Consequences of avian parental incubation behavior for within-clutch variance in incubation temperature and offspring behavioral phenotypes

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ABSTRACT

Parents can have large effects on their offspring by influencing the early developmental environment. In birds, a major way that parents can influence the early developmental environment is through egg incubation. Not only is incubation necessary for hatching success, but small changes of <1°C in average incubation temperature have large effects on post-hatch offspring morphology and physiology. However, incubation is energetically costly and time-consuming for parents, and thus parents must allocate resources between incubation and self-maintenance. This can lead to differences in parental incubation behavior and egg temperatures among and within populations. Understanding which factors influence incubation, and the subsequent effects for offspring, is crucial for understanding parental effects, non-genetic drivers of phenotypic variation, and how environmental changes affect avian populations. I used wood ducks (*Aix sponsa*) as a study species to investigate how factors (disturbance, clutch size, ambient temperature) that influence parental demands may affect parental incubation behavior, physiology, and egg temperatures, and subsequently how egg temperatures affect offspring behavior and physiology. In a field experiment, I found that nest disturbance (i.e., capture) reduced both parent prolactin concentrations and the amount of time that parents spent incubating (Chapter 1). Further, ambient temperature was positively and clutch size negatively related to egg temperatures. Notably, in large clutches, differences in average incubation temperature among eggs *within* nests were large enough (i.e., >1°C) to lead to different offspring phenotypes within broods (Chapter 2). Then, in a series of experiments in which I controlled incubation temperature, I provided evidence that lower average incubation temperatures lead to a reduced ability of ducklings to exit the nest cavity (Chapter 3), a more proactive behavioral phenotype (Chapter 4), a smaller body size, and a reduced efficiency in food consumption (Chapter 5), compared to those incubated at higher temperatures. Together, my dissertation illustrates how disturbances, clutch size, and ambient temperature can influence an important aspect of avian parental care, which has wide-ranging effects on offspring traits and fitness. This has broad implications for understanding the evolution of clutch size, development of behavior, and the effects of anthropogenic changes on wildlife.

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GENERAL AUDIENCE ABSTRACT

Animal parents can have large effects on the development of their offspring. In birds, an important way that parents affect their offspring is through incubation, where parents physically warm their eggs to stimulate embryo development. Eggs must be incubated in order to hatch, but recent research has shown that small changes (<1°C/2°F) in average incubation temperature have major consequences for the quality (e.g., size, ability to thermoregulate) of offspring after they hatch. However, parents must balance how they spend their time and energy between incubation and other important activities (e.g., eating, avoiding predators), and thus incubation behavior and temperature can vary among birds. Understanding which factors affect incubation, and the consequences of altered incubation temperatures for the offspring, will help us to better understand how animals care for their offspring and how environmental changes may influence offspring development. I investigated how human disturbance, environmental temperature, and the number of eggs in the nest influenced parental incubation behavior and egg temperatures, and subsequently how egg temperatures affected offspring behaviors. By studying wood ducks (*Aix sponsa*) in the wild, I found that parents spent less time incubating after a human disturbance (i.e., capture) than before (Chapter 1), egg temperatures increased as environmental temperatures increased, and egg temperatures decreased as the number of eggs in the nest increased (Chapter 2). Further, in nests with many eggs (>12), some eggs experienced much lower average incubation temperatures (>1°C/2°F difference) than others in the same nest (Chapter 2). Then, by studying wood duck ducklings in an aviary, I found that ducklings incubated at lower temperatures were less successful at exiting a nest (Chapter 3), exhibited bolder and more

exploratory behaviors (Chapter 4), were smaller, and consumed less food (Chapter 5), than those incubated at a higher temperature. Together, my dissertation shows that the number of eggs in a nest, environmental temperatures, and human disturbances can influence parental behaviors, which then affect offspring. This has broad implications for understanding why birds lay the number of eggs that they do, how animal behaviors develop, and how environmental changes (including those caused by humans) can affect wildlife.

DEDICATION

To my Mama Duck, Kate Hope

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ATTRIBUTION

Chapter 1 was coauthored by Sarah DuRant, Frédéric Angelier, John Hallagan, Ignacio Moore, Charline Parenteau, Robert Kennamer, and William Hopkins. SD, RK, and WH conceived and designed the study. RK provided access to the field site. FA, IM, and CP performed laboratory analyses. SD, JH, and WH conducted fieldwork. All coauthors provided comments and approved the manuscript.

Chapter 2 was coauthored by Sarah DuRant, John Hallagan, Michelle Beck, Robert Kennamer, and William Hopkins. SD, JH, RK, and WH conceived and designed the study. RK provided access to the field site. SD, JH, and WH conducted fieldwork. MB helped with statistical analyses. All coauthors provided comments and approved the manuscript.

Chapter 3 was coauthored by Robert Kennamer, Schuyler van Montfrans, and William Hopkins. RK and WH helped conceive and design the study. RK provided access to the field site. SVM helped analyze behavioral data. All coauthors provided comments and approved the manuscript.

Chapter 4 was coauthored by Robert Kennamer, Ignacio Moore, and William Hopkins. RK and WH helped conceive and design the study. RK provided access to the field site. IM performed laboratory analyses. All coauthors provided comments and approved the manuscript.

Chapter 5 was coauthored by Robert Kennamer, Alexander Grimaudo, John Hallagan, William Hopkins. RK, JH, and WH helped conceive and design the study. RK provided access to the field site. JH and AG helped conduct the experiment. AG helped analyze behavioral data. All coauthors provided comments and approved the manuscript.

INTRODUCTION

Across animal taxa, parental care is crucial for offspring development, but energetically-costly and time-consuming for parents (Trivers, 1972, 1974). Thus, to maximize lifetime reproductive success, parents must trade-off investments in time and energy between parental care and self-maintenance (Stearns, 1992). Many factors can influence this trade-off, including offspring number, parental age, external disturbances, parental body condition, ambient temperature, and food availability (Alonso-Alvarez and Velando, 2012; Angelier and Chastel, 2009; Clutton-Brock, 1991; Stearns, 1992). These parental investment decisions are critical for offspring because changes in parental care behavior can affect the early developmental environment, which can have long-term consequences for offspring quality (Lindström, 1999). For example, parents can regulate the temperature, humidity, oxygen availability, and nutrients for developing offspring (pre- and post-natal), and engage in other behaviors such as grooming, carrying, and brooding (Bernardo, 1996; Mousseau and Fox, 1998a). Small changes in these aspects of the early developmental environment can affect offspring morphology, physiology, behavior, sex ratio, reproductive success, and survival (Dixon et al., 2016; Francis et al., 1999; Hepp et al., 2015; Monaghan, 2008; Mousseau and Fox, 1998b). Understanding factors that influence parental care, as well as the consequences of altered parental care on offspring phenotype, is crucial to understanding life history evolution, non-genomic drivers of phenotypic variation, and the consequences of environmental (including anthropogenic) changes on animal reproductive success.

In birds, one of the most critical aspects of parental care that influences the early developmental environment is egg incubation. Avian parents must keep eggs within a narrow temperature range to ensure hatch success (Deeming and Ferguson, 1991; Hepp et al., 2015).

Although once thought to be a passive process (Williams, 1996), it is now known that incubation is energetically costly and time-intensive for parents (Nord and Williams, 2015; Tinbergen and Williams, 2002). During incubation, parental metabolic rate increases, body mass decreases, and the incubating parent must spend large portions of the day without foraging (Thomson et al., 1998; Vleck, 1981). Further, these time and energy costs can be influenced by various factors, such as weather, predation risk, or clutch size, which can lead to changes in parental incubation behavior (Amininasab et al., 2016; Basso and Richner, 2015; Coe et al., 2015; Wiebe and Martin, 2000). As a result, incubation temperatures can vary substantially among individuals within a population (Aldrich and Raveling, 1983; Ardia et al., 2010; Coe et al., 2015; Conway and Martin, 2000; Haftorn and Reinertsen, 1985; Nord et al., 2010). Although it is known that incubation behavior varies in response to altered parental demands, little is known about the physiological mechanisms that underlie these behavioral changes, nor are the subsequent effects on egg temperatures fully understood. In particular, because most studies to date only use a single, stationary temperature logger to measure avian nest temperature, almost nothing is known about how incubation temperature may vary among eggs *within* nests. Filling these knowledge gaps is essential for complete understanding of how avian parents manage the trade-off between incubation and self-maintenance, the subsequent consequences for the early developmental environment of their offspring, and how the variation by which natural selection acts upon could be produced.

Importantly, the ability of the parent to regulate incubation temperature can have large consequences for the offspring after hatching. Recent research has revealed that small changes in average incubation temperature (<1°C) can have substantial and long-lasting effects on avian offspring traits, including growth rates (DuRant et al., 2010; Nord and Nilsson, 2011; Ospina et al., 2018; Wada et al., 2015), hormone levels (DuRant et al., 2010, 2014; Wada et al., 2015), thermoregulatory ability (DuRant et al., 2013a), immune function (DuRant et al., 2012), metabolism (Nord and Nilsson, 2011; Wada et al., 2015), locomotor ability (Belnap et al., 2019; Hopkins et al., 2011), and survival (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; Nord and Nilsson, 2016). However, almost nothing is known about how incubation temperature may influence important avian offspring behaviors. It is likely that incubation temperature influences avian offspring behavior because non-avian reptiles incubated at different temperatures exhibit different behaviors (Amiel and Shine, 2012; Putz and Crews, 2006; Sakata and Crews, 2003, 2004). Further, many of the physiological characteristics that are affected by incubation temperature in birds, such as hormone levels, growth rate, and metabolic rate, are related to behaviors across taxa (Biro and Stamps, 2010; Careau et al., 2008; Cockrem, 2007; Koolhaas et al., 1999; Stamps, 2007). Behaviors during early-life are critical for survival because they can influence the performance of important tasks such as food acquisition, predator avoidance, and intra-specific competitive outcomes. Understanding how incubation temperature affects behavior is crucial to fully understand the consequences of altered incubation for avian offspring, and may reveal a way in which a non-genetic parental effect could influence the development of behavior.

Thus, the overarching goal of my dissertation was to investigate how factors (e.g., clutch size, ambient temperature, disturbance) that can alter parental time and energy demands may affect parental incubation behavior and physiology, how this affects within-clutch variance in egg incubation temperatures, and how egg temperatures affect offspring behaviors (Fig. 1).

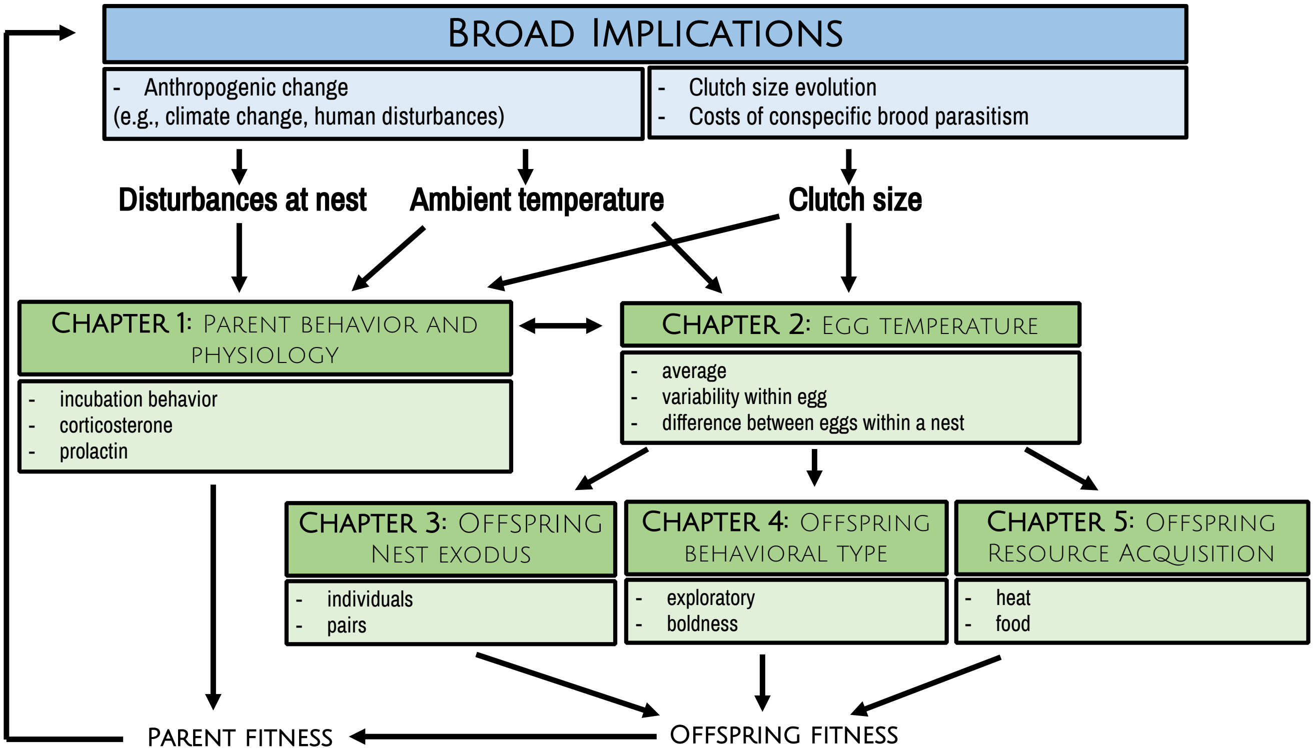

Figure 1. Conceptual framework of the relationships among questions that I addressed in my dissertation chapters, and their broad implications.

Study Species

The wood duck (*Aix sponsa*) is a dabbling duck that is widely distributed throughout North America (Hepp & Bellrose 2013), and nests in tree cavities but readily uses nest boxes (Hepp et al., 1987). Their breeding season extends from mid-February to mid-July (Hepp & Bellrose 2013) and females typically lay one egg per day to produce an average clutch size of 12 (Bellrose and Holm, 1994). Conspecific brood parasitism is common in this species, affecting up to 85% of nests; parasitized clutch sizes of >40 eggs occur in some populations (Hepp & Bellrose 2013). Females are solely responsible for incubation, which lasts an average of 30 days (Hepp & Bellrose 2013), although developmental rate is temperature-dependent (DuRant et al., 2011). Females spend most of their time incubating and usually take one recess in the morning and one in the evening, each lasting ~1-2 h, to forage (Manlove and Hepp, 2000).

Hatching is usually synchronous and occurs within 24 hours (Bellrose and Holm, 1994). Shortly after hatching, ducklings must respond to their mother's call and jump out of the nest cavity, and failure to complete this nest exodus can result in abandonment by the mother and, ultimately, death (Bellrose and Holm, 1994). Offspring are precocial and can feed themselves once they leave the nest, but the mother stays with the brood to protect them from predators and guide them to sources of food (Bellrose and Holm, 1994). The hen-brood bond in the southern portion of the breeding range lasts about 5 weeks, but ducklings are known to survive alone in the wild (Bellrose and Holm, 1994).

Wood ducks are an excellent study species for meeting the objectives of my dissertation. First, because they are a precocial species, incubation is one of the most important aspects of parental investment. Further, because females incubate without any help from the male, and they maintain high incubation constancy for a long incubation period, incubation is a large maternal investment. Additionally, the large variation in wood duck clutch sizes and the tendency of females to accept and incubate eggs that are not their own (i.e., as a result of conspecific brood parasitism) makes this species particularly suitable for conducting studies involving clutch size manipulations. Thus, parental care demands can be altered by manipulating clutch size. Lastly, wood ducks are one of the most well-studied species in regards to the effects of incubation temperature on offspring phenotype (DuRant et al., 2013b). Thus, they can be used as a model species to pursue potential effects on phenotype that are not yet studied.

Chapter Objectives

My dissertation is divided into five core chapters (Fig. 1). I began by investigating how human disturbance affects incubation behavior, how changes in two important hormones (prolactin and corticosterone) may underlie changes in parental incubation behavior, and how this affects egg temperatures (Chapter 1). This revealed a link between parental physiology, parental incubation behavior, and egg temperatures. Next, I investigated how clutch size and ambient temperature influence egg incubation temperatures (Chapter 2). By using multiple mobile artificial egg temperature loggers within nests, this chapter revealed that, in large clutches, average incubation temperatures differ among eggs *within* the same nest, and that temperature differences were great enough that differences in offspring phenotypes should result (i.e., >1°C).

My remaining three chapters focused on how different egg temperatures influence offspring behaviors. I began by investigating how incubation temperature affected the first crucial behavior for wood duck ducklings: the nest exodus (Chapter 3). This study has direct implications for offspring fitness because, if ducklings do not successfully exit the nest, they will die. Because Chapter 2 revealed that wood duck broods in the wild should consist of ducklings that were incubated at different average incubation temperatures, I tested the ability of ducklings incubated at different temperature to leave the nest both individually and in mixed-incubation temperature pairs. Next, I studied the effects of incubation temperature on behavioral traits of ducklings that were 5-15 days-old (Chapter 4). This study revealed that ducklings incubated at lower temperatures displayed more proactive behavioral types (e.g., quicker to leave a shelter, more active in open field arena) than those incubated at higher temperatures. Lastly, I studied how incubation temperature may affect the ability of ducklings to gain access to food or heat sources (Chapter 5). Because I predicted that within-clutch differences in average incubation temperatures (found in Chapter 2) could result in differential resource acquisition within broods, I tested the ability of ducklings to acquire food or heat resources in broods composed of ducklings incubated at different temperatures. Because Chapter 4 revealed that incubation temperature affected behavioral type, and individuals with different behavioral types may have different competitive abilities depending on the context (Cole and Quinn, 2012; Ward et al., 2004), I tested broods within different environmental contexts (i.e., novel environment, familiar environment).

Because my dissertation focuses on how parents trade-off between parental care and self-maintenance, and how subsequently altered parental care can influence offspring behaviors that are important for survival, my results contribute to our understanding of how altered parental demands translate to effects on avian fitness. By focusing on the effects of clutch size, ambient temperature, and nest disturbance, my results have broad implications for understanding the evolution of clutch size, costs of enlarged clutch sizes due to conspecific brood parasitism, and the effects of anthropogenic changes on avian parental care behaviors, the developmental environment of avian embryos, and offspring phenotypes (Fig. 1). Further, my dissertation improves our understanding of the physiological mechanisms underlying parental care decisions, the causes and consequences of variation in parental effects within a breeding attempt, and the non-genetic drivers of individual variation in behavior.

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CHAPTER 1: Prolactin is related to incubation constancy and egg temperature following a disturbance in a precocial bird

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ABSTRACT

To maximize fitness, parents may trade-off time and energy between parental care and self-maintenance. In vertebrates, prolactin and corticosterone are two important hormones that regulate parental investment because they stimulate parental care and mobilize energy, respectively. Further, concentrations of both hormones change in response to disturbances. One of the most important parental care behaviors in birds is incubation, since small changes in egg temperature have large effects on offspring. We investigated how prolactin and corticosterone may mediate parental incubation behavior and regulation of egg temperature. We collected blood samples from female wood ducks (*Aix sponsa*) near the start and end of the incubation period to measure baseline and stress-induced (30 min after capture and restraint) hormone concentrations. We also quantified incubation behavior and egg temperature using artificial egg temperature loggers. As expected, prolactin decreased and corticosterone increased after 30 min of capture and restraint. Corticosterone concentrations (baseline and stress-induced) were negatively related to body mass, but were not related to any aspect of incubation behavior. Interestingly, prolactin concentrations (baseline and stress-induced) were higher at the end than the start of the incubation period, and stress-induced prolactin concentrations were positively related to the daily percentage of time hens spent incubating eggs following nest disturbance (i.e., capture). Further, prolactin (baseline and stress-induced) concentrations were positively related to egg temperatures, but only after the disturbance. These results suggest that prolactin may be associated with the regulation of parental incubation behavior and resulting heat-transfer after a disturbance, which may ultimately affect offspring development.

Keywords: incubation, parental care, prolactin, corticosterone, wood duck, *Aix sponsa*, stress-response, egg temperature

INTRODUCTION

Parental care is necessary for successful offspring development in most bird species, and one of the most important aspects of avian parental care is egg incubation. Adequate regulation of egg temperature is crucial because small changes in average incubation temperatures not only affect hatch success, but also influence a range of fitness-related offspring traits (DuRant et al., 2013c) including thermoregulation (DuRant et al., 2013a), immune function (DuRant et al., 2012), growth rate (DuRant et al., 2010; Nord and Nilsson, 2011; Ospina et al., 2018; Wada et al., 2015), metabolic rate (Nord and Nilsson, 2011; Wada et al., 2015), and behavior (Hope et al., 2018b, 2019). Further, there is evidence that incubation temperature affects long-term survival (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; Nord and Nilsson, 2016). Thus, there should be selection pressure for parents to optimize incubation temperature. However, incubation is active, energetically costly compared to resting, and time-consuming for parents (Nord and Williams, 2015; Thomson et al., 1998; Tinbergen and Williams, 2002), especially for species in which only one sex incubates. This might create a trade-off for parents in how they invest time and energy towards incubation behaviors and important self-maintenance behaviors, such as foraging. Further, this trade-off can be affected by various disturbances (e.g., predators, human disturbance; Fontaine and Martin, 2006; Ghalambor and Martin, 2002; Martin et al., 2015), which are particularly significant in relation to incubation because incubating birds spend a large portion of their time on the nest, which could make it difficult to avoid disturbances. Thus, a complete understanding of the trade-off between parental incubation behavior and self-maintenance is crucial to understand how different parental care strategies evolve and how species respond to disturbances during parental care. The latter is especially important as sources of anthropogenic disturbances (e.g., urban development, introduced predators) increase.

The energetic demands and behavioral decisions necessary to manage the trade-off between parental care and self-maintenance are mediated by various physiological signals, including hormones (Ricklefs and Wilkelski, 2002; Wingfield and Sapolsky, 2003). Prolactin and corticosterone are two hormones of particular importance because they stimulate parental care and mobilize energy reserves, respectively (Buntin, 1996; McEwen and Wingfield, 2003). During periods of avian parental care, baseline prolactin concentrations increase as a result of longer photoperiods (Dawson et al., 2001; Dawson and Sharp, 1998; Hall, 1986), and in response to nest, egg, and offspring stimuli (Hall, 1987, 1986; Leboucher et al., 1993; Sharp et al., 1998; Silver, 1984). Baseline corticosterone concentrations either increase or decrease during parental care, depending on the species and the type of care (Romero, 2002). However, when birds are faced with an acute disturbance, corticosterone concentrations typically increase and prolactin concentrations typically decrease, and the resulting hormone concentrations after the disturbance are commonly referred to as 'stress-induced corticosterone' and 'stress-induced prolactin', respectively (Angelier and Chastel, 2009; Sapolsky et al., 2000). These post-disturbance high concentrations of corticosterone and low concentrations of prolactin may be related to a shift in investment from parental care to immediate survival, which can manifest as reduced parental care behaviors or, in extreme cases, offspring abandonment (Groscolas et al., 2008; Ouyang et al., 2012; Spée et al., 2010). Thus, maintaining high prolactin concentrations and/or low corticosterone concentrations in the face of stressors could be a mechanism by which animals maintain parental care behaviors despite disturbances (prolactin: Angelier et al., 2016; Angelier and Chastel, 2009; corticosterone: Bókony et al., 2009; Holberton and Wingfield, 2003; O'Reilly and Wingfield, 2001; Wingfield et al., 1995).

Although parental behavior has often been studied in relation to stress-induced corticosterone concentrations, relatively little is known about how it may be related to stress-induced prolactin concentrations (Angelier et al., 2016; Angelier and Chastel, 2009). The hypothesis that the suppression of the corticosterone stress-response serves to maintain parental behaviors is supported in some species, where lower stress-induced corticosterone concentrations are related to a greater investment in parental care behaviors (Ouyang et al., 2012; Silverin, 1986; Spée et al., 2011). However, some species exhibit greater stress-induced corticosterone concentrations as parental investment increases, suggesting that corticosterone mobilizes energy without causing abandonment (DuRant et al., 2013b; Perfito et al., 2002; Romero, 2002). Prolactin is classically known as the 'parental care hormone' (Buntin, 1996; Hall, 1986; Sockman et al., 2006; Vleck, 1998). Many studies show that baseline prolactin concentrations are positively related to parental effort (reviewed in Angelier et al., 2016), and that experimental reductions in circulating baseline prolactin concentrations lead to a reduced frequency of parental care behaviors, including incubation behavior (e.g., Angelier et al., 2009a; Smiley and Adkins-Regan, 2018; Thierry et al., 2013). To date, however, there has only been one study investigating whether prolactin concentrations after a disturbance are related to parental behavior, which found that snow petrels (*Pagodroma nivea*) with low stress-induced prolactin concentrations were more likely to abandon their nest than those with higher concentrations (Angelier et al., 2015).

Nothing is known about how stress-induced changes in prolactin may be related to subtle changes in the frequency of day-to-day parental care behaviors, or how these changes may affect developing offspring. Understanding the role of both corticosterone and prolactin in mediating changes in incubation will shed light on the mechanism by which animals modulate their behavior to cope with disturbances during parental care.

In this study, we investigated whether prolactin and corticosterone are associated with parental behavior and resultant changes to egg temperatures after birds are confronted with a disturbance during incubation. We investigated this question by studying a population of wood ducks (*Aix sponsa*) nesting in nest boxes. Because the current study was a part of a larger study investigating the effects of clutch size on the costs of incubation, we first manipulated clutch size. This manipulation also strengthened the current study because it resulted in a range of clutch sizes and decoupled investment in egg-laying from investment in incubation behavior. We installed artificial egg temperature loggers in the nest boxes to quantify parental incubation behavior (e.g., on- and off-bouts, constancy) and egg temperatures throughout incubation. We captured hens at two timepoints during the ~30-day incubation period (early and late) and collected blood samples to determine baseline and stress-induced (after 30 min of a standardized capture/hold stressor) prolactin and corticosterone concentrations. We investigated the relationships among hormones, behavior, and egg temperature both during the 24 h before and the 24 h after this disturbance because we expected that hormones and behavior would change over a short time period in response to the disturbance. We hypothesized that the secretion of high prolactin concentrations during incubation, especially when faced with a disturbance, is an indicator of high parental investment (Angelier and Chastel, 2009). Thus, we predicted that both baseline and stress-induced prolactin concentrations would be positively correlated with the amount of time that hens spent incubating (incubation constancy) and egg temperatures. Specifically, a positive relationship between stress-induced prolactin and incubation constancy would support the hypothesis that maintaining high prolactin concentrations despite a disturbance is an indicator of increased parental investment (Angelier and Chastel, 2009). We had two alternative hypotheses for corticosterone. First, elevated concentrations of corticosterone may help mediate incubation behavior through increasing energy availability (DuRant et al., 2013b), in which case we would predict that baseline and stress-induced corticosterone concentrations would be positively related to incubation constancy and egg temperatures. Alternatively, elevated concentrations of corticosterone may inhibit parental care behavior through shifting investment toward self-maintenance (e.g., O'Reilly and Wingfield, 2001; Ouyang et al., 2012; Silverin, 1986; Spée et al., 2011), in which case we would predict that there would be a negative relationship between corticosterone concentrations and incubation constancy and egg temperatures.

METHODS

Study species and site

The wood duck is a dabbling duck that is widely-distributed throughout the United States and nests in tree cavities and nest boxes (Hepp and Bellrose, 2013). Females lay one egg per day and produce an average clutch size of 12 (Bellrose and Holm, 1994). Conspecific brood parasitism is common in both natural and artificial nest cavities (Roy Nielsen et al., 2006a; Semel et al., 1988; Semel and Sherman, 1986), affecting up to 85% of nests and resulting in parasitized clutch sizes that average over 40 eggs in some populations (Hepp and Bellrose, 2013; Morse and Wight, 1969). Hatch success is high in parasitized clutches, and is normally only slightly lower, if at all, than in un-parasitized clutches (Roy Nielsen et al., 2006b; Semel et al., 1988).

Female wood ducks are solely responsible for incubation, which begins with partial night incubation about 4 days before the end of egg-laying. After clutch completion, females progress to full incubation, which lasts an average of 30 days (Hepp and Bellrose, 2013). Females have high incubation constancy and usually take one recess in the morning and one in the evening to forage (Hepp and Bellrose, 2013; Manlove and Hepp, 2000), although behavior is variable among individuals (Bellrose and Holm, 1994; Manlove and Hepp, 2000). Ducklings are precocial and can feed themselves shortly after exiting the nest (Hepp and Bellrose, 2013), and thus egg incubation is one of the most important aspects of parental care in this species. Further, small changes in average incubation temperature have large effects on the morphology, physiology, behavior, and survival of wood duck offspring (DuRant et al., 2013c; Hepp and Kennamer, 2012), which suggests that females should be under selective pressure to optimize incubation temperature.

We studied a population of wood ducks breeding in nest boxes at the Department of Energy's Savannah River Site in South Carolina (33.1°N, 81.3°W) on a series of 12 ephemeral wetlands, where wood ducks have been monitored for over 35 years. We monitored nest boxes from 21 February – 20 June 2014. All methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC #11-056 and 14-083).

Clutch size manipulation

As part of a larger study, we manipulated clutch sizes. Once 3 – 5 eggs were laid, we began adding or removing one egg per day to achieve both reduced and augmented clutch sizes.

Occasionally, to create very large clutch sizes (e.g., 33 eggs), we added more than one egg per day, which is similar to what happens in heavily parasitized nests in the field (Hepp and Bellrose, 2013; Morse and Wight, 1969). We ensured that the eggs that we added to a clutch were always of a similar lay-date to the eggs of that clutch. All eggs were moved before incubation had started. To measure incubation behavior and temperature, we replaced seven eggs in each nest with seven artificial (one stationary and six free-moving) eggs containing temperature loggers (see *Incubation behavior* and *Incubation temperature*). Out of 40 total nests in this study, we installed free-moving loggers into 33 nests (this number was limited by the number of loggers). The other 7 nests contained only a stationary logger. Out of the 33 nests, we collected full temperature data from the six free-moving loggers in 29 nests, but due to technological difficulties, three nests contained five and one nest contained four functional loggers (i.e., the other 1-2 loggers did not successfully record temperature data). Final clutch sizes ranged from 8 – 33 eggs (mean ± SD = 17.6 ± 5.2 eggs; including artificial eggs) and clutch sizes were equally distributed among wetlands and across the breeding season.

Capture and blood sampling

We captured females at their nest boxes once during early (range = 3 – 8 days after the start of full incubation; mean ± SD = 5.3 ± 1.4 d) and once during late (range = 21 – 32 d; mean ± SD = 24.4 ± 1.9 d) incubation to take blood samples for baseline and stress-induced prolactin and corticosterone concentrations. To capture birds, we slowly and quietly approached the nest box while the hen was incubating, covered the opening of the nest box, and then removed the hen from the nest. All birds were captured after their morning recess between 9:09 – 15:57 h (mean ± SD = 12:07 ± 1:49 h). We collected blood from the femoral vein using a 25 G needle.

We collected up to 300 μl of blood to measure baseline hormone concentrations and 200 μl for stress-induced hormone concentrations using 100 μl heparinized capillary tubes. For baseline samples, the blood that was collected the quickest was allocated to corticosterone analyses. Baseline samples were collected within ~4 min of covering the opening of the nest box (samples used for corticosterone were collected within: range = 2.7 – 4.0 min; mean ± SD = 3.5 ± 0.5 min; samples used for prolactin were collected within: range = 2.7 – 7.0 min; mean ± SD = 4.2 ± 1.2 min). There was no relationship between the amount of time it took to collect the blood sample and baseline corticosterone ($R^2 = 0.01$; $p = 0.36$) or baseline prolactin ($R^2 = 0.04$; $p = 0.14$). After the baseline sample, we placed a cloth hood over the hen’s head and upper torso to cover its eyes and then placed the hen in a cloth bag for 30 min, as a standardized acute stressor (Wingfield et al., 1992). Then, blood samples for stress-induced hormone concentrations were taken, and we changed which leg the blood was collected from, if necessary. We then measured hen size (mass and tarsus length), and immediately placed the hen back on the nest. Blood samples were placed on ice in the field, and after transport to the laboratory they were centrifuged at 3.5*g* for 5 min and plasma was stored at -80°C until it was analyzed.

Incubation behavior

To determine hen incubation behavior, we equipped each nest with a stationary temperature logger, which was contained within an artificial egg, as described in (Hope et al., 2018a). Briefly, we mounted one thermal probe from the logger in a stationary, but flexible, position in the middle of the clutch. This logger also had a second probe, which we attached to the inside wall of the nest box to record ambient temperature. We programmed the logger to simultaneously record ambient and egg temperatures every 2 min. This high temporal resolution enabled us to infer hen behaviors from the temperature data (e.g., beginning of full incubation, active incubation vs. recesses). We analyzed incubation behavior during the 24 h before capture and the 24 h after release for each time that a hen was captured. We used this short time window to investigate the short-term relationships among hormones and behavior because both hormone concentrations and behavior can rapidly respond to perturbations, and return to normal levels relatively quickly.

We used Rhythm/RAVEN software (Cooper and Mills, 2005) to analyze the temperature data to determine when the hen was on and off of the nest each day. We determined that the hen was off of the nest when there was a temperature drop of at least 2°C that lasted for at least 13 minutes (Manlove and Hepp, 2000), or if there was a temperature drop of <2°C but the drop followed a clear daily pattern of off-bouts for that hen. From these data, we calculated the total number of hours that each hen was on the nest (i.e., incubation constancy) during the 24-hour periods surrounding capture.

Incubation temperature

To monitor incubation temperature, we used six free-moving artificial eggs containing data loggers as described in (Hope et al., 2018a). These artificial eggs mimic the cooling and warming properties of real eggs and were calibrated before using (methods fully described in Hope et al., 2018a). Temperature data from these six free-moving eggs, rather than those from the centrally located stationary logger, were used to calculate incubation temperature because they more accurately represented the temperature that real eggs experience as they are rotated by hens throughout the course of incubation (Hope et al., 2018a). We calculated the average incubation temperature for each egg, and then calculated the average of these values to obtain an overall average incubation temperature. We calculated this for each 24-hour period for which we had incubation behavior data.

Hormone assays

To determine the concentrations of prolactin and corticosterone in the blood samples, we used radioimmunoassays. The corticosterone assay was conducted at Virginia Tech, using the protocol of DuRant et al. (2010) and the B3-163 anti-corticosterone antibody (Esoterix Endocrinology). Mean plasma volumes (±SD) used to measure baseline and stress-induced hormone concentrations, respectively, were 48.2 ± 6.1 µl and 20.0 ± 0.4 µl. All samples were run in one assay and samples were run singly to increase the probability of detection. We corrected for the individual extraction efficiency of each sample in the final calculations. The mean extraction efficiency was 72.5%. Intra-assay variation was 6.5% and the assay limit of detection was ~1 ng/ml (varied based on plasma volume and extraction efficiency of each sample). The prolactin assay was conducted at the Centre d'Etudes Biologiques de Chizé, using the protocol of (Cherel et al., 1994) and the antibody against chicken prolactin (supplied by Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). All samples were run in one assay and samples were run in duplicate, with 25µl of plasma for each duplicate. The intra-assay variation was 9.1% and the limit of detection was 0.43 ng/ml.

Statistical analyses

All statistical analyses were conducted in R v. 3.3.1 using the *lme4* package. We assured that all models met the assumptions of normal and homoscedastic residuals by examining fitted vs. residuals plots, normal quantile plots, and histograms of residuals. We reduced all models using stepwise backward elimination of non-significant terms. If not otherwise specified, we defined significance as $p < 0.05$. When two models contained the same response variable and thus were not independent, we used Bonferroni corrections to adjust p-values, which are noted below. We report p-values using type III Wald chi-square tests (*Anova*; *car* package) for each model. We only report terms that remained in the models, however results from all full and reduced models are reported in the Appendix. For all analyses, bird ID was included as a random effect to account for multiple observations from each individual.

In our analyses, we use baseline and stress-induced hormone concentrations as variables, not the magnitude of change between these concentrations. In regard to corticosterone, the difference or percentage change in hormone concentrations is typically of little biological relevance because baseline and stress-induced concentrations of corticosterone bind to different receptors (type 1 and type 2), and thus are not completely comparable (Romero, 2004). In regard to prolactin, there is evidence that behavioral changes associated with prolactin may only be observed after crossing a certain threshold (Angelier et al., 2016; Boos et al., 2007; Spée et al., 2010). If this is the case, an individual that has crossed this threshold and one that has not could both have the same value for the 'change in prolactin'. Thus, for our study, examining baseline and stress-induced concentrations separately is most biologically relevant.

To determine whether prolactin or corticosterone varied throughout the incubation period or in response to a nest disturbance, we constructed two linear mixed-effects models. Prolactin and corticosterone were the dependent variables for the two models and both were log-transformed to meet model assumptions. For both models, stage of incubation (early or late), bleed (baseline or stress-induced), and their interaction were included as categorical predictors, bird ID was included as a random factor, and clutch size, day of year, time of day, and body condition (residuals of mass vs. tarsus linear regression) were included as continuous covariates in initial models. Stage of incubation, bleed, and time of day were retained in the final model structure for the model investigating prolactin. Bleed and hen body condition were retained in the final model structure for the model investigating corticosterone.

To determine if incubation constancy (hours spent on the nest during a 24 h period) varied throughout the incubation period or as a result of a nest disturbance, we constructed one linear mixed-effects model. Incubation constancy was the dependent variable and was cube-transformed to meet model assumptions. The stage of incubation (early or late), the time period with respect to the day of capture (either 24 h before capture or 24 h after release), and their interaction were included as categorical predictors. Bird ID was included as a random factor. Clutch size, day of year, time of day, and body condition were included as continuous covariates in the initial model. Stage of incubation, the time period with respect to the day of capture, and time of day were retained in the final model structure.

