2006

Behaviorally-induced periodic cooling of avian embryos

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Behaviorally-induced periodic cooling of avian embryos

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Ecology and Evolutionary Biology

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Iowa State University
Ames, Iowa
2006

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This dissertation is dedicated to my mom

Judith Ann Olson
1945-2004
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CHAPTER 1: INTRODUCTION

INTRODUCTION

For heterothermic organisms, rates of metabolism and development are strongly temperature-dependent, and temperature plays a key role in determining development time (Deeming and Ferguson 1991, Gillooly and Dodson 2000, Gillooly et al. 2002). For bird eggs it is widely accepted that egg temperature ($T_e$) must be maintained within a narrow range for successful embryonic development (Lundy 1969, White and Kinney 1974, Drent 1975, Webb 1987). Eggs of many species, however, are rarely held at stable temperatures, but rather experience frequent episodes of periodic cooling as $T_e$ approaches ambient temperature ($T_a$) when the incubating parent leaves the nest to forage. In particular, many songbirds (order Passeriformes) have uniparental, female incubation, and must leave the nest frequently to forage. They also have small eggs with low thermal inertia, which results in rapid cooling when they are exposed to cool $T_a$ (White and Kinney 1974, Drent 1975, Morton and Pereyra 1985, Weathers and Sullivan 1989). During these behaviorally-induced drops in $T_e$, embryonic development and metabolism are presumably delayed until the adult returns and rewarms the eggs (Ewert 1991). Such temperature variation may result in ~15% variation in observed incubation periods for passerines (Kendeigh 1952, Boersma 1982), and many seabirds are more variable (Boersma 1982). Temperature therefore plays a critical role in determining avian embryonic development rates, but the temperature variation that eggs experience in nature is not well studied. My dissertation is designed to study how periodic cooling of bird eggs affects embryo growth and development. Adult incubation behavior is thought to be based on optimizing the balance between providing care to the young and adult maintenance (Hainsworth et al. 1998). A better understanding of the effects of egg neglect on embryonic growth and sensitivity to temperature will enhance our understanding of parental
incubation behavior and clarify how embryonic thermal requirements may constrain the evolution of parental incubation strategies.

