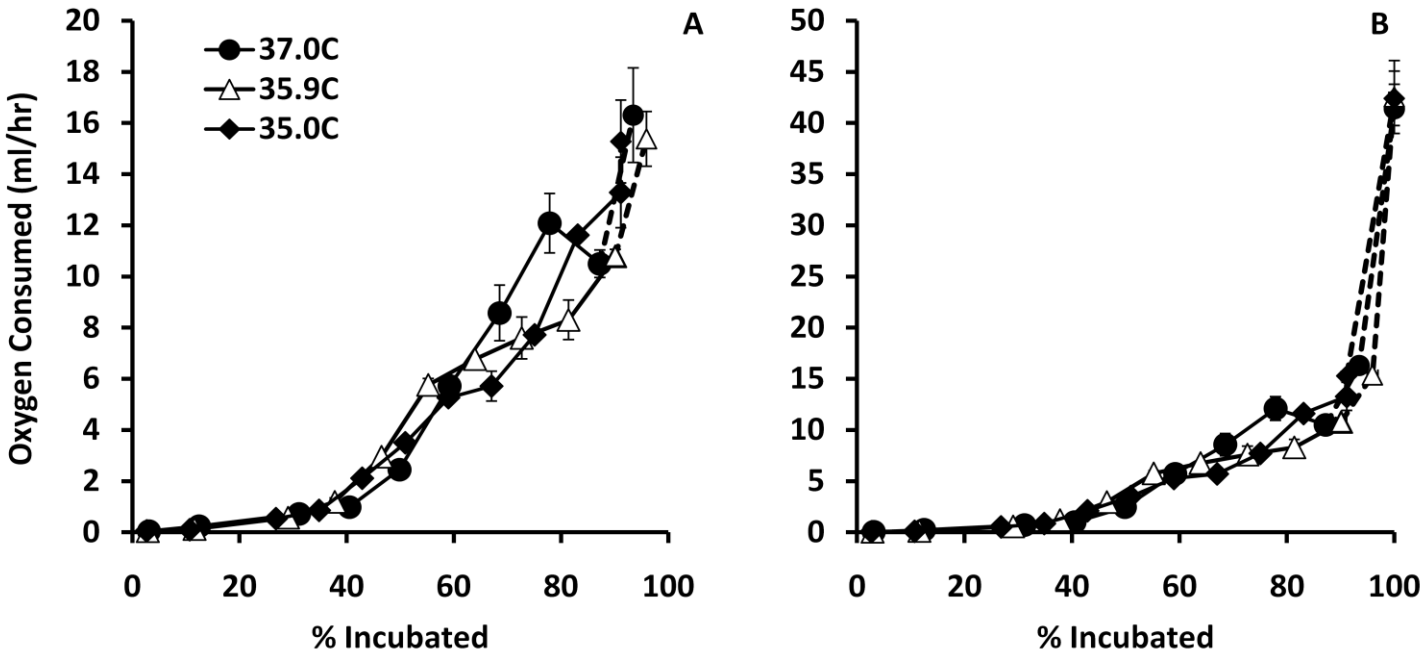

Figure 4.2. Oxygen consumption rates (ml/hr) of wood duck (*Aix sponsa*) embryos during incubation to external pipping (A) and to hatching (B) plotted as a function of percent incubation. Embryos were incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Dotted lines connect respiration points associated with pipped eggs and ducklings after hatching. Error bars represent ± 1 SE of the mean.

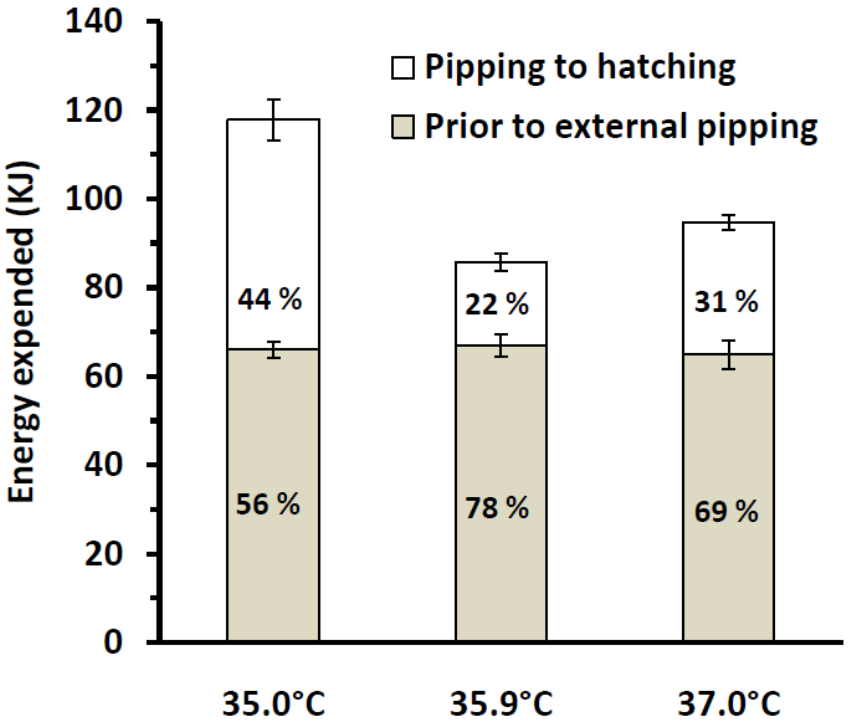

Figure 4.3. Total energy expended (kJ) during incubation (to hatching) in wood duck embryos (*Aix sponsa*) incubated at one of three temperatures (35.0, 35.9, or 37.0°C). The gray portion of each bar represents energy expended up to external pipping, whereas the white portion represents energy expended during the hatching process. Percentages within bars represent the percent of total embryonic energy expenditure allocated to embryonic development up to pipping v. energy needed for hatching. Error bars represent ± 1 SE of the mean.