To investigate whether there were individual relationships among baseline/stress-induced prolactin or corticosterone and incubation constancy (time spent on the nest) during either the 24 h before capture or the 24 h after release, we constructed four linear mixed-effects models with bird ID as the random factor. For the first two models, incubation constancy during the 24 h before capture was the dependent variable. Since these two models used the same response variable, significance was defined as $p < 0.025$. For the second two models, incubation constancy during the 24 h after release was the dependent variable and was cube-transformed to meet the model assumptions. Similarly, because these two models used the same response variable, significance was defined as $p < 0.025$. For one model of each set, baseline prolactin and baseline corticosterone were included as independent variables and in the other, stress-induced prolactin and stress-induced corticosterone were included as independent variables. To avoid issues of autocorrelation, baseline and stress-induced concentrations of each hormone were not included in the same models because they were correlated with each other (prolactin: r(spearman) = 0.75, $p < 0.001$; corticosterone: r(spearman): 0.34, $p = 0.005$). For the same reason, body condition and stage of incubation (early or late) were not included in these models because they were significantly related to corticosterone and prolactin, respectively (see Results). For all models, clutch size, day of year, and time of capture were included as initial covariates. The terms that were retained in each of these four models are reported in Tables A4-A7.

Lastly, we investigated whether hormone concentrations were related to incubation temperature by constructing four linear mixed-effects models with bird ID as the random factor. Two models included incubation temperature during the 24 h before capture as the dependent variable and the other two models included incubation temperature during the 24 h after release as the dependent variable. Incubation temperature during the 24 h after release was cube-transformed to meet model assumptions. Because the same response variable was used for two models, significance was defined as $p < 0.025$ for these models. One model in each set contained baseline prolactin and corticosterone concentrations as predictors, and the other model contained stress-induced prolactin and corticosterone as predictors. All models contained average ambient temperature during the 24 h period and clutch size as initial covariates. To avoid issues of autocorrelation, body condition and stage of incubation (early or late) were not included in these models. All incubation temperatures were calculated from the free-moving temperature loggers. The terms that were retained in each of these four models are reported in Tables A8-A11.

RESULTS

Factors related to hormone concentrations

Plasma prolactin concentrations were affected by capture/handling stress and differed with the stage of incubation (early or late). Specifically, prolactin concentrations were lower after 30 min of capture and handling stress than they were at baseline ($X^2 = 41.2$, $p < 0.001$; $N_{individuals} = 35$, $N_{observations} = 124$; Fig. 1A; Table A1). In addition, prolactin concentrations were greater later during the incubation period compared to earlier ($X^2 = 36.0$, $p < 0.001$; Fig. 1A; Table A1). The time of day of capture also explained some of the variation in prolactin, where prolactin concentrations were slightly higher later in the day ($X^2 = 3.86$, $p = 0.0496$; Table A1).

In contrast to prolactin concentrations, corticosterone concentrations remained similar throughout early and late incubation (Fig. 1C; Table A2), but were related to hen body condition and were affected by handling. Body condition was negatively related to both baseline and stress-induced corticosterone concentrations ($X^2 = 10.9$, $p < 0.001$; Table A2). Further, as expected, corticosterone concentrations were higher after 30 min of capture/handling than they were at baseline ($X^2 = 940$, $p < 0.001$; $N_{individuals} = 37$, $N_{observations} = 132$; Fig. 1C; Table A2).

Factors related to incubation constancy

Incubation constancy was affected by capture/handling stress and differed with the stage of incubation. On average, hens spent 27% less time on the nest during the 24 h after release than the 24 h before capture ($X^2 = 103$, $p < 0.001$; $N_{individuals} = 35$, $N_{observations} = 128$; Fig. 1B; Table A3) and spent more time on the nest later during the incubation period compared to earlier ($X^2 = 20.9$, $p < 0.001$; Fig. 1B; Table A3). Further, the time of day of capture also explained some of the variation in incubation constancy. The total time that birds spent on the nest during the 48 h of our study was slightly greater for birds that were captured later in the day compared to those captured earlier in the day ($X^2 = 5.40$, $p = 0.020$; Table A3).

Relationships among hormones and behavior

Stress-induced prolactin was related to incubation constancy after the disturbance, but there were no other relationships among hormones and behavior. Stress-induced prolactin was positively related to the amount of time that hens spent incubating during the 24 h after release ($X^2 = 6.18$, $p = 0.013$; $N_{\text{individuals}} = 35$, $N_{\text{observations}} = 63$; Fig. 2; Table A7). However, baseline prolactin was not related to incubation constancy during the 24 h after release (Table A6), and neither stress-induced nor baseline prolactin concentrations were related to incubation behavior before capture (Tables A4-5). Corticosterone concentrations were not related to incubation constancy (Tables A4-7). Further, prolactin and corticosterone were not correlated either at baseline (r(spearman) = 0.05, $p = 0.7$) or stress-induced concentrations (r(spearman) = -0.06, $p = 0.6$).

Relationships among hormones and incubation temperature

Prolactin concentrations were related to incubation temperature during the 24 h after release, but not before capture. During the 24 h after release, average incubation temperature was positively related to both baseline ($X^2 = 7.94$, $p = 0.005$; Fig. 3A; $N_{observations} = 56$; $N_{individuals} = 31$; Table A10) and stress-induced (X^2 5.49, $p = 0.019$; Fig. 3B; $N_{observations} = 58$; $N_{individuals} = 32$; Table A11) prolactin concentrations. However, during the 24 h before capture, there were no relationships between incubation temperature and prolactin concentrations (Tables A8-9). Further, corticosterone was not related to incubation temperature (Tables A8-11). Ambient temperature was positively related to incubation temperature both before capture and after release (all $p < 0.01$; Tables A8-11; Fig. 3). Clutch size was negatively related to incubation temperature before capture ($p < 0.001$; Tables A8-9), but not after release (Tables A10-11).

DISCUSSION

In this study, we found evidence that inter-individual variation in prolactin concentrations could be important for the regulation of incubation behavior and egg temperature after a disturbance. Although prolactin is classically known as the parental care hormone (Buntin, 1996; Hall, 1986; Sockman et al., 2006; Vleck, 1998), surprisingly little is known about how it is related to parental decisions in a life-history context (Angelier et al., 2016). We found that both prolactin concentrations and incubation constancy increased as the incubation period progressed, but decreased after a nest disturbance. Further, incubating wood ducks with higher concentrations of stress-induced prolactin spent more time on the nest incubating during the 24 h after a nest disturbance (capture) than those with lower concentrations. Additionally, both baseline and stress-induced prolactin concentrations were positively related to egg incubation temperature during the 24 h after disturbance, but not during the 24 h before. In contrast, corticosterone concentrations were not related to incubation behavior or temperature in any case. By revealing a possible role of prolactin in governing commitment to incubation after a stressor, this study improves our understanding of the proximate mechanisms that underlie how animals make parental decisions when faced with disturbances during reproduction.

The relationships that we observed between prolactin, incubation behavior, and incubation temperature provide insight into how this hormone may be related to the regulation of incubation. First, we only found relationships among prolactin and incubation constancy and egg temperature during the 24 h after a disturbance, not before. Before the disturbance, there was little variation in incubation constancy among individuals (Fig. 1B). Thus it may be expected, statistically, that there would not be a significant relationship between these small behavioral differences and hormone concentrations. However, we found that incubation constancy was much more variable among individuals following a disturbance. Moreover, the concentration of prolactin that a parent was able to maintain in the face of a disturbance was positively associated with both the time spent incubating and egg temperatures after that disturbance. This suggests that stress-induced prolactin concentrations may mediate parental behavior decisions when faced with environmental disturbances, which then influence egg temperatures. In contrast, baseline prolactin concentrations were not related to incubation constancy, but they were positively related to egg temperatures after the disturbance. It is possible that baseline prolactin could indicate the parent's ability to transfer heat (e.g., regulation of vascularization of the brood patch; Clapp et al., 2012; Jones, 1971) once they resume incubation, rather than the time spent incubating, after the disturbance. Together, these results provide evidence that small differences in prolactin concentrations among individuals can be an indicator of parental investment after being faced with a disturbance.

Our results involving corticosterone also shed light on the relationships between corticosterone, prolactin, and parental care in this species. In contrast to prolactin, corticosterone concentrations were not related to parental behavior. Thus, we did not find support for either of our alternative hypotheses concerning corticosterone, which suggests that corticosterone neither mediates upregulation nor downregulation of incubation constancy in this species. Further, we found no relationship between prolactin and corticosterone. This is surprising in light of studies that show that experimentally increased corticosterone concentrations within the physiological range result in decreased prolactin concentrations, which then lead to decreased parental care behavior (e.g., Angelier et al., 2009). However, a review of the literature shows that the relationship between prolactin and corticosterone is complex and context- and species-dependent (reviewed in Angelier et al., 2013), and it is possible that these two hormones mediate different aspects of the behavioral and physiological stress response in some cases (Angelier and Chastel, 2009). Indeed, we found a negative relationship between corticosterone concentrations and female body condition, suggesting that a primary role of corticosterone during wood duck parental incubation behavior may be to physiologically regulate energy use. Altogether, our results suggest that, when wood ducks face disturbances during incubation, prolactin mediates the parental care response (including behavior and heat-transfer) while corticosterone mediates the allocation of energy reserves throughout the entire body (Angelier and Chastel, 2009).

It is possible that the relationship that we found between stress-induced prolactin and incubation constancy was influenced by either the stage of incubation or the presence of visual or tactile stimuli from the eggs. Both prolactin and the amount of time that the hen spent incubating increased as the incubation period progressed and cannot be disentangled in our study. Thus, the relationship that we found between incubation constancy and stress-induced prolactin concentrations after the disturbance may have been driven by incubation stage. However, if this relationship was solely driven by the relationship with incubation stage, we would have also expected to find relationships between stress-induced prolactin and incubation constancy before the disturbance, and between baseline prolactin and incubation constancy both before and after the disturbance, none of which were the case. Additionally, classic studies show that prolactin is positively related to stimuli provided by visual cues and tactile contact with nests and eggs (Book et al., 1991; Hall, 1987, 1986; Leboucher et al., 1993; Sharp et al., 1998; Silver, 1984).

Thus, it is possible that a decrease in prolactin concentrations in response to our standard experimental capture and restraint protocol may be, in part, a result of a lack of nest-related stimuli. However, it is unlikely that this is the sole explanation for a capture-related decrease in prolactin because there is evidence that stressors themselves influence prolactin concentrations (Angelier et al., 2016, 2015; Angelier and Chastel, 2009; Delehanty et al., 1997; Riechert et al., 2014). Further, if visual cues and tactile contact with eggs were driving hormone concentrations, we may have expected to find a positive relationship between clutch size and prolactin concentrations. However, clutch size was not a significant covariate in any of our models investigating hormone concentrations or incubation behavior. Nevertheless, our results call for future studies on incubation behavior where prolactin is manipulated to decouple the potential influence of sensory and breeding stage factors from prolactin concentrations.

Our study demonstrates that the behaviors of incubating parents after being faced with a disturbance are related to prolactin concentrations. From a proximate perspective, this suggests that the plasma concentration of prolactin that an individual maintains after a stressor plays a role in regulating incubation constancy after a disturbance, and that both baseline and stress-induced prolactin concentrations may help regulate heat transfer after an individual resumes incubation after being disturbed. However, the ultimate reason for why prolactin concentrations, incubation behavior, and temperature vary among individuals remains unknown. Because decreased incubation constancy and incubation temperature can have negative fitness consequences for offspring, the baseline and stress-induced prolactin concentrations and the related behavioral and energetic responses should be under selective pressure. Thus, the finding that individuals have different physiological and behavioral responses to the same disturbance, some of which may be disadvantageous for offspring development, suggests that individuals face other constraints or trade-offs. Future studies could further investigate the role of prolactin concentrations in a life history context by manipulating prolactin and determining if plasma concentrations are related to incubation constancy following repeated disturbance, probability of nest abandonment, thermal conditions of eggs over the entire incubation period, and offspring quality. Because disturbances that affect animals are becoming more common in the current era of rapid global change (e.g., urbanization, extreme weather events), it is important to fully understand how parents respond physiologically and behaviorally to disturbances.

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FIGURE LEGENDS

Figure 1. Prolactin concentrations (A) of incubating wood duck hens decrease after 30 min of capture stress (open = baseline; closed = stress-induced) and are higher during late rather than early incubation. The amount of time that a hen spends incubating (B) is higher during the 24 h before capture than during the 24 h after release (open = before; closed = after), and hens spend more time on the nest during late compared to early incubation. Corticosterone concentrations (C) increase after 30 min of capture stress (open = baseline; closed = stress-induced), but are similar during early and late incubation. Large points are mean ± SE. Prolactin and corticosterone were log-transformed and incubation constancy was cube-transformed for statistical tests, but raw values are plotted for clarity.

Figure 2. Stress-induced prolactin concentrations are positively related to the amount of time that wood duck hens spend incubating during the 24 h following capture. Hens were captured both during early (gray points) and late (black points) incubation. The shaded areas show SE. Time spent incubating was cube-transformed for statistical tests, but raw values are plotted for clarity.

Figure 3. Average incubation temperature during the 24 h following capture is positively related to both (A) baseline prolactin concentrations and (B) stress-induced prolactin concentrations, while taking ambient temperature into account. Hens were captured both during early (gray points) and late (black points) incubation. Incubation temperature was cube-transformed for statistical tests, but raw values are plotted for clarity. Planes were generated using predicted values from simple linear models, but data were analyzed using linear mixed effects models.

FIGURES

Figure 1

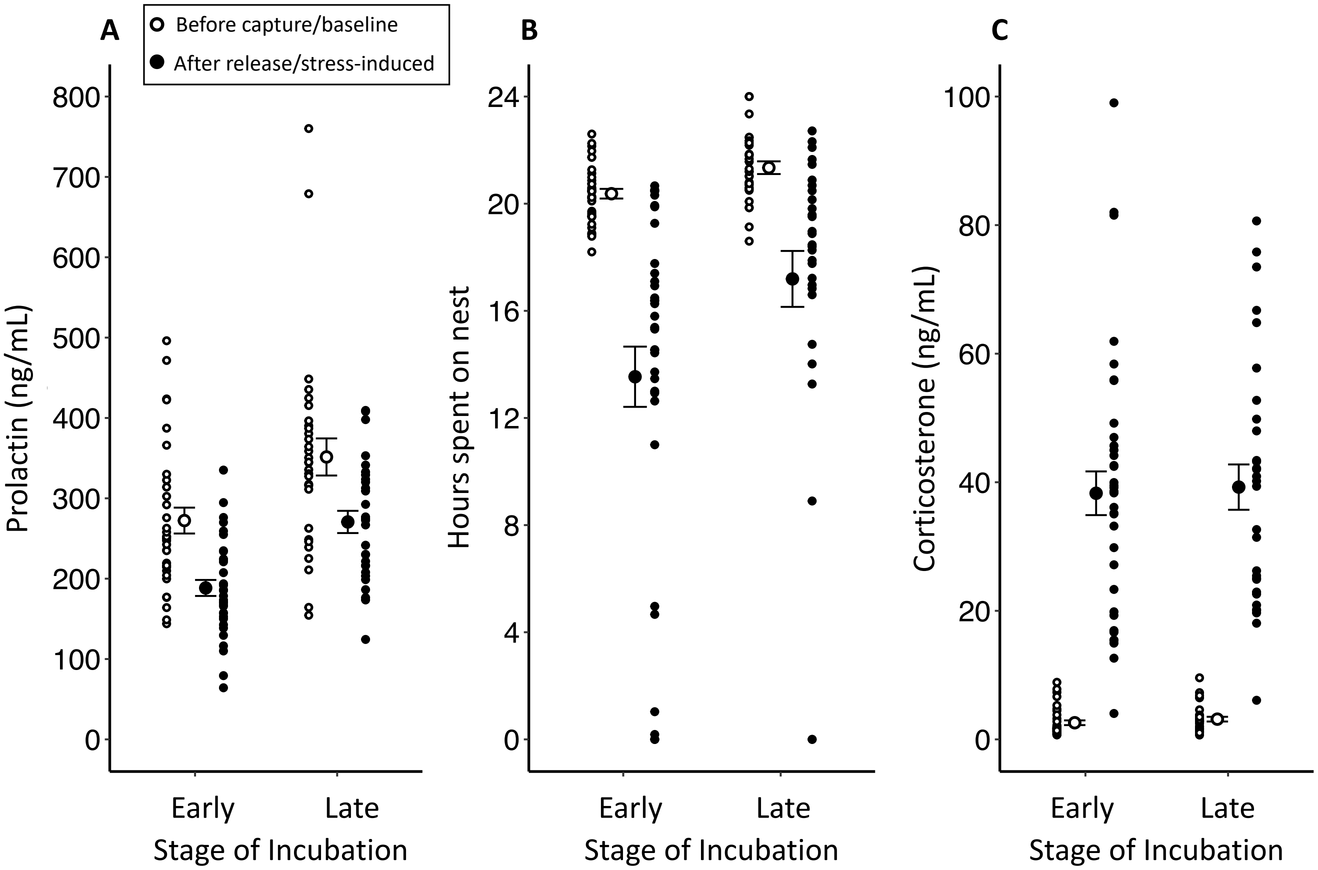


Figure 2

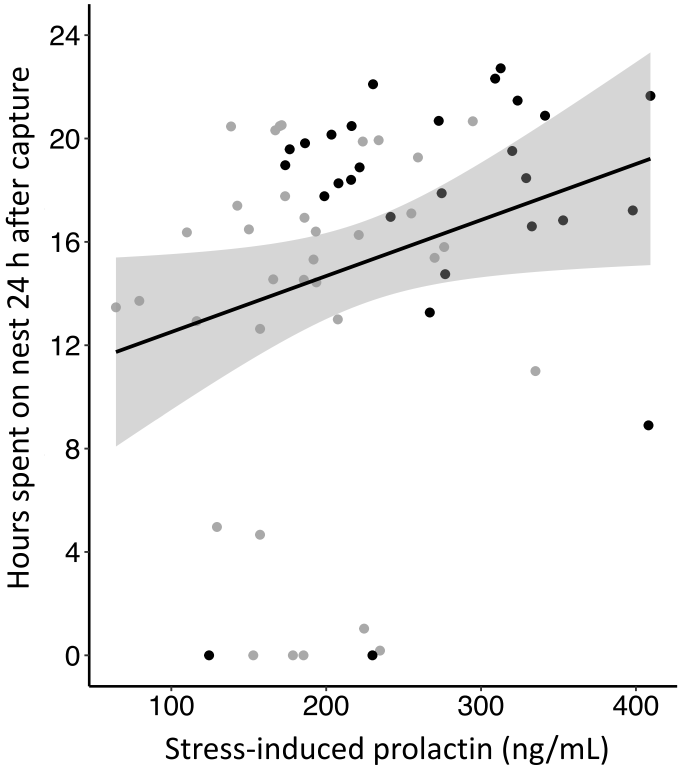
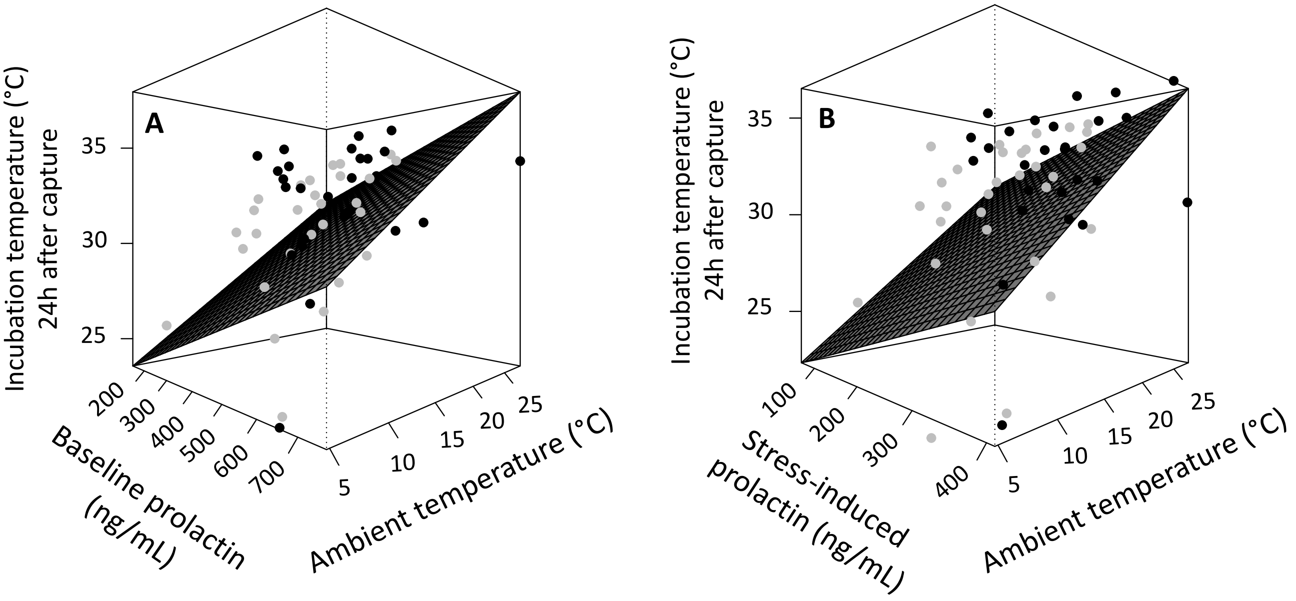

Hours spent on nest 24 h after capture
24
20
16
12
8
4
0
100
200
300
400
Stress-induced prolactin (ng/mL)

Figure 3


A
Incubation temperature (°C)
24h after capture
25
30
35
200
300
400
500
600
700
Baseline prolactin
(ng/mL)
5
10
15
20
25
Ambient temperature (°C)
B
Incubation temperature (°C)
24h after capture
25
30
35
100
200
300
400
Stress-induced
prolactin (ng/mL)
5
10
15
20
25
Ambient temperature (°C)

APPENDIX A: Supplementary tables

Table A1. Results from full and reduced linear mixed effects models investigating which factors are related to prolactin concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.05$.

Response: prolactin (log-transformed)			
Full Model $N_{observations} = 124$; $N_{individuals} = 35$; $R^2 = 0.68$			
Term	**X^2**	**df**	**P**
Clutch size	0.19	1	0.66
Incubation stage (early or late)	5.84	1	0.016
Bleed (baseline or stress-induced)	32.6	1	<0.001
Time of day	4.10	1	0.043
Day of year	2.78	1	0.10
Hen body condition	0.43	1	0.51
Incubation stage X Bleed	1.91	1	0.17
Reduced model $N_{observations} = 124$; $N_{individuals} = 35$; $R^2 = 0.65$			
Incubation stage (early or late)	36.0	1	<0.001
Bleed (baseline or stress-induced)	41.2	1	<0.001
Time of day	3.86	1	0.0496

Table A2. Results from full and reduced linear mixed effects models investigating which factors are related to corticosterone concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.05$.

Response: corticosterone (log-transformed)			
Full Model $N_{observations} = 126$; $N_{individuals} = 35$; $R^2 = 0.92$			
Term	**X^2**	**df**	**P**
Clutch size	0.29	1	0.59
Incubation stage (early or late)	0.12	1	0.73
Bleed (baseline or stress-induced)	572	1	<0.001
Time of day	1.10	1	0.29
Day of year	1.90	1	0.17
Hen body condition	7.79	1	0.005
Incubation stage X Bleed	1.77		0.18
Reduced model $N_{observations} = 132$; $N_{individuals} = 37$; $R^2 = 0.91$			
Bleed (baseline or stress-induced)	940	1	<0.001
Hen body condition	10.9	1	<0.001

Table A3. Results from full and reduced linear mixed effects models investigating which factors are related to incubation behavior before and after acute capture stress in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Incubation behavior was calculated as the time spent incubating during either the 24 hours before capture or the 24 hours after release. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.05$.

Response: hours spent on nest (cube-transformed)			
Full Model $N_{observations} = 128$; $N_{individuals} = 35$; $R^2 = 0.59$			
Term	**X^2**	**df**	**P**
Clutch size	0.40	1	0.53
Incubation stage (early or late)	15.2	1	<0.001
Time period (before or after capture)	71.2	1	<0.001
Time of day	4.53	1	0.034
Day of year	0.07	1	0.79
Hen body condition	0.008	1	0.98
Incubation stage X Time period	1.77	1	0.18
Reduced model $N_{observations} = 128$; $N_{individuals} = 35$; $R^2 = 0.58$			
Incubation stage (early or late)	20.9	1	<0.001
Bleed (baseline or stress-induced)	103	1	<0.001
Time of day	5.40	1	0.020

Table A4. Results from full and reduced linear mixed effects models investigating whether incubation behavior (i.e., time spent on the nest) during the 24 hours before capture stress was related to baseline prolactin and/or corticosterone concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A4 and A5.

Response: hours spent on nest during 24 hours before capture			
Full Model $N_{observations} = 60$; $N_{individuals} = 34$; $R^2 = 0.13$			
Term	**X^2**	**df**	**P**
Baseline prolactin	0.24	1	0.63
Baseline corticosterone	0.12	1	0.73
Clutch size	0.56	1	0.46
Day of year	3.34	1	0.07
Time of day	2.60	1	0.11
Reduced model $N_{observations} = 64$; $N_{individuals} = 35$			
No significant terms in final model			

Table A5. Results from full and reduced linear mixed effects models investigating whether incubation behavior (i.e., time spent on the nest) during the 24 hours before capture stress was related to stress-induced prolactin and/or corticosterone concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A4 and A5.

Response: hours spent on nest during 24 hours before capture			
Full Model $N_{observations} = 63$; $N_{individuals} = 34$; $R^2 = 0.47$			
Term	**X^2**	**df**	**P**
Stress-induced prolactin	2.60	1	0.11
Stress-induced corticosterone	0.03	1	0.87
Clutch size	0.74	1	0.39
Day of year	3.29	1	0.07
Time of day	3.59	1	0.06
Reduced model $N_{observations} = 64$; $N_{individuals} = 35$			
No significant terms in final model			

Table A6. Results from full and reduced linear mixed effects models investigating whether incubation behavior (i.e., time spent on the nest) during the 24 hours after capture stress was related to baseline prolactin and/or corticosterone concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A6 and A7.

Response: hours spent on nest during 24 hours after release (cube-transformed)			
Full Model $N_{observations} = 60$; $N_{individuals} = 34$; $R^2 = 0.15$			
Term	**X^2**	**df**	**P**
Baseline prolactin	1.73	1	0.19
Baseline corticosterone	0.50	1	0.48
Clutch size	0.03	1	0.86
Day of year	0.14	1	0.71
Time of day	2.21	1	0.14
Reduced model $N_{observations} = 61$; $N_{individuals} = 35$			
No significant terms in final model			

Table A7. Results from full and reduced linear mixed effects models investigating whether incubation behavior (i.e., time spent on the nest) during the 24 hours after capture stress was related to stress-induced prolactin and/or corticosterone concentrations in incubating wood ducks. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A6 and A7.

Response: hours spent on nest during 24 hours after release (cube-transformed)			
Full Model $N_{observations} = 63$; $N_{individuals} = 35$; $R^2 = 0.22$			
Term	$\mathbf{X^2}$	**df**	**P**
Stress-induced prolactin	5.57	1	0.018
Stress-induced corticosterone	0.04	1	0.85
Clutch size	0.16	1	0.69
Day of year	0.03	1	0.87
Time of day	1.31	1	0.25
Reduced model $N_{observations} = 63$; $N_{individuals} = 35$; $R^2 = 0.09$			
Stress-induced prolactin	6.18	1	0.013

Table A8. Results from full and reduced linear mixed effects models investigating whether baseline prolactin or corticosterone concentrations were related to average incubation temperature in incubating wood ducks during the 24 h before a nest disturbance (capture). Incubation temperature was calculated from 6 free-moving artificial egg temperature loggers in each nest. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A8 and A9.

Response: average incubation temperature 24 hours before capture			
Full Model $N_{observations} = 55$; $N_{individuals} = 31$; $R^2 = 0.89$			
Term	**X^2**	**df**	**P**
Baseline prolactin	1.39	1	0.24
Baseline corticosterone	0.28	1	0.60
Ambient temperature 24 hours before capture	17.8	1	<0.001
Clutch size	13.5	1	<0.001
Reduced model $N_{observations} = 59$; $N_{individuals} = 32$; $R^2 = 0.89$			
Ambient temperature 24 hours before capture	25.2	1	<0.001
Clutch size	14.2	1	<0.001

Table A9. Results from full and reduced linear mixed effects models investigating whether stress-induced prolactin or corticosterone concentrations were related to average incubation temperature in incubating wood ducks during the 24 h before a nest disturbance (capture). Incubation temperature was calculated from 6 free-moving artificial egg temperature loggers in each nest. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A8 and A9.

Response: average incubation temperature 24 hours before capture			
Full Model $N_{observations} = 58$; $N_{individuals} = 32$; $R^2 = 0.90$			
Term	**X^2**	**df**	**P**
Stress-induced prolactin	1.22	1	0.27
Stress-induced corticosterone	0.02	1	0.90
Ambient temperature 24 hours before capture	17.2	1	<0.001
Clutch size	13.9	1	<0.001
Reduced model $N_{observations} = 59$; $N_{individuals} = 32$; $R^2 = 0.89$			
Ambient temperature 24 hours before capture	25.2	1	<0.001
Clutch size	14.2	1	<0.001

Table A10. Results from full and reduced linear mixed effects models investigating whether baseline prolactin or corticosterone concentrations were related to average incubation temperature in incubating wood ducks during the 24 h after a nest disturbance (capture). Incubation temperature was calculated from 6 free-moving artificial egg temperature loggers in each nest, and was cube-transformed to meet model assumptions. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A10 and A11.

Response: average incubation temperature 24 hours after release (cube-transformed)			
Full Model $N_{observations} = 55$; $N_{individuals} = 31$; $R^2 = 0.74$			
Term	**X^2**	**df**	**P**
Baseline prolactin	7.86	1	0.005
Baseline corticosterone	0.17	1	0.68
Ambient temperature 24 hours after capture	6.16	1	0.013
Clutch size	1.66	1	0.20
Reduced model $N_{observations} = 56$; $N_{individuals} = 31$; $R^2 = 0.74$			
Baseline prolactin	7.94	1	0.005
Ambient temperature 24 hours after capture	7.71	1	0.005

Table A11. Results from full and reduced linear mixed effects models investigating whether stress-induced prolactin or corticosterone concentrations were related to average incubation temperature in incubating wood ducks during the 24 h after a nest disturbance (capture). Incubation temperature was calculated from 6 free-moving artificial egg temperature loggers in each nest, and was cube-transformed to meet model assumptions. Birds were captured twice during incubation (early and late), so bird ID was included as a random effect in all models. Analyses were conducted in R v. 3.3.1 using the *lme4* package. P-values are reported using type III Wald chi-square tests, using the *car* package. R^2 values were calculated using the *sjstats* package based on lme-models. The model was reduced using stepwise backward elimination of non-significant terms. Significance was defined as $p < 0.025$, using a Bonferroni correction for the lack of independence among the response variables for the models outlined in Tables A10 and A11.

Response: average incubation temperature 24 hours after release (cube-transformed)			
Full Model $N_{observations} = 58$; $N_{individuals} = 32$; $R^2 = 0.70$			
Term	$\mathbf{X^2}$	**df**	**P**
Stress-induced prolactin	5.53	1	0.019
Stress-induced corticosterone	0.24	1	0.62
Ambient temperature 24 hours after capture	6.17	1	0.013
Clutch size	1.88	1	0.17
Reduced model $N_{observations} = 58$; $N_{individuals} = 32$; $R^2 = 0.72$			
Stress-induced prolactin	5.49	1	0.019
Ambient temperature 24 hours after capture	8.34	1	0.004

CHAPTER 2: Incubation temperature as a constraint on clutch size evolution and cost of brood parasitism

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ABSTRACT

Elucidating factors that limit the number of offspring produced is fundamental to understanding life-history evolution. Here, we test the hypothesis that parental ability to maintain an optimal physical developmental environment for all offspring constrains clutch size via effects on offspring quality. In birds, a <1°C difference in average incubation temperature has diverse effects on fitness-related post-hatching offspring phenotypes, and thus the inability of parents to maintain optimal incubation temperatures could constrain clutch sizes. However, a fundamental question that has not been sufficiently addressed is whether within nest variation in egg temperature varies sufficiently to alter phenotypic expression within a brood. Such variation would lead to differential survival within the clutch, and create a tradeoff between offspring number and quality. To test this, we manipulated clutch size in free-living wood duck nests and measured incubation temperature among and within clutches using multiple temperature loggers. Average incubation temperatures were lower and more variable as clutch size increased. Notably, the range in *average* incubation temperature among eggs *within* nests increased with clutch size and exceeded 1°C in large clutches. This provides empirical evidence to support the hypothesis that suboptimal incubation temperatures limit clutch size through diminishing returns on offspring quality.

Keywords: clutch size evolution, incubation temperature, life-history, wood duck, offspring number vs. quality, brood parasitism

INTRODUCTION

A fundamental goal of the study of life-history evolution is to determine what limits the number of offspring produced in a given reproductive attempt. Some of the earliest considerations of this life history trait focused on avian clutch size evolution (Lack, 1947; Moreau, 1944; Skutch, 1949). This paved the way for decades of avian clutch size research (Ashmole, 1963; Cody, 1966; Jetz et al., 2008; Klomp, 1970; Monaghan and Nager, 1997; Winkler and Walters, 1983), which continues to provide insight into broader life-history evolution today (Boyle et al., 2016; Hořák et al., 2015; Martin, 2014; Ricklefs, 2000). The most prominent hypothesis for the determination of clutch size is the Lack clutch (Lack, 1947), which is the clutch size that produces the maximum number of offspring that parents can successfully feed and care for post-hatch. However, many species have an average clutch size that is smaller than the Lack clutch, and thus produce fewer offspring than they are able to when clutch or brood sizes are experimentally increased (Vander Werf, 1992). Such findings have been interpreted as resulting from tradeoffs between the number and quality of offspring or between current and future reproduction, and largely focus on the costs of post-hatch offspring care or parental condition and survival (Lessells, 1991; Stearns, 1989, 1992). However, there are still species where empirical studies reveal no apparent tradeoffs associated with offspring number (Hare and Murie, 1992; Humphries and Boutin, 2000; Lepage et al., 1998; Orell et al., 1996; Stearns, 1992; Winkler and Walters, 1983), leaving the central life-history question of what limits offspring number in a given reproductive attempt unresolved.

The factors constraining clutch size in precocial birds, especially waterfowl, have historically been a conundrum, and even Lack stated that “the problem in ducks is highly puzzling” (Lack, 1947). Precocial birds do not spend energy feeding their young, and post-hatch parental care generally does not increase with brood size (Lessells, 1987; Seddon and Nudds, 1994). Lack (Lack, 1967) postulated that food availability during egg production limited clutch size in waterfowl, however, egg production is not reliably related to resource availability or stored nutrients (Arnold and Rohwer, 1991; Winkler and Walters, 1983). Incubation was also originally disregarded as a constraint on clutch size for birds because it was thought to be a passive process. However, subsequent research demonstrated that incubation is an active and energetically costly process and may limit clutch size (Monaghan and Nager, 1997; Nord and Williams, 2015; Thomson et al., 1998; Vleck, 1981). Indeed, incubating larger clutch sizes results in greater parental energy expenditure, longer incubation periods, and lower egg hatchability in both altricial and precocial birds (DuRant et al., 2013a; Engstrand and Bryant, 2002; Haftorn and Reinertsen, 1985; Hepp et al., 1990; Larsen et al., 2003; Moreno et al., 1991; Nielsen et al., 2006; Nord and Nilsson, 2012; Reid et al., 2000; Siikamäki, 1995; Wiebe and Martin, 2000). However, in some species, parents successfully incubate experimentally enlarged clutches without any negative consequences to their condition or to hatch success (Rohwer, 1985; Sandercock, 1997; Thomson et al., 1998). In fact, many waterfowl species commonly engage in conspecific brood parasitism, where females readily and successfully incubate extra eggs laid by other females (Lyon and Eadie, 2008; Sorenson, 1992), and hatch success in these cases is generally quite high (Roy Nielsen et al., 2006b; Semel et al., 1988). Thus, constraints on clutch size for precocial species are still not fully understood, and it is often assumed that the costs of brood parasitism for host females are either relatively minor or nonexistent (Lyon and Eadie, 2008; Sorenson, 1992).

The costs of incubation, however, may manifest in ways other than parental energy expenditure or changes in hatch success. An understudied hypothesis is that parental ability to maintain optimal incubation temperatures for the entire clutch constrains the evolution of clutch size by affecting offspring quality and post-hatch survival. Recent studies show that a decrease in average incubation temperature of ~1°C affects post-hatch offspring phenotypes including growth rate, hormone levels, thermoregulation, immune function, locomotor performance, behavior, survival, adult body size, and recruitment, in altricial and precocial species (Berntsen and Bech, 2016; DuRant et al., 2014, 2012b, 2012a, 2010; Eiby and Booth, 2009; Hepp and Kennamer, 2012; Hope et al., 2018b; Hopkins et al., 2011; Nord and Nilsson, 2011; but see Nord and Nilsson, 2016). Because large clutches are more energetically costly for parents to incubate (DuRant et al., 2013a; Haftorn and Reinertsen, 1985; Moreno et al., 1991) and may be more physically difficult for parents to adequately rotate eggs and distribute heat evenly, some or all offspring in a large clutch may be incubated at suboptimal temperatures. This would lead to reduced post-hatch quality and survival, and thus, diminishing returns on any increases in clutch size. Indeed, a few studies have shown that larger clutch sizes lead to lower incubation temperatures (Niizuma et al., 2005; Nord et al., 2010; Nord and Nilsson, 2012; Verboven et al., 2009), although results are mixed (Boulton and Cassey, 2012; Reid et al., 2000). However, because most studies to date measure incubation temperature using a single central stationary temperature logger, almost nothing is known about *within-clutch* variation in temperature. The few studies that have investigated within-clutch variation in egg temperature only measured incubation temperature during a short time period (Beatty, 2015; Boulton and Cassey, 2012;

Niizuma et al., 2005; Reid et al., 2000). Thus, a fundamental question that has not yet been addressed sufficiently is whether average egg temperatures within nests vary enough (i.e., >1°C over the entire course of incubation) to alter phenotypic expression within a brood, leading to differential survival, and ultimately limiting clutch size and being an unrecognized cost of brood parasitism.