**Ecological Context of Avian Incubation**

For birds in general, mean $T_e$ ranges from 30.7°C to above 40°C (reviewed in Webb 1987), yet how the range and frequency of fluctuations in $T_e$ affect embryo growth has typically received little attention. Presumably the best conditions for development from the perspective of the developing embryo would be high, constant $T_e$, resulting from uninterrupted attentiveness. Strategies to accomplish this include sharing incubation between the male and the female (Reid et al. 2002), or mate-feeding, which would allow the parent to spend more time at the nest (Lyon and Montgomerie 1985, Lyon and Montgomerie 1987). For species in which only the female incubates, the male may increase female foraging efficiency by being vigilant for predators while she forages (Dobbs and Martin 1998), thus shortening the time eggs are left exposed. In many passerines the male plays no role in incubation other than patrolling the territory, thus the incubating female is left to balance her time between providing heat to her eggs and foraging to maintain her own nutritional state. In terms of reproductive fitness, favoring foraging should enhance the incubating female’s long-term survival and future reproduction, while favoring nest attentiveness should enhance her current reproductive fitness. From the point of view of the eggs, however, fitness is enhanced through high nest attentiveness by the incubating female. This represents a classic parent-offspring conflict, where the best interests of the incubating female do not necessarily correspond to those of the eggs. In such a situation the incubating female would be expected to optimize her incubation strategy based on the relative value of current versus future reproduction (Winkler 1987).
Despite the perceived negative effects of neglect on the viability of avian embryos (Lundy 1969, Drent 1975), eggs regularly survive deviations from optimal $T_e$. Cold exposure may include periods of cooling (minutes to days, depending on species) when the parent remains away from the nest (Baldwin and Kendeigh 1932, Boersma and Wheelwright 1979, Haftorn 1988), or when the parent enters a state of torpor (Calder and Booser 1973, Vleck 1981a). House wrens (*Troglodytes aedon*) are an example of a small passerine whose eggs experience variable $T_e$ over the course of incubation (Baldwin and Kendeigh 1932, Kendeigh 1952). Figure 1 shows the thermal profile of a house wren egg in a natural nest in central Iowa (Story Co.). I recorded $T_e$ using implanted thermocouples beginning during the egg laying period prior to the onset of the incubation stage, and through the entire 12-day incubation period until the eggs hatched. Temperature variation prior to the completion of the clutch (arrow) is marked by sporadic on-bouts and frequent off-bouts. Following clutch completion $T_e$ varies less as incubation behavior becomes more consistent, but during the daytime when the incubating parent leaves the nest to forage $T_e$ continues to vary. Except at night when the female remains in the nest on the eggs, the temperature of the eggs is nearly always in a state of flux—$T_e$ regularly drops to $\sim$30°C and returns to $\sim$36-38°C before cooling again. Occasionally the eggs cool for a more prolonged period of time, as on day 143 when the eggs cooled to ambient temperature for several hours during the day. Although the effects of such a long-term cooling event may be more severe than the cumulative effects of several cooling events (Kendeigh 1952), in this nest there was no negative effect on the survivorship of the clutch—all the eggs had hatched by day 152.
Figure 1 (next page). Example of the thermal profile from a house wren (*Troglodytes aedon*) nest showing egg temperature and ambient temperature at the Hind’s Research Farm. Recording was begun during the laying period, continued over the entire incubation period, and ended on the day the first chick was found in the nest. The arrow denotes when the final egg in the clutch of seven eggs was laid. Temperatures were recorded by a miniature thermocouple wire placed in the center of a real egg and glued in place. Another thermocouple wire was placed in the shade beneath the nest box. Temperature was recorded at 1 minute intervals.
The degree of periodic cooling in a nest affects the mean $T_e$ over the incubation period. House wren nests in central Iowa vary in mean $T_e$ (34°-37°C; Chapter 4) and the distribution of temperatures encountered by eggs varies among nests (Figure 2). If there are fitness costs to the embryo associated with reductions in $T_e$, adults should compensate by changing their incubation behaviors. Other ecological constraints, however, also affect adult incubation behavior. For example, reduced food availability, or high energy requirements may force parents to spend more time foraging. Avian incubation is costly to parent birds in both energy (Vleck 1981b, Biebach 1986, Williams 1996) and survival (Visser and Lessells 2001). Incubating birds can detect lower temperatures of eggs and respond by increasing attentiveness (Zerba and Morton 1983, Davis et al. 1984). Conway and Martin (2000a), however, pointed out that bout length changed with ambient temperature in a non-linear way for orange-crowned warblers ($Vermivora celata$). Correlation between ambient temperature and bout length was present between 9° and 26°C, but above 26°C the relationship disappeared, and incubation behaviors were highly variable. If 26°C marks the lower limit of the thermal-neutral zone of the incubating orange-crowned warbler, this may represent a threshold of physiological importance for either female energetics or the developing embryos.

The risk of nest predation from diurnal predators is also thought to drive parental behavior to favor fewer, but longer off-bouts in order to reduce activity at the nest (Skutch 1949, Martin 1995, Conway and Martin 2000b, Ghalambor and Martin 2001, Fontaine and Martin 2006). Thus, embryos may also have been selected to tolerate periods of neglect (Martin 2002, Martin et al. 2006). At the same time natural selection should favor the evolution of rapid embryonic development and shorter incubation periods because of high risk of predation during incubation (Ricklefs 1993, Bosque and Bosque 1995). Adult survival
Figure 2. Frequencies of house wren (*Troglodytes aedon*) egg temperatures recorded simultaneously from two separate nests located at Iowa State University’s Hind’s Research Farm over the entire length of their incubation periods. Recordings were made from 20 May to 5 June and the recording frequency was at 1-min intervals.
is expected to determine the amount of parental care if there is a tradeoff between current and future reproduction (Roff 1992, Sterly 1992). Martin (2002) suggested that if adult survival and prospects for future reproduction are high (often the situation for tropical species), then longer incubation periods that are characteristic of tropical species may be due to low adult nest attentiveness that reduces $T_c$. If so, this may mean that variation in incubation periods across species (a major life-history trait) is more directly determined by temperature (Gillooly and Dodson 2000), than by differences in the intrinsic rates of embryonic growth (Arendt 1997, Tieleman et al. 2004).