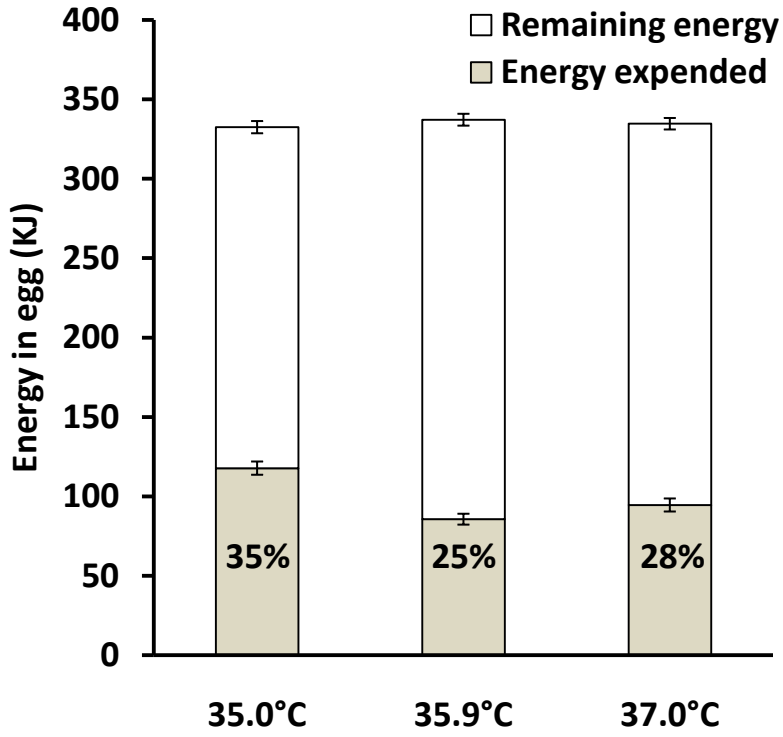

Figure 4.4. Energy consumed (kJ) during incubation (gray portion of the bar) by wood duck embryos in relation to the energy content of freshly-laid wood duck eggs (full bar). Egg energy content was calculated by assuming 8.37 kJ/g of freshly-laid egg mass (average egg mass = 35.0°C: 39.7 ± 0.46g; 35.9°C: 40.3 ± 0.45g; 37.0°C: 40.0 ± 0.43g). Embryos were incubated at one of three temperatures (35.0, 35.9, or 37.0°C). The percentages within the gray bars represent the percentage of energy in an egg that was consumed during incubation. Error bars represent ± 1 SE of the mean.

Conclusions: Incubation temperatures role in determining avian phenotype: implications for avian ecology, life history evolution, and conservation.

Co-authors: William A. Hopkins, Gary Hepp, Jeffrey Walters

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Abstract

Despite the wealth of studies demonstrating the importance of incubation temperature to phenotype of reptilian offspring and its implications for their survival and reproduction, this relationship largely has been ignored in birds. Although birds physically incubate their eggs, thus controlling nest temperature, temperatures experienced during embryonic development do vary within species and populations. Recent research on megapodes and waterfowl demonstrates that differences in incubation temperature have substantial effects on hatchling phenotype, affecting traits important for future development, survival, and reproduction. The nature of these observed phenotypic changes suggest that incubation temperature is an important parental effect in birds that may have acted as a major selective force in the evolution of avian life history characteristics and reproductive ecology. To begin to investigate these questions, however, a better understanding of what drives differences in incubation temperature and its effects on phenotype (e.g., latent effects, persistence of effects into adulthood, etc.) is needed. Such insights will not only provide foundational information regarding avian evolution and ecology, but also contribute towards avian conservation.

Avian Incubation Temperature and Its Influence on Phenotype

Since the discovery of temperature dependent sex determination in reptiles there has been a great deal of research evaluating how incubation temperature influences other aspects of reptile phenotype (Text box 1). Indeed, temperature has been shown to affect a slew of traits important for survival and reproduction (Valenzuela and Lance, 2004). Although the importance of incubation temperature in determining reptilian phenotypes is now clear, surprisingly little is known about how temperature influences the phenotypic characteristics of birds despite their similarly structured amniotic egg. As one researcher put it, "rather belatedly it has been realized that incubation temperature can influence more than hatchling morphology in poultry (Deeming 2004)."

Perhaps relationships between temperatures experienced during development and phenotype have been considered unlikely in birds because avian parents physically incubate their eggs, thus tightly controlling nest temperatures, whereas most reptilian parents do not. However, similar to reptiles, avian nest temperatures do fluctuate, and differences of only 1 °C can affect phenotype (DuRant et al. 2010). Avian nest temperatures typically range between 32 and 38°C (Huggins 1941), and vary within species and populations. However, natural variation in nest temperature is known for only a few species. For example, mean nest temperatures of wood ducks (*Aix sponsa*) in a population in South Carolina, USA range between 34.5—38.5°C. (Hepp et al. 2006, Folk and Hepp 2003) and mean nest temperatures of American robin in OH, USA (*Turdus migratorius*) ranged between 31.6—39.9 °C (Huggins 1941). Even megapodes, whose eggs are laid in soil mounds full of decomposing materials and incubated with chemical heat, maintain remarkably constant nest temperatures by adding organic materials to their nest or

changing the shape of the mound (average temperature: 34°C; range: 30-38°C; Booth and Jones, 2002; Goth and Booth 2005). Reptilian nests differ from birds in that average incubation temperatures are much cooler than avian nest temperatures because most reptile nests are subject to ambient air temperature and are not typically incubated by the parents (e.g., the scincid lizard *Nannoscincus maccoyi*: ~15-20˚C; the skink (*Bassiana duppereyi*: 17-21˚C, 16-23˚C [Shine 2001; Shine et al. 2003]; the Pacific leatherback turtles *Dermochelys coriacea*: 29-31˚C [Binckley et al. 1998]).

Nest attendance by incubating birds is the source of much of the variation in nest temperatures, and avian embryos are sensitive to these fluctuations. In passerine birds, embryos develop optimally between 36 and 40°C, temperatures above 40.5°C are lethal, and development is suspended below 24-26 °C (i.e., physiological zero) (Cooper et al., 2005). If eggs are exposed for extended periods to temperatures between physiological zero and optimum incubation temperatures, embryos experience unsynchronized tissue growth, abnormal development and mortality (Deeming and Ferguson 1992). Work by Stoleson and Beissinger (1999) suggests that three days may be the minimum exposure to such temperatures that can produce mortality, but sensitivity varies among species (Webb 1987). Reptilian embryos appear to be more resilient to prolonged exposures to below optimum temperatures with some reptile embryos even undergoing developmental diapause during the winter months (Ewert 1991; Andrews 2004). Diapause does not occur in birds and suggests that avian developmental rates are less flexible than reptilian developmental rates, perhaps because birds transition from ectothermy as an embryo to endothermy as a hatchling. Given that the lethal consequences of exposure to temperatures outside the optimum incubation range to avian embryos have received considerable

attention, it is remarkable that effects of non-lethal exposures on avian phenotypic quality largely have been ignored.