To investigate whether parental ability to maintain optimal incubation temperatures for all eggs in a clutch could constrain clutch size and be a cost of brood parasitism in a precocial species, we experimentally manipulated clutch size and installed one stationary and six mobile artificial eggs containing temperature loggers in wood duck (*Aix sponsa*) nests. This approach allowed us to estimate the average incubation temperature for the entire nest and *within-clutch* variation in egg temperatures throughout the entire incubation period. Because clutch size influences the amount of energy required for incubation (DuRant et al., 2013a; Moreno et al., 1991; Reid et al., 2000) and likely influences the ability of the hen to rotate her eggs to attain a consistent temperature throughout the clutch, we predicted that larger clutches would have: 1) lower average incubation temperatures, 2) larger fluctuations in temperature throughout incubation, and 3) greater within-clutch variation in incubation temperature than smaller clutches. Subsequently, because low incubation temperatures lead to longer incubation periods and lower hatch success (Engstrand and Bryant, 2002; Hepp et al., 2006, 2005; Moreno et al., 1991; Reid et al., 2000; Siikamäki, 1995; Wiebe and Martin, 2000), we predicted that larger clutches would have longer incubation periods and lower hatch success than smaller clutches. However, an effect of clutch size on incubation parameters but not on hatch success would provide evidence for a tradeoff between offspring number and post-hatch quality, without an effect on immediate survival.

METHODS

Study species and site

The wood duck is a widely distributed dabbling duck (Hepp and Bellrose, 2013) that nests in tree cavities but readily uses nest boxes (Hepp et al., 1987). Their average clutch size is 12 (Bellrose and Holm, 1994), but clutch sizes can reach >40 eggs in some populations (Morse and Wight, 1969) due to conspecific brood parasitism in populations that nest in both boxes (Semel et al., 1988; Semel and Sherman, 1986) and natural cavities (Roy Nielsen et al., 2006a). Such broad natural variation in clutch size makes wood ducks an excellent species for testing hypotheses about the costs of physically incubating enlarged clutch sizes. Further, hatch success is high in parasitized clutches, and is normally only slightly lower, if at all, than un-parasitized clutches (Roy Nielsen et al., 2006b; Semel et al., 1988).

Female wood ducks are solely responsible for incubation (~ 30-35 d) (Hepp and Bellrose, 2013). Hens engage in partial incubation at night for ~4 d before beginning full incubation. During full incubation, hens incubate both day and night and have high incubation constancy, but usually take one recess in the morning and one in the afternoon to forage (Hepp and Bellrose, 2013; Manlove and Hepp, 2000). However, incubation period, temperature, and behavior vary within and among populations (Bellrose and Holm, 1994; Manlove and Hepp, 2000).

We studied a population of wood ducks breeding in nest boxes on the Department of Energy's Savannah River Site (SRS) in South Carolina (33.1°N, 81.3°W; elevation: 157 m) on a series of 12 ephemeral wetlands. Our study population has an average clutch size of 11.2 eggs (range: 4-16) for non-parasitized nests and 18.6 eggs (range: 6-41) for nests that experience conspecific brood parasitism (R.A. Kennamer, unpubl. data). We checked each nest box every 3-

4 days and once an egg was found, we checked the nest box every day and marked individual eggs for lay-date and -order. All procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Clutch size manipulation and field methods

Once 3-5 eggs were laid, we began manipulating clutch size to achieve both reduced and enlarged clutch sizes. To do this, we added or removed one egg per day, but occasionally added multiple eggs to achieve large clutch sizes. We moved un-incubated eggs among nests of similar age and temporarily stored unused eggs as needed at room temperature for $\leq$4 days to augment nests. Storing un-incubated wood duck eggs in this manner for <10 days does not affect hatchability (Arnold et al., 1987; DuRant et al., 2010; Walls et al., 2011). Final clutch sizes ranged from 11-28 eggs (including seven artificial eggs with loggers; see *Incubation monitoring*) and clutch sizes were equally distributed among wetlands and across the breeding season. Note that this range of clutch sizes includes those that would naturally result both from the egg-laying of a single female, and from that of multiple females (i.e., conspecific brood parasitism).

To determine the body condition of female wood ducks associated with each nest, we captured females in their nest boxes in early incubation (3-8 days) to measure body mass. Body mass was not related to day of capture ($r = 0.17$; $p = 0.40$). We also took photos of the positions of eggs in the nest when we measured the females, and opportunistically any time that we saw a female leave the nest. Finally, once ducklings had fledged, we quantified hatch success by counting the number of eggs that remained unhatched.

Incubation monitoring

We used a novel method to monitor incubation temperature, which is fully described previously (Hope et al., 2018a). Briefly, each nest was equipped with seven artificial eggs containing data loggers. One egg was fixed and stationary while six were mobile (data loggers used: mobile: iButtons©; stationary: Onset® HOBO®). Early in the egg-laying sequence (≤5 eggs), we installed the stationary temperature logger, which contained two probes. One probe was attached to the inside wall of the nest box to record ambient temperature. For each nest, we calculated ambient temperature as the average ambient temperature recorded throughout the incubation period. The other probe was fixed inside of an artificial egg. This egg was secured in the center of the clutch, but attached to a spring, which kept the egg stationary but flexible. When the temperature data from the stationary logger indicated that the hen had started partial incubation at night and thus, would shortly begin full incubation, we replaced six eggs in the nest with our mobile temperature loggers (Fig. B1), which could be freely moved and rotated by the hen. Wood ducks are an ideal species for this type of study because they are known to readily accept and incubate eggs from other hens (brood parasitism), as well as free-moving wooden eggs, even with imperfections and holes (S. F. Hope, R. A. Kennamer, J. J. Hallagan, W. A. Hopkins, pers. observ.)

Photos of the nests taken throughout the incubation period revealed that the mobile temperature loggers were evenly distributed throughout the nests and were accepted by the hens (see also Fig. B2). However, in 3 (out of 26) nests, 1-2 (out of 6) mobile loggers were pushed to the corners of the nest box. Since these eggs were pushed, it shows that hens have the ability to exclude eggs from the clutch. However, because these hens did not push all 6 mobile eggs, pushing occurred either at the middle or end of the incubation period, and the 23 other nests had no mobile eggs pushed, it appears that hens generally accepted the mobile loggers. Excluding these “pushed” eggs from statistical analyses did not change any of our results (Table A4). Because of this, and because all hens accepted the majority or all artificial eggs for at least part of the incubation period, we retained these eggs in our analyses.

Incubation parameters

We determined the incubation period (days) of full incubation by investigating data from the stationary logger, as described previously (Hope et al., 2018a). Then, we used the temperature data from the six mobile loggers to calculate three incubation temperature parameters throughout the entire incubation period. We calculated incubation temperature parameters as described previously (Hope et al., 2018a). The *average incubation temperature* was calculated by first calculating the average incubation temperature for each mobile logger, and then averaging the values of the six mobile loggers (i.e., average of the averages). The *temperature fluctuation* was calculated similarly, but was the average of the standard deviations in temperature of the six mobile loggers (i.e., average of the standard deviations). Lastly, the *range in temperature among eggs* was the range of the average temperatures (highest – lowest) of the six mobile loggers. This measure represents the difference in average incubation temperature that eggs *within* the same nest experience. We used only the mobile loggers for this because their mobility within the nest better mimics the incubation temperature that real eggs experience, compared to the stationary logger (Hope et al., 2018a). We chose to focus on average egg temperatures for our incubation parameters because all studies on the effects of incubation temperature on wood duck duckling phenotype in captivity have used average incubation temperature (including daily temperature drops to simulate hens leaving the nest to forage) as the independent variable (DuRant et al., 2013b and references therein; DuRant et al., 2016; Hope et al., 2018b, 2019).

Statistical analyses

We used R v 3.3.1 (R Core Team, 2016) for all analyses. In this study, we included nests in which at least one duckling hatched ($N = 31$ nests). For our incubation temperature analyses, we included nests in which we were able to install six mobile loggers ($N = 26$ nests). For our incubation period analyses, we included nests in which we were able to accurately determine incubation start date ($N = 26$ nests; different subset). We assured that all data met the assumptions of normal and homoscedastic residuals by examining fitted vs. residuals plots, normal quantile plots, and histograms of residuals. We reduced all models using backward elimination of nonsignificant terms, starting with non-significant interaction terms, and defined significance as $p < 0.05$. We only report terms from the final models, but full and reduced models are reported in the Appendix (Tables A1-A3).

To examine the effect of clutch size on incubation temperature parameters, incubation period, and hatch success, we constructed five models. For all models, the predictors were clutch size, ambient temperature, female body mass, and their interactions. We used multiple linear regressions for the models with incubation temperature parameters and incubation period as dependent variables. The range in temperature among eggs was natural log-transformed to meet model assumptions. We used a generalized linear model with a binomial error distribution to examine hatch success (success = hatched; fail = unhatched). To avoid autocorrelation, we did not include nest initiation date in our models as it was correlated with ambient temperature ($p < 0.0001$).

To determine if incubation temperature parameters were associated with incubation period or hatch success, we individually assessed the relationship of all incubation temperature parameters with incubation period and hatch success using simple linear regressions and generalized linear models (binomial error distribution), respectively.

RESULTS

Incubation temperature

Clutch size and ambient temperature both influenced the average incubation temperature of the clutch. As clutch size increased and as ambient temperature decreased, the average incubation temperature decreased (clutch size: $p = 0.001$; ambient: $p = 0.026$; Table A1; Fig. 1). Therefore, small clutches at high ambient temperatures had the highest average incubation temperatures and large clutches at low ambient temperatures had the lowest average incubation temperatures.

Similar to average incubation temperature, clutch size and ambient temperature influenced the extent to which the incubation temperature of each clutch fluctuated throughout incubation. As clutch size increased and as ambient temperature decreased, the temperature fluctuation (the average of the standard deviations in temperature of the six mobile loggers) increased (clutch size: $p = 0.009$; ambient: $p = 0.016$; Table A1; Fig. 2). Therefore, egg temperatures of small clutches at high ambient temperatures fluctuated the least and egg temperatures of large clutches at low ambient temperatures fluctuated the most.

Clutch size also influenced the range of the average incubation temperatures among eggs within a clutch. As clutch size increased, the temperature range among eggs increased (clutch size: $p < 0.0001$; Table A1; Fig. 3), but ambient temperature was not related to the temperature range among eggs (Table A1). Thus, in large clutches, some eggs experienced much colder average temperatures than other eggs within the same nest (Fig. B1, B2). Using the standard deviation of the average egg temperatures within a nest, instead of the range, produced similar results (clutch size: $p < 0.0001$). By using the regression equation (ln(range among eggs) = -2.42 + 0.17(clutch size)) from the final model (Table A1), we can predict the range in average temperature among eggs for different clutch sizes. For example, when the clutch size is 12, the range among eggs is predicted to be 0.68°C, and when the clutch size is 16, the range among eggs is predicted to be 1.35°C.

Incubation period and hatch success

Clutch size affected the duration of incubation. As clutch size increased, incubation period increased (clutch size: $p = 0.0024$; Table A2; Fig. 4). Incubation period was negatively related to the average incubation temperature ($p = 0.0057$; Table A2) and positively related to both temperature fluctuation ($p = 0.0018$; Table A2) and range among eggs ($p = 0.012$; Table A2).

Hatch success was not related to clutch size, ambient temperature, nor any incubation temperature parameter (Table A3). Therefore, the proportion of eggs that hatched remained constant across clutch sizes. However, hatch success was related to female body mass, with hatch success increasing as female body mass increased (body mass: $p = 0.0002$; Table A3).

DISCUSSION

We demonstrated that clutch size influences incubation temperature among and *within* nests in a precocial species, which reveals a previously underrecognized constraint on the evolution of clutch size and cost of conspecific brood parasitism. We found that larger clutches had lower average incubation temperatures, greater temperature fluctuations and longer incubation periods than smaller clutches. Importantly, we found that the range in average incubation temperature among eggs in the same nest increased with clutch size, to an extent that is sufficient to alter phenotypic expression *within* a brood. Because birds move and rotate their eggs throughout incubation, within-clutch differences in average incubation temperature have previously been largely dismissed. Here, we not only provide evidence that this variation exists, but that it is of the same magnitude that has been previously shown to result in disadvantageous phenotypes (Berntsen and Bech, 2016; DuRant et al., 2014, 2012b, 2012a, 2010; Eiby and Booth, 2009; Hepp and Kennamer, 2012; Hope et al., 2018b; Hopkins et al., 2011; Nord and Nilsson, 2011), and thus may lead to diminishing returns on increases in clutch size. Factors that limit clutch size in precocial species are not fully understood (Winkler and Walters, 1983) and, although many precocial birds engage in conspecific brood parasitism, the potential costs to host females remain largely unstudied (Lyon and Eadie, 2008; Sorenson, 1992). This is the first study to investigate incubation temperature as a key constraint on the evolution of clutch size in a precocial species with large and variable clutch sizes, and also reveals an unrecognized cost of brood parasitism.

The relationships that we found among clutch size and incubation temperature parameters suggest that incubation may impose a tradeoff between number and quality of offspring. We found that large clutches had lower average and more variable incubation temperatures than smaller clutches, although hatch success was not affected by clutch size. Many recent studies have shown that low average incubation temperatures result in offspring phenotypes that are associated with lower post-hatch survival (DuRant et al., 2014, 2012b, 2012a, 2010; Eiby and

Booth, 2009; Hope et al., 2018b; Hopkins et al., 2011; Nord and Nilsson, 2011). Indeed, low incubation temperatures were associated with lower apparent or true post-hatch survival in two species (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; but see Nord and Nilsson, 2016). Further, there is recent evidence that fluctuations in incubation temperature can influence avian offspring phenotype, even when average temperatures are the same (Ben-Ezra and Burness, 2017; but see Carter et al., 2014). Therefore, our results provide evidence for a tradeoff because, although the proportion of ducklings that hatched remained constant across clutch sizes, some or all offspring from large clutches were likely of low post-hatch quality.

Notably, we found differences in average incubation temperatures among eggs *within* the same nest, which, based on prior studies, were large enough to lead to differences in offspring phenotype (i.e., ≥0.9°C; DuRant et al., 2013b). Remarkably, modeling our data (i.e., using the regression equation: ln(range among eggs) = -2.42 + 0.17(clutch size); Table A1; Fig. 3) predicts that at the average clutch size for wood ducks (12 eggs), the range in average incubation temperature among eggs is <0.9°C. However, the range becomes >0.9°C, when clutch size reaches ≥14 eggs. Because we found that large clutches have lower average temperatures and larger ranges in temperature among eggs than small clutches, this >0.9°C difference in large clutches likely results in some eggs being incubated at optimal average temperatures and some being incubated at colder, suboptimal temperatures. Thus, in large clutches, at least some ducklings will likely be of low phenotypic quality and have a lower probability of post-hatch survival. Aside from leading to low quality offspring, temperature differences within nests could also lead to differential offspring survival due to within brood competition or could lead to hatching asynchrony (Hepp et al., 2006), which is maladaptive in waterfowl (Arnold et al., 1987). These consequences could be exacerbated for species such as wood ducks that experience high levels of conspecific brood parasitism, and thus, have large and highly variable clutch sizes. Our study therefore provides evidence that incubation temperature may be a constraint on the evolution of clutch size and a heretofore unrecognized cost of conspecific brood parasitism.

After decades of research on avian clutch size evolution (Ashmole, 1963; Cody, 1966; Jetz et al., 2008; Klomp, 1970; Lack, 1947; Monaghan and Nager, 1997; Moreau, 1944; Skutch, 1949; Winkler and Walters, 1983), we still do not have a complete understanding of what constrains clutch size in precocial species (Arnold and Rohwer, 1991; Winkler and Walters, 1983). Our results provide empirical evidence for the hypothesis that subtle changes in average incubation temperature constrain the evolution of clutch size in precocial birds because parents cannot incubate larger than average clutches such that all eggs are maintained at the optimal average temperature. Our conclusions are based on the many experimental studies that show that low average incubation temperatures lead to suboptimal offspring phenotypes (Berntsen and Bech, 2016; DuRant et al., 2014, 2012b, 2012a, 2010; Eiby and Booth, 2009; Hepp and Kennamer, 2012; Hope et al., 2018b; Hopkins et al., 2011; Nord and Nilsson, 2011). However, incubation temperatures in the field (Fig. B1) are much more variable than those simulated in a laboratory setting, and other aspects of incubation temperature (e.g., minimum, maximum, variance) or different time periods during incubation (e.g., beginning, end) could also be important for determining offspring phenotype (Carter et al., 2018). We encourage future studies to investigate how different aspects of incubation temperature, other than the average, could influence avian offspring phenotype, in order to bridge this knowledge gap between the lab and the field. Nevertheless, our study highlights the importance of parental ability to maintain an optimal physical developmental environment as a constraint on clutch/litter size evolution across taxa. Further, our results shed new light on the drivers of the tradeoff between offspring number and quality and, thus, strengthens our understanding of a fundamental aspect of life-history evolution.

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FIGURE LEGENDS

Figure 1. Manipulated clutch size and ambient temperature influenced the average incubation temperature of wood duck clutches. As clutch size increased and ambient temperature decreased, the average incubation temperature decreased. Each point represents one wood duck nest. All average temperatures were calculated over the entire incubation period.

Figure 2. Manipulated clutch size and ambient temperature influenced the fluctuation in temperature (standard deviation) of wood duck clutches. As clutch size increased and ambient temperature decreased, the temperature fluctuation increased. Each point represents one wood duck nest. All values were calculated over the entire incubation period.

Figure 3. The range among the average temperatures that each mobile egg temperature logger experienced throughout incubation increased with manipulated clutch size. Each point represents one nest. The range among eggs was natural log-transformed to meet model assumptions. Confidence intervals are SE. Note that the average clutch size for non-parasitized nests of this species is 12 eggs, and that phenotypic changes in offspring are well documented at temperature differences of 0.9°C ($\ln(0.9) = -0.1$).

Figure 4. As manipulated clutch size increased, the incubation period (days) increased in wood duck nests. Incubation period is the number of days from incubation start date to hatch date. Confidence intervals are SE. Each point represents one nest.

FIGURES

Figure 1

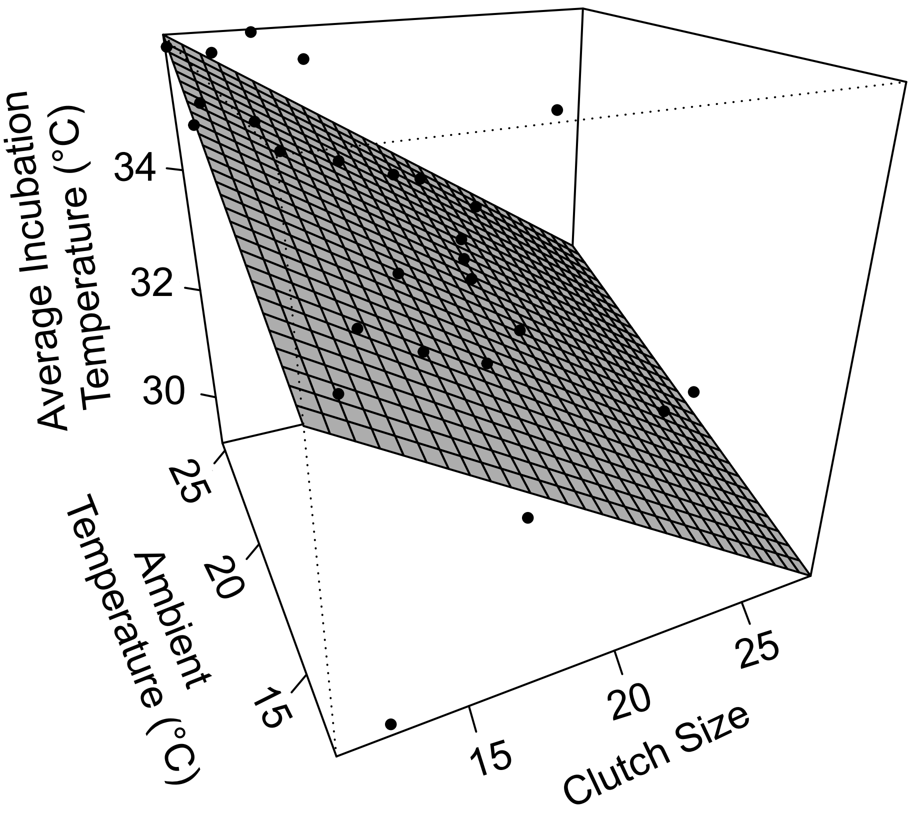


Figure 2

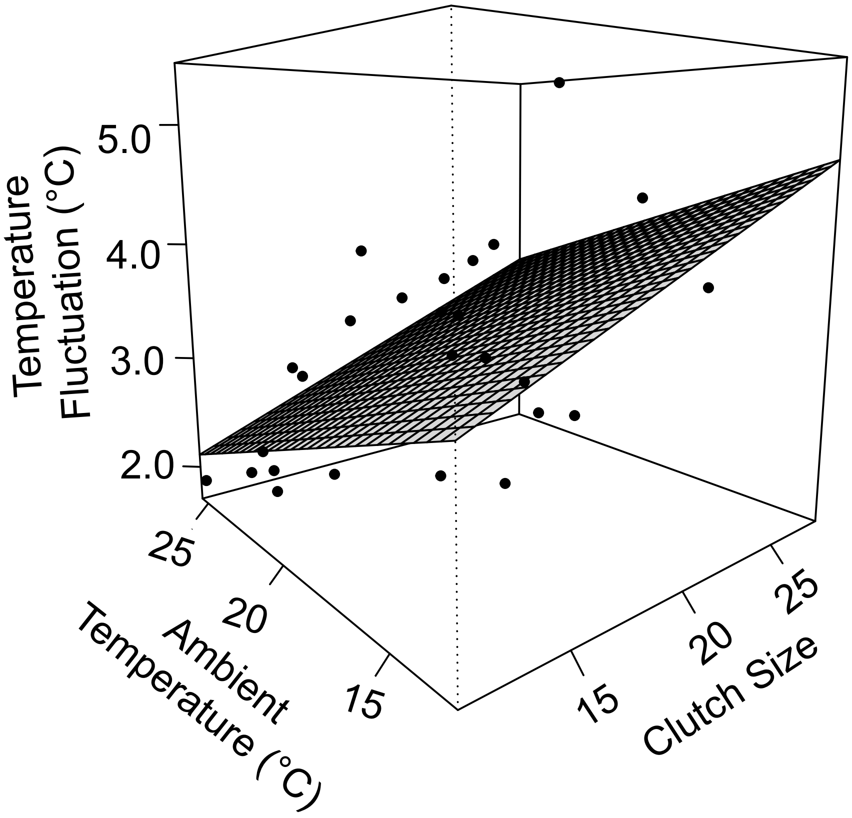

Temperature Fluctuation (°C)
5.0
4.0
3.0
2.0
25
20
15
Ambient Temperature (°C)
15
20
25
Clutch Size

Figure 3

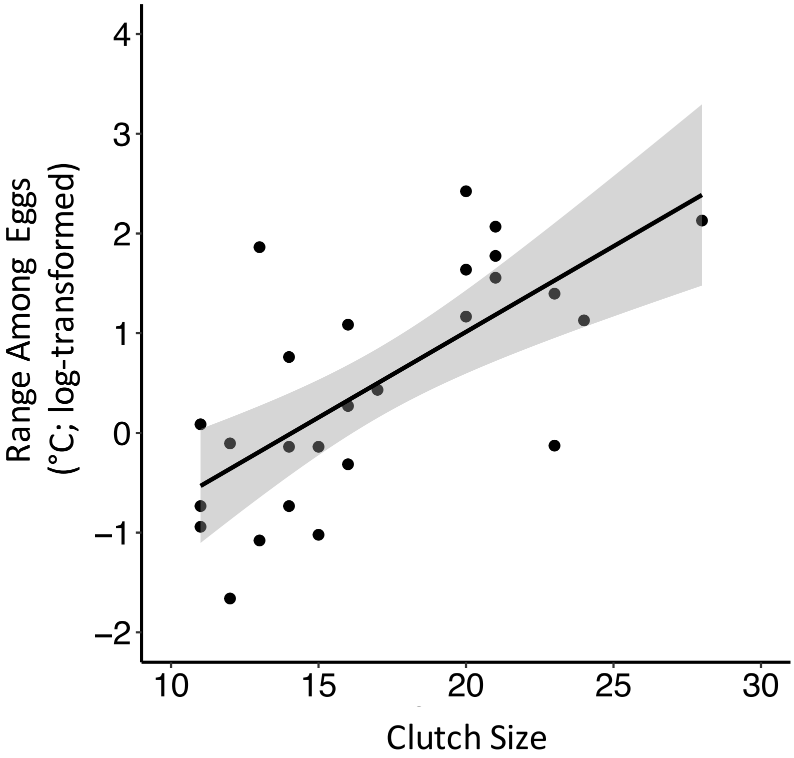
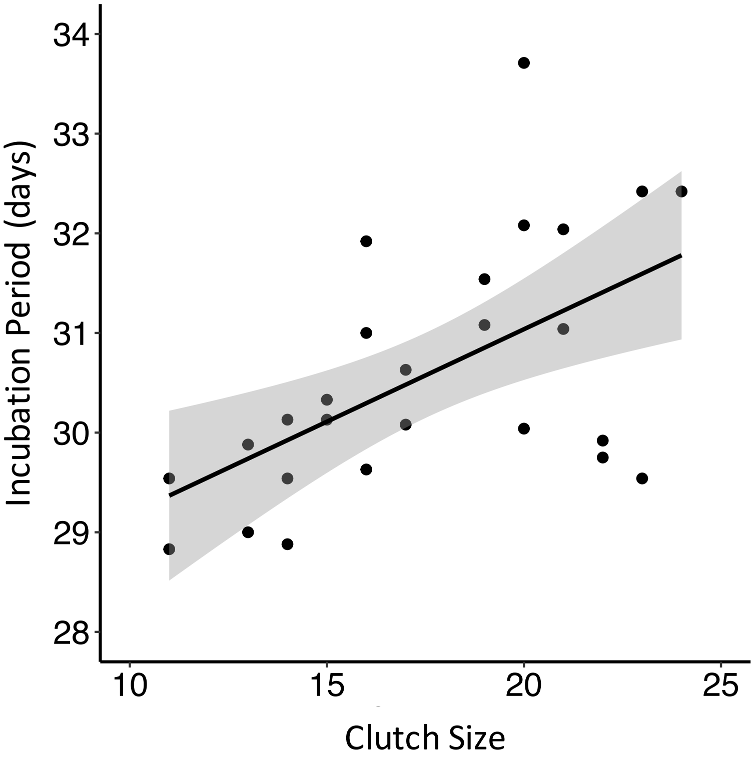

Range Among Eggs
(°C; log-transformed)
4
3
2
1
0
−1
−2
10
15
20
25
30
Clutch Size

Figure 4



34
33
32
31
30
29
28
Incubation Period (days)
10
15
20
25
Clutch Size

APPENIDIX A: Supplementary Tables

Table A1. Incubation Temperature Parameters

Term	β	*P*
Average Temperature full model		
$R^2 = 0.55$, $F_{7, 15} = 2.66$, $p = 0.053$		
Intercept	15.9	0.18
Clutch Size	-9.11	0.31
Ambient Temperature	-7.10	0.35
Mass	-1.19	0.24
Clutch Size x Ambient	1.30	0.40
Clutch Size x Mass	0.21	0.30
Ambient x Mass	0.18	0.32
Clutch x Ambient x Mass	-0.12	0.38
Average Temperature final model		
$R^2 = 0.45$, $F_{2, 23} = 9.27$, $p = 0.0011$		
Intercept	33.8	<0.0001
Clutch Size	-0.26	**0.0014**
Ambient Temperature	0.20	**0.026**
Temperature Fluctuation full model		
$R^2 = 0.47$, $F_{7, 15} = 1.88$, $p = 0.14$		
Intercept	14.8	0.83
Clutch Size	-1.04	0.79
Ambient Temperature	-1.14	0.73
Mass	-0.02	0.90
Clutch Size x Ambient	0.08	0.68
Clutch Size x Mass	0.002	0.80
Ambient x Mass	0.002	0.78
Clutch x Ambient x Mass	<0.001	0.71
Temperature Fluctuation final model		
$R^2 = 0.39$, $F_{2, 23} = 7.25$, $p = 0.0037$		
Intercept	3.51	0.00052
Clutch Size	0.08	**0.0094**
Ambient Temperature	-0.09	**0.016**
Range Among Eggs full model		
$R^2 = 0.54$, $F_{7, 15} = 2.50$, $p = 0.065$		
Intercept	-19.5	0.39
Clutch Size	3.46	0.46
Ambient Temperature	3.20	0.43
Mass	0.47	0.37
Clutch Size x Ambient	-0.56	0.49
Clutch Size x Mass	-0.30	0.45
Ambient x Mass	-0.29	0.40
Clutch x Ambient x Mass	0.05	0.46
Range Among Eggs final model		
$R^2 = 0.47$, $F_{1, 24} = 21.7$, $p < 0.0001$		
Intercept	-2.42	0.0010
Clutch Size	0.17	**<0.0001**

Table A1. Results from full and reduced models using backward elimination multiple linear regression to examine the influence of clutch size, ambient temperature and their interaction on incubation temperature parameters of wood duck nests equipped with temperature loggers. Range among eggs was natural log-transformed to meet model assumptions.

Table A2. Incubation Period Models

Term	β	*P*
Clutch Size full model		
$R^2 = 0.44$, $F_{7,15} = 1.68$, $p = 0.19$		
Intercept	42.1	0.74
Clutch Size	-2.03	0.78
Ambient Temperature	-0.06	0.99
Mass	-0.02	0.92
Clutch Size x Ambient	0.08	0.80
Clutch Size x Mass	0.004	0.77
Ambient x Mass	<0.001	0.99
Clutch x Ambient x Mass	<0.001	0.80
Clutch Size final model		
$R^2 = 0.32$, $F_{1,24} = 11.5$, $p = 0.0024$		
Intercept	27.3	<0.0001
Clutch Size	0.19	**0.0024**
Average Temperature full model		
$R^2 = 0.34$, $F_{1,19} = 9.7$, $p = 0.0056$		
Intercept	43.4	<0.0001
Average Temperature	-0.38	**0.0057**
Temperature Fluctuation full model		
$R^2 = 0.41$, $F_{1,19} = 13.2$, $p = 0.0018$		
Intercept	27.4	<0.0001
Temperature Fluctuation	1.04	**0.0018**
Range Among Eggs full model		
$R^2 = 0.29$, $F_{1,19} = 7.78$, $p = 0.012$		
Intercept	29.9	<0.0001
Range Among Eggs	0.25	**0.012**

Table A2. Results from full and reduced models using backward elimination multiple linear regression to examine the influence of clutch size, ambient temperature and their interaction on incubation period of wood duck nests, and also to examine the relationship between individual incubation temperature parameters and incubation period.

Table A3. Hatch Success Models

Term	**β**	***P***
Clutch Size full model		
N = 27		
Intercept	-63.9	0.49
Clutch Size	4.02	0.38
Ambient Temperature	3.35	0.45
Mass	0.11	0.49
Clutch Size x Ambient	-0.22	0.31
Clutch Size x Mass	-0.007	0.40
Ambient x Mass	-0.006	0.47
Clutch x Ambient x Mass	0.0004	0.32
Clutch Size final model		
N = 27		
Intercept	-9.43	0.001
Mass	0.019	**0.0002**
Average Temperature full model		
N = 26		
Intercept	-0.11	0.97
Average Temperature	0.05	0.55
Temperature Fluctuation full model		
N = 26		
Intercept	1.97	0.003
Temperature Fluctuation	-0.14	0.47
Range Among Eggs full model		
N = 26		
Intercept	1.66	<0.0001
Range Among Eggs	-0.04	0.46

Table A3. Results from full and reduced models using backward elimination generalized linear models with a binomial distribution to examine the influence of clutch size, ambient temperature, female body mass and their interaction on hatch success of wood duck nests, and also to examine the relationship between individual incubation temperature parameters and hatch success.

Table A4. Incubation Temperature Parameters with Pushed Eggs Excluded

Term	β	*P*
Average Temperature full model		
$R^2 = 0.55$, $F_{7,15} = 2.64$, $p = 0.054$		
Intercept	207	0.15
Clutch Size	-8.87	0.27
Ambient Temperature	-6.85	0.32
Mass	-0.31	0.20
Clutch Size x Ambient	0.35	0.35
Clutch Size x Mass	0.02	0.26
Ambient x Mass	0.01	0.28
Clutch x Ambient x Mass	-0.001	0.32
Average Temperature final model		
$R^2 = 0.42$, $F_{2,23} = 8.19$, $p = 0.0021$		
Intercept	33.7	<0.0001
Clutch Size	-0.22	**0.0027**
Ambient Temperature	0.18	**0.030**
Temperature Fluctuation full model		
$R^2 = 0.50$, $F_{7,15} = 2.18$, $p = 0.10$		
Intercept	26.8	0.65
Clutch Size	-1.82	0.58
Ambient Temperature	-1.62	0.57
Mass	-0.03	0.73
Clutch Size x Ambient	0.11	0.50
Clutch Size x Mass	0.003	0.61
Ambient x Mass	0.002	0.63
Clutch x Ambient x Mass	<0.001	0.54
Temperature Fluctuation final model		
$R^2 = 0.38$, $F_{2,23} = 6.92$, $p = 0.0044$		
Intercept	3.58	0.00017
Clutch Size	0.07	**0.015**
Ambient Temperature	-0.09	**0.013**
Range Among Eggs full model		
$R^2 = 0.51$, $F_{7,15} = 2.25$, $p = 0.089$		
Intercept	-64.4	0.40
Clutch Size	3.15	0.46
Ambient Temperature	2.97	0.42
Mass	0.12	0.37
Clutch Size x Ambient	-0.14	0.48
Clutch Size x Mass	-0.006	0.44
Ambient x Mass	-0.006	0.38
Clutch x Ambient x Mass	<0.001	0.44
Range Among Eggs final model		
$R^2 = 0.43$, $F_{1,24} = 18.0$, $p = 0.0003$		
Intercept	-2.11	0.0021
Clutch Size	0.15	**0.0003**

Table A4. Results from full and reduced models using backward elimination multiple linear regression to examine the influence of clutch size, ambient temperature and their interaction on incubation temperature parameters of wood duck nests equipped with temperature loggers. In three nests (out of 26 nests), 1-2 artificial egg temperature loggers were pushed to the corner of the nest box during the middle or end of the incubation period. In these models, data from these "pushed" temperature loggers were excluded from analyses. (Table A1 includes all temperature loggers.) Range among eggs was natural log-transformed to meet model assumptions.

APPENDIX B: Supplementary Figures

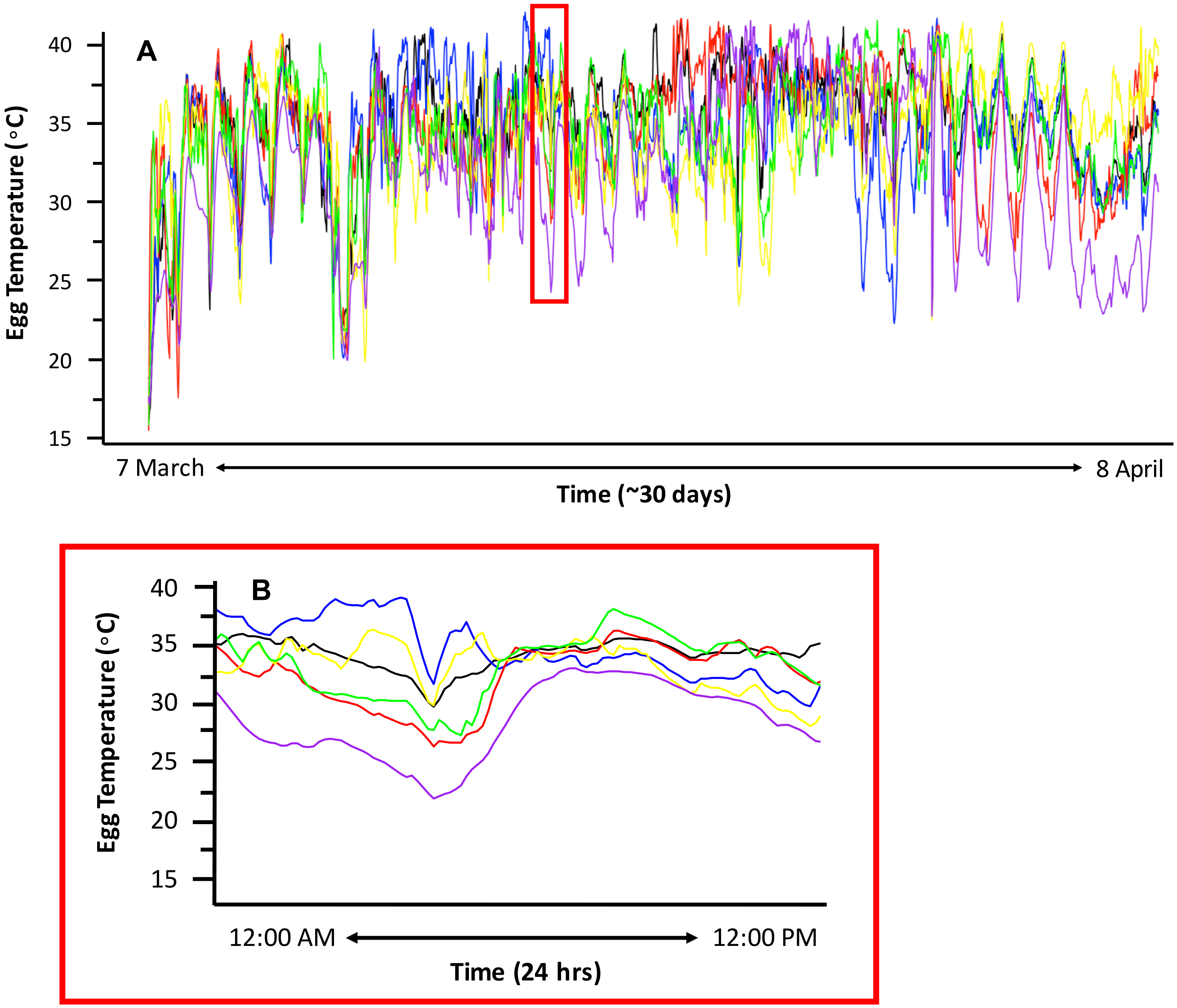


Figure B1. An example of the temperature readings of the 6 mobile temperature loggers in a large (24 eggs) clutch throughout the entire incubation period (A). Each color represents a different mobile logger. Notice that eggs fluctuate between being warmer and cooler than others, showing that eggs are rotated throughout the clutch during incubation. The area that is boxed in Panel A is shown in Panel B, which shows one day of temperature data. Notice the simultaneous temperature drop in all eggs, which indicates that the hen left the nest.