To assess the validity of these ideas, quantitative information on how temperature variation during incubation affects embryonic growth and physiology is needed.

**Phenotypic consequences of temperature variation**

In many heterothermic organisms temperature variation commonly induces phenotypic differences during development, thus creating variation on which natural selection can act (Kingsolver and Huey 1998, Travis et al. 1999, Kaplan and Phillips 2006). For altricial birds the evidence for this is scant and observational (e.g. Sockman and Schwabl 1998), but many organisms, including fish (Egginton and Sidell 1989, Egginton et al. 2000), insects (Kingsolver and Huey 1998), reptiles (Rhen and Lang 1999a, Rhen and Lang 1999b, Booth et al. 2000, Flatt et al. 2001, Shine and Olsson 2003, Shine 2004) and amphibians (Kaplan and Phillips 2006) display phenotypic changes in response to temperature variation. Both physiological and morphological consequences of temperature variation need to be considered as phenotypic changes in avian development. Numerous physiological traits are known to be affected by temperature, including enzyme kinetics (Podrabsky et al. 2000, Hochachka and Somero 2002), membrane fluidity (Cossins and Prosser 1978), rates of
protein synthesis (Storch et al. 2003) and gene expression (Podrabsky and Somero 2004). These may be manifest in avian embryos as differences in metabolic intensity, rates of nutrient absorption, growth rates and growth efficiencies. Morphological differences may come about as a direct result of temperature-sensitive changes in the underlying gene expression or protein synthesis and/or through changes in the timing of ontogenetic development due to temperature-sensitive alterations in nutrient supply.

Avian embryos are ectothermic organisms which do not produce adequate body heat to elevate body temperature significantly above body temperature. Embryo temperature instead depends on the supply of heat from the brood patch of the incubating female (Turner 1997), or tracks the local thermal environment (Webb and King 1983, Turner 1985). Despite the popular notion that ectotherms in general are capable of functioning at a wide range of body temperatures, most are found in thermally stable microclimates, are able to adjust their body temperatures behaviorally by moving to more favorable locations or by altering their body posture to control the rate of heat flux (McNabb 2002). Bird embryos, in contrast, have no control over the temperatures to which they are exposed. Instead, parental incubation behaviors control embryo temperature, and thus embryos often develop under a fluctuating regime of temperatures. The degree of cooling depends on the species and habitat. For example, eggs of white-crowned sparrows (Zonotrichia leucophrys) breeding at high elevation commonly cool to ~16°C (Webb and King 1983), whereas biparental incubators, such as the zebra finch (Taeniopygia guttata; Zann and Rossetto 1991), generally show little temperature variation resulting from adult foraging activity.

Thermal adaptation can be defined as the evolution of an altered physiology to function more efficiently at a given temperature. The cellular machinery of organisms adapted to live at a wide range of body temperatures is more costly to produce and maintain
than that of an organism adapted to live only over a narrow range of body temperatures (Hochachka and Somero 2002, Clarke 2003). This is because temperature directly alters numerous physical properties, including cellular and extracellular pH, membrane fluidity, molecular diffusion rates, and molecular configurational states. Adaptation to a wide range of temperature therefore requires a broad repertoire of cellular machinery capable of maintaining coordination over a range of temperatures (Hochachka and Somero 2002, Clarke 2003). Consider the temperature sensitivity of enzymes that allow the unfavorable chemical reactions in respiratory pathways to move forward (Hochachka and Somero 2002). These enzymes exist in an ensemble of conformational states with differing levels of ligand binding ability. Changes in temperature alter enzyme function in different ways. Increased temperatures broaden the diversity of configurational states of a given protein and may reduce the probability of competent ligand binding sites, thus reducing enzyme activity at higher temperatures. On the other hand, decreased temperatures tend to narrow the range of possible configurational states of a given enzyme, but if the enzyme adapts a configurational state at a low temperature with few competent ligand binding sites it will function poorly at this low temperature as well. Enzymes therefore have maximal activity at a relatively narrow range of temperatures. Organisms that are eurythermic (thrive at a wide range of temperatures) must compensate for temperature-driven changes in enzyme function, usually by increasing the amount of a given enzyme to allow for adequate metabolic function across a wide range of temperature (Hochachka and Somero 2002). Alternatively, the production of a wide array of isozymes with different kinetic properties can allow an organism to function over a wide range of temperature (Hochachka and Somero 2002). Either way, for avian embryos to synthesize a greater quantity of enzymes, or a greater diversity of isozymes to maintain homeostasis over a broad range of temperatures, would require increased ATP. This
ATP used to power the synthesis of enzymes could otherwise go towards tissue synthesis. With this in mind, eurythermy in avian embryos may be costly in terms of increased demand on nutrients derived from yolk stores.