We know that temperatures of naturally-incubated nests vary among and within avian species (Webb 1987), but we know very little about the consequences of this variation for avian phenotypes. What is known about the latter comes primarily from studies on domesticated species. In studies on domesticated turkeys and chickens, incubation temperature is typically manipulated during a portion of the incubation period, and has been shown to affect thermoregulation, post-hatch growth, metabolism, feed conversion efficiency and hatchling morphology (Hill, 2001; Hulet et al. 2000; Lourens and can Middelkoop, 2000; Lourens, 2001; Nichelmann and Tzchentke, 2003). These studies, which are primarily grounded in aspects of commercial production, suggest that incubation temperatures may influence a broad array of avian phenotypic traits and warrant study in wild birds by ecologists and evolutionary biologists.

Only a few studies have looked at the effects of natural variations in incubation temperature on offspring phenotype in non-domesticated species. The majority of these studies have been conducted in wood ducks (Text box 2) and several mound nesting birds (Text box 3). In those studies eggs were artificially incubated at temperatures that fall within the range of naturally incubated nest temperatures. Incubation temperature was shown to affect an array of phenotypic traits important for early survival in wood ducks (Hepp et al. 2006; DuRant et al. 2010; Hopkins et al. 2011; DuRant et al. In Review a,b), and sex ratios of Australian brush turkeys via temperature dependent embryonic mortality (Goth and Booth 2005; Eiby et al. 2008). An interesting trend in the work on wood ducks is that physiological performance of ducklings is poorest at the lowest incubation temperature, but ducklings that hatched from eggs at the intermediate temperatures perform either similarly or slightly better than ducklings from the

highest incubation temperature (See Figure 1). If birds produced from intermediate incubation temperatures are truly of higher quality, then females may choose between incubating eggs at higher temperatures to reduce the incubation period, thus reducing nest-predation risks, versus incubating eggs at medium temperatures and producing hatchlings of slightly higher quality.

There is also some less direct evidence that temperature may influence offspring phenotype in birds, but these studies either did not directly measure nest temperatures, do not control for other parental effects (feeding behavior immediately after hatching, female heart rate during incubation, etc.), or do not control for other aspects of developmental microclimate (e.g., humidity). In the first study female European Starlings (*Sturnis vulgaris*) were exposed to thirty minute stressors four times a day for eight days while they were incubating eggs, but nests were not disturbed after hatching (Cyr et al. 2007). Sixteen days after hatching nestlings from disturbed mothers had altered stress hormone profiles relative to those from unstressed mothers. Although the authors did not measure nest temperatures, they did find that stressed mothers spent more time away from their nest than unstressed mothers suggesting that incubation temperature may have influenced offspring phenotype. Another study demonstrated that Tree Swallow (*Tachicyneta bicolor*) nestlings that developed in cooled nest boxes had lower innate immune responses than nestlings from unmanipulated nest boxes (Ardia et al. 2010). While this study suggests that incubation temperatures may have been the stimuli affecting swallow immunity, other differences in female behavior or physiology during incubation (e.g., heart rate; Walker et al. 2005) or nest microclimate may also have contributed to their results. Studies have also shown that exposure to contaminants can alter parental incubation behavior and may contribute to lower hatching success of offspring (Bennett et al. 1991; Fisher et al., 2006). Again, these

studies cannot rule out other parental effects such as maternal deposition of contaminants into the eggs.

For now it remains unclear how incubation temperature is affecting offspring phenotype. In reptiles, there is evidence that gene expression may be the underlying mechanism for temperature dependent sex determination (Lance 2008). At this time a relationship between gene expression and effects on phenotypic traits of avian offspring has not been explored. However, several studies have demonstrated that incubation temperature can influence both developmental rates and energy expended during the incubation period which could contribute to the effects of incubation temperature on phenotypic traits. For instance, wood duck and mallee fowl embryos incubated at cooler temperatures expended more energy during incubation than embryos incubated at higher temperatures (Booth et al. 1987; DuRant et al. In Press). Longer incubation periods result in embryos expending more energy during development leaving fewer remaining energy reserves in hatchlings, and could have implications for post-hatching traits of young birds. Temperature also affected developmental rates of zebra finches (*Taeniopygia guttata*) with embryos that experienced periodic drops in temperature to 20°C developing more slowly than embryos incubated constantly at 37°C (Olson et al. 2006). Finch embryos experiencing periodic cooling also had higher mass-specific metabolic rates, incurred greater metabolic costs, and reduced efficiency at converting nutrients into tissue than embryos incubated at constant temperatures. Taken together, effects of temperature on avian developmental rates and embryonic energy expenditure could have a number of consequences for hatchling phenotypes.

Effects of incubation temperature on offspring phenotype greatly expand the previously known influence of incubation on reproductive success, Historically, the efficiency of incubation has been viewed in terms of hatching success. Temperatures above the optimal incubation range

and prolonged exposure to temperatures below the optimal range can be lethal and thus impact egg viability (Cooper et al. 2005). Brief exposure to temperatures below the optimum range retards development and prolong incubation period (Boersma 1982) and hence possibly increase vulnerability to nest predation. Thus, to maximize hatching success incubation behavior should be structured firstly to avoid exposure of eggs to lethally high temperatures, and secondly to avoid exposure to low temperatures sufficiently to keep eggs viable and achieve a suitable length of incubation period. Yet, this is still somewhat of an all or none point of view. In light of what has recently been discovered regarding the effects of temperature on phenotype, effectively hatching a brood may not be the only selection pressure for maintaining nest temperatures within a narrow range. The additional selection pressure of maintaining nest temperatures that optimize hatchling phenotypic quality has implications for many aspects of avian reproductive biology and life history (Text box 4).

In addition to providing important insights into the reproductive ecology, life history, and evolution of birds, a better understanding of how incubation temperature influences avian development will also have practical implications for their conservation (Text Box 5). Variables that directly influence the nest environment such as climate change or nest site quality could directly influence egg incubation temperatures and exert additional selective pressures on adults to maintain optimal incubation conditions (Matthysen et al., 2011). Additionally, anthropogenic factors such as environmental pollutants, reduced habitat quality, and nest site disturbance that reduce nest attendance by adults could cause deviations from optimal incubation conditions. Although the relationships between these factors, incubation conditions, and offspring phenotype remain largely unexplored, they should provide fruitful research avenues.

What Influences Avian Incubation Temperature

A number of factors can contribute to variations in nest temperature including nest-site microclimate, parental condition, differences in parental physiology (e.g., size or vascularization of the brood patch), nest initiation date, and of course incubation behavior (e.g., duration of on/off bouts, onset of incubation). For many species a combination of these factors may dictate the temperatures that embryos experience during incubation. Currently, the contribution of each of the above variables to variations in nest temperatures is unknown, and is an area of research in much need of attention.