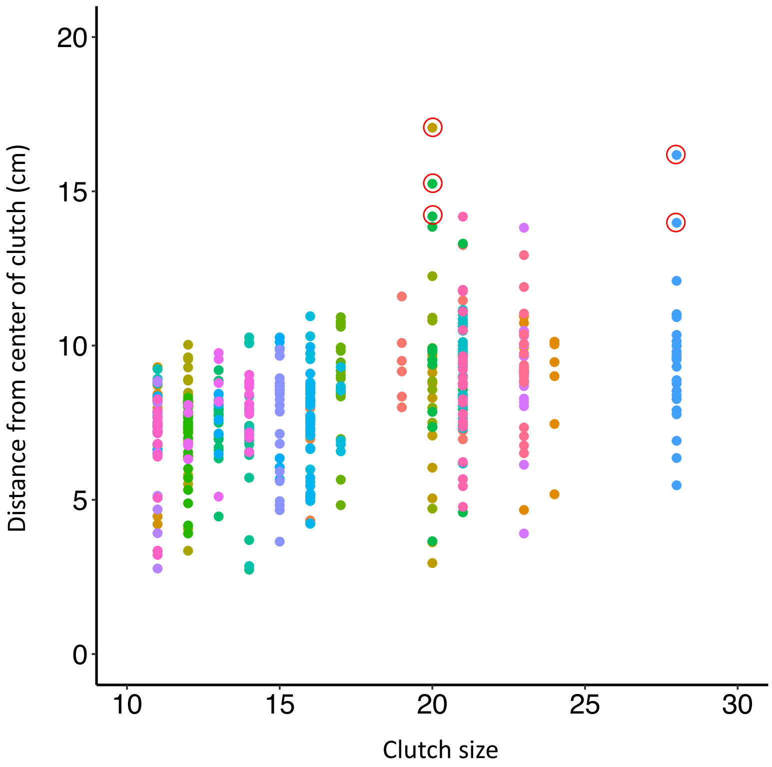


Figure B2. Illustration of the distance of each artificial mobile egg temperature logger from the center of the clutch. Each point represents one artificial egg. Each clutch is a different color. Distances were calculated using ImageJ for each mobile egg for every photo that was taken of a clutch (range: 1-5 photos). The center point of each clutch was calculated by drawing an ellipse around the clutch and calculating the center coordinates using the Measure function. Distances were from the center of the clutch to the outermost point of each artificial egg. The length of each egg is ~5cm. Thus, a distance of <5 cm indicates that the artificial egg was in the center of the clutch, and distances between 5-10 cm indicate that the artificial egg is adjacent to the center of the clutch. Note that artificial eggs are distributed evenly throughout the clutches, regardless of clutch size. Points circled in red are artificial eggs that were noticeably pushed to the corner of the nest box at the time that the photo was taken. When these eggs were excluded from analyses (Table A4) there were no changes in significance to any statistical model.

CHAPTER 3: Incubation temperature and social context affect the nest exodus of precocial ducklings

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ABSTRACT

The environments that animals experience during development have important fitness consequences. In birds, parents influence the developmental environment of their offspring through incubation. Subtle changes in incubation temperature affect offspring morphology and physiology, such as growth, immune function, and thermoregulation, yet little is known about how it may affect critical early-life behaviors. Because expression of behavior can be influenced by the social environment, the effect of incubation temperature on behavior may be context-dependent. We investigated whether incubation temperature and social context influence a critical early-life task in wood ducks (*Aix sponsa*). Wood ducks nest in tree cavities and, shortly after hatching, ducklings must jump and climb out of the cavity. Failure to exit the nest is fatal. In two experiments, we incubated eggs at different mean temperatures and examined the nest exodus of ducklings individually and in mixed-incubation temperature pairs. When tested individually, ducklings incubated at 35.8°C and 37.0°C were ~2.5 times more successful at exiting the nest, and jumped and climbed more often, than those incubated at 35.0°C. However, in an experiment conducted the following year, we found that social interactions mitigated these effects and there was no difference in nest exodus success when ducklings incubated at 35.0°C and 36.0°C were tested together in pairs. This may be because, when in pairs, ducklings incubated at the low temperature experience social enhancement while those incubated at the high temperature maintain similar behaviors. These results advance our understanding of how parental effects influence offspring behaviors and performance within different social contexts.

Keywords: early development, wood duck, social environment, offspring behavior, parental effect, *Aix sponsa*

INTRODUCTION

The environment that animals experience during development can have long-term fitness consequences (Lindström 1999). Parents can influence the early developmental environment of their offspring through various parental effects, such as nutrient or toxicant deposition to the propagule, alteration of the nest environment, grooming, and food provisioning (Bernardo, 1996; Mousseau and Fox, 1998a). Even small changes during development can have lasting effects on offspring morphology, physiology, behavior, reproductive success, and survival (Southwick 1955; Williams 1994; Mousseau and Fox 1998b; Lindström 1999; DuRant, Hopkins, Hepp, et al. 2013; Dixon et al. 2016).

In birds, the regulation of incubation temperature is one of the most important parental effects influencing offspring development (Webb 1987; Deeming and Ferguson 1991; DuRant, Hopkins, Hepp, et al. 2013; Hepp et al. 2015). Parents must maintain egg temperatures within a narrow range to ensure proper development. However, incubation is energetically costly for the parent and limits the amount of time available for foraging, which is especially demanding for uniparental incubators that do not receive food from their mates (Tinbergen and Williams 2002; Nord and Williams 2015). Thus, parents face trade-offs in time and energy investments between maintaining incubation temperatures and maintaining their own body condition (Monaghan and

Nager 1997; Reid et al. 2002). Internal and external factors, such as weather or body mass, can shift this tradeoff and influence how much time and energy parents allocate towards incubation, as well as directly influence incubation temperature (Aldrich and Raveling 1983; Haftorn and Reinertsen 1985; Conway and Martin 2000; Coe et al. 2015). Thus, average incubation temperature can vary among clutches of eggs, among clutches from different breeding attempts of the same individual, and even within one clutch (Reid et al. 2000; Hepp et al. 2006; Boulton and Cassey 2012; Coe et al. 2015; Hope, DuRant, et al. 2018). Even if eggs hatch successfully, variation in incubation temperature may still influence offspring condition. Recent studies show that subtle changes in average incubation temperature can influence avian offspring growth rate, immune function, hormone levels, metabolic rate, thermoregulation, and long-term survival (DuRant et al. 2010; DuRant et al. 2011; Nord and Nilsson 2011; DuRant, Hopkins, Hawley, et al. 2012; DuRant, Hopkins, Wilson, et al. 2012; Hepp and Kennamer 2012; DuRant, Hopkins, Carter, et al. 2013; DuRant et al. 2014; Hepp et al. 2015; Berntsen and Bech 2016; Nord and Nilsson 2016).

Incubation temperature can also have considerable effects on behavior and performance metrics that are important for survival. Here, we use ‘behavior’ to describe what an animal does, and ‘performance’ to describe a quantifiable measure of how well (e.g., how quickly) the animal does it (Irschick and Higham 2015). In non-avian reptiles, many studies have shown that incubation temperature influences behavior, including activity level, foraging, aggression, and cognition, as well as locomotor performance (Deeming and Ferguson 1991; van Damme et al. 1992; Flores et al. 1994; Booth 2006; Burgess et al. 2006; Amiel and Shine 2012; Ballen et al. 2015). In birds, evidence is limited to two studies. One found that 15-20 day-old wood duck ducklings (*Aix sponsa*) incubated at a lower temperature had reduced running and swimming performance compared to those incubated at a higher temperature (Hopkins et al. 2011). More recently, others found that wood duck ducklings incubated at a low temperature displayed more proactive behaviors than those incubated at two higher temperatures (Hope, Kennamer, et al. 2018). However, much remains unknown about how incubation temperature influences other aspects of performance or behavior that are critical for early survival in birds. For many species, a large amount of mortality occurs during the transition between life in the nest and independence. Thus, behaviors and performance metrics associated with begging, fledging, and natal dispersal are particularly important because they can have major fitness implications (Godfray 1991; Leonard and Horn 1998; Visser and Verboven 1999; Forero et al. 2002).

Although incubation temperature is a key determinant of offspring phenotype, the post-hatching environmental context ultimately influences which phenotypes are advantageous or disadvantageous and thus, may further influence the expression of alternative phenotypes. The social environment may be a particularly important context for shaping offspring behavior because the fitness consequences of certain behaviors frequently depend on the actions of other individuals (Moore et al. 1997). For example, in altricial species, most broods fledge synchronously despite asynchronous hatching and individual differences in size (Nilsson and Svensson 1993; Bowers et al. 2013; Radersma et al. 2015). This suggests that, although it may be advantageous for each nestling to wait until they are optimally developed before fledging, it may be more advantageous for them to follow the actions of their siblings so they are not abandoned by their parents. Similarly, an individual’s behavior may change in relation to the phenotype or condition of other individuals in the group. For example, great tit (*Parus major*) nestlings beg more when they are paired with a food-limited sibling than when paired with a well-fed control sibling (Carere et al. 2005), suggesting that it is advantageous to beg more when there is increased competition from a hungry nestling. Indeed, this ‘social enhancement’, where the behavior of one individual amplifies the same behavior in others, has been shown in multiple species (Leonard and Horn 1998; Rodríguez-Gironés et al. 2002; Carere et al. 2005), and may play a role in shaping offspring behaviors when individuals with different phenotypes are in the same nest. Because there is evidence that average incubation temperatures vary within avian nests of some species (Beatty 2015; Hope, DuRant, et al. 2018), incubation temperature and social context may interact to influence avian offspring behavior.

To investigate whether incubation temperature and the social environment influence critical early-life behavior and performance, we conducted two experiments using wood ducks (*Aix sponsa*). In the wild, wood duck ducklings must climb out of the nest cavity and jump down to their mother shortly after hatching. This behavior is crucial because ducklings that do not exit quickly may be left behind by their mother and siblings, and those that fail to exit die in the nest (Bellrose and Holm 1994). Most nests exhibit synchronous hatching and nest exodus (Gottlieb 1963; Hepp and Bellrose 2013). However, in large clutches, substantial developmental asynchrony is common (Kennamer et al. 1990), which may result in a single or multiple ducklings that must exit by themselves. In the wild, it is common to find nests in which 1-2 fully hatched ducklings are dead (S. Hope, R. Kennamer, and W. Hopkins, pers. observ.; nests with >2 dead ducklings occur, but are rare), suggesting that situations where 1-2 ducklings must exit the nest by themselves are common. Further, average incubation temperature varies among eggs within wood duck nests (Hope, DuRant, et al. 2018), suggesting that there are situations in which ducklings incubated at different temperatures must exit the nest singly, as well as together. We incubated eggs at different mean temperatures and tested nest exodus behavior and performance (i.e., speed) of ducklings individually (Individual Experiment) and in mixed-incubation temperature pairs (Pairs Experiment). Because wood duck ducklings incubated at a lower temperature have reduced locomotor performance compared to those incubated at a higher temperature (Hopkins et al. 2011), we predicted that, when tested individually, ducklings incubated at the low temperature would take more time to exit the nest and fewer ducklings would successfully exit the nest box compared to those incubated at the higher temperatures. When tested in pairs, we predicted that ducklings incubated at the higher temperature would more frequently exit the nest box before those incubated at the lower temperature. However, we also hypothesized that social interactions would amplify exodus behavior because, in the wild, it is advantageous for ducklings to leave at the same time as their siblings (Bellrose and Holm 1994). Thus, we predicted that when tested in pairs, ducklings incubated at different temperatures would have similar success rates, and once one duckling exited the nest, the other duckling would attempt to follow. Alternatively, if there was no social enhancement, we expected that our results would be similar to those in the Individual Experiment, and that ducklings incubated at the higher temperature would be more successful at exiting the nest than those incubated at the lower temperature.

METHODS

Study species

The wood duck is a common species of waterfowl that nests in tree cavities and nest boxes across the United States (Hepp and Bellrose 2013). Their average clutch size is 12 (Bellrose and Holm 1994), but clutches can reach >40 eggs in some populations due to conspecific brood parasitism (Morse and Wight 1969; Eadie et al. 1998). Recent work has shown that average incubation temperatures vary both among and within nests (Hope, DuRant, et al.

2018), and that consistent differences in temperatures among eggs within nests increase with clutch size, from a difference of 0.5°C among average egg temperatures in small nests (12 eggs) to a difference of 3.1°C in large nests (24 eggs; Hope et al., unpublished data). Thus, wood duck broods are normally composed of ducklings that have hatched from eggs that have been incubated at different average temperatures.

Hatching within small clutches is synchronous and usually occurs 6-18 h after the first egg pips (Gottlieb 1963), although eggs in large clutches experience over 3 d of developmental asynchrony (Kennamer et al. 1990) likely due to a combination of differences in the date eggs were laid and to within-clutch differences in incubation temperatures (Hope, DuRant, et al. 2018). Eggs take, on average, 32 h to hatch after pipping (Bellrose and Holm 1994), but this also varies with incubation temperature (DuRant et al. 2011). Once the first egg pips, the hen begins to vocalize while in the nest and continues to do so for 20-36 h (Gottlieb 1963). Ducklings are precocial and are active within about 7 h after hatching. After the ducklings hatch and when the environment is suitable (e.g., no visible predators), the hen leaves the nest and vocalizes at a fast rate from below (Gottlieb 1963). Ducklings must respond to their mother's call, climb up and out of the nest cavity, and jump down to meet their mother and siblings. Ducklings usually perform this nest exodus within 24 h of hatching (Hepp and Bellrose 2013), but will still perform this behavior until 4 d after hatching (Siegfried 1974). The hen will vocalize beneath the nest until most of her brood has joined her, which usually takes ~5 min (Gottlieb 1963; Siegfried 1974; Bellrose and Holm 1994), but can occasionally take longer (Bellrose and Holm 1994). This is a crucial event because ducklings that cannot exit die in the nest (usually only 1-2 ducklings; S. Hope, R. Kennamer, W. Hopkins, pers. observ.). Further, because there are many predators that eat ducklings, it is common for a female to flee from a predator with only the ducklings that have already exited (Bellrose and Holm 1994). Thus, those that are too slow at exiting are left behind and have a lower chance of survival without their brood mates and maternal care (Bellrose and Holm 1994). On the other hand, ducklings that exit too quickly before their nest mates may be vulnerable to lurking predators, so exiting simultaneously with nest mates may be the optimal strategy.

Egg collection and incubation

We collected eggs from a population of wood ducks breeding in nest boxes on the Department of Energy's Savannah River Site (SRS) in South Carolina, USA (33.1°N, 81.3°W) from 6 – 29 March 2015 for the Individual Experiment and 29 February – 16 March 2016 for the Pairs Experiment. We checked nest boxes daily on a series of 12 ephemeral wetlands, which have nest boxes that have been maintained for >30 yrs. We marked eggs for lay-date, collected up to 10 eggs from each nest, and replaced eggs with wooden eggs to prevent hens from abandoning (Hepp et al. 1987). Eggs were transported at ambient temperature to Virginia Tech, rotated twice daily, and incubated within 10 d (Walls et al. 2011) in Grumbach incubators (model BSS 420, Asslar, Germany). For the Individual Experiment, eggs were incubated at three different overall mean temperatures: 35.0, 35.8, and 37.0°C. We chose these temperatures because they are within the natural range for wood ducks and they have been shown to produce ducklings with different phenotypes in previous experiments (DuRant, Hopkins, Hepp, et al. 2013). For the Pairs Experiment, eggs were incubated at two different overall mean temperatures: 35.0 and 36.0°C. We chose these temperatures because a 1°C difference in mean temperature is enough to produce different phenotypes, and is also a realistic temperature difference among eggs within natural nests (Hope, DuRant, et al. 2018). Eggs from the same nest and the same lay-date were distributed among treatments. Incubators were programmed to reach maximum temperatures that were higher than the mean temperatures (listed above), and had two 75 min cool-down periods (~3°C decrease in temperature) at 8:15 and 18:30 h to simulate hens leaving the nest for foraging (Manlove and Hepp 2000). This allowed incubators to maintain the overall mean temperatures. The temperatures reported here for each experiment are the average temperatures recorded using *i*Buttons® in the incubators. Due to a minor discrepancy in incubator performance in the second year of the study, the actual temperatures that were recorded (36.0°C) were slightly higher than what we programmed (35.8°C). This 0.2°C discrepancy in mean incubation temperature between the two experiments and the fact that they were conducted in two different years prevented direct statistical comparisons between experiments, but neither of these factors detracts from our overall conclusions. The average humidity for all incubators was kept between 60-65%. Once pipped, eggs were placed in a hatcher with constant temperature of 36°C and humidity kept between 72-82%. We placed a speaker inside the hatcher that played wood duck hen vocalizations, to mimic auditory cues that ducklings would experience in a nest once pipped, and to stimulate auditory imprinting (Gottlieb 1963).

General husbandry

Once hatched, we recorded the hatch time, and then weighed and color-banded the ducklings. During the Individual Experiment, we checked for hatching at least every 3 h between 8:00-17:00 and during the Pairs Experiment we checked at least every 2 h and videotaped the hatcher while we were not present to record precise time of hatch. We placed newly hatched ducklings together (no more than 12 per cage) under a 50W infrared heat lamp in a covered cage to simulate dark, communal nest conditions. Ducklings stayed in this environment until they were at least 7 h old, but not older than 27 h, and we then performed the nest exodus trial (overall average age ± SD during trial = 14.8 ± 4.6 h). Although ducklings did not hatch in the nest boxes in which we conducted behavioral trials, we think it is likely that keeping ducklings in these conditions before the trial was sufficient to mimic natural nest conditions. After the trial, we measured tarsus length and ducklings were housed in cages in groups of 2 or 3 to undergo a series of trials for other studies. Differences in hatch success, incubation period, hatch mass, and tarsus length are reported in Table 1. After all studies were complete, ducklings were humanely euthanized and sex was determined by inspecting both external genitalia and internal gonads. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Individual experiment

During the Individual Experiment, ducklings were tested individually on their ability to exit the nest box, the speed at which they did so, and their associated behaviors (i.e., latency of first movement, jump, call, and climbing attempt, and the number of jumps and climbs). The trial was conducted in a wooden nest box (20 x 20 x 50 cm) identical to nest boxes in the field, but with one Plexiglas wall which allowed for behavior to be videotaped. Wooden planks surrounded the Plexiglas wall and left only a small opening for a video camera, so that the wall looked dark to the duckling. A light was attached outside and above the nest box exit hole and pointed down into the box to simulate natural daylight and facilitate better video recording. Thus, the only light sources were coming from directly above and outside the exit hole, which mimics natural conditions. Wire mesh was attached below the hole (11.5 x 34.5 cm) to aid the ducklings in climbing, which is common for nest boxes in the field. The nest box was on the ground and a trough of water was placed below the hole for ducklings to land in once they jumped out.

Each duckling (*N* = 144 ducklings from 36 different nests) was placed in the box and, outside of the box, we played a recording of a wood duck hen call mixed with duckling calls to simulate natural conditions and motivate the duckling to exit. Both hen call (https://www.youtube.com/watch?v=0nFIIPtm844) and duckling call (https://www.youtube.com/watch?v=XhTEk31kzuI) MP3s were downloaded from YouTube. The hen call was recorded from a wood duck nest in Smithfield, NC. Portions of each MP3 were mixed together using the program Audacity® version 2.1.2 (Audacity Team, 2015). The hen call played in a pattern of 2 min on/2 min off and the duckling call played in a pattern of 30 s on/30 s off for the duration of the trial. The recording was played, at the same volume setting for each trial, from an iPod© (Apple Inc.) on an iHome© (Apple Inc.) speaker placed ~0.5 m away from the nest box. The trial ended when the duckling jumped out of the nest box, or after 30 min. Although Gottlieb (1963) found that all ducklings in eight natural nests exited within 4 min from when the hen began to vocalize from beneath the nest, and a previous lab-study only gave ducklings 5 min to exit the nest (Siegfried 1974), we chose 30 min as a conservative time limit. In this study, 39% of ducklings did not exit within the 30 min time frame. From the videos, one person (SFH) later recorded the time of the duckling's first movement, jump, call, and climbing attempt, the number of jumps and climbs, and the latency to leave the box.

Pairs experiment

During the Pairs Experiment, ducklings were tested in pairs (*N* = 54 pairs; 108 ducklings from 31 different nests), with one duckling from each treatment (35°C and 36°C). Ducklings that were similar in age (h) were paired (average ± SD difference in age = 3.39 ± 3.44 h, range = 0 – 15.7 h). We tried to avoid pairs of ducklings that originated from eggs from the same nest, but 1 out of the 54 pairs consisted of ducklings from the same nest because we prioritized similarity in age. Ducklings were individually marked with numbers on their heads using non-toxic white correcting fluid, so they were identifiable in the video recording. The trial was conducted with the same nest box configuration and audio recording as in the Individual Experiment.

The trial ended when both ducklings exited the nest box, or after 30 min. In this study, 46% of ducklings did not exit within the 30 min time frame. From the videos, one person (SGVM) recorded the same behaviors as in the Individual Experiment, along with noting which duckling exited the nest first, and quantifying the number of jumps and climbs that the duckling left behind in the box made before and after the first duckling exited.

Statistical analyses

All analyses were conducted in R version 3.3.1 (R Core Team 2016) and we used the package *lmerTest* (Kuznetsova et al. 2016). For all models, incubation temperature was the categorical independent variable. The age of the duckling at the time of the trial (h), sex, lay-date, and body condition (the residuals of body mass vs. tarsus length linear regression) were originally included in all analyses as covariates, but we used backward elimination for insignificant terms and only report significant or marginally significant covariates. We consider $P < 0.05$ as significant, but also report $0.05 < P < 0.10$ as trends. Because ducklings from the same clutch were used, clutch was included as a random effect in each model. Also, for analyses of the Pairs Experiment, the pair (ducklings tested together) was included as a random effect to account for any effect of pairing. We visually inspected graphs of the residuals of our models to ensure they met the assumptions of normality and homoscedasticity. When models did not meet assumptions, we first attempted to transform the data to meet assumptions, and if transformations did not work, we used general linear models with non-normal error distributions.

To determine if incubation temperature affected the proportion of ducklings that were able to exit the nest box in each experiment, we used generalized linear mixed models (*glmer*) with a binomial error distribution. Whether or not the duckling exited the box (binary: yes or no) was the response variable in both models.

Next, to determine if incubation temperature affected the latency for ducklings to exit the nest box, we used linear mixed effects models (*lmer*). All ducklings that exited the nest box on their own were included in these analyses (Individual Experiment: $N = 88$ ducklings from 33 nests; Pairs Experiment: $N = 58$ ducklings from 29 nests). The latency to exit (s) was used as the response variable. Latency to exit was log-transformed for the Individual Experiment model to meet the assumptions of normally-distributed and homoscedastic residuals. The data from the Pairs Experiment met model assumptions and did not require transformation.

To further investigate nest exodus performance in the Pairs Experiment, we tested whether incubation temperature influenced which duckling first exited the nest by using a generalized linear mixed model (*glmer*) with a binomial error distribution. Whether or not the duckling was the first of its pair to exit (binary: yes or no) was the dependent variable. Only pairs in which at least one duckling exited were used in this analysis ($N = 37$ pairs with ducklings from 29 nests).

To examine whether incubation temperature influenced duckling behavior during the trial, we used principal components analyses (PCA; *princomp*) with correlation matrices for each experiment. For both PCAs, the latency time to first move, jump, call, and climb, the number of jumps per minute and the number of climbs per minute were included. If a duckling did not perform any of these behaviors, they were given a score of 30 min for the latency of that behavior. For both PCAs, scree plots indicated that PC1 explained most of the variation (59% in both experiments), so we used PC1 for each experiment as the dependent variable in the model. The PC1 scores for the Individual Experiment were highly left-skewed, so we transformed them to be right-skewed (multiplied by -1 and added 3) and used a generalized linear mixed model (*glmer*) with a Gamma distribution. We used a linear mixed effects model (*lmer*) for the Pairs Experiment because the data met all assumptions.

Lastly, to investigate whether the second duckling's behavior changed after the first duckling exited, we used two models to compare the number of jumps and number of climbs that each duckling took before and after the first duckling exited. Our sample size for these analyses was 37 ducklings from 22 different nests because we did not have before/after behavior data for pairs where neither duckling exited. For both models, the time (categorical: before or after), incubation temperature, and their interaction were the independent variables and duckling ID was a random effect. The first model used the number of jumps as the dependent variable and we used a linear mixed effects model (*lmer*) with a log+1 transformation to meet model assumptions. The second model used the number of climbs as the dependent variable and we used a generalized linear mixed model (*glmer*) with a Gamma distribution because the data were highly left-skewed.

RESULTS

Nest exodus

When ducklings were tested individually (Individual Experiment), a larger proportion of ducklings incubated at the higher temperatures were successful at exiting the nest than those incubated at the lower temperature ($F_{2, 140} = 12.1$; $P < 0.0001$; Fig. 1A). *Post-hoc* analysis (*lsmeans with tukey adjustment*) revealed that the proportion of successful ducklings incubated at the two higher temperatures did not differ significantly ($P = 0.08$), but both had greater success (35.8°C: 39 out of 60 successful; 37°C: 39 out of 45 successful) than those incubated at the lower temperature (35°C: 10 out of 37 successful; intermediate-low temperature: $P = 0.003$; high-low temperature: $P < 0.0001$). When ducklings were tested in pairs (Pairs Experiment), incubation temperature was not related to exit success ($F_{1, 103} = 2.0$; $P = 0.17$; Fig. 1B). However, the relationship between lay-date and exit success was marginally significant ($F_{1, 103} = 4.4$; $P = 0.053$), where ducklings hatched from eggs laid at a later date tended to be less likely to successfully exit than those laid earlier in the season (effect size = -13.25; *effsize*; Torchiano, 2017).

For ducklings that successfully exited, latency to exit the nest box tended to be negatively related to incubation temperature when ducklings were tested individually (Individual Experiment; $F_{2, 85} = 3.1$; $P = 0.052$; Fig. 2A). In pairs, latency to exit was not related to incubation temperature (Pairs Experiment; $F_{1, 27} = 1.0$; $P = 0.32$; Fig. 2B), but it was negatively related to duckling age in hours ($F_{1, 53} = 5.9$, $P = 0.02$; effect size = -2.13).

Contrary to our predictions, incubation temperature did not affect whether a duckling was the first to exit the nest box in the Pairs Experiment ($F_{1, 70} = 0.94$; $P = 0.35$; Fig. 3).

Duckling behaviors

In the Individual Experiment, PC1 loaded negatively with latencies to move (-0.448), call (-0.458), jump (-0.500), climb (-0.437), and positively with the number of jumps per minute (0.274) and the number of climbs per minute (0.271). Thus, a higher PC1 score indicated that the duckling was quicker to move, call, jump, and climb, and jumped and climbed more. Duckling activity levels increased as incubation temperature increased ($F_{2,\ 138} = 4.6$; $P < 0.001$; Fig. 4A). A post-hoc test (*lsmeans with tukey adjustment*) revealed that ducklings incubated at the lowest temperature were significantly less active than those incubated at the both of the higher temperatures (high-low temperature: $P = 0.0008$; intermediate-low temperature: $P = 0.03$). However, ducklings incubated at the intermediate and high temperatures did not differ in activity levels ($P = 0.3$). There was also a trend towards females being more active than males ($F_{1,\ 138} = 2.6$, $P = 0.057$; effect size = -0.27).

In the Pairs Experiment, PC1 loaded negatively with latencies to move (-0.419), call (-0.453), jump (-0.490), climb (-0.434), and positively with the number of jumps per minute (0.277) and the number of climbs per minute (0.338). As observed in the Individual Experiment, a higher PC1 score indicated that the duckling was quicker to move, call, jump, and climb, and jumped and climbed more. In contrast to the Individual Experiment, however, incubation temperature was not related to behavior ($F_{1,\ 52} = 0.055$; $P = 0.81$; Fig. 4B). However, there was a marginal relationship between behavior and lay date ($F_{1,\ 64} = 3.2$, $P = 0.08$; effect size = 4.95), where ducklings hatched from eggs laid at a later lay date tended to be less active than those laid earlier in the season.

In the Pairs Experiment, once the first duckling exited the nest box, the second duckling jumped ($F_{1,35} = 13.5$; $P < 0.001$; Fig. 5A) and climbed ($F_{1,35} = 27.7$; $P < 0.001$; Fig. 5B) > 4 times more than they did before the first duckling exited, regardless of their incubation temperature (in all cases, $F \leq 0.22$ and $P \geq 0.77$ for incubation temperature main effect and interaction with time [before or after]).

DISCUSSION

We demonstrated that a change in incubation temperature of <1°C and the early social environment interacted to affect the ability of precocial ducklings to exit the nest, a critical early-life event. When tested individually, ducklings incubated at the lowest temperature were less successful at exiting the nest than those incubated at slightly higher temperatures. However, social interactions mitigated these effects and, when tested in pairs, ducklings incubated at different temperatures had similar exodus success rates. It appears that success rates converged because ducklings incubated at the low temperature experienced social enhancement and increased motivation to exit when in the presence of a duckling incubated at the higher temperature. In contrast, those incubated at a higher temperature displayed consistent behavior, or even slightly poorer performance, in the presence of social interactions with a duckling from the cooler incubation temperature. Regardless of incubation temperature, all ducklings appeared to have increased motivation to exit once they were alone because after one duckling in the pair exited the nest, the duckling that remained behind jumped and climbed more often. These results advance our understanding of how parental effects may differentially influence offspring behavior and performance depending on their early social context.

We found that when tested individually, ducklings incubated at 35°C were less active, slower, and less successful at exiting the nest than those incubated at higher temperatures. We did not find a significant difference in exodus success between ducklings incubated at the higher two temperatures, suggesting that there may be a thermal threshold for promoting exodus performance. Lower performance in low temperature-incubated ducklings is consistent with previous studies that showed that a small decrease in average incubation temperature can produce a diverse array of phenotypic differences in birds. In wood ducks, ducklings incubated at a lower temperature grow slower (DuRant et al. 2010), have reduced locomotor performance (Hopkins et al. 2011), inefficient thermoregulatory abilities (DuRant, Hopkins, Wilson, et al. 2012; DuRant, Hopkins, Carter, et al. 2013), reduced immunocompetence (DuRant, Hopkins, Hawley, et al. 2012), altered glucocorticoid and thyroid hormone levels (DuRant et al. 2010; DuRant et al. 2014), and reduced survival (Hepp and Kennamer 2012), compared to those incubated at a higher temperature. Further, altricial blue tits incubated at lower temperatures have slower growth rates and higher metabolic rates than those incubated at higher temperatures (Nord and Nilsson 2011). Unlike some alternative phenotypes that may be advantageous depending on the environmental context (e.g., a small body size may reduce the total energy costs of self-maintenance), the effect of a low incubation temperature on the ability to exit the nest is almost certainly disadvantageous, since failure to complete a timely exodus dramatically decreases the chances of survival (Bellrose and Holm 1994).

Our behavioral (i.e., jumps and climbs) and performance metrics (i.e., latency to exit) reveal possible correlates that may help explain the variance in nest exodus success. For example, ducklings incubated at a low temperature may have been less successful when tested individually due to lower persistence or less motivation compared to those incubated at higher temperatures. Indeed, in the Individual Experiment, ducklings incubated at the lowest temperature made fewer jumps and climbs, and had longer latencies to begin activity than those incubated at higher temperatures (Fig. 4A). This agrees with Siegfried (1974), who found that wood duck ducklings that successfully exited an artificial cavity jumped more per minute than those that did not exit successfully. Further, ducklings incubated at the lower temperature may have been less motivated to exit the nest than those incubated at the higher temperatures when there was no social stimulus. Our results from the Pairs Experiment support this possibility. Once there was a source of social motivation, ducklings incubated at the lower temperature were just as successful at exiting the nest as those incubated at the higher temperature (Fig. 1B). Additionally, ducklings that were left behind in the box jumped and climbed more once the first duckling left, likely due to increased motivation to exit (Fig. 5A, B).

It is also possible that differences in exodus success were due to energetic or morphological constraints. For example, it may be more energetically demanding for a duckling incubated at a lower temperature to jump or climb than it is for a duckling incubated at a higher temperature. DuRant, Hopkins, Wilson, et al. (2012) found that wood ducks incubated at a lower temperature expend more energy during a thermoregulatory challenge than those incubated at higher temperatures, and thus, it is possible that similar inefficiencies exist when jumping and climbing. It is also possible that ducklings incubated at a lower temperature had expended more energy during incubation (DuRant et al. 2011) or depleted more of their yolk reserves before hatching (Olson et al. 2006). However, if this were the case, we would have expected ducklings incubated at the lower temperature to have a lower body mass at hatching than those incubated at the higher temperature, which was not the case (Table 1). Another possibility is that structural size contributed to the differences in exit success. However, we think that this is unlikely because neither body mass nor tarsus length were related to exit success in either experiment (all $F \leq 2.3, p \geq 0.13$).

Interestingly, social interactions mitigated the effects of incubation temperature on nest exodus success that were present when ducklings were tested individually. When tested in mixed-incubation temperature pairs, there was no effect of incubation temperature on nest exodus success, nor on the probability of which duckling exited the nest first. This convergence was primarily due to an increase in success by the ducklings incubated at the low temperature, while those incubated at the higher temperature (35.8-36.0°C) displayed similar, or even slightly lower, success in the presence of social interactions. For low temperature-incubated ducklings (35.0°C), success increased from 27% in the Individual Experiment to 48% in the Pairs Experiment (Fig. 1). In contrast, exodus success for high temperature-incubated ducklings (35.8 and 36.0°C) varied from 65% in the Individual Experiment to 59% in the Pairs Experiment (Fig. 1). Thus, our results collectively suggest that ducklings from different incubation temperatures responded differently to social stimuli.

The enhanced success of ducklings incubated at the low temperature while in pairs may be due to social enhancement (Carere et al. 2005), where the behavior of one duckling causes another duckling to increase the frequency of the same behavior. This has been documented in altricial species, where nestlings alter their begging rates in response to the begging rates of their nest mates (Leonard and Horn 1998; Kitaysky et al. 2001; Rodríguez-Gironés et al. 2002; Carere et al. 2005) and fledge synchronously despite asynchronous hatching and differing body sizes (Nilsson and Svensson 1993; Bowers et al. 2013; Radersma et al. 2015). It makes sense that social enhancement would play a role in wood duck nest exodus because, even if a duckling is not in the best condition to leave the nest, it is still usually more advantageous to leave with their siblings to avoid being left behind (Bellrose and Holm 1994).

In contrast to outcomes produced from low incubation temperatures, ducklings incubated at higher temperatures appear to have relatively similar exit success both while in pairs and individually. This may be because ducklings incubated at the higher temperature are less influenced by social interactions, and their probability of exit success depends primarily on physiological traits. Interestingly, however, a few lines of evidence suggest that ducklings incubated at the higher temperature may in fact display lower exodus performance when paired with a duckling incubated at a lower temperature. First, a slightly lower proportion of ducklings incubated at the higher temperature exited while in pairs than when tested individually (Fig. 1). Second, of those that successfully exited, ducklings incubated at higher temperatures displayed longer latencies to exit while in pairs than when tested individually (Fig. 2). Third, we investigated this further by determining the frequency of pairs in which both ducklings exited, both failed to exit, or only one duckling exited (Fig. 6). This comparison revealed that ducklings performed the same way (either both succeeded or both failed) 70% of the time. Importantly, ducklings incubated at the higher temperature almost never failed if their low-temperature incubated partner succeeded (only 9% of the time), suggesting that the performance of ducklings incubated at the higher temperature is indeed influenced to some degree by social interactions. The high temperature-incubated ducklings may be displaying social conformity because, although they are physiologically capable of exiting when alone, many fail to exit when in pairs, and this happens almost exclusively when the other duckling also fails. However, it is still unknown why both ducklings failed so often (31%) while in pairs. It is possible that ducklings are physically interfering with each other (e.g., pushing) or engaging in beneficial social interactions instead of attempting to exit (e.g., preening).