DISSERTATION ORGANIZATION

I have approached studying the consequences of intermittent incubation to avian embryos at three levels. First I performed artificial incubation experiments which incorporate periodic cooling to examine changes that occur in embryo phenotype. Chapter 2 examines the effects of periodic cooling on survival, growth rate, growth efficiency and metabolic rate of house wren and zebra finch embryos, while chapter 3 examines the morphological consequences of periodic cooling to zebra finch embryos. Temperature variation has been found to alter the developmental phenotypes of numerous non-avian species, but despite a widespread perception that avian embryos are highly sensitive to deviations from optimal incubation temperature, quantification of the developmental consequences of temperature variation for avian embryos is lacking. If temperature variation during embryonic development alters embryo phenotypes, it will create additional variation on which natural selection may act. Chapter 3 establishes the phenotypic context on which selection acts to determine egg temperature maintenance and adult incubation behavior. If embryo phenotype is altered by temperature, this effect must be incorporated into current theory (along with predation and food limitation) proposed to explain adult incubation behavior.

I then extended the artificial incubation experiments to a field study with house wrens to demonstrate the effect of variation in temperature on embryonic phenotypes and adult incubation behavior in free-living birds. In Chapter 4 I describe the use and effects of a solid-state cooling device that reduced the temperature of all eggs in naturally incubated clutches.
of house wrens. Embryos in these nests therefore developed under conditions of periodic cooling determined by house wren incubation, but at a lower mean temperature than control nests. Many species show a seasonal decline in incubation period which corresponds to warmer ambient temperatures later in the season relative to cooler temperatures early in the breeding season. This seasonal temperature gradient results in faster embryo growth later in the season. I experimentally reduced mean incubation temperature of late-season nests and extended their incubation periods to those comparable to early-season nests, and examined the phenotypic consequences to the embryos. In addition, I quantified the incubation behaviors of incubating females to evaluate how they respond to cooling of their eggs. Incubation is energetically costly to the incubating female and thus cooler egg temperatures should translate into increased energy demands and more time required for foraging. Cooler egg temperatures, however, should also require the incubating female to allocate more time to warming her eggs. If the incubating female increases the foraging time in response to cooler egg temperatures, this would indicate that self-maintenance and future reproductive prospects take precedence over those of her current clutch. However, increased attentiveness to the nest and shorter off-bout lengths when eggs are cooled would indicate maintaining high egg temperatures to ensure her current reproductive prospects takes precedence.

In Chapter 5 I quantified egg metabolic responses to thermal fluctuations in real time to better understand the energetic consequences of cooling in eggs of two species (zebra finch and house wren). Tissue synthesis requires metabolism; therefore knowledge of how embryos respond metabolically to episodes of periodic cooling will indicate the energetic cost of development at a range of temperature. Rates of simple chemical reactions change with temperature in highly predictable ways. Whole organisms, on the other hand, represent a complex amalgamation of multiple biochemical reactions that are contained in a
compartmentalized body where the supply of chemical reactants and products depends on their movement across cell membranes. Thus the response of the organism cannot easily be predicted in any simple way. However, for organisms that aerobically respire (e.g. avian embryos), the rate of oxygen consumption ($V_{O_2}$) indicates the rate of ADP/ATP turnover. The extent to which embryonic metabolic rates changes with temperature will indicate the thermal limits of organismal function (maintenance and/or growth), as well as the extent of adaptation to those temperatures. On one hand, avian embryos must be able to survive unpredictable interruptions in incubation, and embryo survival at lower temperatures suggests the ability to maintain adequate growth at these temperatures. However, the high nest attentiveness of incubating birds suggests that extreme interruptions to incubation are bad for embryos, possibly leaving them outside of the normal range of homeostatic function.