The most obvious and best studied factor contributing to nest temperature variation is incubation behavior (Deeming and Ferguson 1991; Zicus et al. 1995; Martin et al. 2007). For example, a study on Common Goldeneye (*Bucephala clangula*) found that an incubation period of 29 days instead of 30 could be attributed to 13 minutes less parental nest attendance per day (Zicus et al. 1995). In Common Eiders (*Somateria mollissima*), unsuccessful nests were linked to longer off-bouts taken by females exposing embryos to greater temperature fluctuations (Zicus et al. 1995). Similarly, other studies have demonstrated that disturbance causing incubating parents to spend more time away from the nest leads to reductions in hatching success, presumably due to decreases in nest temperatures (Lord et al. 2001; Verhulst et al. 2001; Bolduc and Guillemette 2003; Verboven et al. 2001). Incubating parents also modify incubation behavior with changes in climate (Deeming 2002). As ambient temperatures fluctuate incubating parents will adjust the duration of on and off bouts, keeping their nests at relatively similar average temperatures in both cold and hot conditions (Yerkes 1998; Deeming 2002; Hepp and Kennamer 2011). Birds

tend to take longer nest breaks when it is warm, but shorter breaks during cold weather when nest temperatures are more likely to drop when not incubated (Caldwell and Cornwell 1975; Afton and Paulson 1992; Hepp and Kennamer 2011). Surprisingly, however, few of these studies have looked at subtle variation in average, minimum, and maximum nest temperatures and how these variables correlate with parental behavior. Presumably parents that take longer breaks could produce lower mean incubation temperatures than parents that take shorter breaks. Alternatively, parents taking longer breaks may incubate their eggs at slightly higher temperatures than parents that take shorter or less frequent breaks resulting in similar mean incubation temperatures. Since a multitude of previously published studies have used temperature loggers to quantify the duration of on and off bouts, average nest temperatures and the frequency at which they drop below optimal temperatures could be calculated for nests in those studies to begin to answer questions regarding the relationship between parental incubation behavior and temperatures experienced by the embryos.

Nest-initiation date can also influence temperatures that embryos experience. Embryos laid earlier in the season experience greater drops in temperature when parents leave the nest to forage. However, as previously mentioned, parents tend to modify the duration of off bouts when ambient temperatures are cooler, thus reducing the length of time eggs are exposed to the cooler temperatures (Bentzen et al. 2010). Even though parents modify their behavior when nesting in cold versus warm conditions, embryos laid later in the season experience smaller decreases in temperature when parents are off the nest (Hepp and Kennamer 2011). However, it is unknown whether brief periodic exposure to colder minimum temperatures influences hatchling phenotype. Exposure to higher temperatures late in incubation adversely affect a chick's thermal preference and ability to withstand heat stress in domesticated species

(Nichelmann 2004), thus experiencing slightly cooler temperatures could have similar adaptive benefits for hatchlings early in the breeding season.

Nest location, the primary factor affecting nest temperatures in reptiles (Congdon and Gibbons 1990; Wilson 1998; Weisrock and Janzen 1999), also can affect nest temperatures in birds. Birds tend to select sites that offer shelter from direct sunlight and wind, and tend to provide some measure of insulation (Gloutney and Clark 1997; Deeming 2002 chapter; other citations). In megapodes, parents select areas that are protected from direct exposure to sunlight and with leaves from plant species that are suitable for rapid decomposition (Booth and Jones, 2002). There is some evidence that microclimate of avian nests also vary depending on where they are located and/or how they are constructed (Hansell and Deeming 2002; Weibe 2001). A study on Northern flickers (*Colaptus auratus*) revealed that cavity temperatures differed dramatically and that cavity temperatures positively correlated with clutch size (Weibe 2001), suggesting that the most fit flickers procure better nest sites.

Clutch size and location of the egg in the nest can also produce variability in temperatures experienced during incubation. Maintaining high incubation temperatures for all the eggs in a large clutch can be difficult, resulting in substantial differences in temperatures experienced by eggs in the center of the nest versus the periphery (Huggins 1941; Caldwell and Cornwell 1975). Differences in nest temperature in the center versus periphery of the nest are most noticeable early in incubation and disappear as incubation progresses due to metabolic heat production of later staged embryos (Caldwell and Cornwell 1975). Parents can negate some of these differences by shifting eggs around, but in very large clutches it may be difficult to keep all eggs at optimal temperatures for a significant proportion of the incubation period. For species with wide variability in clutch size there may be fairly high within nest variation in incubation

temperature. This problem could be exacerbated by brood parasitism. For example, in wood ducks the average clutch size is approximately 12 eggs, but can exceed 30 eggs when nests are parasitized by other hens (Bellrose and Holm, 1994). At this time, however, it is unclear how much variation in incubation temperature exists within clutches in most bird species.

Variation in incubation temperature also might be due to differences in the ability of individual birds to incubate eggs at optimal temperatures. For example, adults in poorer condition might be less effective incubators than those in good body condition. Parents in poor body condition should have greater foraging demands particularly in colder weather when it can be metabolically costly to provide heat for their embryos. Incubating birds have been known to increase their breathing rate, oxygen consumption, and heart rate and induce shivering to generate heat in response to artificial cooling of their eggs (Lea and Klandorf 2002 and citations therein). Since heating or cooling of eggs requires the ability of the parent to sense the temperature of their eggs via thermoreceptors (Tøien 1993), variability could exist among individuals within a species to detect small variations in egg temperature, causing them to incubate eggs at slightly higher or cooler temperatures than conspecifics. Likewise, variability could exist among individuals in their ability to transfer heat to their eggs. For example, vascularization and size of the brood patch could affect temperatures experienced by embryos during incubation. In response to egg cooling localized vasodilatation occurs in the brood patch (Midtgård et al. 1985). To our knowledge, no studies have examined incubation behavior and incubation efficiency of adults in relation to embryonic developmental temperatures and offspring phenotype.