Our finding that social interactions mitigated the effects of incubation temperature raises interesting questions regarding how our observations translate to nest exodus performance under natural circumstances. For instance, if social interactions lead to ducklings incubated at both high and low temperatures exiting the nest at the same time, there may be post-exodus fitness consequences for individuals experiencing lower temperatures during development. Because average egg temperatures vary within a clutch (Hope, DuRant, et al. 2018) and there are numerous developmental asymmetries among ducklings incubated at different temperatures (DuRant, Hopkins, Hepp, et al. 2013), ducklings that may have exited the nest too early in order to follow their nest mates may be at a disadvantage once out of the nest. For example, lower incubation temperatures produce ducklings that are poor thermoregulators during the first day of life compared to those produced from higher incubation temperatures (DuRant, Hopkins, Wilson, et al. 2012), and the environment outside of the nest box most certainly poses greater thermal challenges than within the box. Thus, following nest mates may have its own fitness consequences if an individual has deficits due to suboptimal developmental conditions.

Additionally, ducklings may not always experience social interactions under natural circumstances, and incubation temperature may be especially important for shaping nest exodus behavior when there is hatching asynchrony. Eggs incubated at the lowest temperatures take the longest to hatch (Hepp et al. 2006) and developmental asynchrony increases with clutch size (Kennamer et al. 1990) likely due to within-clutch variance in incubation temperature (Hope, DuRant, et al. 2018). This problem is exacerbated in wood ducks because of high rates of conspecific brood parasitism (Eadie et al. 1998). Thus, it may be common for ducklings incubated at the lowest temperatures within enlarged clutches to hatch last, and thus exit last, and in some cases singly. Then, once alone, these ducklings will likely be unsuccessful. This may result in ducklings being abandoned and dying in the nest, which we commonly observe each nesting season at our field sites (S. Hope, R. Kennamer, and W. Hopkins, pers. observ.).

Because we focused on mixed-temperature pairs in this study, we were not able to fully disentangle the influence of simply the presence of a partner from the influence of the temperature at which that partner was incubated. However, we have some evidence that the temperature at which each partner was incubated influences the outcome of their social interactions. During the Pairs Experiment, we opportunistically tested some same-temperature pairs. We tested nine low-low temperature pairs and found that, in seven of those pairs, both ducklings failed to exit. We also tested two high-high temperature pairs and found that in one pair both ducklings exited and in the other pair one duckling exited. Although these sample sizes are small, it provides further anecdotal evidence that social facilitation alone does not determine exit success, and that the temperature at which the partner was incubated matters. These observations suggest that additional experimentation may be needed to fully disentangle the relative importance of social interactions and incubation temperature for exodus success.

In addition to the observed effects of incubation temperature and social context, we also found a noteworthy trend suggesting that lay date was related to both nest exodus success and activity levels. Ducklings hatching from eggs laid later in the season were less likely to exit the nest and were less active in their attempts to exit. This is consistent with other studies that found lower hatching success and poorer quality offspring as the reproductive season progressed (Hochachka 1990; Verhulst et al. 1995; Brinkhof and Cave 1997; Harriman et al. 2017). These differences in quality may be due to either individual differences among hens, where different hens invest differentially in egg quality, or to differences in environmental conditions as the season progresses.

In conclusion, our study sheds light on how parental effects and the early social environment can interact to influence a critical early-life event. Incubation temperature influenced exodus performance differently depending on whether ducklings were tested individually or in pairs. This shows how parental effects can be context-dependent and highlights the importance of taking the early social environment into account when studying parental effects on offspring behaviors. In wood ducks, incubation temperatures vary both among and within nests (Hope, DuRant, et al. 2018), and our study shows how variation in incubation temperature within nests may influence offspring behavior both directly, and indirectly by shaping the composition of the post-hatch social environment. Further, because within-clutch temperature variation (Hope, DuRant, et al. 2018) and developmental asynchrony (Kennamer et al. 1990) increase as clutch sizes increase, our study has implications for understanding constraints on the evolution of clutch size and the costs of brood parasitism.

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FIGURE LEGENDS

Figure 1. Incubation temperature and social context influence nest exodus success. When tested individually, fewer ducklings that hatched from eggs incubated at the lowest temperature (35°C: *N* = 37 ducklings from 24 nests) successfully exited the nest than those that hatched from eggs incubated at the two higher temperatures (35.8°C: *N* = 60 ducklings from 34 nests; 37°C: *N* = 47 ducklings from 30 nests) (A). When tested in pairs (B; one low [35°C] and one high [36°C] incubated duckling in each pair), incubation temperature did not affect nest exodus success (35°C: *N* = 54 ducklings from 25 nests; 36°C: *N* = 54 ducklings from 27 nests). Numbers indicate the proportion of ducklings that successfully exited the nest box within 30 min, out of the number tested from each incubation temperature.

Figure 2. Effect of incubation temperature on the latency to leave the nest box. Latency to exit the nest (min; means ± SE) tended to decrease with incubation temperature when ducklings were tested individually (A; 35°C: *N* = 10 ducklings from 10 nests; 35.8°C: *N* = 39 ducklings from 26 nests; 37°C: *N* = 39 ducklings from 26 nests), but there was no difference in latency to leave the nest when tested in pairs (B; 35°C: *N* = 26 ducklings from 20 nests; 36°C: *N* = 32 ducklings from 19 nests). Only ducklings that successfully exited on their own were included in the analysis.

Figure 3. Incubation temperature does not influence which duckling exited first when ducklings were tested in pairs. Pairs consisted of one low (35°C) and one high (36°C) incubated duckling (*N* = 37 pairs consisting of ducklings from 29 nests). Numbers indicate the proportion of ducklings incubated at each temperature that exited the nest first during the trial.

Figure 4. Incubation temperature and social context influence duckling behaviors during exodus from the nest. Ducklings that hatched from eggs incubated at higher temperatures (35.8°C: $N =$ 60; 37°C: $N = 47$) were more active in the nest box than those incubated at the lowest temperature (35°C: $N = 37$) when tested individually (A). Incubation temperature did not affect activity when ducklings were tested in pairs (B; 35°C: $N = 54$; 36°C: $N = 54$). Separate PCAs were conducted for the Individual Experiment (A) and the Pairs Experiment (B), and in both cases, a higher PC1 score (means ± SE) indicates that the duckling had a shorter latency to move, jump, call, and climb, and jumped and climbed more often.

Figure 5. Ducklings left in the nest box jump and climb more often once the first duckling in the pair exits. When ducklings were tested in pairs, the duckling that was left in the nest box jumped (A; 35°C: $N = 21$ ducklings from 14 nests; 36°C: $N = 16$ ducklings from 15 nests) and climbed (B; 35°C: $N = 21$; 36°C: $N = 16$) more per min (means ± SE) after the first duckling exited the box than before it exited. Jumps and climbs before and after were similar among incubation temperature treatments.

Figure 6. Ducklings in pairs frequently behave similarly. The frequency of times that, within a duckling pair in the Pairs Experiment, either both ducklings succeeded in exiting the nest, both failed to exit, only the duckling incubated at the low temperature failed, or only the duckling incubated at the high temperature failed. Note that ducklings behave similarly (either both succeed or both fail) 70% of the time.

TABLES

Table 1. Means (± SD) of hatching success, incubation period, hatch mass, and tarsus length for ducklings incubated at different temperatures in two different experiments

	Individual Experiment			Pairs Experiment	
	Incubation temperature treatment (°C)				
Variable	35.0	35.8	37.0	35.0	36.0
Hatch success (%)	48	80	83	62	74
Incubation period (d)	39.5 ± 1.1	36.0 ± 1.0	32.6 ± 0.8	38.5 ± 0.9	35.7 ± 1.0
Hatch mass (g)	28.0 ± 2.7	27.8 ± 2.4	28.9 ± 2.5	27.8 ± 3.2	27.9 ± 3.4
Tarsus length (mm)	19.4 ± 0.7	18.9 ± 0.7	18.6 ± 0.7	19.6 ± 0.9	19.4 ± 0.9

FIGURES

Figure 1

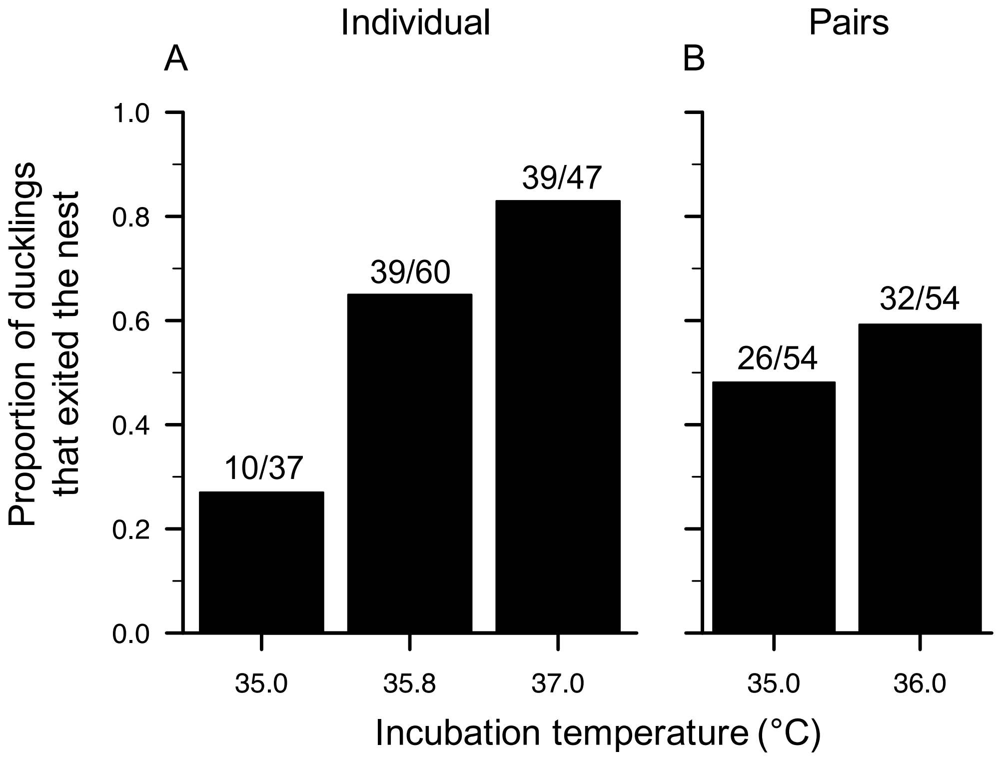


Figure 2

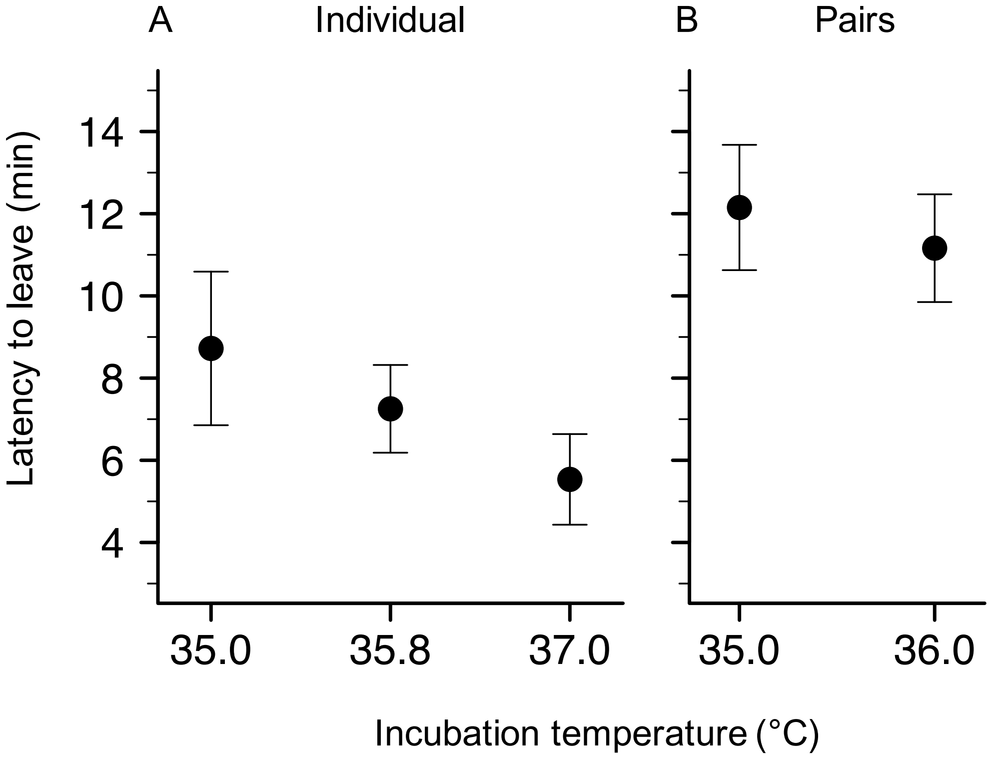
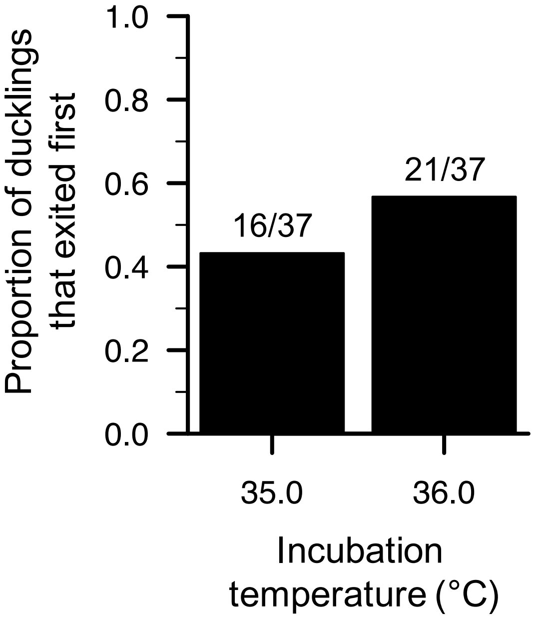

A
Individual
B
Pairs
Latency to leave (min)
14
12
10
8
6
4
35.0
35.8
37.0
35.0
36.0
Incubation temperature (°C)

Figure 3



Proportion of ducklings that exited first
1.0
0.8
0.6
0.4
0.2
0.0
16/37
21/37
35.0
36.0
Incubation temperature (°C)

Figure 4

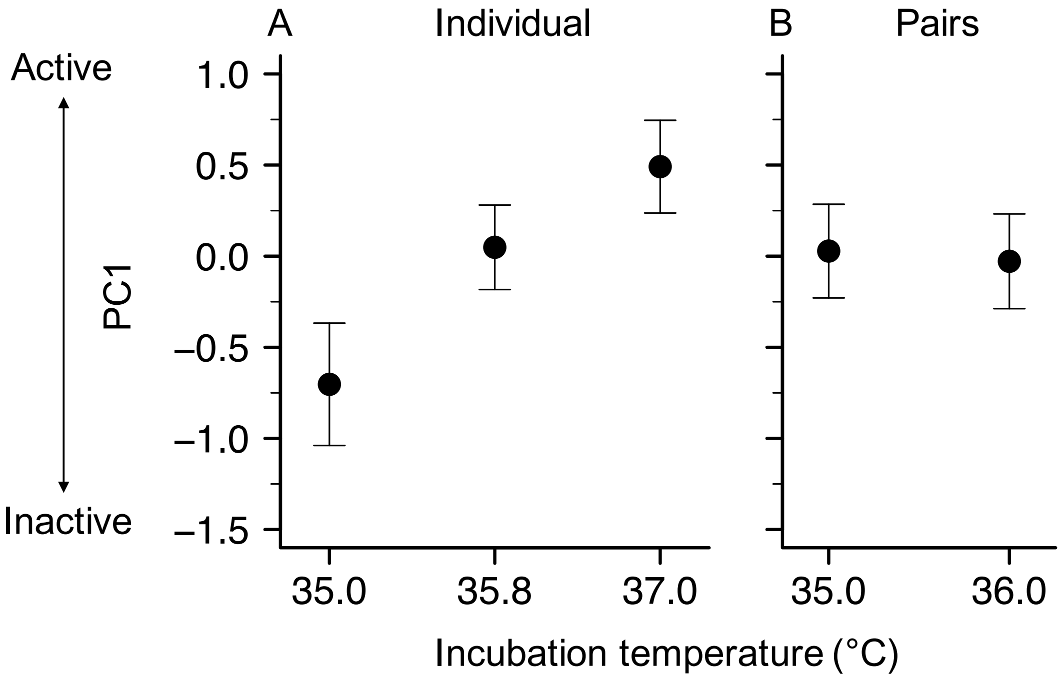

A
Individual
B
Pairs
Active
Inactive
PC1
1.0
0.5
0.0
−0.5
−1.0
−1.5
35.0
35.8
37.0
35.0
36.0
Incubation temperature (°C)

Figure 5

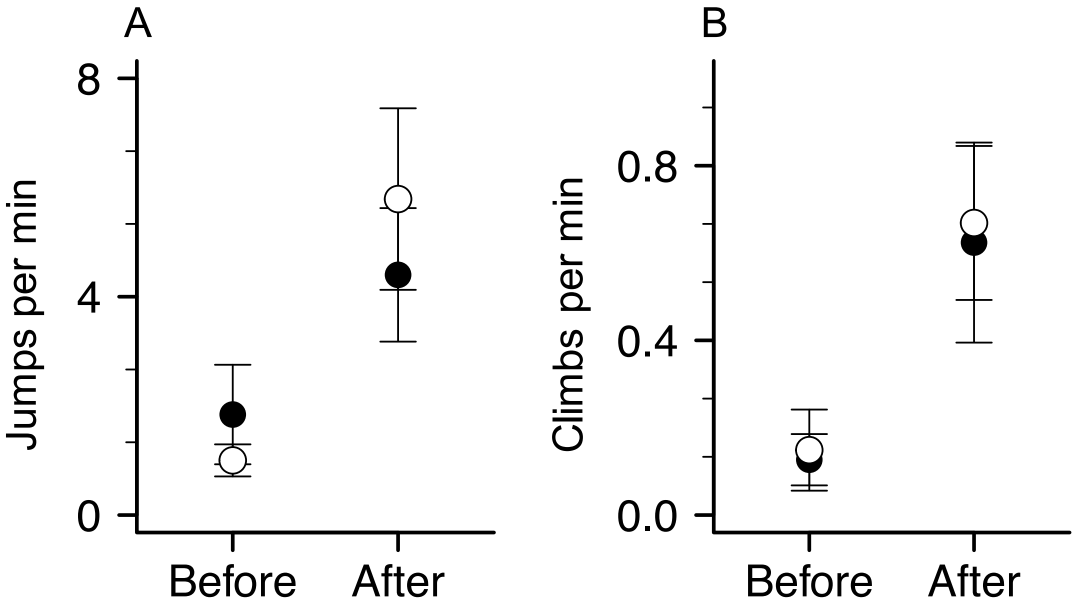
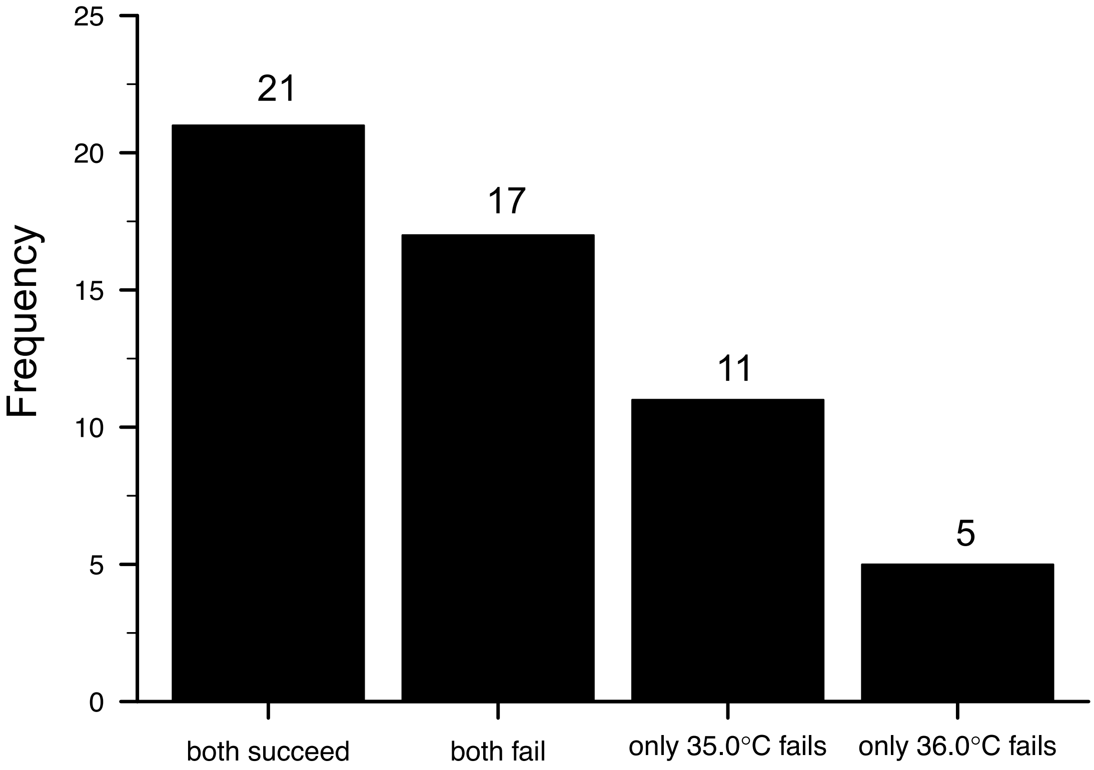

A
8
4
0
Jumps per min
Before
After
B
0.8
0.4
0.0
Climbs per min
Before
After

Figure 6


Frequency
25
20
15
10
5
0
21
17
11
5
both succeed
both fail
only 35.0°C fails
only 36.0°C fails

CHAPTER 4: Incubation temperature influences the behavioral traits of a young precocial bird

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ABSTRACT

The environment in which animals develop can have important consequences for their phenotype. In reptiles, incubation temperature is a critical aspect of the early developmental environment. Incubation temperature influences morphology, physiology, and behavior of non-avian reptiles, however, little is known about how incubation temperature influences offspring phenotype and behaviors important to avian survival. To investigate whether incubation temperature influences avian behaviors, we collected wood duck (*Aix sponsa*) eggs from the field and incubated them at three naturally occurring incubation temperatures (35.0, 35.8, 37.0°C). We conducted multiple repeated behavioral trials on individual ducklings between 5-15 days post-hatch to assess activity, exploratory, and boldness behaviors, classified along a proactive/reactive continuum. We measured growth rates and circulating levels of baseline and stress-induced corticosterone levels to investigate possible physiological correlates of behavior. Ducklings incubated at the lowest temperature displayed more proactive behaviors than those incubated at the two higher temperatures. We also found that younger ducklings exhibited more proactive behavior than older ducklings and males exhibited more proactive behavior than females. Further, duckling behaviors were repeatable across time and contexts, indicative of a proactive-reactive continuum of behavioral tendencies. However, neither corticosterone levels nor growth rates were related to behavior. This provides some of the first evidence that incubation temperature, a critical parental effect, influences avian offspring behaviors that may be important for survival. Our results identify incubation temperature as a mechanism that contributes to the development of behavioral traits and, in part, explains how multiple behavioral types may be maintained within populations.

Keywords: parental effect, behavioral syndrome, *Aix sponsa*, corticosterone, wood duck, early developmental environment, coping style

INTRODUCTION

The environment in which animals develop can lead to a substantial amount of phenotypic variation (Lindström, 1999). This developmental plasticity can be adaptive if the effect of the environment on a trait increases offspring fitness. For example, temperature-dependent sex determination in reptiles can be adaptive because the temperature that produces each sex results in the maximum fitness outcome for that sex (Warner and Shine, 2008a), and because parents can manipulate sex ratios to match environmental conditions (Trivers and Willard, 1973; Warner and Shine, 2007, 2008b). However, developmental plasticity can also negatively affect offspring fitness if poor developmental conditions result in suboptimal phenotypes (Stearns, 1989; Monaghan, 2008; DuRant et al. 2013a; Nettle and Bateson, 2015). For example, offspring that develop in polluted habitats often have suboptimal phenotypes compared those that develop in unpolluted habitats (Nagle et al., 2001; Hopkins et al., 2004; Eisenreich et al., 2009). Understanding how the early developmental environment influences offspring phenotype is thus important for understanding parental effects, non-genetic sources of trait variation within populations, epigenetics, and the impacts of human-induced environmental change.

In reptiles, a crucial aspect of the early developmental environment is the temperature at which eggs are incubated (Deeming and Ferguson, 1991). In non-avian reptiles, parents can influence the incubation temperature of their clutches based on nesting location (Shine and Harlow, 1996). Subsequent to nest site selection and egg deposition, incubation temperature is then largely determined by the environment and thus, can fluctuate considerably among and within nests (Thompson et al., 2018). This is important because incubation temperature can affect sex, morphology, physiology, and behavior of reptilian offspring (Deeming and Ferguson, 1991; Sakata and Crews, 2003, 2004; Deeming 2004; Booth 2006; Putz and Crews, 2006; Amiel and Shine, 2012). In contrast, avian parents actively regulate incubation, which is thought to make developmental temperatures more consistent than those of many non-avian reptiles. However, avian incubation requires considerable time and energy (Tinbergen and Williams, 2002) and thus, parents must allocate time and energy between incubation and self-maintenance. This trade-off can be influenced by variation in environmental conditions, clutch size, and nest insulation (Mainwaring et al., 2014; Deeming and Mainwaring, 2015; Reid et al., 2000a, b), which can lead to variation in incubation temperature among and within nests (Reid et al., 2000a; Hepp et al., 2006; Boulton and Cassey, 2012; Coe et al., 2015; Hope et al., 2018). As with non-avian reptiles, small differences in average incubation temperature have major impacts on avian phenotype and fitness (wood ducks: DuRant et al., 2010, 2011, 2012a; b, 2013a, 2014, 2016, 2016; Hepp and Kennamer, 2012; Hepp et al., 2015; blue tits: Nord and Nilsson, 2011; zebra finches: Berntsen and Bech, 2016). Unlike most of their non-avian counterparts, however, parental behavior, including elaborate nest construction and time spent incubating, provides a mechanism for avian parents to buffer their developing embryos from fluctuating environmental conditions, or to actively manipulate offspring phenotype during the course of incubation.

Although considerable research has demonstrated the influence of developmental temperatures on morphology, performance, physiology, and sex ratios of both avian and non-avian reptiles (Deeming and Ferguson, 1991; DuRant et al. 2013b), comparatively less is known about how these temperatures affect avian offspring behaviors. Across taxa, animals possess suites of correlated behaviors that are consistent across time and contexts, often called 'personalities', 'coping styles', or 'behavioral syndromes' (Koolhaas et al., 1999; Gosling, 2001; Sih et al., 2004). These suites of behaviors are commonly classified using a continuum from 'proactive', 'fast', or 'bold', to 'reactive', 'slow', or 'shy' (Koolhaas et al., 1999; Groothuis and Carere, 2005). Proactive individuals quickly and incompletely explore novel environments, actively seek out others (in social species), are less responsive to environmental changes, and are risk-prone (Koolhaas et al., 1999). In contrast, reactive individuals explore their environment slowly and thoroughly, are more sedentary, are flexible and responsive to environmental changes, and are risk-averse (Koolhaas et al., 1999). A major question in animal behavior is how these behavioral types, and the associated lack of behavioral plasticity within individuals, are maintained within populations. Recent studies have shown that suites of behavioral traits are heritable (great tits: Dingemanse et al., 2002; Groothuis and Carere, 2005; bluefin killifish: McGhee and Travis, 2013; house mice: Benus et al., 1991; Japanese quail: Mills and Faure 1991; domestic chicken: Komai et al., 1959) and may be maintained by fluctuating selection (Dingemanse and Réale, 2005) because behaviors can affect fitness differently depending on the environmental context (Dall et al., 2004; Dingemanse et al., 2004; Biro and Stamps, 2008; Smith and Blumstein, 2008; Nicolaus et al., 2012). For example, proactive brown trout (*Salmo trutta*) have higher growth rates in simple habitats, but slower growth rates in complex habitats, compared to reactive individuals (Hojesjo et al., 2004). Further, proactive/reactive behavioral traits may be mechanistically linked to physiological traits that consistently differ among individuals (Wolf and Weissing, 2010). Indeed, glucocorticoid levels, metabolic rate, and growth rate may be related to behavioral traits (Koolhaas et al., 1999; Cockrem, 2007; Stamps, 2007; Careau et al., 2008; Biro and Stamps, 2010). However, despite the importance of behavioral traits along a proactive/reactive continuum, their non-genetic determinants are poorly studied (Stamps and Groothuis, 2010a; b; Groothuis and Trillmich, 2011). Understanding how conditions during development may influence suites of proactive/reactive behaviors may reveal a heretofore unknown mechanism by which consistent differences in behavior are maintained within animal populations.

To investigate whether incubation temperature influences suites of avian offspring behaviors, as well as a potential hormonal mechanism, we conducted an experiment using wood ducks (*Aix sponsa*). We incubated eggs at three different mean temperatures within the natural range of incubation temperatures and assayed behaviors that can be classified along a proactive/reactive continuum multiple times in four different contexts, between 5 and 15 d after hatching. We also measured baseline and stress-induced corticosterone levels as a potential hormonal mediator, and growth rate, as a possible correlate of behavior. We hypothesized that incubation temperature would influence proactive/reactive behaviors for three reasons. First, other avian parental effects, such as hormone deposition to the egg, influence behavioral traits in birds (Forstmeier et al., 2004; Daisley et al., 2005). Second, incubation temperature is known to influence boldness and fear-related behaviors in non-avian reptiles (Trnik et al., 2011; Siviter et al., 2017), and we suspect a similar effect exists for birds. Third, incubation temperature influences avian corticosterone levels (DuRant et al., 2010; Wada et al., 2015), growth rates (DuRant et al., 2010), and metabolic rates (Nord and Nilsson, 2011; DuRant et al., 2012b), all of which are related to proactive/reactive behavioral traits in other species (Carere et al., 2003; Cockrem, 2007; Stamps, 2007; Careau et al., 2008; Biro and Stamps, 2010; Stöwe et al., 2010).

METHODS

Study species

The wood duck is a common North American dabbling duck that nests in tree cavities and nest boxes in or near wooded bodies of water (Hepp et al., 1987; Hepp and Bellrose, 2013). Their breeding season extends from mid-February to mid-July (Hepp and Bellrose, 2013). Females typically lay one egg per day to produce an average clutch size of 12 (Bellrose and Holm, 1994). However, clutches may reach >40 eggs (Morse and Wight, 1969) due to conspecific brood parasitism in populations that nest in nest boxes (Semel and Sherman, 1986; Semel et al., 1988) and natural cavities (Roy Nielsen et al., 2006). Females are solely responsible for incubation (Hepp and Bellrose, 2013) and spend most of the day incubating, but usually take two 1-2 h recesses each day to forage (Manlove and Hepp, 2000). However, incubation temperature varies among and within populations (Bellrose and Holm, 1994; Manlove and Hepp, 2000; Hepp et al., 2006), and within nests (Hope et al., 2018).

Hatching is usually synchronous and complete within 24 h (Bellrose and Holm, 1994), although large clutches can experience up to 3 d of developmental asynchrony (Kennamer et al., 1990) possibly due to differences in incubation temperature within nests. Because of hatching asynchrony, along with high duckling mortality during the first two weeks of life, small broods (as small as 1-3 ducklings) can occur in the wild. Offspring are precocial and can feed themselves once they leave nests, but females stay with broods to protect them from predators and guide them to sources of food (Bellrose and Holm, 1994). Ducklings are highly social and the hen-brood bond in the southern portion of the breeding range lasts about 5 weeks, but ducklings are known to survive alone in the wild (Bellrose and Holm, 1994). Ducklings spend much of their time in the water, but commonly warm themselves on land when the water is cold, and many make long journeys on land when initially dispersing from the nest site (Bellrose and Holm, 1994). Because ducklings are faced with many predators, it is common for them to become separated from their mother and brood when fleeing from a predator or other disturbance. When this happens, even for a short amount of time, behavior can be crucial for duckling survival. Duckling behavior is especially crucial when they are very young because almost 50% of ducklings die before fledging, and 90% of those mortalities occur within the first two weeks of life (McGilvrey, 1969). Especially relevant to this work, female ducklings hatching from eggs artificially incubated at low temperatures experienced lower apparent survival to recruitment into the breeding population than ducklings incubated as eggs at higher temperatures (Hepp and Kennamer 2012), which we hypothesize may be due in part to differences in behavior.

Egg collection and incubation

We collected eggs from 6 – 29 March 2015 from a population of wood ducks on the Department of Energy's Savannah River Site (SRS) in South Carolina (33.1°N, 81.3°W; elevation: 157 m) on a series of 12 ephemeral wetlands, which have nest boxes that have been maintained for >30 years. We checked nest boxes daily to mark eggs for lay date, collected eggs from 42 nests (avg ± SD: 6.2 ± 2.4 eggs from each nest; range: 1-11 eggs), and replaced eggs with wooden eggs to prevent hens from abandoning the nest (Hepp et al. 1987). All eggs were collected before the hen began to incubate. Eggs were transported at room temperature to

Virginia Tech, rotated twice daily, and incubated within 10 days (Walls et al., 2011). Eggs were incubated in Grumbach incubators (model BSS 420, Asslar, Germany) at three different mean temperatures within the natural range (35.0, 35.8, and 37.0°C; DuRant et al., 2013b), with eggs from the same nest and the same lay date distributed among treatments. Incubators had two 75 min cool-down periods at 8:15 and 18:30 h daily to simulate hens leaving the nest for foraging (Manlove and Hepp, 2000), but incubators maintained the above mentioned overall mean temperatures. The average humidity for all incubators was kept between 60-65%. Overall hatch success was 70% (hatch success by temperature treatment: 35.0°C: 48%, 35.8°C: 80%, and 37.0°C: 83%) and incubation periods were 39 ± 2 d (35.0°C), 36 ± 1 d (35.8°C), 33 ± 1 d (37.0°C), which are within the natural range for wood ducks (Hepp et al., 2006). For this study, we used 114 ducklings from 36 nests. Of these ducklings, 34 were incubated at 35.0°C (18 female and 16 male), 43 were incubated at 35.8°C (17 female and 26 male), and 37 were incubated at 37.0°C (17 female and 20 male).

General husbandry

Once hatched, ducklings were weighed and color-banded. Because we collected and incubated eggs over the course of one month, hatching was staggered. Duckling body masses at hatch were 28.0 ± 3.0 g (35.0°C), 28.2 ± 2.5 g (35.8°C), and 28.8 ± 2.7 g (37.0°C). We placed newly hatched ducklings together, as they hatched (groups of ~2-10), in a covered cage with a 50W infrared heat lamp. When ducklings were at least 7 h old and fully mobile, they performed a trial that tested their performance at exiting a nest box (Hope et al., in prep) and once complete, were assigned to a cage. Plastic cages (46 x 32 x 24.5 cm) were set up in a rack system in an indoor hallway that separates two rows of aviaries. A heat lamp (50W) was suspended 32.5 cm above each cage, heating the cages and providing a thermal gradient within cages of ~30-35°C. Because cages were located in an aviary hallway that received natural light, the photoperiod mimicked ambient conditions (approximate photo period during study = 13:11 day:night). Cages were cleaned daily, and *ad lib* food and water were provided.

The nature of our gradual egg collection prevented synchronous hatching of large numbers of ducklings and made it impossible to construct large broods of similarly-aged and incubated ducklings. Therefore, ducklings were housed in groups of two or three with others from the same incubation temperature. Although these group sizes are smaller than some natural broods, our approach still allowed ducklings to have social interactions with brood mates. Because all ducklings experienced similar housing conditions, any effect of rearing them in small broods was equivalent across the three treatment groups.

Ducklings underwent a series of behavioral trials and blood sampling (Fig. 1) between 19 April – 23 May 2015. We chose to conduct trials in the same order for each duckling to keep any effect of one trial on the behavior in a subsequent trial consistent among individuals (similar to many other behavioral studies; e.g., van Oers et al., 2004; Reyes-Meza et al., 2011; Butler et al., 2012; Pittet et al., 2012). Body mass and tarsus length were measured on 6, 9, 12, and 15 days post-hatch (dph). Ducklings were humanely euthanized on 15 dph via carbon dioxide asphyxiation and cervical dislocation, and sex was determined by examining external (i.e., the presence or absence of a penis) and internal genitalia. All procedures were approved by Virginia Tech IACUC.

Behavioral trials

Isolation Trials

The behavior of social species while alone in a familiar environment is commonly used to quantify social behavior (Bertin and Richard-Yris, 2005; Formanek et al., 2008; Pittet et al., 2014b). More movement and vocalization indicates a more active response to social isolation and thus, a more proactive style of seeking out others (Koolhaas et al., 1999). On 5 and 12 dph, each duckling was individually placed in a cage (46 x 32 x 24.5 cm) identical to the cage within which the ducklings were communally housed, and thus was a familiar environment, but was visually and acoustically isolated from the other ducklings. The bottom of the cage had gridlines, creating 5 x 5 cm squares, so movement could be recorded. Trials were conducted from 5:56-10:03 h and lasted 3 min, similar to the timeframe given in other studies (Bertin and Richard-Yris, 2005; Formanek et al., 2008; Pittet et al., 2014b). The temperature of the room was 22.6 ± 1.7°C (mean ± SD).