In chapter 5 I test this by cooling and rewarming eggs over a range of ecologically relevant temperatures, while concurrently measuring their oxygen consumption.

    I have integrated the three approaches outlined above to examine how avian embryo thermal biology may constrain life-history evolution, adult incubation behavior and avian breeding biology. My goal is to define clearly how thermal effects as encountered by eggs in nature determine embryo phenotypes. A complete understanding of the importance of thermal biology must incorporate the proximate metabolic response of embryos to cooling, the phenotypic consequences during development at different temperatures, and the degree to which incubating adults respond to cool egg temperatures. Subsequent work should address how the embryo thermal tolerance has evolved along with adult incubation behaviors. Such an analysis will require extensive comparative studies, for which my research provides a solid foundation.
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CHAPTER 2: PERIODIC COOLING OF BIRD EGGS REDUCES EMBRYONIC GROWTH EFFICIENCY

A paper published in *Physiological and Comparative Zoology*

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ABSTRACT

For many bird embryos, periodic cooling occurs when the incubating adult leaves the nest to forage, but the effects of periodic cooling on embryo growth, yolk use and metabolism are poorly known. To address this question I conducted incubation experiments on eggs of zebra finches (*Taeniopygia guttata*) that were frequently cooled and then rewarmed or were allowed to develop at a constant temperature. After 12 days of incubation, embryo mass and yolk reserves were less in eggs that experienced periodic cooling, compared to controls incubated constantly at 37.5°C. Embryos that regularly cooled to 20°C had higher mass-specific metabolic rates than embryos incubated constantly at 37.5°C. Periodic cooling delayed development and increased metabolic costs, reducing the efficiency with which egg nutrients were converted into embryo tissue. Avian embryos can tolerate periodic cooling possibly by adjusting their physiology to variable thermal conditions, but at a cost to growth efficiency as well as a decrease in the rate of development. This reduction in

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¹ Running Head: Periodic Cooling Affects Growth Efficiency in Finch Eggs
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embryo growth efficiency adds a new dimension to the fitness consequences of variation in adult nest attentiveness.

INTRODUCTION

Rates of growth, metabolism and development are strongly temperature dependent (Gillooly et al. 2001, Gillooly et al. 2002). For developing bird embryos, maintenance of egg temperature ($T_e$) by the incubating adult has long been thought to be essential for proper development (White and Kinney 1974, Webb 1987). In a few species it is known that prolonged cold exposure on the scale of hours to days reduces metabolic rate and rate of development (Tazawa et al. 1989), reduces hatching success (Baldwin and Kendeigh 1932, Williams and Ricklefs 1984, Feast et al. 1998, Reid et al. 1999), extends incubation periods (Sealy 1984, Lyon and Montgomerie 1985), and may negatively influence post-hatch growth (Sockman and Schwabl 1998). Yet eggs of many species experience frequent thermal fluctuations, particularly in small passerines with uniparental incubation in which the incubating female regularly leaves the nest to forage, and $T_e$ begins to approach ambient temperature (Zerba and Morton 1983, Davis et al. 1984, Morton and Pereyra 1985, Haftorn 1988, Weathers and Sullivan 1989). Embryonic development is delayed when parents leave the nest to forage, and upon return of the adult and rewarming of the eggs, embryonic development accelerates (Boersma and Wheelwright 1979, Lyon and Montgomerie 1985).

Although short-term cooling is expected to have costs in terms of extended incubation (Lyon and Montgomerie 1987, Martin 2002), it is not clear whether short-term cooling imposes other developmental costs like those associated with long-term neglect. Growth rate and the developmental state of the embryo co-vary (Ricklefs and Stark 1998), but egg cooling may affect the relationship between these two aspects of embryo development. The
mean $T_e$ has been reported for various species (Webb 1987), but the range and frequency of fluctuations in $T_e$, and their ramifications for development have received little attention. Wild bird eggs clearly survive cooling well below 25°C (Zerba and Morton 1983, Morton and Pereyra 1985), and embryos appear to be metabolically capable of tolerating short bouts of cooling (Bennett and Dawson 1979). How frequent, but short term periodic cooling episodes affect development, post-hatching fitness, and phenotype is largely unknown (Reid et al. 2002).