Future Research Priorities

As we continue to explore the role incubation temperatures play in shaping avian offspring phenotype, a multitude of research opportunities will arise. Perhaps most pertinent is how widespread are the effects of incubation temperature on offspring phenotype in birds; are some species more sensitive than others? Before investigating temperature effects in a new species, a better understanding of the natural variation in incubation temperature for that species is needed. In addition to continuing to explore different species and various phenotypic traits that incubation temperature could affect, researchers should begin to search for mechanisms by which these effects are mediated. Does temperature alter when certain genes are expressed, having lasting impacts on development or could temperature affect production of hormones that are important in orchestrating developmental processes? Similarly, we know nothing about the long term or latent effects of incubation temperature on avian phenotype. In wood ducks, some performance deficits remain and can even be exacerbated 20 days post hatch, but we do not know whether these or other effects persist into adulthood. Likewise, it is possible that the influence of incubation on certain traits may not be evident until later in ontogeny. For instance, age at sexual maturity, clutch sizes, sperm viability, plumage coloration, flight performance, and incubation behavior could all be traits influenced by early developmental experiences.

Understanding the various aspects of temperatures experienced throughout incubation and whether they contribute to phenotypic variation should also be evaluated. For instance, as demonstrated by the reptile literature, certain windows in development are more sensitive to warmer or colder temperatures than others (Shine and Elphick 2001; Andrews 2004; Nichelmann 2004; Tzschentke 2008). Identifying if these windows of development exist in wild bird species may help unveil the underlying mechanisms for the effects of incubation temperature on phenotype. Another aspect of temperature that could be important for avian development is the

maximum and minimum temperatures eggs are exposed to and diel fluctuations in incubation temperatures. Research in reptiles suggests that fluctuations in temperature can be instrumental in determining phenotype (Birchard 2004). For example, a study on smooth softshell turtles (*Apalone mutica*) revealed that thermal fluctuations influenced both swimming speed of hatchlings and incubation period, with both variables increasing with increasing thermal variance (Ashmore and Janzen 2003).

Lastly, birds produce offspring that span the altricial-precocial spectrum. There could be some interesting differences in the effects of temperature on offspring phenotype for birds at opposing ends of this spectrum. Precocial hatchlings undergo most of their development in ovo whereas altricial birds continue to rapidly develop after hatching. Therefore altricial species may respond differently to nest temperatures pre hatching versus post hatching. Or, precocial birds could be more sensitive to nest temperature early in incubation when the majority of development of physiological systems is occurring and less sensitive in the latter portion of incubation when embryos are mostly maturing tissues (Vleck et al. 1980). Because altricial embryos continue to develop, parents may be able to make up for delays in embryonic development due to lower nest temperatures during the post-hatch period by brooding young at optimal temperatures and increasing feeding rates.

Text Box 1: Effects of incubation temperature on reptile phenotype

The discovery of temperature dependent sex determination in reptiles over 50 years ago (Charnier, 1966) spawned decades of intensive molecular, physiological, and ecological research on the influence of temperature on early reptilian development (Bull, 1983; Valenzuela and

Lance, 2004). It is now well established that incubation temperature is the predominant factor determining sex in some turtles and squamates, and all crocodilians (Valenzuela, 2004). The molecular mechanisms by which temperature influences sex ratios of reptiles have gradually been revealed, and the evolutionary and ecological consequences of environmental sex determination have become a primary focus in recent years (Valenzuela, 2004; Shine, 2004; Warner and Shine, 2008). In addition, much theoretical and empirical research has been directed at determining the consequences of global climate change on reptile development and population dynamics (Booth, 2006).

In addition to determining sex, incubation temperature can have a wide array of other more subtle effects on phenotypic characteristics and life history traits of reptiles (Table 1). For example, body mass, shape, and condition (i.e., mass relative to structural size) of hatchlings are influenced by incubation temperature in lizards and turtles. Likewise, early growth, locomotion, and physiological performance are influenced by nest temperatures in a wide array of species and in some cases these effects may be long-lasting. Most importantly, a few studies have demonstrated that incubation temperatures influence the survival and reproduction of reptilian offspring in the wild. For example, Parker and Andrews (2006) artificially incubated lizard (*Sceloporus undulatus*) eggs at 22, 24, and 27 ˚C and released them in the field. They found significant reductions in early survival in hatchlings from eggs incubated at the coolest temperature that were also correlated with differences in locomotor performance. In a seminal study by Warner and Shine (2008), eggs of the lizard *Amphibolurus muricatus* were incubated across a range of temperatures and hatchlings were then released into field enclosures where their lifetime reproductive success was monitored. Incubation temperature influenced fitness of lizards, but affected male and female offspring differently. These findings suggest that early

developmental experiences may influence the fitness of reptiles, and that temperature-dependent sex determination may control sex ratios in a manner that is adaptive given particular environmental conditions that preferentially favor one sex over the other (Warner and Shine, 2008).

Text Box 2: Wood ducks: A case study in temperature induced phenotypic variation

Recent studies on Wood Ducks (*Aix sponsa*) provide the most comprehensive example of how incubation temperature can influence the phenotype of avian offspring. Wood ducks are intermittent incubators with a prolonged breeding season and only the female cares for the eggs (Bellrose and Holm 1994). Females typically leave the nest twice a day to forage (~1.5 hrs in the morning and early evening), which creates regular fluctuations in nest temperatures (Manlove and Hepp 2000). Average incubation temperatures in a population of wood ducks in South Carolina, USA range from 34.8-37.7°C (Manlove and Hepp, 2000; Folk and Hepp, 2003; Hepp et al., 2006).

Based on the natural variation in wood duck nests, a series of laboratory studies were conducted to simulate the daily average nest temperatures and the daily variability in nest temperature generated by natural female behavior (incubators were programmed with two cool down periods to mimic drops in temperature that occur when females leave the nest to forage).Differences in mean incubation temperatures of < 1˚C influenced hatching success of eggs, duckling body composition (e.g., protein content), immune responses, growth, body condition, thermoregulatory ability, locomotor performance, and stress endocrinology (Figures Conclusion.2 and Conclusion.3; Hepp et al., 2006; DuRant et al. 2010; Hopkins et al. 2011;

DuRant et al. In Review a,b). In most cases, ducklings that had been incubated at the lowest temperature performed more poorly than ducklings incubated at higher temperatures. Qualitative comparisons also suggest that in most cases physiological performance was best at the intermediate temperature, suggesting that females can behaviorally generate an optimal incubation temperature for their young. Importantly, effects on the immune system were found 6 days post hatch (dph) and on locomotor performance at 15 dph, and effects on growth and body condition persisted until at least 20 dph. These findings suggest that the effects on early phenotype could persist. Furthermore, because the traits affected by incubation temperature in ducklings have implications for future development, survival, and reproduction, incubation temperature produces phenotypic variation in birds upon which natural selection can act, with implications for many aspects of avian life history evolution.