Through video analysis, we measured the number of unique and non-unique grid-system squares that the duckling occupied or traversed, the number of jumps, the amount of time spent vocalizing, and the latency to their first step and first vocalization. If they did not move or vocalize, which happened in 18 trials (8%), that behavior was given a latency of 3 min.

Open Field Trials

The Open Field trial is a common way to measure exploratory behavior by quantifying the behavior of an animal in an unfamiliar open environment, where more movement and vocalization indicates more proactive behavior (Bertin and Richard-Yris, 2005; Daisley et al., 2005; Formanek et al., 2008; Pittet et al., 2014b). On 6 and 13 dph, each duckling was individually placed in an empty outdoor aviary room (5.5 x 2.5 m) with a grid system on the floor forming 0.25 x 0.25 m squares. The room was visually isolated from outside disturbances.

Each duckling was placed under a large bucket and was allowed to acclimate for 2 min (Butler et al., 2012). Then, the bucket was lifted remotely by a string and pulley system so the duckling could explore the room. A GoPro camera was used to videotape the entire arena and the trial lasted for 12 min, which is the same amount of time (Daisley et al., 2005) or 7 min longer than the timeframe given for this trial in other studies (Bertin and Richard-Yris, 2005; Formanek et al., 2008; Pittet et al., 2014b). Trials were conducted from 6:38-16:12 h. The temperature of the room was 16.9 ± 4.7°C (mean ± SD).

Through video analysis, we measured the number of unique and non-unique grid system squares that the duckling occupied or traversed, the number of jumps, the amount of time spent vocalizing, and the latency to their first step and first vocalization. If they did not move or vocalize, which happened in 14 trials (6%), that behavior was given a latency of 12 min.

Emergence Trials

The Emergence trial is a common trial used to quantify boldness, where proactive individuals are more likely to emerge quickly from a sheltered area into an unknown environment than reactive individuals (Formanek et al., 2008; Pittet et al., 2012, 2014b). On 8 and 15 dph, each duckling was placed in a wooden box (0.5 x 0.5 x 0.5 m) and given 1 min to acclimate (Pittet et al., 2012, 2014b). The door to the box was lifted remotely by a string and the duckling was allowed 10 min to emerge, which is 7 min greater, and thus more conservative, than the timeframe given in similar studies (Formanek et al., 2008; Pittet et al., 2012, 2014b). On 8 dph, the setup of this trial was combined with the Maze trial (see below), but the two trials were analyzed separately. From video analysis, we measured the time it took for each duckling's feet to cross over a line that was drawn across the door opening of the box. If they did not emerge, which happened in 80 trials (37%), they were given a time of 10 min. Trials were conducted from 6:23-19:38 h. The temperature of the room was 16.8 ± 3.9°C (mean ± SD).

Maze Trials

The Maze trial measured the motivation to explore an unknown environment to reach a social stimulus (Formanek et al., 2008; Pittet et al., 2012, 2014a; b). Proactive individuals should be more likely to explore unknown areas to actively seek out conspecifics than reactive individuals (Koolhaas et al., 1999). On 8 dph, the Maze trial began once the duckling emerged from the Emergence trial (see above) into a wooden enclosure (2.0 x 0.5 x 0.5 m) with lines drawn on the bottom every 10 cm and three small wooden walls inside, on alternating sides and perpendicular to the emergence path, to create a simple maze. A piece of Plexiglas was placed on top of the enclosure so the duckling could not jump out. Once the duckling emerged, we played a continuous recording of a wood duck duckling vocalization from a speaker placed outside of the enclosure, behind the final (fourth) wall, as a social stimulus. The duckling vocalization MP3 was downloaded from YouTube (https://www.youtube.com/watch?v=XhTEk31kzuI) and modified using Audacity® version 2.1.2 (Audacity Team, 2015). If the duckling did not emerge from the Emergence trial within 10 min (the time limit for the Emergence trial), a small wooden plank was remotely lowered behind the duckling to gently move the duckling out of the box and into the Maze trial arena. The trial lasted for 10 min, which is 5 min longer than the time limit used in similar studies (Formanek et al., 2008; Pittet et al., 2014a; b), and trials were conducted from 7:10-19:38 h. The temperature of the room was 17.0 ± 4.9°C (mean ± SD).

Through video analysis, we measured the number of lines crossed going toward and away from the duckling recording, the number of jumps, the farthest line crossed, the latency to pass the third wall, and the latency to vocalize. If they did not pass the third wall or vocalize, which happened in 32 trials (28%), that behavior was given a latency of 10 min.

Blood sampling

Sample Collection

On 4 and 11 dph, we collected a blood sample from the femoral vein of each duckling within 3 min of removal from their cage to measure baseline corticosterone. Then, another blood sample was taken after the duckling was held in a cloth bag for 30 min, to measure stress-induced hormone levels. Blood samples were kept on ice until they were centrifuged (within 3 h) at 3.5*g*. The plasma was separated and stored at -80°C until analysis. To control for circadian variation in circulating hormone levels, all samples were taken between 12:00-13:35 h.

Radioimmunoassay

We determined corticosterone concentrations of each sample (mean plasma volume = 31 μl) in three radioimmunoassays (RIA), using the protocol of DuRant et al. (2010). Samples were run singly to increase the probability of detection. We determined the individual extraction efficiency of each sample and corrected for it in final calculations. The mean extraction efficiency was 72.4% (range: 70.7 – 74.0%). Intra-assay variation averaged 15.4% (range: 10.3 – 18.1%) and inter-assay variation was 24.0%. The assay limits of detection ranged between 0.68 - 1.74 ng/ml.

Statistical Analyses

Effects of incubation temperature

Data from one duckling from the Isolation trial, four ducklings from the Open Field trial, and ten ducklings from the Emergence trial were excluded from analyses due to technical malfunctions with video or audio equipment. All analyses were performed in R version 3.3.1 (R Core Team, 2016). Because we measured multiple behavioral parameters on each individual duckling, we condensed these non-independent measures from all trials into a single value using a z-score analysis, as described in Guilloux et al. (2011) and Labots et al. (2018). This statistical technique has been used in studies to measure emotionality in mice from multiple trials (e.g., Mendez-David et al., 2015; Piantadosi et al., 2016; Shepard et al., 2016; Zemdegs et al., 2016). It is an improved way to quantify behavior when using multiple trials because it reduces variance and increases reliability of behavioral measures, compared to using the raw behavioral data (Guilloux et al., 2011). We calculated a z-score for each behavior (e.g., number of steps, latency to call) in each trial by subtracting the value from the mean of all individuals for that behavior and dividing by the standard deviation. Z-scores were averaged within each trial, and then z-scores from each trial were averaged to create two z-scores per individual. The first Isolation, Open Field, Maze, and Emergence trials were averaged for each individual as its 'younger' z-score (5-8 dph) and the 'older' z-score (12-15 dph) was the average of the second Isolation, Open Field, and Emergence trials. In all cases, a larger z-score indicated a more proactive behavior (i.e., shorter latency to move, more steps taken, etc.) than a smaller z-score. Z-scores for ducklings where data from certain trials were excluded were calculated using the remaining data.

We used these z-scores as the response variable in a linear mixed effects model (*lmerTest*; Kuznetsova et al., 2016), with incubation temperature, age (younger or older), and their interaction as the predictors, body condition (residuals of mass vs. tarsus regression), sex, and lay date as covariates, and nest ID ($N = 36$ nests) and duckling ID as random factors. Normal quantile plots of residuals, histograms of residuals, and predicted vs. residuals plots were used to ensure that the model met the assumptions of normal and homoscedastic residuals. We used backward elimination (*step*) for non-significant terms, and only values from the final model are reported. We used post-hoc analyses (Tukey test; *lsmeans*; Lenth, 2016) to determine which means differed.

Consistency across time and contexts

To determine if ducklings exhibited consistent behavior across time and contexts, we calculated repeatability using the mean squares of ANOVAs, as described in Lessells and Boag (1987). First, to examine consistency across time, we calculated the repeatability between trials that were repeated twice (i.e., Isolation, Open Field, and Emergence). To examine consistency across contexts, we calculated the repeatability among behaviors in all 7 trials. The ANOVAs included the overall z-scores for the trials as the dependent variable and duckling ID as the predictor. To calculate confidence intervals, we followed the methods of Wolak et al. (2012).

Physiological correlates

To determine whether corticosterone levels and growth rates were related to incubation temperature and age, we used three linear mixed effects models (*lmerTest*) with baseline corticosterone, stress-induced corticosterone, and growth rate as the response variables. All corticosterone levels were log-transformed to meet the model assumptions of normal and homoscedastic residuals. Incubation temperature, age, and their interaction were the predictors and nest and duckling ID were random factors. Growth rate was calculated as the difference in mass between the target day (e.g., 6 dph) and the hatch day, divided by the total number of days (e.g., 6 days).

To determine if corticosterone levels ($N = 420$ samples from 114 ducklings) were related to behaviors, we used Spearman's rank correlations (*cor.test*) to relate baseline corticosterone levels and stress-induced corticosterone levels to the two z-scores for each individual. Separate correlations were performed for 'younger' and 'older' z-scores. Corticosterone levels measured on 4 dph were compared to the 'younger' z-scores and levels measured on 11 dph were compared to the 'older' z-scores. Baseline samples ($N = 35$) that were not obtained within 3 min were excluded from this analysis.

To determine if growth rates were related to behaviors, we also used Spearman's rank correlations (*cor.test*). The growth rates to 6 dph were compared to the 'younger' z-scores and the growth rate to 12 dph were compared to the 'older' z-scores.

RESULTS

Effects on behavior

Incubation temperature, age, and sex influenced the behavior of ducklings. Duckling behaviors were dispersed across a continuum, with higher z-scores indicating more proactive behaviors than lower z-scores. Incubation temperature affected duckling behavior ($F_{2, 206} = 9.6$, $p < 0.0001$; Fig. 2A) and a Tukey test revealed that ducklings that hatched from eggs incubated at the lowest temperature were more proactive than those incubated at the two higher temperatures, but those incubated at the higher temperatures did not differ from each other. Further, younger ducklings exhibited more proactive behavior than older ducklings ($F_{1, 184} = 16.1$, $p < 0.0001$; Fig. 2B), and males exhibited more proactive behavior than females ($F_{1, 217} = 7.6$, $p = 0.006$; Fig. 2C).

Importantly, sexes were distributed across incubation temperature treatments, so these predictors were not confounded with each other.

Consistency across time and contexts

Individual duckling behavior was repeatable across time and contexts. No confidence intervals overlapped zero, although repeatability differed across trials (Table 1). These repeatabilities are within the range of those reported in other behavioral studies (Dingemanse, 2002; Bell et al., 2009).

Physiological correlates

Growth rate was related to both incubation temperature and age, but both baseline and stress-induced corticosterone levels were only related to age. Duckling growth rate increased as both incubation temperature and age increased (Incubation temperature: $F_{2, 293} = 5.15$, $p = 0.006$,; Age: $F_{1, 337} = 907$, $p < 0.0001$; Incubation temperature X Age: $F_{2, 337} = 2.11$, $p = 0.12$; Fig. 3). Ducklings had lower baseline and stress-induced corticosterone levels on 11 dph than on 4 dph (Baseline: Incubation temperature: $F_{2, 101} = 0.81$, $p = 0.45$; Age: $F_{1, 98} = 27.9$, $p < 0.0001$; Incubation temperature X Age: $F_{2, 98} = 0.26$, $p = 0.77$; Stress-induced: Incubation temperature: $F_{2, 93} = 0.96$, $p = 0.38$; Age: $F_{1, 109} = 23.4$, $p < 0.0001$; Incubation temperature X Age: $F_{2, 109} = 0.76$, $p = 0.47$; Table 2). Behavioral z-scores were not related to corticosterone or growth rate in any case (all $p \geq 0.14$).

DISCUSSION

In this study, we demonstrated that incubation temperature, a critical parental effect in birds, influenced suites of behaviors that may be important for the early survival of wood duck ducklings. Ducklings incubated at the lowest temperature displayed more proactive behavior than those incubated at the two higher temperatures. These effects on behavior are especially important for ducklings in their first two weeks of life because this is the age where individuals have their highest mortality risk. Because we reared ducklings in small broods and then tested behavior of ducklings individually, our results are particularly applicable to instances when a duckling temporarily or permanently becomes separated from its mother and/or brood, which is a time when behaviors related to survival are particularly crucial. Further, we found evidence that duckling behaviors were consistent in different contexts. Although ducklings became more reactive as they grew older, the consistent differences that we found *among* individuals are indicative of behavioral types (Dingemanse et al., 2010). Thus, our study identifies incubation temperature as a parental effect that contributes to differences in behavioral traits and, in part, explains how multiple behavioral types may be maintained within populations.

The effect of incubation temperature on proactive/reactive behavioral traits has important implications for avian populations. First, unlike most of their reptilian counterparts, avian parents actively regulate incubation temperature. Thus, any factor that influences parental incubation or nest-building behavior, including for example, weather (Conway and Martin, 2000b; Coe et al., 2015), predation (Conway and Martin, 2000a), pollution (Fisher et al., 2006), and available nesting materials (Mainwaring et al., 2014) has the potential to influence egg temperature and thus, the development of individual behavioral types within a population. Further, much like other reptiles, there is some evidence that incubation temperatures vary consistently *within* the nests of some avian species, including wood ducks (Beatty 2015; Hope et al., 2018), suggesting that broods in the wild likely contain individuals exposed to thermal conditions that promote the development of different behavioral traits. This variation in proactive/reactive behavior within broods may be beneficial for the parent's reproductive success in unpredictable or shifting environments because the fitness of individuals displaying different behavioral traits often varies depending on the environmental context (Dall et al., 2004; Dingemanse et al., 2004; Biro and Stamps, 2008; Smith and Blumstein, 2008; Nicolaus et al., 2012). Additionally, because proactive/reactive behaviors can influence competitive ability (Ward et al., 2004; Cole and Quinn, 2012), individual differences in behavioral tendencies within broods may influence within-brood social interactions.

Since consistent differences in behavior among individuals may result from consistent differences in physiology (Wolf and Weissing, 2010), we investigated corticosterone levels and growth rates as possible physiological mediators of variation in behavior. Previous studies have found mixed evidence for the relationship between corticosterone levels and behavioral traits (Jones et al., 1994; Koolhaas et al., 1999; Carere et al., 2003; Mignon-Grasteau et al., 2003; Martins et al., 2007; Thomson et al., 2011; Baugh et al., 2013; Grace and Anderson, 2014), and between growth rate and behavior (Clobert et al., 2000; Vøllestad and Quinn, 2003; Adriaenssens and Johnsson, 2011; Biro et al., 2014). In our study, neither circulating corticosterone levels nor growth rates were related to any behavior, suggesting that incubation temperature is exerting an effect on behavior independently of these physiological factors. More work is needed to determine if other potential physiological correlates, such as metabolic rate (Careau et al., 2008; Biro and Stamps, 2010), other hormones (e.g., vasopressin and oxytocin, Kelly and Goodson, 2014; thyroid hormone, Fundaro, 1989; Sala-Roca et al., 2002; Pilhatsch et al., 2010) or other components of the neuroendocrine system (e.g., hormone receptors, size of brain regions, Duckworth, 2015; number and density of neurons, Amiel et al., 2017) underlie differences in behavioral traits.

The observed differences in behavior may be associated with other known effects of incubation temperature on physiology and performance. For example, ducklings incubated at a low temperature (35ºC) have greater energy expenditure while in the egg (DuRant et al., 2011), lower immune function (DuRant et al., 2012a), higher metabolic rates when faced with a thermoregulatory challenge (DuRant et al., 2012b), decreased locomotor performance (Hopkins et al., 2011), reduced thermoregulatory ability (DuRant et al., 2013a), and altered thyroid hormone levels (DuRant et al., 2014) compared to those incubated at higher temperatures (~36-37ºC). The proactive behaviors that we observed in ducklings incubated at the low temperature may be a way for these ducklings to behaviorally compensate for physiological and performance deficits. Indeed, proactivity may be beneficial for seeking out food (Biro and Stamps, 2008; Luttbeg and Sih, 2010), or seeking out a duckling's mother and broodmates (Koolhaas et al., 1999; Aplin et al., 2013; Snijders et al., 2014) to gain needed care, protection, warmth, and cues to optimize foraging success. Yet, the lower apparent survival of ducklings incubated at low temperatures in the wild (Hepp and Kennamer, 2012) suggests that behavior does not fully compensate for physiology. Alternatively, if proactive behaviors are not beneficial to ducklings incubated at low temperatures, then the effects of incubation temperature on behavioral traits may represent another negative consequence of suboptimal incubation. To determine whether the developmental plasticity of proactive/reactive behavior is adaptive or not, more studies are needed to investigate the fitness of individuals displaying proactive and reactive behaviors in different natural environments.

In addition to the possibility of allowing for behavioral compensation, the developmental plasticity of proactive/reactive behavior might also be beneficial if it results in duckling behaviors that are matched to current environmental conditions (Monaghan, 2008). When environmental conditions are poor (e.g., food is scarce), incubating parents may need to spend more time foraging and less time incubating, resulting in a lower incubation temperature (e.g., Coe et al., 2015). Thus, ducklings incubated at low temperatures may benefit from exhibiting proactive behaviors to seek out food in resource scarce conditions (Biro and Stamps, 2008). In contrast, ducklings incubated at warmer temperatures, and thus likely living in resource-rich environments, may not require high activity levels to gain adequate amounts of food, and may instead benefit most by being reactive to avoid predators, or to invest resources in growth rather than activity levels (Vøllestad and Quinn, 2003; Wolf et al., 2007; Careau et al., 2008; Luttbeg and Sih, 2010; Adriaenssens and Johnsson, 2011).

Duckling age and sex also were related to behavioral traits, regardless of incubation temperature. Ducklings displayed more reactive behaviors as they grew older. It is possible that younger ducklings, similar to ducklings incubated at a low temperature, may need to be more proactive to behaviorally compensate for low energy reserves and weak thermoregulatory abilities. Additionally, because all older ducklings had already experienced each trial, it is possible that behavioral changes could have been a result of experience or habituation. Further, females displayed more reactive behaviors than males, which was surprising because ducklings do not show other sexual dimorphisms at this age. This difference might be expected in adult wood ducks because males may need to be more proactive to actively seek and compete for mates, while females may benefit from being reactive to hide from predators and save energy reserves for reproduction. It would be interesting to investigate whether behavioral differences between male and female ducklings are consistent until adulthood, and whether behavioral tendencies of ducks while they are juveniles ultimately benefit their survival and reproductive success as adults.

Conclusions

In this study, we demonstrated that incubation temperature is a non-genetic mechanism that influences the development of proactive/reactive behavioral types in young birds. A large question in animal behavior is how consistent individual differences in behavior can persist within populations, and our results show that variation in the developmental environment may be one such reason. Because incubation temperature varies seasonally as well as among and within nests, this has major implications for how individuals with different behavioral traits may be distributed spatially and temporally within the population, and among and within broods. We suggest that the developmental plasticity of proactive/reactive behavior may be adaptive if it allows ducklings to behaviorally compensate for physiological and performance deficits, or matches offspring behavior with the current environment. Alternatively, trait differences due to this developmental plasticity may be an additional negative consequence of suboptimal incubation. Further, because incubation temperature is an avian parental effect, our work shows how slight differences in investment in, and efficiency of, parental care can influence the behavior of offspring. It also suggests that changes in the environment that influence parental behavior, such as human disturbances (Carney and Sydeman, 1999), pollution (Fisher et al., 2006), and weather (Conway and Martin, 2000b; Coe et al., 2015), could also influence suites of behavioral traits in avian offspring.

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FIGURE LEGENDS

Figure 1. Timeline of trials and sampling.

Figure 2. Proactive/reactive behaviors differed among wood duck ducklings incubated at different temperatures (A), changed as ducklings grew older (B), and differed between sexes (C). Data are expressed a z-scores, which are mean-standardized values. We calculated a z-score for each behavior (e.g., number of steps, latency to call) in each trial by subtracting the value from the mean of all individuals for that behavior and dividing by the standard deviation. Z-scores were averaged within each trial, and then z-scores from each trial were averaged to create two z-scores per individual: one from behavioral trials conducted from 5-8 days post-hatch (dph) and the other from trials conducted from 12-15 dph. The LS-means ± SE from a linear mixed effects model are plotted, and thus values are corrected for all covariates and random effects retained in the final model.

Figure 3. Growth rate (g d^{-1}; mean ± SE) increased with duckling age (days post-hatch) and incubation temperature. Different types of points indicate incubation temperature treatment. Closed circles = 35°C, $N = 34$; open circles = 35.8°C, $N = 43$; squares = 37°C, $N = 37$. Growth rate was calculated as the difference in mass between the target day and the hatch day, divided by the total number of days. Duckling ID was included as a random effect in the model.

TABLES

Table 1. Repeatability across time and contexts

Comparison	Repeatability (r)	Confidence interval	*F*	df	*P*
Isolation 1 & 2	0.244	0.063-0.409	1.645	112, 113	0.004
Open Field 1 & 2	0.269	0.087-0.433	1.735	109, 110	0.002
Emergence 1 & 2	0.467	0.303-0.605	2.756	103, 104	<0.0001
All trials	0.265	0.195-0.349	3.482	113, 669	<0.0001

Table 1. Repeatability of duckling behaviors across time and contexts, calculated using one-way ANOVAs, according to Lessels and Boag (1987). Confidence intervals were calculated following Wolak et al. (2012). The *F*-values, degrees of freedom, and *P*-values reported are from the ANOVAs. The Isolation, Open Field, and Emergence trials were each conducted twice for each duckling. 'All trials' includes seven total trials for each duckling: the repeated Isolation, Open Field, and Emergence trials, plus one Maze trial.

Table 2. Least-squares means of corticosterone levels

Age (dph)	Baseline corticosterone levels (ng ml^{-1})	Stress-induced corticosterone levels (ng ml^{-1})
4	1.31 ± 0.09	2.68 ± 0.09
11	0.71 ± 0.08	2.20 ± 0.09

Table 2. The least-squares means (± SE) for baseline and stress-induced levels of corticosterone for ducklings at 4 and 11 days post-hatch (dph).

FIGURES

Figure 1

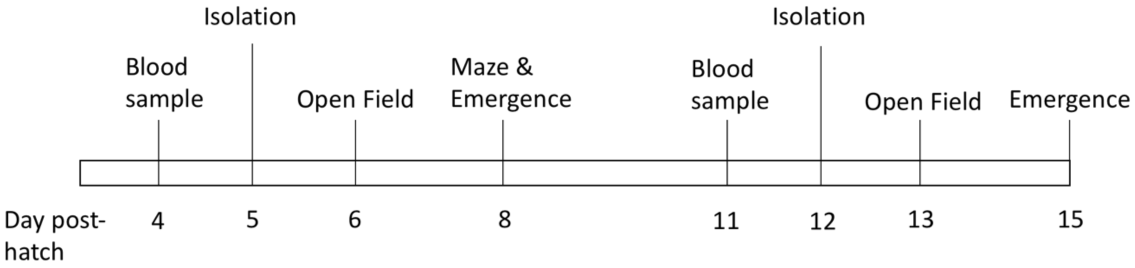


Figure 2

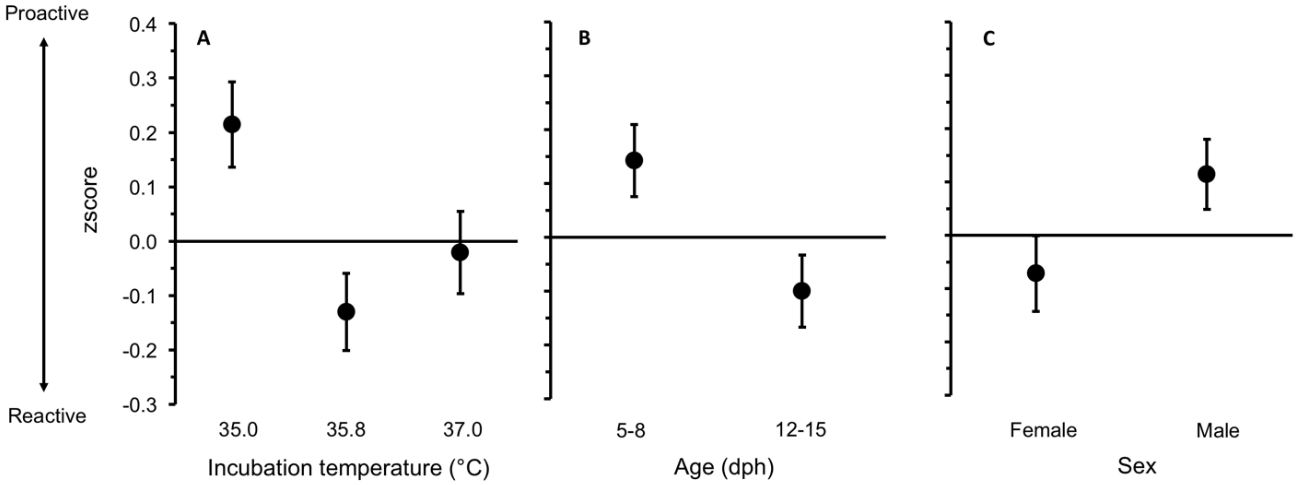
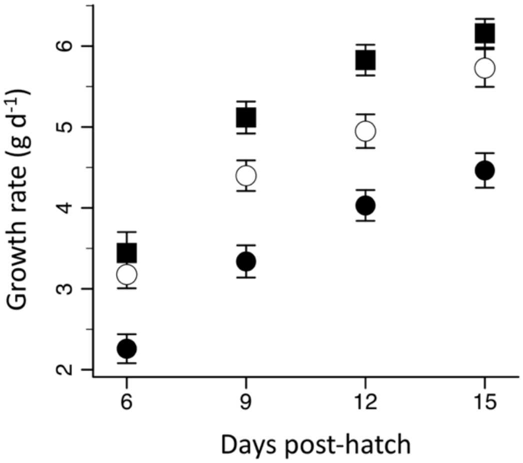

Proactive
Reactive
zscore
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
A
35.0
35.8
37.0
Incubation temperature (°C)
B
5-8
12-15
Age (dph)
C
Female
Male
Sex

Figure 3



CHAPTER 5: Incubation temperature affects duckling body size and food consumption despite no effect on associated feeding behaviors

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ABSTRACT

Developmental conditions can have consequences for offspring fitness. For example, small changes (<1°C) in average avian incubation temperature have large effects on important post-hatch offspring phenotypes. Further, recent research has shown that average incubation temperatures differ among eggs *within* the same nest, to the extent (i.e., >1°C) that differences in offspring phenotypes within broods should result. A potential consequence of within-nest incubation temperature variation is inequality in behaviors that could cause differences in resource acquisition within broods. To investigate this, we incubated wood duck (*Aix sponsa*) eggs at one of two ecologically-relevant incubation temperatures (35 or 36°C), formed mixed-incubation temperature broods after ducklings hatched, and conducted trials to measure duckling behaviors associated with heat (one trial) or food (three trials) acquisition. Contrary to our predictions, we found no effect of incubation temperature on duckling behaviors (e.g., time spent occupying heat source, frequency of feeding bouts). However, we found evidence that ducklings incubated at the higher temperature consumed more food during the 1-hour feeding trials, and grew faster in body mass and structural size (culmen and tarsus) throughout the study, than those incubated at the lower temperature. Apparent food consumption during the 1-hour trials was positively related to culmen length, suggesting that differences in food consumption may be driven by structural size. This could result in positive feedback, which would amplify size differences between offspring incubated at different temperatures. Thus, our study identifies incubation temperature as a previously unrecognized mechanism by which fitness-related phenotypic differences could be amplified within avian broods.

Keywords: *Aix sponsa*, early developmental environment, food consumption, growth, parental effect, sibling interactions

INTRODUCTION

Across taxa, parents can have non-genomic effects on the phenotype and fitness of their offspring. Parental effects such as nest site choice, differential allocation of hormones/nutrients to embryos, food provisioning, and grooming have long-lasting consequences for offspring phenotype (Bernardo, 1996; Lindström, 1999; Mousseau and Fox, 1998). In addition to affecting offspring morphology, physiology, and behavior, parents can also affect offspring through influencing the potential for differential resource acquisition within the brood/litter. For example, if parents distribute resources (e.g., nutrients, hormones) unequally among embryos within the same brood/litter, it can create variation in individual offspring phenotypes, with consequences for offspring ability to acquire additional resources (Correa et al., 2013; Eising and Groothuis, 2003; Müller et al., 2012). Those individuals that are able to maximize their resource acquisition, either from their parent or from the environment, will have an advantage. In turn, this can either amplify or reduce differences in phenotype and survival among offspring within a brood (Drummond et al., 2000; Groothuis et al., 2005; Hofer et al., 2016; Muller and Groothuis, 2013). Understanding how parental effects can influence offspring fitness through influencing differences among siblings is necessary for a comprehensive understanding of the consequences of parental effects.

In birds, one of the most important ways that parents can affect offspring phenotype is through egg incubation temperature (Deeming and Ferguson, 1991; Hepp et al., 2015). Incubation is an energetically costly and time-consuming aspect of parental care (Nord and Williams, 2015; Tinbergen and Williams, 2002), and thus avian parents must tradeoff time and energy between incubation and self-maintenance. Further, parental incubation behavior varies depending on factors such as weather, parental body condition, and clutch size, and this leads to differences in egg incubation temperatures among nests in the same population, and even among different breeding attempts of the same individual (Aldrich and Raveling, 1983; Ardia et al., 2010; Coe et al., 2015; Conway and Martin, 2000; Haftorn and Reinertsen, 1985; Hope et al., 2018a; Nord et al., 2010). This temperature variation is important for the offspring because small differences in average egg incubation temperature (<1°C) have large effects on post-hatch avian offspring phenotypic traits (DuRant et al., 2013b), such as growth rate (DuRant et al., 2010; Nord and Nilsson, 2011; Ospina et al., 2018; Wada et al., 2015), thermoregulatory ability (DuRant et al., 2013a), hormone levels (DuRant et al., 2014, 2010; Wada et al., 2015), and proactive/reactive behavior (Bertin et al., 2018; Hope et al., 2018b). Further, incubation temperature is related to long-term survival (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; Nord and Nilsson, 2016), suggesting that these phenotypic differences have fitness consequences.

Interestingly, incubation temperature is a parental effect that also has the potential to influence within-brood differences in resource acquisition. Recent evidence shows that average incubation temperatures can consistently differ among eggs *within* nests (Beatty, 2015; Hope et al., 2018a; Reid et al., 2000). Notably, in wood ducks (*Aix sponsa*), average egg temperatures throughout the entire incubation period differ sufficiently to create broods containing individuals with different phenotypes (i.e., >1°C; Hope et al., 2018a). Because incubation temperature-induced differences in traits such as offspring size, hormone levels, and proactive/reactive behavior can influence competitive ability (Cole and Quinn, 2012; Greig-Smith, 1985; Kitaysky et al., 2001; Oddie, 2000; Ruppli et al., 2012; Ward et al., 2004), within-nest variation in average incubation temperature could result in differential ability to acquire resources within broods. Until now, the idea of differential resource acquisition within avian broods has been focused on altricial species. For example, parental effects such as hormone allocation to embryos or hatching asynchrony can influence offspring ability to monopolize resources from the parent after hatching, and lead to differential growth and survival within broods (Krebs et al., 1999; Morandini and Ferrer, 2015; Ostreiher, 1997; Ploger and Medeiros, 2004; Schwabl, 1996). If incubation temperature affects the ability of precocial offspring to access resources, this would reveal a previously unrecognized way by which within-brood differences in resource acquisition, and possibly differential survival, could be created within both altricial and precocial broods.

To investigate if differential resource acquisition within avian broods could be a consequence of variation in average incubation temperature among eggs within nests, we conducted an experiment to determine whether differences in incubation temperature affect the ability of wood duck ducklings to gain access to heat and food sources. We incubated wood duck eggs at either 35 or 36°C, which are within the natural range for wood ducks (Hepp et al., 2006; Hope et al., 2018a). We chose these two temperatures because they have been shown to produce a wide array of differences in duckling traits in previous studies (DuRant et al., 2013b), such as different growth rates (DuRant et al., 2010), metabolic efficiency (DuRant et al., 2012), and thermoregulatory ability (DuRant et al., 2013a). Further, a difference of 1°C in average incubation temperature among eggs within the same clutch is realistic and likely common in the wild (Hope et al., 2018a). We formed mixed-incubation temperature broods, and conducted one trial to measure behaviors associated with the ability to gain access to a source of heat and three trials to measure behaviors associated with the ability to gain access to food. For the trials involving food, we measured duckling body mass before and after the trials to estimate food consumption and verify that feeding behavior correlated with food acquisition. We also measured aspects of duckling morphology throughout the experiment to determine whether differences in resource acquisition could either amplify or reduce morphological differences within broods. We had two alternative predictions. First, because higher incubation temperatures produce ducklings with faster growth rates (DuRant et al., 2010), greater locomotor abilities (Hopkins et al., 2011), and a greater probability of survival (Hepp and Kennamer, 2012), we predicted that ducklings incubated at higher temperatures would be physically advantaged (e.g., larger, faster, stronger) and thus would outperform ducklings incubated at the lower temperature. If this is the case, we would expect that differences in incubation temperature within broods would amplify phenotypic (morphological) differences among offspring. Alternatively, because lower incubation temperatures produce ducklings with less efficient metabolisms (DuRant et al., 2012) and weaker thermoregulatory abilities (DuRant et al., 2013a), it is possible these ducklings may be more inclined to take risks to meet their thermoregulatory and nutritional needs. Indeed, ducklings incubated at a lower temperature display more proactive (i.e., risky, bold, exploratory) behaviors than those incubated at higher temperatures (Hope et al., 2018b). Because there is evidence that proactive behavior is positively related to competitive ability (Cole and Quinn, 2012; Ward et al., 2004), we predicted that it may be possible for ducklings incubated at the lower temperature to acquire equivalent or more resources than those incubated at higher temperatures, especially within a novel environmental context. If this is the case, it would indicate that ducklings incubated at lower temperatures could buffer their physiological disadvantages by using behavior.

METHODS

Study species

The wood duck (*Aix sponsa*) is a dabbling duck that is widely distributed throughout North America and nests in tree cavities and nest boxes (Hepp and Bellrose, 2013). Wood ducks lay an average of 12 eggs per clutch (Bellrose and Holm, 1994). However, conspecific brood parasitism is common both in natural cavities (Roy Nielsen et al., 2006) and nest boxes (Semel et al., 1988; Semel and Sherman, 1986), and thus clutches can reach >40 eggs in some populations (Eadie et al., 1998; Morse and Wight, 1969). Only the females incubate (Hepp and Bellrose, 2013). The incubation period lasts ~30 d and females usually take two 1-2 h recesses per day to forage (Manlove and Hepp, 2000). Incubation temperature is a crucial aspect of parental care in this species because small differences in average incubation temperature (<1°C) affect a wide array of traits (DuRant et al., 2013b), as well as long-term survival (Hepp and Kennamer, 2012). Average incubation temperature varies both among and within nests in the field, and average egg temperatures can differ by >3°C among eggs within the same clutch (Hope et al., 2018a). Thus, natural wood duck broods consist of ducklings that have hatched from eggs that were incubated at different average temperatures.

Wood duck ducklings are precocial and leave the nest within 24 h of hatching. Ducklings stay with their mother for ~5 weeks, and the mother provides warmth, guides ducklings to sources of food, and provides protection from predators (Bellrose and Holm, 1994). However, ducklings are not completely dependent on their mother. They can feed themselves once they leave the nest, and can seek other ducklings in the same brood to huddle with for warmth. Indeed, duckling broods that are separated from their mother are known to sometimes survive in the wild (Bellrose and Holm, 1994). Ducklings spend much of their time in the water, but also frequently spend time on land to warm themselves, especially in early spring when the water is cold. Further, because hens do not always nest directly over a body of water, it is common for ducklings to travel long distances on land to reach a body of water (Bellrose and Holm, 1994). Ducklings are most vulnerable to starvation, cold temperatures, and predators during the first two weeks of life (Bellrose and Holm, 1994). A large proportion (50-75%) of ducklings die before they can fly (~day 60), and 90-99% of these mortalities occur within the first 1-2 weeks of life (McGilvrey, 1969; Sedinger et al., 2018). Thus, duckling behaviors related to seeking warmth or food during this early-life period are critical for survival.

Egg collection and incubation

Eggs were collected from a wood duck population breeding in nest boxes, which have been maintained for >35 years, on the Department of Energy's Savannah River Site (SRS) in South Carolina (33.1°N, 81.3°W) from 29 February – 16 March 2016. We checked nest boxes daily on 12 ephemeral wetlands, marked each egg for lay –date and –order, and collected up to 10 eggs from each nest before the hen began to incubate. We replaced eggs with wooden eggs to prevent hen abandonment (Hepp et al., 1987). We transported the unincubated eggs to Virginia Tech at room temperature. We held eggs for no longer than 10 days before beginning incubation (mean ± SD holding time = 6.9 ± 1.8 days; range = 4-10 days). During this time, eggs were held at room temperature and rotated twice daily. Keeping eggs in this way before beginning incubation does not affect hatchability (Walls et al., 2011).