Lipids in yolk are the main energy source for avian embryonic development (Romanoff 1967) with ~35% of the energy content of the egg lost to metabolic processes prior to hatching (Sotherland and Rahn 1987, Vleck 1991). Lengthening development time increases the amount of energy needed for development (Ackerman et al. 1980, Williams and Ricklefs 1984, Vleck and Vleck 1987, Booth and Jones 2002), and differences in the energy content of eggs of different species vary with incubation period for a given egg size. For example, the total energy cost of development for wedge-tailed shearwaters ($Puffinus pacificus$) with a 52 day incubation period is 156 kJ, while similar-sized chicken eggs that hatch after only 21 days use only 104 kJ (Ackerman et al. 1980). Within species, differences in $T_e$ during incubation can also affect development time, and therefore use of available yolk. Megapodes exhibit considerable variation in the incubation temperature that their eggs can tolerate, probably because of their unusual methods of obtaining heat to warm eggs (Frith 1956). In two species of megapode birds, a 4°C reduction in egg temperature lengthened incubation periods by 12-16 days and resulted in 55-76% more total energy used over the incubation periods (Booth and Jones 2002).

Periodic cooling may impose similar energy costs, particularly if tissue growth slows more rapidly than maintenance metabolism as $T_e$ drops. To decipher the significance of
periodic cooling to avian embryos I measured growth and metabolism of zebra finch
(Taeniopygia guttata) embryos over the first 12 days of development under either constant or
fluctuating artificial incubation. I also compared remaining yolk reserves to assess energy
allocation by embryos under different thermal regimes.

MATERIAL AND METHODS

Study Population

Zebra finches are passeriform birds that lay eggs weighing 0.9-1.2 grams and readily
breed in laboratory conditions. Their normal incubation period is ~14 days (Zann and
Rossetto 1991). Zebra finches were maintained in a captive breeding colony in large
communal flight cages provided with water, seed and grit. Their diet was supplemented
thrice weekly with fresh spinach and scrambled chicken egg, including shell. Finches usually
initiated breeding within a few days after gaining access to a nestbox and nest material, so
egg production was synchronized by supplying boxes and nesting material.

Eggs were collected 0 – 2 hours after they were laid, and replaced with non-fertile
eggs laid by females with no access to males to encourage birds to continue laying. Two
flight cages contained a total of 12 pairs of zebra finches and 12 nestboxes, all of which were
used. I labeled eggs by the nestbox from which they were collected and order of appearance
with a non-toxic felt-tip pen. Eggs were weighed to the nearest 0.001g and their length and
width measured to the nearest 0.1mm with digital calipers. Maternity was not known with
certainty, because some females occasionally laid eggs in different boxes on different days.

To best control for possible effects of maternity and laying order, eggs laid within a single
nestbox were distributed evenly across treatments and eggs were assigned to experimental
treatments randomly with respect to laying order. All work reported here was approved by the Iowa State University Committee on Animal Care, log number 6-2-5145-Q.