Energy expended during the incubation period could help explain how differences in duckling phenotype arise. Ducklings incubated at the lowest temperature expended more energy during the hatching process, and also exhibited different developmental trajectories late in incubation relative to ducklings incubated at higher temperatures (DuRant et al. In Press). Thus, greater energy expenditure and developmental delays induced by incubation temperature could contribute to differences in duckling condition, composition, and performance.

Text Box 3: Megapodes: The mound-nesters

Megopodiidae, a family of distributed throughout the Indo-Austrailian islands (Jones et al. 1995), are the only birds to use environmental heat instead of body heat to incubate their eggs (Booth and Jones 2002). Many megapodes compile damp litter, lay their eggs on top of this

material, then cover the eggs with more organic materials, producing nest mounds ranging in size from 0.8-4 m high (Jones 1988). Heat is produced by the many micro-organisms, via microbial respiration, present in the organic material (Booth and Jones 2002). To help regulate nest temperatures, megapodes will continue to add and remove insulating organic material on the nest throughout the incubation period (Booth and Jones 2002). Although mounds keep fairly stable temperatures, variation does occur. Optimal incubation temperature of the megapode, malleefowl (*Leipoa ocellata*), is 34˚C but average mound temperatures can range from 30-38˚C. Some megapodes do not build mounds, but use heat from either solar radiation or geothermal sources by nesting near hot springs or gases associated with volcanic areas (Booth and Jones 2002). Megapode embryos, unlike many other avian embryos, are tolerant to long-term exposure to suboptimal incubation temperatures (Booth and Jones 2002), resulting in wide variation in incubation periods (45-90 days; Bellchambers 1916*; Meyer 1930; Fleay 1937; Frith 1956; Baltin 1969).*

Probably because of their unique nesting behavior, some of the first studies to explore the effects of incubation temperature on avian development and phenotype were conducted in Megapode birds. Studies on Australian brush turkey (*Alectura lathami*) and malleefowl found that embryonic oxygen consumption patterns and total energy expended during incubation differed between embryos incubated at low temperatures versus those incubated at higher temperatures (Booth 1987; Vleck et al. 1984). Embryos incubated at the lower temperatures expended more energy during incubation largely due to a prolonged plateau phase in oxygen consumption that occurs in the late stages of incubation (Booth 1987; Vleck et al. 1984; Booth and Jones 2002). In addition to these effects, more recent studies revealed that incubation temperature also influences sex ratios of hatchlings (Goth and Booth 2005; Eiby et al. 2008). In

the Australian brush turkey temperature dependent embryonic mortality occurs, causing more males to hatch at lower incubation temperatures and more females to hatch at higher incubation temperatures (Goth and Booth 2005; Eiby et al. 2008).

Whether incubation temperature influences other aspects of phenotype of megapode hatchlings remains to be tested, but is probably the case. An interesting area of research in the megapodes, as well as other avian species that might exhibit temperature induced sex-dependent embryonic mortality, is if temperature differentially affects phenotype of males (or females) at male-producing v. female producing temperatures (Warner and Shine 2008).

Text Box 4: Ecological and Evolutionary Implications of Variable Phenotypes Produced by Incubation Temperature

Incubation temperature appears to play an important role in providing variation in avian offspring phenotype, variability upon which natural selection acts. Thus, incubation temperature could be an important selective agent for many aspects of avian ecology and evolution via its effects on offspring quality. We explore several aspects of avian life history that may be influenced by incubation temperature.

The selective force imposed by effects on offspring quality might be a factor in the evolution of nest-site selection and more elaborate nest construction. Most work on effects of incubation temperatures on offspring quality has been done on hole-nesting species, and it is clear that differences between nest sites exist in these species (Stoleson and Beissinger 1999; Weibe 2001; Hepp et al. 2006). There is an extensive literature on nest site selection related to microclimate indicating that such differences also exist in species that build platform and cup

nests, as well as ground-nesting birds, but this literature is focused on the energetics of incubating adults (e.g., Walsberg 1983) rather than effects on embryo development. Variation in microclimate is probably a function of not only the placement of the nest but also the quality of its construction. Effects of incubation temperature on offspring quality might reveal that nest microclimate has a larger role, relative to predation, in nest site selection than previously suspected.

Optimizing offspring phenotypic quality may also play a role in determining the onset of incubation. The degree of synchrony of hatching of eggs within a clutch depends on incubation behavior, asynchrony being maximized when incubation begins with the laying of the first egg and minimized when incubation begins with the laying of the last egg. Asynchronous hatching leads to size hierarchies among the young, and the smaller, last-hatched young suffer from competition for food with their older siblings, often starving or being killed. Hatching is synchronous in many species, and among those in which it is not, explanations of asynchrony have focused on identifying an adaptive function of these nestling size disparities (Stoleson and Beissinger 1995). For example they might function to reduce brood size to match feeding conditions after hatching. But asynchronous hatching has also been postulated to be a non-adaptive byproduct of other selective forces that compel some species to begin incubating with the first or second egg. Primary among these ideas is the hypothesis that eggs must be incubated to avoid temperatures that could reduce egg viability (Stoleson and Beissinger 1995; 1999; Cooper et al. 2005; Ardia et al. 2006). If delaying incubation until the last egg is laid has an effect on the phenotypic quality of the young, this could create an additional selective force leading to early incubation and thus asynchronous hatching.

Incubation temperature may also play an important role in clutch size evolution. Other researchers have proposed that both egg size and clutch size could be influenced by incubation temperatures due to the differing thermal properties of small versus large eggs and clutches (Martin et al. 2008; Cooper et al. 2005). These newly proposed hypotheses focus on how incubation temperature affects hatching success. However, given the effects of incubation temperature on hatchling traits important for survival, clutch and egg size could also be influenced by the phenotypic quality of offspring produced at different temperatures.

Clearly if constancy of incubation and frequency and duration of off-nest periods affect offspring quality due to the temperatures experienced by those eggs, the resulting selective force could influence incubation patterns. It could also impact mating systems by increasing the cost of polygamy, as incubation constancy is limited when parental duties fall to only one parent. There will be variation in the strength of this selective force with factors that determine the potential for incubation temperatures to affect offspring quality, such as climate and the nest environment. Thus incubation temperatures could be a factor in latitudinal and altitudinal gradients in avian life history.