Eggs were then incubated for the entire incubation period in Grumbach incubators (model BSS 420, Asslar, Germany) at two different overall mean temperatures: 35.0 and 36.0°C, within the natural range for wood ducks (Hepp et al., 2006; Hope et al., 2018a). Eggs from the same nest and the same lay date were distributed evenly between treatments. Incubators had two 75 min cool-down periods at 8:15 and 18:30 to simulate hens leaving the nest for foraging (Manlove and Hepp, 2000), but incubators maintained the above-mentioned overall mean temperatures. The average humidity for both incubators was kept between 60-65%. Hatch success was 62% (35.0°C) and 74% (36.0°C) and average (±SD) incubation periods were 38.5 ± 0.9 d (35.0°C) and 35.7 ± 1.0 d (36.0°C). In total, 114 ducklings were used in this study. All ducklings were tested in every trial. Because some ducklings died before the end of the experiment ($N = 6$), specific sample sizes for each trial are stated in the *Results*.

General husbandry

Once ducklings hatched, hatch date/time was recorded, and ducklings were weighed and color-banded. We assured that hatch time (and thus, duckling age) was accurate by checking the hatcher at least every 2 h and videotaping the hatcher when we were not present to record the precise time of hatch. As part of a different study, ducklings used in the current trials first performed a test of their ability to exit the nest within 24 h of hatching (Hope et al., 2019). Then, ducklings were housed in pairs or groups of three (mixed-incubation temperatures) in plastic cages assembled in a rack system. Each cage had a 50W infrared light and *ad-lib* food (DuMOR Chick Starter/Grower 20% Feed, Tractor Supply Co. ®) and water. To allow for individual identification during all behavioral trials, ducklings were marked with numbers on their heads and dots on their backs using non-toxic white correcting fluid (Fig. B1A).

Once ducklings were 4 days old, broods of 6 ducklings were formed and housed in outdoor aviary rooms. Broods consisted of 3 ducklings from each incubation temperature. We chose a brood size of 6 because it is a realistic size for wood ducks in the wild, and it was small enough to both maximize sample size and be logistically feasible given the difficulty of attaining sufficient hatching synchrony using artificial incubation. Occasionally, a brood of 5 was used for trials because some ducklings died before completing all trials (see *Feeding Trials in Three Contexts*). The aviary rooms (5.5 m x 2.5 m) were semi-outdoor, with mesh on three walls, covering the top half of each wall. Each room had a 100W infrared heat lamp, food, and water. The feeding area was assembled specifically to acclimate ducklings to eating from a dish similar to those used in the Familiar Environment and Novel Object Feeding Trials (see below). The dish was a plastic cylinder with multiple openings, so food was replenished as the ducklings fed (Fig. B1B). There was also a metal grate (40 x 42 cm) underneath the food, so that spilled food was not accessible (Fig B1C).

Duckling body mass and culmen length were measured on days 0, 2, 4, 6, 8, and 10, and tarsus length was measured on days 0, 3, 6, 8, and 10. Tarsus was measured in triplicate and the average of these measurements was taken. Duckling body mass was also measured before and after each feeding trial to calculate change in mass, as an indirect measure of their food consumption during the trial. After all trials were complete, ducklings were euthanized using carbon dioxide asphyxiation followed by cervical dislocation, and sex was determined by inspecting both external genitalia and internal gonads. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Heat trial

When ducklings were 2-3 days old, a trial was conducted to assess ability to gain access to a concentrated heat source. For each trial, 6 ducklings (3 high temperature and 3 low temperature) were transported from their cages to the trial arena (Fig. B2). Trials were conducted on 19 broods with 6 ducklings each. Trials were conducted in the morning, starting between 0508 and 0617 hours, and in an air-conditioned room (mean ± SD room temperature = 14.9 ± 1.4°C; range = 12.3 – 17.4°C) so that the cold room temperature would encourage ducklings to seek the heat source and induce huddling. The temperature of the room was recorded before each trial. The arena was a circular (diameter = 50 cm) wooden platform with tall walls and two cameras (GoPro©) mounted above to record behaviors. One 50W infrared heat lamp was suspended above the arena and was fitted with metal flashing so only a small, concentrated amount of heat was emitted into the center of the arena. Another 50W infrared heat lamp was fitted beneath the arena to emit heat in the exact spot as the suspended heat lamp. A piece of mesh was laid over the heated spot (diameter = 4 cm). This spot of direct heat was about 35°C, and there was a considerable drop in heat in the spaces farther away from the heat source (e.g., the temperatures at 4, 8, and 15 cm away from the center of the heat source were ~ 21, 19, and 17°C, respectively). Thus, ducklings were predicted to seek this heat source. Ducklings were allowed to acclimate in the arena for 15 min without the heat lamps on. After 15 min, the heat lamps were turned on remotely and the trial lasted for 45 min.

Concentric circles were drawn on the floor of the arena to quantify duckling behavior. Videos were analyzed and the position of each duckling was recorded for each minute of the trial, starting when the light turned on. Position 1 indicated that the duckling was in the heat spot, the next concentric circle was recorded as position 2, and so on, until position 12. Position was determined based on in which circle the majority of the duckling's body was located. The latency of each duckling to step onto the heat spot was also recorded. Several broods did not huddle under the heat source (4 out of 19 broods), and instead, huddled in a different part of the arena. Thus, we also quantified the number of ducklings that were directly surrounding (i.e., making direct contact) each duckling for each minute of the trial, using a scan sampling approach. A higher number indicated a warmer location. We then calculated the average position, average number of surrounding ducklings, number of minutes spent directly under the heat source (i.e., position 1), and number of minutes spent within a 6 cm radius from the center point of the heat source (i.e., either position 1, 2, or 3) for each duckling for the entire trial.

Feeding trials in three contexts

Because ducklings incubated at different temperatures exhibit different exploratory and boldness behaviors (Hope et al., 2018b), we conducted feeding trials in three different contexts (*Novel Environment, Familiar Environment, and Novel Object; see sections below*). These trials were conducted after broods had already been formed and housed in aviary rooms. Each trial was conducted on 19 broods consisting of either 5 or 6 ducklings, with 2-3 high temperature-incubated ducklings and 2-3 low temperature-incubated ducklings (number of broods consisting of 5 ducklings: Novel Environment Feeding Trial = 3; Familiar Environment Feeding Trial = 5; Novel Object Feeding Trial = 6). To stimulate feeding during the trials, food was removed from the aviary room (but water remained) 10.5 h before each trial would start the next morning. The temperature of the trial room was recorded before the start of each trial (for all trials: mean ± SD = 14.3 ± 2.5°C; range = 7.9-17.4°C). Trials lasted 1 h and there was enough food in each trial so that ducklings could eat during the entire hour. A feeding bout was defined as a discrete up-and-down head movement into and out of the food dish. To verify that feeding behavior was related to food consumption, we measured duckling body mass immediately before (fasted) and immediately after each of the three trials in order to calculate the change in body mass. Because we used the change in duckling body mass as a proxy of food consumption, we cannot discount the possibility that this measurement is confounded by digestive ability of ducklings or defecation rate. However, it is unlikely that ducklings would have fully digested the food ingested during the 1 h trial, so body mass differences should not reflect the ability to convert food to body mass or excrement.

Novel environment feeding trial

The purpose of this trial was to investigate whether incubation temperature affected the ability of ducklings to acquire food while in a novel environment. When ducklings were 6-7 days old, broods were transported from their aviary rooms to the Novel Environment Feeding Trial arena (Fig. B3). This arena was in a separate aviary room that was set-up in a different way than the home aviary rooms, and thus was a novel environment. The arena (2.0 x 2.5 m) had gridlines taped to the floor (0.25 x 0.25 m squares), and 18 small food dishes spaced evenly throughout and secured to the ground. A potted plant was placed in front of each food dish so that the dishes were not immediately visible to ducklings. Trials started between 0555 and 0831 hours. Broods were placed under a bucket to acclimate in the dark for 5 min. Then, the bucket was lifted remotely and ducklings were allowed to explore and forage for 1 h. Cameras (GoPro©) were mounted above the arena to record duckling behavior. From the videos, we quantified the latency to first feed, the number of unique dishes visited (i.e., if the duckling visited the same dish twice, the dish was *not* counted twice), the number of non-unique dishes visited (i.e., if the duckling visited the same dish twice, the dish *was* counted twice), the total number of feeding bouts (possible to have multiple feeding bouts per dish), and the total time spent at the dishes for each duckling.

Familiar environment feeding trial

The purpose of this trial was to investigate whether incubation temperature affected the ability of ducklings to acquire food in a familiar environment. The Familiar Environment Trial was conducted when ducklings were 8-9 days old, and was conducted in the home aviary room (i.e., familiar environment) that the duckling broods had lived in since day 4. Trials began between 0655 and 0722 hours. On the morning of the trial, the metal grate below the food dish was replaced with one that had gridlines (10.0 x 10.5 cm squares) drawn on it and ducklings were given a food dish that was similar to the dish that they were accustomed to, but had only one opening instead of four (Fig. B4A). The opening on the food dish was covered by a piece of plastic attached to a string. After the trial was set-up, ducklings were allowed 15 min to re-acclimate and then the string was pulled from outside of the aviary room to reveal the food. Ducklings were given 1 h to access the food. Trials were video-recorded and the latency to first feed, the latency to first enter the feeding area (metal grate), the total number of feeding bouts, the total number of times a duckling entered the feeding area, and the total amount of time spent in the feeding area were recorded for each duckling.

Novel object feeding trial

The purpose of this trial was to investigate whether incubation temperature affected the ability of ducklings to acquire food that was in a familiar environment, but had a novel object near it. The Novel Object Feeding Trial was conducted when ducklings were 10-11 days old in the home aviary room (i.e., familiar environment), in which the duckling broods had lived since day 4. Trials began between 0648 and 0716 hours. This trial was the same as the Familiar Environment Trial, with two exceptions. First, there was a novel object (9 cm tall pink plastic cone; Fig. B4B) placed in front of the food dish. Second, a cardboard box (30 x 30 x 30 cm) covered both the food dish and the novel object during the acclimation period (15 min), and was lifted remotely by a string when the trial began. Through video analysis, we quantified the same behaviors as in the Familiar Environment Trial for each duckling.

Statistical analyses

Because we measured 4-5 behaviors for each trial, we condensed behavioral measures using z-score analysis (Guilloux et al., 2011; Hope et al., 2018b; Labots et al., 2018). For each individual behavior recorded, a z-score was calculated by subtracting the value from the mean value of all individuals for that behavior, divided by the standard deviation. Each z-score was calculated so that a higher value indicated a more active behavior (e.g., quicker to go to the heat source, quicker to begin feeding, more feeding bouts, etc.). We then calculated the average z-score of each individual for each trial, resulting in one z-score per individual per behavioral trial. The z-score for the Heat Trial included the average position, number of minutes spent directly under the heat (i.e., position 1), number of minutes spent within 6 cm of the center point of the heat source (i.e., either position 1, 2, or 3), and the latency to first go under the heat. A larger z-score indicated that a duckling was quicker to go to and spent more time under the heat source. Ducklings that did not spend any time under the heat source, but were within broods where at least one duckling spent time under the heat source, were included and given a latency of 45 min (i.e., the length of the trial; N = 3 ducklings). Broods in which no ducklings went under the heat source were excluded (N = 4 broods) from this analysis. The z-score for the Novel Environment Feeding Trial included the number of unique dishes visited, number of non-unique dishes visited, latency to feed, total number of feeding bouts, and the total time spent at the dishes. The z-scores for both the Familiar Environment and Novel Object Feeding Trials included the latency to feed, latency to enter the feeding area, total number of times in the feeding area, total number of feeding bouts, and total amount of time spent in the feeding area. For all Feeding Trials, a larger z-score indicated that the duckling was quicker to begin feeding and fed frequently.

We used R version 3.5.1 (R Core Team, 2018) for all analyses. We use the *lme4* package (Bates et al., 2015) for all linear mixed effects models (*lmer*) and report p-values using Type III Wald chi-square tests using the *Anova* function of the *car* package (Fox and Weisberg, 2011). We reduced all models by using stepwise backward elimination of insignificant terms. Because we used the same ducklings in 4 different behavioral trials, we used a Bonferroni correction ($\alpha = 0.05/4 = 0.0125$) and thus set significance at $p < 0.0125$ for all models investigating behavioral endpoints. Here, we only report terms that were left in the models, but we report all full and final models in Appendix A. We examined histograms of residuals, predicted vs. residuals plots, and normal quantile plots to ensure that all models met the assumptions of normality and homoscedasticity.

To examine the ability to gain access to a heat source, we conducted two analyses using linear mixed effects models. The first model included the Heat Trial z-score as the dependent variable. The second model included the average number of surrounding ducks (defined as the number of ducklings making direct contact with each duckling) as the dependent variable, to examine how central each duckling's position was in the brood regardless of proximity to the heat source. For both of these models, incubation temperature was the independent variable, brood was included as a random effect, and body mass (g), sex, lay date, age (hours old), and room temperature were included as covariates.

We built linear mixed effects models to answer two questions for each Feeding Trial: 1) what factors affect feeding behavior (z-score)?, and 2) is feeding behavior (z-score) an accurate indicator of food consumption? For the first question, feeding behavior (z-score) was the dependent variable (separate model for each of the three feeding trials). For these models, incubation temperature was the independent variable and brood was included as a random effect. Duckling age (hours) at the time of the trial, body mass (g), lay date, sex, and the ambient temperature during the trial were all included as covariates. For the second question, the change in body mass during the 1-hour trial (i.e., body mass after – body mass before trial) was the dependent variable, feeding behavior (z-score) was the independent variable (separate model for each of the three feeding trials), and brood was included as a random effect. We also included incubation temperature as a covariate, to determine if there might be an effect of incubation temperature on apparent food consumption that was not explained by behavioral differences (i.e., z-score). After finding that this was indeed the case (see *Results*), we also added duckling culmen length as a covariate because we predicted that differences in culmen length might result in differences in food consumption independently of behavior. Two extreme and influential (>20 times the mean Cook's distance) outliers were excluded from our models and figures, one from the Novel Environment Feeding Trial and one from the Familiar Environment Feeding Trial.

To investigate effects of incubation temperature on duckling body mass, tarsus length, and culmen length throughout the experiment, we constructed 3 linear mixed effects models. Because we used the same ducklings to measure these 3 aspects of morphology, we used a Bonferroni correction ($\alpha = 0.05/3 = 0.0167$) and thus set significance at $p < 0.0167$ for all models investigating morphology. For two of these models, either body mass (g) or culmen length (mm) was the dependent variable and data were included for days 0, 2, 4, 6, 8, and 10. For the third model, tarsus length (mm) was the dependent variable and data were included for days 0, 3, 6, 8, and 10. Only ducklings that survived until day 10 were used in these morphological analyses (35°C: $N = 54$; 36°C: $N = 54$ ducklings). For all models, incubation temperature, age (day; categorical), and their interaction were included as independent variables. Duckling ID was included as a random effect to account for repeated measures. Pairwise comparisons were investigated using estimated marginal means, using the *emmeans* (Lenth, 2018) package.

RESULTS

Gaining access to a heat source

Contrary to our predictions, incubation temperature did not influence duckling behaviors related to seeking a concentrated heat source, either in the model investigating the relationship of incubation temperature to z-score ($p = 0.52$; Fig. 1A; $N = 15$ broods; 45 ducklings from 35.0°C, 44 ducklings from 36.0°C; Table A1) or the model investigating its relationship to the average number of surrounding ducklings ($p = 0.90$; $N = 19$ broods; 57 ducklings from 35.0°C, 56 ducklings from 36.0°C; Table A2). There were also no significant covariates retained in either model (Tables A1 and A2).

Food acquisition in different contexts

Contrary to our predictions, duckling feeding behavior (z-score) was not affected by incubation temperature in any of the three trials (effect of incubation temperature: all $p \geq 0.23$; Fig. 1B-D; all trials $N = 19$ broods; Novel Environment: $N = 55$ ducklings from 35.0°C, 55 ducklings from 36.0°C; Familiar Environment: $N = 53$ ducklings from 35.0°C, 55 ducklings from 36.0°C; Novel Object: $N = 54$ ducklings from 35.0°C, 54 ducklings from 36.0°C; Tables A3-A5). However, in both the Familiar Environment and Novel Object Feeding Trials, duckling body mass was negatively related to feeding behavior (z-score) (Familiar Environment: $X^2 = 8.32$, $p = 0.004$, Table A4; Novel Object: $X^2 = 8.87$, $p = 0.003$, Table A5) and thus, individuals with a greater body mass spent slightly less time feeding than those with a lower body mass. However, the correlation coefficients for both of these relationships were quite low (Familiar Environment: $r = -0.19$; Novel Object: $r = -0.11$), and thus the relationships between body mass and behavior were not strong.

As expected, we found that feeding behavior (z-score) was positively related to apparent food consumption (change in duckling body mass between the beginning and end of 1-hour feeding trial) in all three feeding trials (Novel Environment: $p = 0.0005$, $r = 0.37$, Fig. 2A, Table A6; Familiar Environment: $p = 0.0133$, $r = 0.29$, Fig. 2B, Table A7; Novel Object: $p = 0.0003$, $r = 0.40$, Fig. 2C, Table A8). Thus, these results provide evidence that ducklings that were quickest to feed and fed most frequently consumed the most food. Interestingly, incubation temperature also had a significant (or marginally significant, after the Bonferroni correction) effect on apparent food consumption in all three trials (Novel Environment : $p = 0.017$, Fig 2A, Table A6; Familiar Environment: $p < 0.0001$, Fig 2B, Table A7; Novel Object: $p < 0.0001$, Fig. 2C, Table A8) where, in all cases, given the same feeding behavior, high temperature-incubated ducklings appeared to consume more food during a trial than low temperature-incubated ducklings. To investigate this further, we added culmen length (mm) to each model because we expected that ducklings with longer culmen lengths could consume more food per feeding bout. Culmen length was positively related to apparent food consumption in the Familiar Environment

($p < 0.0001$, Fig. 3A, Table A7) and Novel Object Trials ($p < 0.0001$, Fig. 3B, Table A8), but not the Novel Environment Trial ($p = 0.36$; Table A6). Taken together, these results suggest that the difference in apparent food consumption during the 1-hour feeding trials among ducklings incubated at different temperatures could be, at least in part, driven by differences in structural size.

Body mass, structural size, and growth

There was an interactive effect of incubation temperature and age (day) on body mass (incubation temperature: $X^2 = 0.20$, $p = 0.66$; age: $X^2 = 1780$, $p < 0.0001$; interaction: $X^2 = 94.5$, $p < 0.0001$; Table A9; Fig. 4A), tarsus length (incubation temperature: $X^2 = 0.08$, $p = 0.78$; age: $X^2 = 1245$, $p < 0.0001$; interaction: $X^2 = 27.2$, $p < 0.0001$; Table A10; Fig. 4B), and culmen length (incubation temperature: $X^2 = 1.91$, $p = 0.17$; age: $X^2 = 3340$, $p < 0.0001$; interaction: $X^2 = 51.9$, $p < 0.0001$; Table A11; Fig. 4C). Pairwise comparisons among ages revealed that shortly after hatching, all ducklings lost body mass, grew larger culmens, and had no change in tarsus length (pairwise comparisons: day 0 – 2: mass: $p < 0.001$; culmen: $p = 0.0009$; day 1 – 3: tarsus: $p = 0.62$; Fig. 4). After that (day 4 – 10), ducklings grew larger in all aspects of morphology as they aged (all pairwise comparisons: $p < 0.0001$; Fig. 4). As for the interaction with incubation temperature, pairwise comparisons revealed that ducklings incubated at different temperatures were of similar size during the first few days, and then those incubated at the higher temperature grew faster until the end of the experiment. Specifically, there were no differences between ducklings incubated at different temperatures in body mass on day 0 ($p = 0.66$) or day 2 ($p = 0.31$), tarsus length on day 0 ($p = 0.78$) or day 3 ($p = 0.35$), or culmen length on day 0 ($p = 0.17$) or day 2 ($p = 0.08$). However, ducklings incubated at the higher temperature had greater body masses on day 4 ($p = 0.003$), day 6 ($p = 0.0004$), day 8 ($p < 0.0001$), and day 10 ($p < 0.0001$), longer tarsus lengths on day 6 ($p = 0.013$), day 8 ($p = 0.002$), and day 10 ($p = 0.0007$), and longer culmen lengths on day 4 ($p = 0.005$), day 6 ($p < 0.0001$), day 8 ($p < 0.0001$), and day 10 ($p < 0.0001$), than those incubated at the lower temperature (Fig. 4).

DISCUSSION

In this study, we investigated whether incubation temperature affects acquisition of food and heat resources within broods of precocial avian offspring. Because incubation temperature influences multiple fitness-related offspring traits in birds (reviewed in DuRant et al., 2013b) and average incubation temperatures vary among eggs within nests (Hope et al., 2018a), we predicted that this could create differences among brood mates in the ability to acquire resources. Contrary to our predictions, we found no difference in the behaviors related to food or heat acquisition among ducklings incubated at different temperatures when tested in mixed-incubation temperature broods. However, ducklings incubated at the higher temperature consumed more food during the 1-hour feeding trials and had greater body mass and structural size than those incubated at the lower temperature. Thus, our results suggest that individuals incubated at low temperatures may be disadvantaged compared to those incubated at higher temperatures, but this deficit is likely due to differences in structural size rather than behaviors. This reveals a previously unrecognized way by which differential survival could arise within avian broods.

Consistent with previous studies (reviewed in DuRant et al., 2013b), we found evidence that ducklings incubated at low temperatures displayed a physiological deficit compared to those incubated at higher temperatures. Ducklings incubated at different temperatures had similar body masses and structural sizes until day 2-3, after which ducklings incubated at the higher temperature grew faster in body mass, tarsus length, and culmen length than those incubated at the lower temperature until the end of the study (day 10). Further, ducklings incubated at the higher temperature consistently consumed more food during the 1-hour feeding trials than those incubated at the lower temperature. Because the frequency of feeding behavior did not differ among treatments, the differences in apparent food consumption between ducklings incubated at different temperatures seemed to be driven by differences in duckling structural size, rather than by differences in behavior. Specifically, because food consumption was related to culmen length, it is likely that the larger bills of ducklings incubated at high temperatures helped them to consume more food per bite than their counterparts incubated at a cooler temperature. This could create a positive feedback loop, wherein larger ducklings are more efficient at consuming food, which leads to faster growth rates and the ability to consume even more food. This positive feedback could amplify differences in phenotype among offspring incubated at different temperatures and, in part, underlie the incubation temperature-induced differences in body size and growth in the current study, and in previous studies (DuRant et al., 2010; Nord and Nilsson, 2011; Ospina et al., 2018; Wada et al., 2015).

Our observations suggest that ducklings incubated at higher temperatures may have an advantage compared to brood mates that experienced slightly lower incubation temperatures, which could have important consequences in a natural setting. For example, although all ducklings exhibited a similar capacity to secure time near a heat source, ducklings incubated at the higher temperature would likely have a greater chance of surviving cold conditions than those incubated at the lower temperature because of the inherent thermoregulatory advantages of a larger body size (Rhymer, 1988) and because they have more metabolically efficient thermoregulatory abilities (DuRant et al., 2013a, 2012). Similarly, although incubation temperature did not affect the frequency of feeding behavior in our trials, it is likely that the ability of ducklings incubated at the higher temperature to consume more food per feeding bout would allow them to gain access to more food compared to those incubated at the lower temperature when food is limited. A greater feeding efficiency could also allow ducklings to feed more quickly, limiting their time spent in the open and vulnerable to predators. Further, the larger body mass of a high temperature-incubated duckling could increase the chances of recovering from a period of mass loss (Arroyo, 2002), or decrease the chances of predation by gape-limited predators (e.g., fish). Indeed, a recent meta-analysis found that offspring body mass generally has a positive relationship with offspring survival across mammal and bird species (Ronget et al., 2018), and a study on wood ducks also found that survival probability in the wild increased with duckling body mass (Sedinger et al., 2018). Because 50-75% of wood duck mortality in the wild occurs within the first 1-2 weeks of life (McGilvrey, 1969; Sedinger et al., 2018), the effect of incubation temperature on body mass, growth, structural size, and food consumption in 4-10 day-old ducklings could give high temperature-incubated ducklings an early advantage in the most critical days of life. This may explain, in part, why studies have found evidence that avian offspring incubated at higher temperatures have higher long-term survival compared to those incubated at lower temperatures (zebra finches: Berntsen and Bech, 2016; wood ducks: Hepp and Kennamer, 2012). However, it is important to note that a larger body size could also be disadvantageous in some cases. For example, larger bodies have higher metabolic demands, which would be disadvantageous if food is difficult to find. The complexity of the relationship between offspring body mass and survival could be the reason why one study found that nestlings incubated at high temperatures with large body masses experienced lower survival compared to smaller nestlings (blue tits; Nord and Nilsson, 2016).

Incubation is a parental effect that can influence the behavioral, physiological, and morphological phenotype of the individual (DuRant et al., 2013b), and the phenotypic composition of the brood through variation in average incubation temperature within nests (Hope et al., 2018a). Although there has been accumulating evidence over the past decade that incubation temperature affects diverse avian offspring traits, this is the first study to investigate whether these trait differences could be amplified or reduced due to the phenotypic composition of the brood. Our study provides evidence that higher incubation temperatures lead to larger body sizes, which in turn leads to increased efficiency of food consumption, rather than differences in frequency of feeding behavior. This provides insight into how an important avian parental effect could generate positive feedback that amplifies early phenotypic differences among offspring within broods. In altricial species, there is ample research that suggests that parental effects can create differences in offspring growth and size through hatching asynchrony, hormone deposition, or differential food allocation, leading to differential survival within broods (Krebs et al., 1999; Morandini and Ferrer, 2015; Ostreiher, 1997; Ploger and Medeiros, 2004; Schwabl, 1996). Our study reveals a previously unrecognized way by which differential survival among offspring could occur within both altricial and precocial broods.

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FIGURE LEGENDS

Figure 1. Incubation temperature did not affect duckling behavior (mean z-score ± SE) in relation to gaining access to (A) heat, (B) food in a novel environment, (C) food in a familiar environment, or (D) food with a novel object placed next to it. Trials were conducted on mixed-incubation temperature duckling broods (2-3 ducklings from each incubation temperature treatment per brood).

Figure 2. Feeding behavior (z-score) was positively related to change in duckling body mass (g) during the (A) Novel Environment, (B) Familiar Environment, and (C) Novel Object feeding trials. Change in body mass (mass after – mass before trial) is indicative of food consumption. Each trial lasted 1 hour. Trials were conducted on mixed-incubation temperature duckling broods (2-3 ducklings from each incubation temperature treatment per brood). For clarity, data from ducklings incubated at the same temperature are pooled within broods for this figure ($N = 19$ broods), although data analyses were conducted using brood as a random effect. Point color indicates the temperature at which ducklings were incubated (grey = 35.0°C; black = 36.0°C). Note that, in general, ducklings incubated at 36.0°C consume more food than those incubated at 35.0°C, even when z-scores are similar.

Figure 3. Duckling feeding behavior (z-score) and culmen length were both positively related to change in body mass (g) during the Familiar Environment (A) and Novel Object (B) Feeding Trials. Change in body mass (mass after – mass before trial) is indicative of food consumption. Each trial lasted 1 hour. Trials were conducted on mixed-incubation temperature duckling broods (2-3 ducklings from each incubation temperature treatment per brood). For simplicity, data from

ducklings incubated at the same temperature are pooled within broods for this figure (*N* = 19 broods), although data analyses were conducted using brood as a random effect. Point color indicates the temperature at which ducklings were incubated (grey = 35.0°C; black = 36.0°C). Planes were generated using predicted values from simple linear models, but data were analyzed using linear mixed effects models.

Figure 4. (A) Body mass (mean ± SE), (B) tarsus length (mean ± SE), and (C) culmen length (mean ± SE) of ducklings incubated at either 35°C (grey) or 36°C (black) from hatch (day 0) until day 10. (*) indicates that there was a significant difference between incubation temperatures. Note that tarsus was measured on day 3 instead of days 2 and 4.

FIGURES

Figure 1

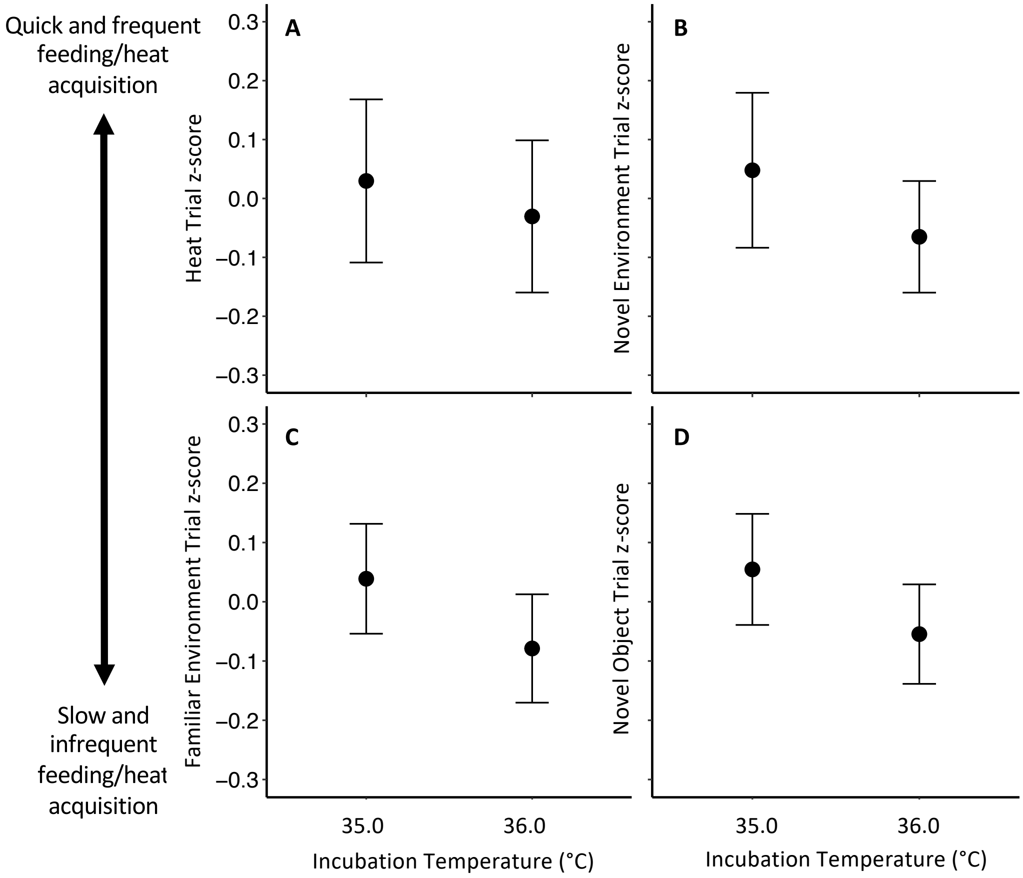
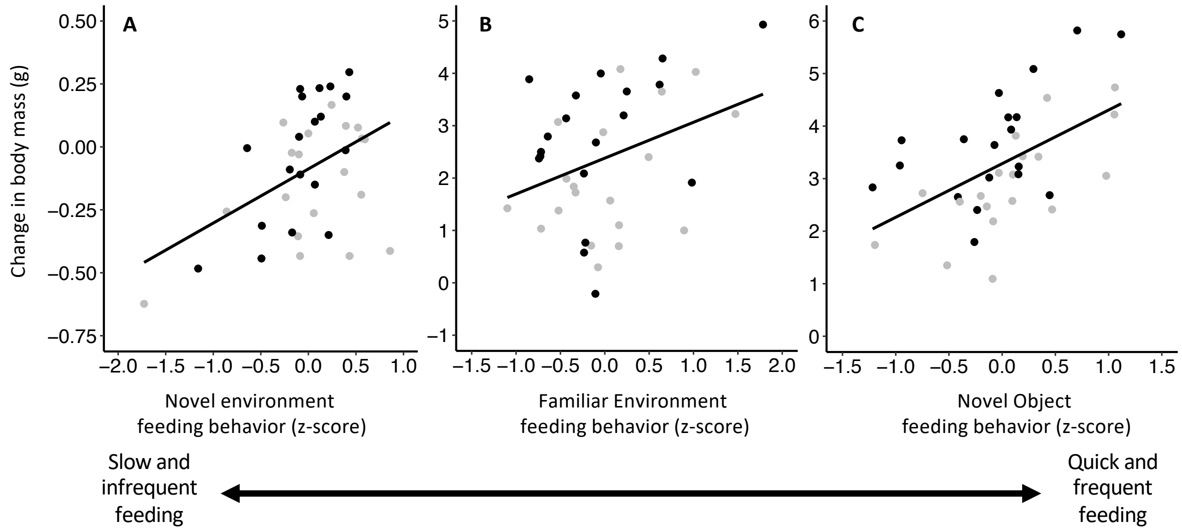


Figure 2



A
B
C
Change in body mass (g)
0.50
0.25
0.00
−0.25
−0.50
−0.75
−2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0
Novel environment feeding behavior (z-score)
5
4
3
2
1
0
−1
−1.5 −1.0 −0.5 0.0 0.5 1.0 1.5 2.0
Familiar Environment feeding behavior (z-score)
6
5
4
3
2
1
0
−1.5 −1.0 −0.5 0.0 0.5 1.0 1.5
Novel Object feeding behavior (z-score)
Slow and infrequent feeding
Quick and frequent feeding

Figure 3

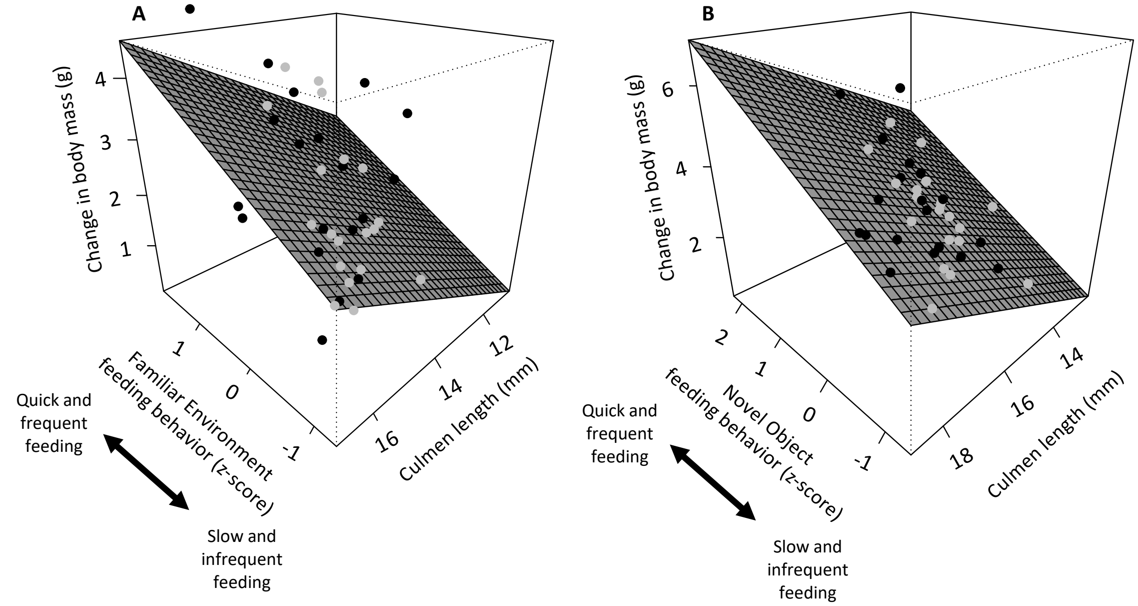
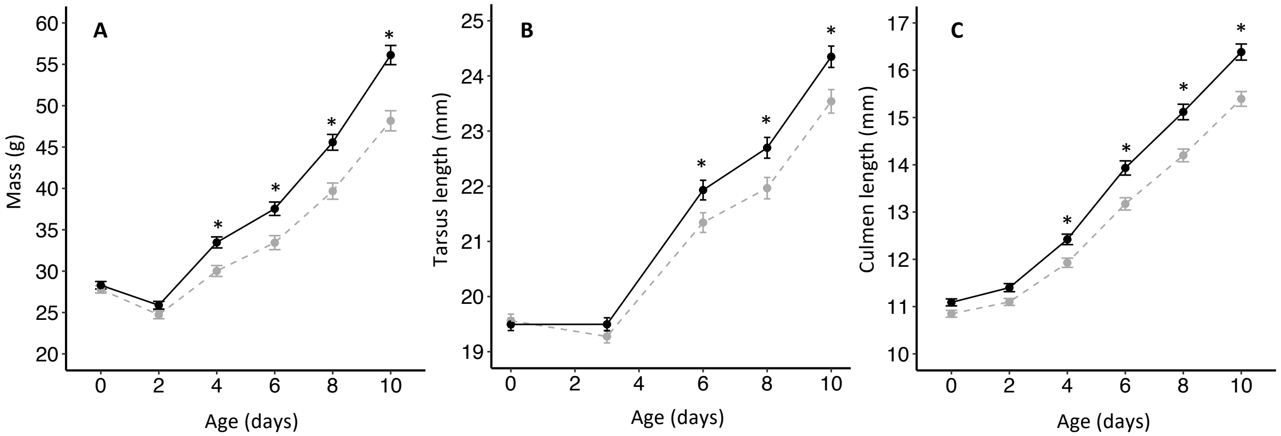

A
Change in body mass (g)
1
2
3
4
Familiar Environment feeding behavior (z-score)
1
0
-1
Culmen length (mm)
12
14
16
Quick and frequent feeding
Slow and infrequent feeding
B
Change in body mass (g)
2
4
6
Novel Object feeding behavior (z-score)
2
1
0
-1
Culmen length (mm)
14
16
18
Quick and frequent feeding
Slow and infrequent feeding

Figure 4


A
Mass (g)
60
55
50
45
40
35
30
25
20
0
2
4
6
8
10
Age (days)
*
B
Tarsus length (mm)
25
24
23
22
21
20
19
0
2
4
6
8
10
Age (days)
C
Culmen length (mm)
17
16
15
14
13
12
11
10
0
2
4
6
8
10
Age (days)

APPENDIX A: Supplementary Tables

Dependent variable: Heat Trial z-score *N = 15 broods; 45 ducklings from 35.0°C, 44 ducklings from 36.0°C*		
Full Model		
Term	X^2	*P*
Incubation temperature	0.41	0.52
Body mass	0.89	0.35
Sex	0.06	0.80
Lay date	3.80	0.051
Age (hours)	0.95	0.33
Room temperature	0.45	0.50
Final Model		
No significant terms		

Table A1. Full and reduced linear mixed effects model investigating whether incubation temperature and other covariates influence duckling behavior when attempting to gain access to a heat source. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. The dependent variable was duckling behavior (z-score), where a higher z-score indicated that the duckling was quick to go to the heat and spent a large quantity of time under the heat. The model was reduced using stepwise backward elimination of insignificant terms (p > 0.0125; Bonferroni correction for reusing same individuals in 4 behavioral trials).