Artificial Incubation

I built environmental chambers designed to maintain constant egg temperatures or permit periodic rapid cooling and rewarming. Chambers were built from identical plastic foam containers and were constantly supplied with either warm (~42°C) or cold (~5°C) air to control egg temperatures. Eggs in each chamber were placed in contact with each other on a platform that rocked mechanically to simulate parental egg-turning. Small thermocouples were inserted into the centers of two eggs in each chamber, and these eggs were placed on opposite ends of the rocking platform, closest to both the cold and warm air intake, so that they would experience the extreme values of any thermal gradient that might arise across the width of the environmental chambers. For ~1 g eggs metabolic heat production has little impact on egg temperature (Webb and King 1983), so temperature differences between live eggs and dead thermocouple-implanted eggs were considered negligible. The mean temperature of the two implanted eggs within each chamber was measured at 10 s intervals using a Campbell Scientific CR10 datalogger. The datalogger was programmed to activate fans and open gates to draw in cold or warm air as needed to regulate egg temperature. Mean values of egg temperature were recorded to the datalogger’s memory bank every minute. One chamber (the control) was programmed to maintain a constant incubation temperature of ~37.5°C, near the mean measured for zebra finch eggs incubated in captivity (Vleck et al. 1979; Zann and Rossetto 1991) over the entire development period. The other two chambers (periodic cooling treatments) were programmed to undergo periodic cooling once per hour for 15 hours each day, followed by a 9-hour “night” period at a constant temperature of
Figure 1. Thermal profiles experienced by eggs over two days of incubation. Periodic cooling occurred on an hourly basis 15 times a day, followed by a “night time” of constant temperature held at ~37.5°C. Open circles are eggs periodically cooled to 20°C and closed circles are eggs cooled to 30°C. Eggs in the constant temperature treatment remained at 37.4 ±0.04°C. The inset illustrates two cooling bouts over two hours of incubation.
~37.5°C (Figure 1). The two treatment chambers generally cooled at the same rate, but reached different minimum temperatures of 30°C and 20°C before being rewarmed to ~37.5°C. Cool periods generally lasted 20-24 minutes. Mean egg temperatures calculated over the entire incubation period were 37.4°C ±0.04 SD, 37.0°C ±1.5 and 35.4°C ±4.3 for control eggs and those cooled to 30°C and 20°C, respectively. The temperature difference between reference eggs located on opposite sides of the chamber was small, differing by a mean of 0.1°C, 0.4°C and 0.3°C for control eggs, 30°C and 20°C, respectively. The largest differences occurred during times of cooling or rewarming, and declined when eggs were held at a constant temperature. This temperature gradient should increase variation within a treatment, but its effect was minimized because I frequently moved eggs to different positions within a chamber during an experiment. Relative humidity was controlled by adjusting an open water surface in the warm and cool air sources. Eggs lost an average 11% of their initial mass over a projected 14 day incubation period, which is within the range considered optimal (10-12%) for proper development (Rahn and Ar 1974; Drent 1975). Mass loss did not differ between the three chambers (p = 0.12).

At day 12 of incubation each egg was removed from its chamber, weighed to the nearest 0.001g, and its metabolic rate at 37.5°C measured. Eggs were then dissected and the shell, residual yolk, and yolk-free embryo were weighed to the nearest 0.001g. I dried shell and residual yolk to a constant mass in a 60°C oven overnight to obtain dry mass. I froze embryos at -80°C, later thawed and photographed them for a morphometric analysis (Chapter 3), then dried them to obtain dry embryo mass as described above.

By 12 days of incubation, periodic cooling resulted in developmentally delayed embryos that resembled younger embryos from the constant temperature control treatment. I therefore measured dry, yolk-free embryo mass (E_d), dry residual yolk mass (Y_d) and the rate
of oxygen consumption ($\dot{V}_{O_2}$) at 37.5°C for reference sets of 29 zebra finch eggs (in addition to the 16 control eggs that were allowed to develop for 12 days) and incubated under constant temperature conditions and then sacrificed at 8-13 days. I used these reference data to separate effects of periodic cooling via simply slowing development from effects that altered the way energy was used in tissue growth.

**Measurement of Egg Metabolism**

I always measured egg $\dot{V}_{O_2}$ (ml · hr$^{-1}$) at 37.5°C immediately prior to sacrificing eggs. To monitor general egg health in each group and develop an ontogeny of metabolism curve for the treatment and control eggs, I also measured $\dot{V}_{O_2}$ of a subset of eggs ($n = 9$ eggs; 3 per treatment) on days 7, 9 and 11, and then returned these eggs to their chambers to continue development to day 12. Metabolism measurements were conducted in the early morning before the onset of cooling cycles (Figure 1). $\dot{V}_{O_2}$ was measured in a closed system (Vleck 1987), using 60 cc plastic syringes as metabolism chambers. I controlled temperature during measurement periods by submerging syringes in a circulating water bath held at 37.5°C. Barometric pressure and air temperature at the time chambers were sealed were recorded, and gas volumes are reported at standard temperature (0°C), 1 atm pressure, dry (STPD). I left eggs in the chambers for enough time to reduce the concentration of oxygen available by no more than 2% (20-210 minutes) with older eggs requiring less time in the chambers than younger eggs. Although variation due to embryo activity would affect measurements of $\dot{V}_{O_2}$, I assume that the recording intervals used adequately represent “long-term” metabolic activity for that stage of development. At the end of the sampling interval I removed metabolism chambers from the water bath and placed them in a syringe pump that forced the chamber gas through a column containing silica gel and soda lime to absorb water vapor and
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CO₂, respectively. The fractional concentration of oxygen in the gas samples was measured with an Applied Electrochemistry S3-A oxygen analyzer.