Text Box 5: Conservation Implications of Avian Incubation Temperature

Environmental contaminants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are ubiquitous and known to have an array of adverse effects on the reproductive ecology of birds. Many of these and other contaminants disrupt the avian endocrine system, including reproductive hormones (e.g., androgens and estrogens), thyroid hormones, and prolactin (Fernie et al., 2003; Verreault et al., 2008; Marteinson et al., 2010). As a result, these compounds have the potential to alter parental behaviors including

those that influence the temperature of the nest. For example, American Kestrels (*Falco sparverius*) fed PCBs exhibited reduced nest attendance and poor coordination of incubation duties, largely due to aberrant attendance by males (Fisher et al., 2006). Although egg temperatures were not measured, the result of reduced nest attentiveness were consistent with the predicted relationship between nest cooling and incubation duration; Kestrel eggs from less attentive adults fed PCBs took significantly longer to develop and were less likely to hatch than those from control parents. Moreover, embryonic exposure of male Kestrels to PBDEs later affected their fertility and reproductive behaviors as adults, suggesting that these contaminants may have lifelong and multigenerational consequences for incubation behaviors in birds (Marteinson et al., 2010). However, we know of only one study that has explored the relationship between contaminants and nest temperatures. Verboven et al. (2009) found negative relationships between plasma levels of organic pollutants and nest temperatures in Glaucous Gulls (*Larus hyperboreus*) and postulated that these reductions in temperature were caused by physiological aberrations in incubating adults (e.g., brood patch development). To the best of our knowledge, however, no ecotoxicological studies have explicitly linked changes in adult nest attendance or physiology to incubation temperatures and resulting phenotypic consequences to offspring. Similarly, no studies have examined the interactive consequences of embryonic exposure to maternally transferred contaminants and suboptimal incubation conditions, which could act synergistically or additively to produce effects on the embryo/hatchling that neither would produce alone. Future studies that utilize brood manipulations to disentangle the effects of maternal transfer of contaminants and incubation behavior on early development may shed new light on the hazards of these compounds to birds. Likewise, studies that determine the

physiological (e.g., endocrinological) causes of aberrant incubation behavior are needed to understand the mechanism underlying these anthropogenic effects.

Other environmental factors that force incubating adults to leave their eggs unattended more frequently or for longer periods of time should also influence incubation temperatures and offspring phenotype. Nest disturbances such as those caused by military activities, urbanization, and ecotourism could influence nest attendance. For example, numerous studies have demonstrated that improperly managed ecotourism and outdoor recreation can influence the physiology, behavior, and survival of birds (reviewed in Carney and Sydeman, 1999; Müllner et al., 2004; Ellenberg et al. 2007). Many of these studies indicate that adults are flushed from the nest when they perceive the threat of approaching humans, but the consequences for egg incubation temperatures have not been adequately explored. This problem is further exacerbated by the fact that in many areas, peak visitation by humans coincides with the nesting season due to favorable climate or the attraction of viewing large congregations of birds (Klein et al., 1995).

Alternatively, habitat variables that influence resource availability could force adults to take more frequent or longer recesses from the nest to forage. For example, depleted fisheries and invertebrate prey are known to influence the foraging behavior and success of some seabirds (Kitaysky, 2000; Furness, 2007) and increased competition for fish prey can force birds to travel longer distances to forage (Lewis et al., 2001). However, most studies consider parental foraging in relation to provisioning young, and not incubation patterns per se. Future studies designed to explicitly examine habitat conditions in relation to incubation behavior, particularly in uniparental incubators where adults must balance the competing demands of attending to their eggs and maintaining their own body condition, will prove useful to land managers.

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Table Conclusion.1: Examples of phenotypic traits and life history characteristics of reptilian offspring affected by incubation temperature. Taxa included represent those in which the phenotypic trait is known to be affected in at least one representative species.

Phenotypic Trait/Life History Characteristic	Reptile Taxa[1]
Sex	All crocodilians, tuatara, & some turtles, lizards, & snakes
Hatchling Body Size	Turtles, Lizards, & Crocodilians
Hatchling Body Shape/Scalation	Lizards, Snakes, & Crocodilians
Hatchling Body Condition/Composition	Lizards, Crocodilians
Color	Turtles, Lizards, & Crocodilians
Locomotor Performance	Turtles, Lizards, & Snakes
Behavior	Turtles, Lizards, & Snakes
Metabolism	Turtles
Thermoregulation	Turtles, Lizards, Snakes, & Crocodilians
Growth	Turtles, Lizards, Tuatara, Crocodilians
Immune Function	Turtles
Endocrinology	Turtles, Lizards
Survival	Lizards, Turtles, Crocodilians
Lifetime Reproductive Success	Lizards

[1]Failure to include a taxonomic group is often attributable to the fact that studies have not evaluated that phenotypic trait in that group.

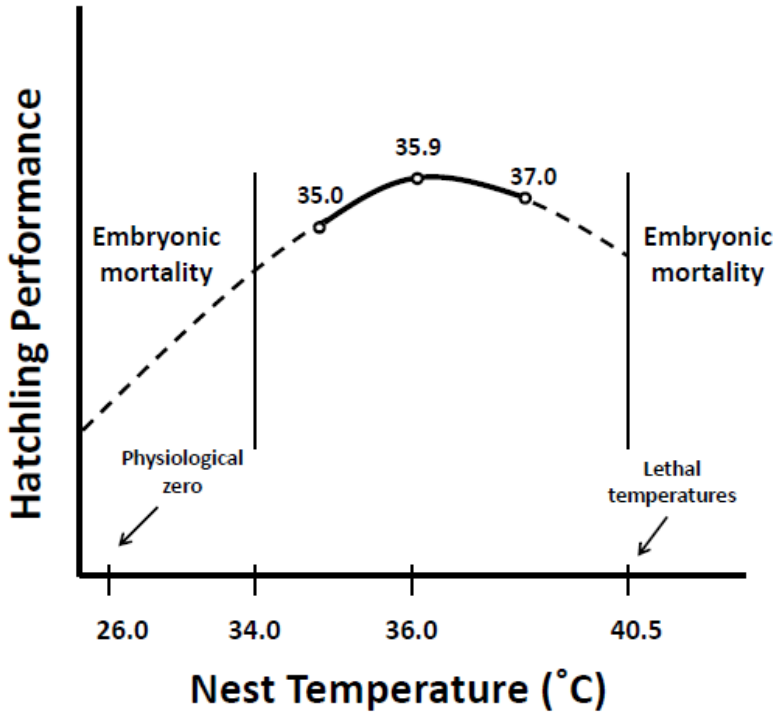

Figure.conclusion.1: The relationship between average temperature experienced during incubation and hatchling performance in wood ducks (*Aix sponsa*). Points on graph represent physiological performance (e.g., immune responses, stress hormone concentrations) of wood duck embryos incubated at either 35.0, 35.9 or 37.0˚C. Based on studies conducted in wood ducks, duckling performance is optimized around ~ 36˚C. Dotted lines represent predicted performance at incubation temperatures that have yet to be tested. Natural nest temperatures in wood ducks are typically not higher than 38.5˚C (Manlove and Hepp, 2000; Folk and Hepp, 2003; Hepp et al., 2006) and research suggests that even short exposure to temperature exceeding 40.5˚C is lethal to most avian embryos (Webb 1987). Temperature below 24-26˚C is considered physiological zero for most avian embryos. Although not tested, we propose that a similar as curve presented here is probably representative of the relationship between nest temperature and hatchling performance for most avian species.