Dependent variable: Heat Trial Average number of ducks surrounding

N = 19 broods; 57 ducklings from 35.0°C, 56 ducklings from 36.0°C

Full Model

Term	X^2	*P*
Incubation temperature	0.02	0.90
Body mass	1.75	0.19
Sex	0.02	0.90
Lay date	3.21	0.07
Age (hours)	0.48	0.49
Room temperature	0.26	0.61

Final Model

No significant terms

Table A2. Full and reduced linear mixed effects model investigating whether incubation temperature and other covariates influence duckling behavior when attempting to gain access to a heat source. The dependent variable was the average number of ducklings that were surrounding each duckling (i.e., measure of optimal, central position within the brood). Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. The model was reduced using stepwise backward elimination of insignificant terms ($p > 0.0125$; Bonferroni correction for reusing same individuals in 4 behavioral trials).

Dependent variable: Novel Environment Feeding Trial z-score
N = 19 broods; 55 ducklings from 35.0°C, 55 ducklings from 36.0°C

Full Model

Term	X^2	*P*
Incubation temperature	1.42	0.23
Body mass	2.22	0.14
Sex	0.11	0.74
Lay date	0.14	0.71
Age (hours)	0.14	0.71
Ambient temperature	0.35	0.56

Final Model

No significant terms

Table A3. Full and reduced linear mixed effects model investigating whether incubation temperature and other covariates influence duckling behavior in relation to gaining access to food in a novel environment. The dependent variable was feeding behavior (z-score), where a high z-score indicates that the duckling was quick to begin feeding and fed frequently. Body mass was measured before the trial. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. The model was reduced using stepwise backward elimination of insignificant terms (p > 0.0125; Bonferroni correction for reusing same individuals in 4 behavioral trials).

Dependent variable: Familiar Environment Feeding Trial z-score *N = 19 broods; 53 ducklings from 35.0°C, 55 ducklings from 36.0°C*		
Full Model		
Term	X^2	P
Incubation temperature	0.46	0.50
Body mass	5.97	0.015
Sex	0.76	0.38
Lay date	2.78	0.10
Age (hours)	0.01	0.91
Ambient temperature	2.85	0.09
Final Model		
Body mass	**8.32**	**0.004**

Table A4. Full and reduced linear mixed effects model investigating whether incubation temperature and other covariates influence duckling behavior in relation to gaining access to food in a familiar environment. The dependent variable was feeding behavior (z-score), where a high z- score indicates that the duckling was quick to begin feeding and fed frequently. Body mass was measured before the trial. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. The model was reduced using stepwise backward elimination of insignificant terms (p > 0.0125; Bonferroni correction for reusing same individuals in 4 behavioral trials).

Dependent variable: Novel Object Feeding Trial z-score *N = 19 broods; 54 ducklings from 35.0°C, 54 ducklings from 36.0°C*		
Full Model		
Term	X^2	*P*
Incubation temperature	0.57	0.45
Body mass	5.04	0.025
Sex	0.07	0.80
Lay date	0.49	0.48
Age (hours)	0.10	0.75
Ambient temperature	0.92	0.34
Final Model		
Body mass	**8.87**	**0.003**

Table A5. Full and reduced linear mixed effects model investigating whether incubation temperature and other covariates influence duckling behavior in relation to gaining access to food that had a novel object placed next to it. The dependent variable was feeding behavior (z-score), where a high z-score indicates that the duckling was quick to begin feeding and fed frequently. Body mass was measured before the trial. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. The model was reduced using stepwise backward elimination of insignificant terms ($p > 0.0125$; Bonferroni correction for reusing same individuals in 4 behavioral trials).

Dependent variable: Novel Environment Feeding Trial change in body mass *N = 19 broods; 55 ducklings from 35.0°C, 55 ducklings from 36.0°C*		
First Model		
Term	X^2	*P*
Feeding behavior (z-score)	**12.1**	**0.0005**
Incubation temperature	5.75	0.017
Second Model (including culmen)		
Feeding behavior (z-score)	**11.6**	**0.0007**
Incubation temperature	3.76	0.052
Culmen length	0.85	0.36

Table A6. Linear mixed effects model investigating whether feeding behavior (z-score) is an accurate indication of food consumption (change in body mass) during a trial where ducklings seek access to food in a novel environment. A high z-score indicates that the duckling was quick to begin feeding and fed frequently. Incubation temperature was included as a covariate to investigate whether there might be an effect of incubation temperature on food consumption that was not explained by behavioral differences. Because of the results of the first model, culmen length (mm) was included as a covariate in the second model in an attempt to explain differences in food consumption. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. Significance was defined as $p < 0.0125$; Bonferroni correction for reusing same individuals in 4 behavioral trials.

Dependent variable: Familiar Environment Feeding Trial change in body mass

N = 19 broods; 53 ducklings from 35.0°C, 55 ducklings from 36.0°C

First Model

Term	X^2	*P*
Feeding behavior (z-score)	6.13	0.0133
Incubation temperature	**20.7**	**<0.0001**
Second Model (including culmen)		
Feeding behavior (z-score)	**9.73**	**0.002**
Incubation temperature	**9.37**	**0.002**
Culmen length	**16.7**	**<0.0001**

Table A7. Linear mixed effects model investigating whether feeding behavior (z-score) is an accurate indication of food consumption (change in body mass) during a trial where ducklings seek access to food in a familiar environment. A high z-score indicates that the duckling was quick to begin feeding and fed frequently. Incubation temperature was included as a covariate to investigate whether there might be an effect of incubation temperature on food consumption that was not explained by behavioral differences. Because of the results of the first model, culmen length (mm) was included as a covariate in the second model in an attempt to explain differences in food consumption. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. Significance was defined as $p < 0.0125$; Bonferroni correction for reusing same individuals in 4 behavioral trials.

Dependent variable: Novel Object Feeding Trial change in body mass

N = 19 broods; 54 ducklings from 35.0°C, 54 ducklings from 36.0°C

First Model

Term	X^2	*P*
Feeding behavior (z-score)	**13.1**	**0.0003**
Incubation temperature	**18.7**	**<0.0001**

Second Model (including culmen)

Term	X^2	*P*
Feeding behavior (z-score)	**20.8**	**<0.0001**
Incubation temperature	**6.35**	**0.0117**
Culmen length	**21.3**	**<0.0001**

Table A8. Linear mixed effects model investigating whether feeding behavior (z-score) is an accurate indication of food consumption (change in body mass) during a trial where ducklings seek access to food that had a novel object placed next to it. A high z-score indicates that the duckling was quick to begin feeding and fed frequently. Incubation temperature was included as a covariate to investigate whether there might be an effect of incubation temperature on food consumption that was not explained by behavioral differences. Because of the results of the first model, culmen length (mm) was included as a covariate in the second model in an attempt to explain differences in food consumption. Ducklings were in groups of 6 (3 incubated at 35°C and 3 incubated at 36°C). Brood was included as a random effect. Significance was defined as $p < 0.0125$; Bonferroni correction for reusing same individuals in 4 behavioral trials.

Dependent variable: Body mass (g) *N = 108 ducklings; 648 measurements*		
Full Model		
Term	X^2	*P*
Incubation temperature	0.20	0.66
Age (day)	**1780**	**<0.0001**
Incubation temperature x Age	**94.5**	**<0.0001**

Table A9. Linear mixed effects model investigating how duckling body mass changed over time (day 0, 2, 4, 6, 8, and 10) and differed depending on incubation temperature. Duckling ID was included as a random effect because each duckling was measured multiple times. Significance was defined as $p < 0.0167$; Bonferroni correction for reusing same individuals to investigate 3 aspects of morphology.

Dependent variable: Tarsus length (mm) *N = 108 ducklings; 540 measurements*		
Full Model		
Term	X^2	*P*
Incubation temperature	0.08	0.78
Age (day)	**1245**	**<0.0001**
Incubation temperature x Age	**27.2**	**<0.0001**

Table A10. Linear mixed effects model investigating how duckling tarsus length changed over time (day 0, 3, 6, 8, and 10) and differed depending on incubation temperature. Duckling ID was included as a random effect because each duckling was measured multiple times. Significance was defined as $p < 0.0167$; Bonferroni correction for reusing same individuals to investigate 3 aspects of morphology.

Dependent variable: Culmen length (mm) *N = 108 ducklings; 648 measurements*		
Full Model		
Term	X^2	P
Incubation temperature	1.91	0.17
Age (day)	**3340**	**<0.0001**
Incubation temperature x Age	**51.9**	**<0.0001**

Table A11. Linear mixed effects model investigating how duckling culmen length changed over time (day 0, 2, 4, 6, 8, and 10) and differed depending on incubation temperature. Duckling ID was included as a random effect because each duckling was measured multiple times. Significance was defined as $p < 0.0167$; Bonferroni correction for reusing same individuals to investigate 3 aspects of morphology.

APPENDIX B: Supplementary Figures

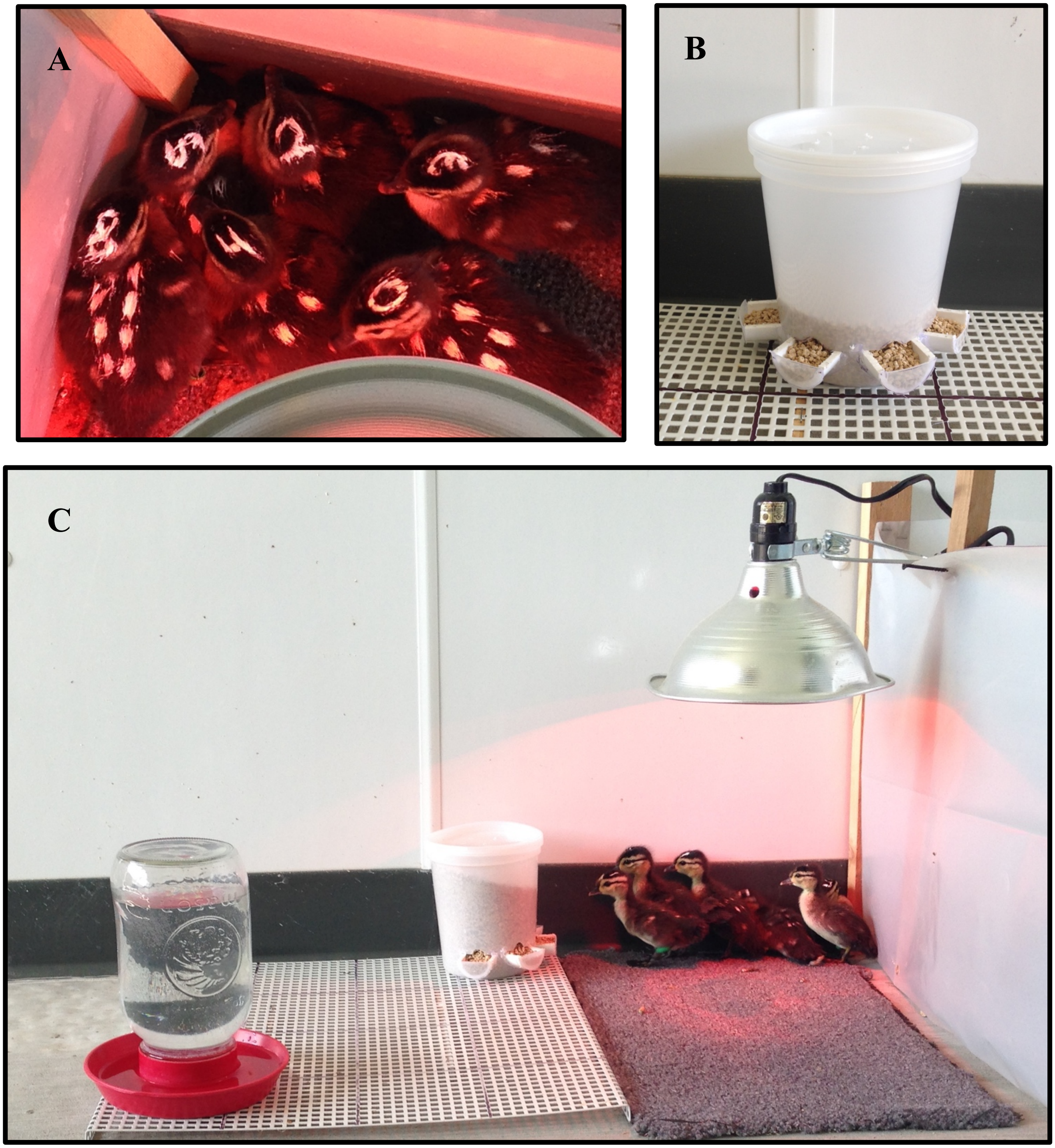


Figure B1. Daily ducking housing set-up (A) Ducklings were numbered for individual recognition in behavioral trials. (B) The food dish used daily had multiple openings and resembled the food dish that was used in feeing trials (Fig. B4). (C) There was a grate under the food dish so ducklings could not eat food spilled on the floor during feeding trials.

Figure B2. The Heat Trial arena (A) Ducklings attempted to gain access to a centralized heat source (infrared heat lamp). Lines in the trial arena were used to calculate the position of each duckling during the trial, where a lower position number indicated that the duckling was closer to the heat source. The position was determined based on the circle in which the majority of the duckling's body was located. Diameter of central position was 4 cm. Length between positions 2, 3, 4, 5, and 6 was 2 cm. For the three most exterior circles, there was a space of 4 cm between each circle, and thus these were given positions of 8, 10, and 12. (B) A zoomed-out picture of the trial arena; GoPro© cameras were used to video record the trials.

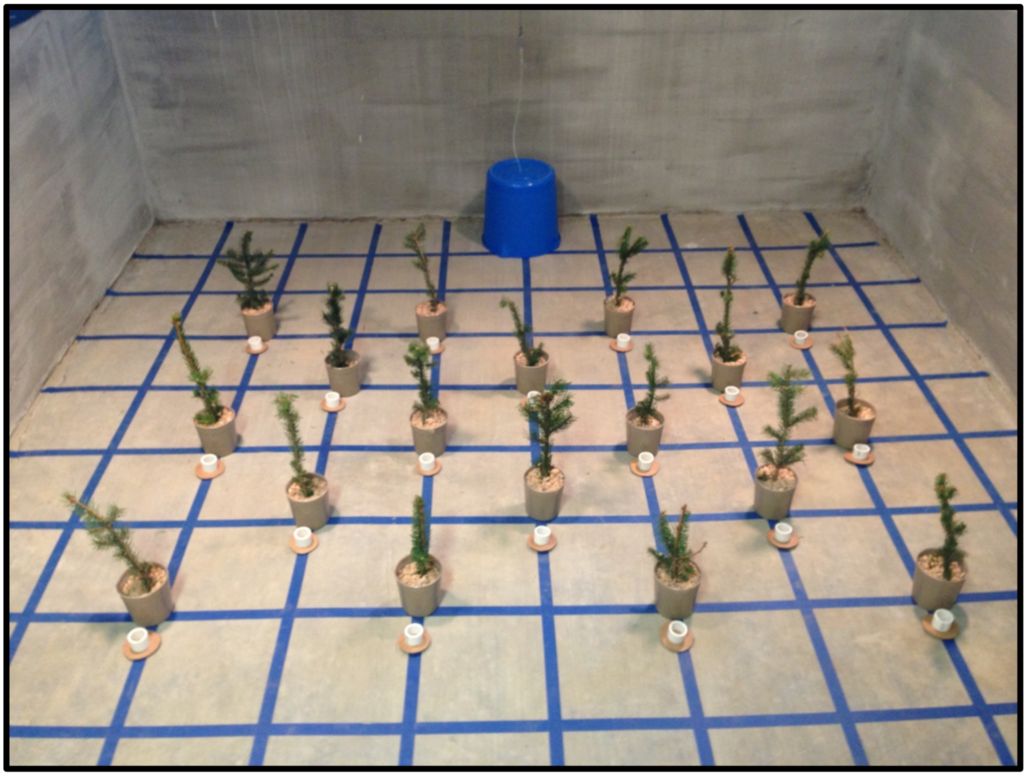


Figure B3. The Novel Environment Feeding Trial arena. Ducklings acclimated in the blue bucket, then explored the arena (novel environment) to find food. Food was placed in white dishes, each behind a small potted plant.

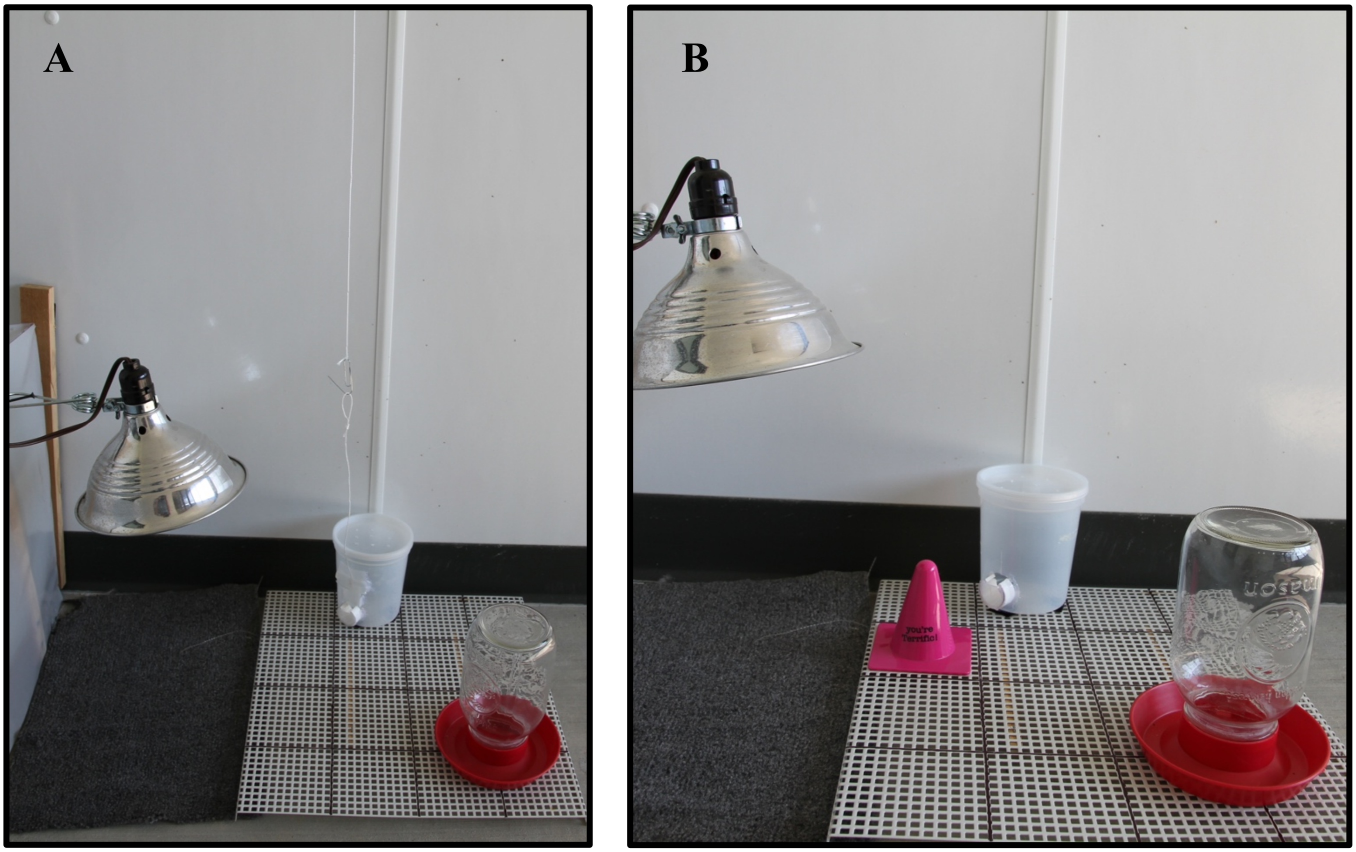


Figure B4. Familiar Environment and Novel Object Feeding Trial set-ups. (A) The Familiar Environment Feeding Trial was conducted in the home aviary (i.e., familiar environment). The normal food dish (Fig. B1B) was replaced by a similar food dish, but with only one opening for food. A string was used to remotely reveal food after letting ducklings acclimate. (B) The Novel Object Feeding Trial was the same as the Familiar Environment Feeding Trial, but there was a novel object (pink cone) placed next to the food.

CONCLUSION

My dissertation advances our understanding of how factors (i.e., clutch size, ambient temperature, disturbance) that alter parental demands influence avian parental incubation behavior, egg temperatures, and subsequently, offspring behaviors that could have fitness consequences. These findings contribute to our understanding of fundamental biological processes such as parental effects, evolution of clutch/litter size, and mechanisms underlying animal behavior. Further, understanding how human-induced environmental changes may affect incubation can inform avian conservation and management decisions, which is particularly important during the current era of rapid global environmental change.

Incubation temperature as an important parental effect

Previously, incubation temperature was an unrecognized factor in determining avian offspring phenotypes, possibly because it was thought that parental regulation of incubation resulted in negligible variation in egg temperatures. However, subsequent work has shown that incubation temperatures vary substantially among nests (Aldrich and Raveling, 1983; Haftorn and Reinertsen, 1985; Conway and Martin, 2000a; Hepp et al., 2006; Ardia et al., 2010; Nord et al., 2010; Coe et al., 2015a). Further, recent studies have shown that even small changes in average incubation temperature (<1°C) can have large effects on offspring phenotype (Chapters 3-5 herein; Hepp et al., 2006; Eiby and Booth, 2009; DuRant et al., 2010, 2011; Hopkins et al., 2011; Nord and Nilsson, 2011; DuRant et al., 2012b, 2012a; Hepp and Kennamer, 2012; DuRant et al., 2013a, 2014; Wada et al., 2015; Berntsen and Bech, 2016; DuRant et al., 2016; Nord and Nilsson, 2016; Bertin et al., 2018; Ospina et al., 2018). However, because birds move and rotate their eggs during incubation, the idea that there might be variation in average incubation temperature among eggs *within* clutches was, nevertheless, largely dismissed. My research provides evidence that average incubation temperatures not only vary among nests, but also vary *within* clutches to the extent (i.e., >1°C) to which different offspring phenotypes should result (Chapter 2). Taken together, these findings highlight incubation temperature as an important and highly variable parental effect that has the potential to not only affect offspring phenotype, but also affect phenotypic differences within broods. This variation is important because natural selection requires phenotypic variation upon which to act. These results also open up a new avenue of research for studying how variation in phenotypes within broods could have further effects. For example, different incubation temperature-induced phenotypes within a brood could lead to differential resource acquisition among siblings (Chapter 5). It is also possible that incubation temperature could be a way by which parents produce variation in offspring phenotypes as part of a bet-hedging strategy in unpredictable environments (Laaksonen, 2004; Marshall et al., 2008; Crean and Marshall, 2009).

Evolution of clutch size and costs of conspecific brood parasitism

The question of what limits the evolution of clutch size is central to the study of life history evolution (Moreau, 1944; Lack, 1947; Skutch, 1949; Ashmole, 1963; Cody, 1966; Klomp, 1970; Winkler and Walters, 1983; Monaghan and Nager, 1997; Jetz et al., 2008), however, the limitations of clutch size in waterfowl are still not fully understood. Further, waterfowl commonly engage in conspecific brood parasitism, where females readily accept and successfully incubate extra eggs laid in their nests by other females. Because this behavior is common, and parasitized broods experience relatively high hatch success (Semel et al., 1988a; Roy Nielsen et al., 2006), it was previously thought that there were little-to-no costs of conspecific brood parasitism to the host (Sorenson, 1992; Lyon and Eadie, 2008). My research reveals that, as clutch sizes increase, overall incubation temperature decreases, and there are also large enough differences in average incubation temperature within nests to produce different offspring phenotypes within broods (Chapter 2). Because low incubation temperatures result in offspring with a decreased ability to exit the nest (Chapter 3), decreased food consumption (Chapter 5), and smaller body sizes (Chapter 5), along with many other suboptimal phenotypes (reviewed in DuRant et al., 2013b), the inability of parents to incubate all eggs in a large nest to an optimal temperature could be a cost of conspecific brood parasitism and limitation on clutch size via diminishing returns on offspring quality. Most studies that investigate costs or limitations of clutch size largely focus either on parental ability to provide nutrition as the limiting factor (as in Lack's hypothesis; Lack, 1947), or on consequences for the parent (e.g., body condition, future breeding attempts) or for hatch success. My work advances life history theory by providing evidence for an understudied alternative hypothesis, that offspring number is constrained by a parent's ability to maintain an optimal *physical* developmental environment, via fitness costs incurred due to effects on post-hatch offspring *quality*. Although my research shows that incubation temperature is affected by clutch size, an important next step is to determine whether experimentally enlarged clutch sizes do indeed lead to at least some offspring with low post-hatch quality and survival. This could be investigated by manipulating waterfowl (e.g., wood duck) clutch sizes in the field and then taking measurements on the offspring once they hatch, followed by capture-mark-recapture methods to estimate survival.

Advancements in animal behavior

Niko Tinbergen identified the four main questions of animal behavior as ‘survival value’ (later called ‘adaptive significance’ or ‘current utility’), ‘ontogeny’, ‘evolution’, and ‘causation’ (or ‘mechanism’) (Tinbergen, 1963; Bateson and Laland, 2013; Nesse, 2013). My dissertation advances our knowledge of the two proximate questions (i.e., mechanism and ontogeny; Nesse, 2013) in relation to avian incubation. First, my research identifies prolactin as an important hormone in regulating short-term behavioral responses to disturbances during incubation (Chapter 1). Although prolactin is known to regulate the onset of parental care behaviors (Hall, 1986; Buntin, 1996; Vleck, 1998; Sockman et al., 2006), my first chapter is one of the first studies to identify prolactin as an important proximate mechanism underlying daily behavioral decisions. Second, my research shows that a non-genetic parental effect (i.e., incubation temperature) affects the development of offspring behaviors (Chapters 3 and 4). This is especially important regarding the study of behavioral types/personality (Chapter 4). Although studies show that animals across taxa display consistent individual differences in behavior across time and contexts (Koolhaas et al., 1999; Gosling, 2001; Sih et al., 2004), it is not fully understood how these behavioral types, and the associated lack of behavioral plasticity within individuals, are maintained within populations (Stamps and Groothuis, 2010a, 2010b; Groothuis and Trillmich, 2011). My research not only reveals that wood duck ducklings exhibit behavioral types, but that variation in the early developmental environment can be a way in which different behavioral types could be maintained within populations (Chapter 4).

Implications for conservation and management

A recent study showed that total bird abundance in North America has decreased by 29% since 1970 (Rosenberg et al., 2019). This is a major problem for society because birds are essential for ecosystem functions upon which we depend. In particular, waterfowl provide crucial ecosystem services such as plant and animal (e.g., aquatic invertebrate) propagule dispersal, nutrient cycling, stimulating decomposition, and pest control, as well as provide economic benefits through recreational hunting, birdwatching, and ecotourism (Green and Elmberg, 2014). Studying avian biology in the context of current environmental changes is critical because humans are interconnected with the environment (Fig. 1), and thus human actions (disturbance or mitigation) will have effects for the entire social-ecological system.

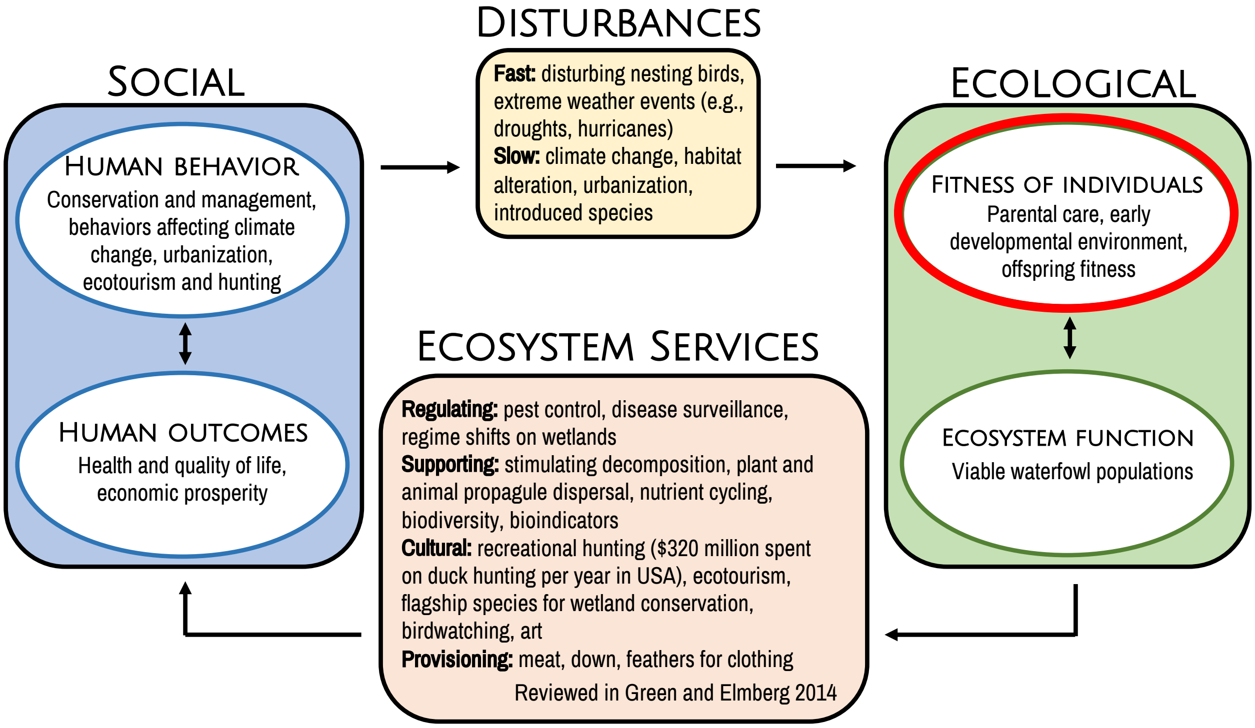


Figure 1. The social-ecological framework for waterfowl (adapted from Collins et al., 2011), describing how humans and waterfowl are interconnected. Social and ecological factors are connected through human-induced disturbances on the ecosystem (or mitigation thereof) and the ecosystem services provided by waterfowl. The circle highlighted in red is the subject of my dissertation.

Avian incubation temperature is a particularly important parental effect in relation to bird conservation and management. Parental incubation behavior can be influenced by human disturbances in nesting areas (Carney and Sydeman, 1999), pollution (Fisher et al., 2006), predation risk (Conway and Martin, 2000b), ambient temperature, and severe weather events (Conway and Martin, 2000c; Coe et al., 2015b). Thus, anthropogenic changes such as urbanization, introduced species, and climate change may affect incubation and, in turn, offspring phenotypes. Indeed, my dissertation shows that, after a nest disturbance, parents spend less time incubating (Chapter 1), which leads to lower incubation temperatures. If disturbances occur frequently enough, this could lead to suboptimal offspring phenotypes (Chapters 3 and 5). Further, studies show that conspecific brood parasitism increases when nest boxes are placed in open areas and in clusters (Semel et al., 1988b; Gowaty and Bridges, 1999; Evans et al., 2002). My research shows that very large clutch sizes, due to brood parasitism, would suffer from lower incubation temperatures (Chapter 2) and, therefore, suboptimal offspring phenotypes (Chapters 3 and 5). Thus, my dissertation can inform management decisions regarding nest box placement practices, and improves our understanding of how human-induced environmental changes may affect offspring fitness through changes in parental behavior.

Future directions

Over the past decade, accumulating evidence has demonstrated that incubation temperature is important for avian offspring phenotype. However, most studies to date, including this dissertation, focus on how average incubation temperature over the entire course of incubation affects offspring phenotype. In a natural setting (as found in Chapter 2), incubation temperatures are much more variable and, in general, lower than those used in laboratory studies, which suggests that avian embryos are tolerant to low and variable temperatures. Indeed, one study found that chicken, pigeon, quail, and budgerigar eggs exhibit no difference in hatch success when incubated under a daily regime of 18 h at standard temperatures (~38°C) and 6 h at

10°C (producing average incubation temperatures of ~28°C), compared to those incubated at constant standard temperatures (Zhao et al., 2017). Therefore, it is possible that certain aspects of temperature (e.g., minimum, maximum, variation) may be more important than the average temperature for influencing offspring phenotype. It is also possible that certain time windows during incubation (e.g., beginning, end) could be more influential for offspring phenotypes than others. For example, a recent study in turtles showed that the incubation temperature during a short time window (~8 days) determines sex, rather than the average incubation temperature over the entire incubation period (Carter et al., 2018). Determining which aspects of incubation temperature have the largest effects on avian phenotype is critical to bridge the knowledge gap between results from laboratory experiments and what occurs in a natural setting. Future work could address this by artificially incubating eggs at the same average incubation temperatures, but changing certain aspects among treatments (e.g., minimum, maximum, temperature changes during different time periods), and then measuring offspring phenotypes.

There is also more research needed to understand how phenotypic differences ultimately influence avian fitness. In general, low incubation temperatures produce ‘suboptimal’ phenotypes (Chapters 3 and 5; DuRant et al., 2013b), and thus we predict that low incubation temperatures would have a negative effect on fitness. Indeed, there is evidence that individuals incubated at lower temperatures experience lower long-term survival compared to those incubated at higher temperatures (zebra finches: Berntsen and Bech, 2016; wood ducks: Hepp and Kennamer, 2012). Although, one study found negative long-term consequences of high incubation temperatures (blue tits; Nord and Nilsson, 2016), suggesting that the relationship between incubation temperature and fitness is likely nuanced and context-dependent. My dissertation identifies some incubation temperature-induced phenotypes that may affect survival differently depending on the environmental context (e.g., behavioral type: Chapter 4, Dingemanse et al., 2004; body size: Chapter 5, Gardner et al., 2011). For example, although some studies suggest that offspring body size is positively correlated with survival (Ronget et al., 2018), a smaller body size, and thus lower absolute metabolic demand, could be an advantage when food resources are limited and unpredictable because it may allow individuals to survive for a longer time without food (e.g., Oksanen et al., 2003). Future studies could investigate whether incubation temperature may interact with food predictability to affect survival using a factorial experimental design, with incubation temperature (low vs. high) and food regime (limited and unpredictable vs. *ad lib*) as the independent variables, and survival as the dependent variable. A precocial species housed in a semi-natural setting (e.g., outdoor aviary) would be an ideal approach, so that food acquisition would not be confounded with parental feeding, and the food regime could be made unpredictable in space and time.

Additionally, my dissertation suggests that the social context may influence the effects of incubation temperature on phenotype, which opens new avenues for research. For example, when ducklings were tested individually, I found an effect of incubation temperature on behavior (Chapter 3 and 4), but when ducklings were tested in mixed-incubation temperature pairs or groups, there were no longer any incubation temperature-induced differences in behavior (Chapter 3 and 5). However, because I only used mixed-incubation temperature groups, it is unknown whether the lack of behavioral differences in groups was due to the effect of the social group (i.e., individual vs. group) or the effect of group composition (i.e., same-temperature groups vs. mixed-temperature groups). To disentangle these, future studies could form both mixed-incubation temperature and same-incubation temperature groups (e.g., all cold, 50:50 cold:hot, all hot) and then measure offspring traits (e.g., exploratory and boldness behaviors, foraging behavior, growth rate). A precocial species (e.g., wood ducks) would, again, be an ideal system because broods can be formed in aviaries and effects of incubation temperature would not be confounded by parental feeding behavior. However, altricial birds that breed well in captivity (e.g., great tit) could also be used, as long as broods are cross-fostered to randomize any effects of parental food provisioning.

Lastly, almost nothing is known about how incubation temperature may influence future reproductive success (but see Hepp and Kennamer, 2012). Given that incubation temperature affects offspring behaviors (Chapters 3 and 4), it would be interesting to investigate whether incubation temperature has long-term effects on reproductive behaviors, such as mate choice, nest building, or indeed, incubation behavior. To investigate this, future studies could incubate eggs at different temperatures, and then raise offspring to adulthood. This would be best done in a short-lived species that matures quickly, and readily breeds in captivity (e.g., zebra finch, house sparrow). Once the offspring are adults, assays to determine mate choice could be conducted (e.g., Y-maze). Then, breeding pairs could be formed, and endpoints such as clutch size, hatch success, nest quality (e.g., mass, height), incubation temperature, and incubation behavior (e.g., on/off-bouts) could be measured. It would be particularly interesting if the incubation temperature that an individual experienced during development affected the incubation temperature that they produce for their offspring, because it would reveal a way by which incubation temperature-induced phenotypic effects could persist throughout generations.

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