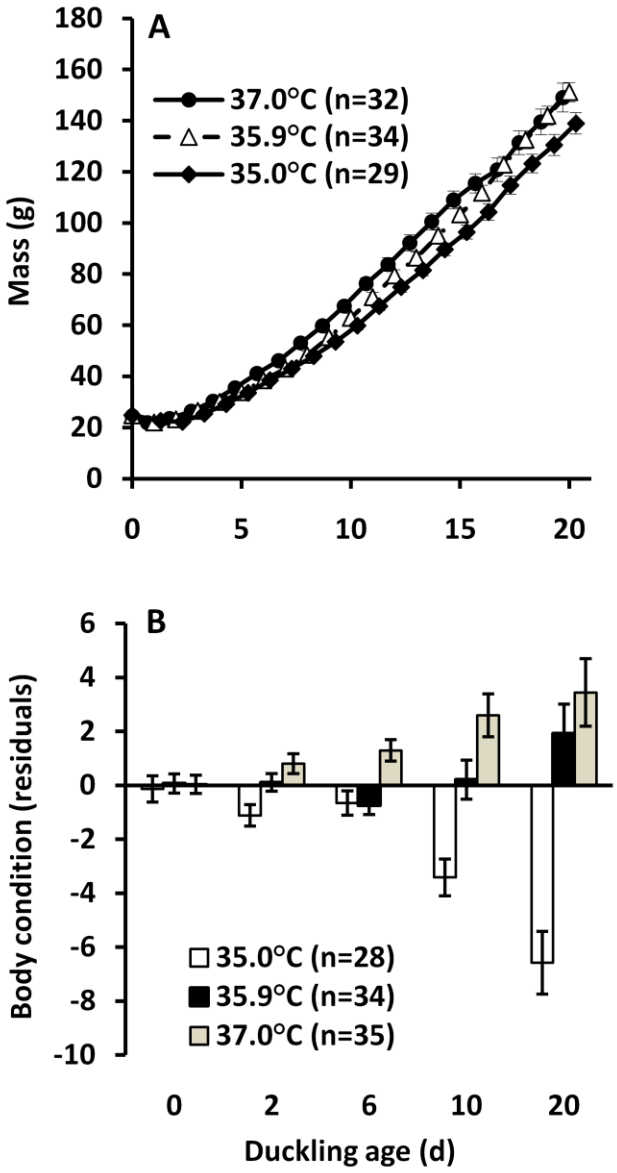

Figure. conclusion.2: Average (± 1 SE) duckling growth (A) and body condition (B) of wood duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). Body condition was estimated form the residuals of a regression of body mass versus tarsus length.

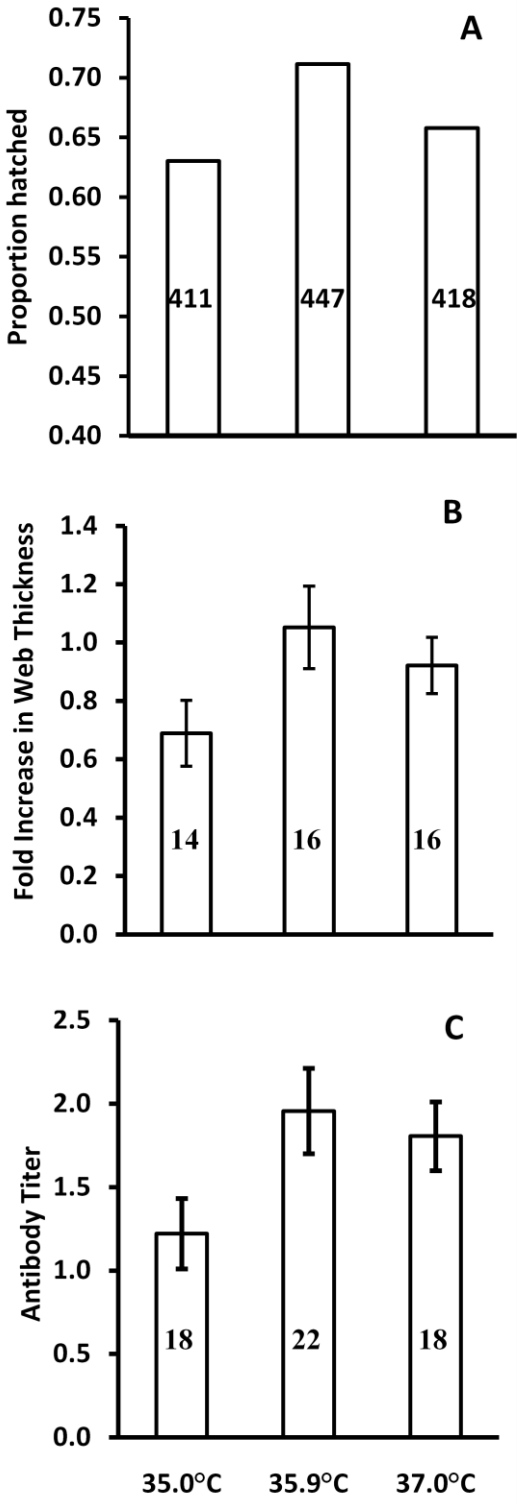

Figure. conclusion.3: A) The effect of incubation temperature on hatching success of wood duck (*Aix sponsa*) eggs incubated at three temperatures. A total of 1276 eggs were incubated

during the reproductive seasons of 2008-2011 (Chi square; p = 0.036). **B & C)** Immune responses (mean ± 1 SE) of wood duck (*Aix sponsa*) ducklings that hatched from eggs incubated at one of three temperatures (35.0, 35.9, or 37.0°C). **B)** Fold increase in foot web thickness of ducklings 24h after exposure to phytohemagglutinin **C)**. Antibody titers of ducklings exposed to sheep red blood cells (SRBC). Samples sizes are on graph. Samples sizes are on the graph. Error bars are ± 1 standard error of the LSmean.