Analysis

Differences in yolk mass and metabolic function were compared among cooling treatments in ANCOVAs with embryo mass as the covariate. Where parametric analyses were performed, data were checked for normality, equal variance and log-transformed as appropriate. Percent water composition of embryos and yolks was arcsine-transformed prior to analysis. I combined data from the control eggs incubated at constant temperature for ~12 days (±0.15 days) with the data from eggs incubated over a range of 8-13 days (measured to the nearest 0.1 day) at the same constant temperature (hereafter all called control eggs). To address how periodic cooling affected Ed and Yd, I compared the treatment eggs that had developed for ~12 days to control eggs. I plotted embryo mass and yolk mass against each egg’s accumulated thermal dose (TD), defined as the product of mean egg temperature and the time the embryo was allowed to develop, in degree · days (Gillooly and Dodson 2000). All eggs incubated to ~12 days accumulated a TD that was highly dependent on the cooling treatment they experienced (One-way ANOVA F₂, 36 = 79.98, p < 0.0001). Eggs that experienced periodic cooling for ~12 days had TDs comparable to eggs incubated at constant temperature, but for fewer days (Figure 2), thus making it possible to tease apart effects of both cumulative TD and periodic cooling on growth, yolk use and metabolism. I examined these differences with non-parametric Wilcoxon Signed Rank tests.
Figure 2. Accumulated thermal dose (TD) of control and treatment eggs. TD is determined by the time incubated and the cooling treatment. Periodic cooling reduced the thermal dose accumulated at any given incubation time. The arrow indicates the cluster of treatment eggs incubated for ~12 days for which I measured tissue masses, yolk masses and metabolic rates. Solid circles are control eggs and open squares and triangles are the 30°C and 20°C treatments, respectively.
RESULTS

Survival

Survival of zebra finch eggs to 12 days did not differ among control and cooling treatments ($X^2 = 0.06, p > 0.5$), and was 70%, 67% and 67% for the constant, 30°C and 20°C eggs, respectively. Eggs did not survive to 12 days because they were infertile or they died after some development took place. Sixteen (out of an original 23) in the control group were fertile, and all survived to day 12, while 7 eggs in this group lacked evidence of development. There were 15 (out of an original 18) fertile eggs in the 30°C treatment, but 3 died before day 12, resulting in 12 eggs that survived to 12 days. Two of 16 (out of an original 21) fertile eggs in the 20°C treatment underwent some development and then died, resulting in 14 viable eggs that reached 12 days of age. Eggs that were infertile or died prior to 12 days of age were removed before subsequent analyses.

Wet and dry mass of egg components

Water content (as a % of total) of both embryos and yolks declined as incubation time increased in eggs incubated at constant conditions (Figure 3; $p < 0.001$ and $p = 0.004$, respectively, $n = 45$). Among eggs incubated to 12 days, mean body water did not differ between control and treatment eggs (Figure 3a, Table 1; 88.8% ±1.9; ANOVA, $F_{2, 39} = 0.12$, $p = 0.89$). Yolk water content also did not differ among treatments (Figure 3b, Table 1; 57.4% ±5.9; $F_{2, 39} = 0.10$, $p = 0.90$). To eliminate the large variation due to water content in tissues, only dried component masses were used in further analyses.

Compared to the constant temperature treatment, periodic cooling resulted in smaller embryos that consumed more yolk over the same development time. At 12 days, $E_d$ of 20°C treatment < constant temperature treatment, while the 30°C treatment was intermediate, but
Figure 3. Changes in water content of zebra finch embryos (A) and residual yolk (B) with time. Regression lines for control eggs are: %H₂O embryo = -0.7216·time + 97.578 (r² = 0.37), and %H₂O yolk = -2.0675·time + 84.665 (r² = 0.19). Symbols are the same as Figure 2.
Table 1. Mean (±SD) dry mass (g), wet mass and percent water composition of zebra finch embryos and residual yolk at 12 days of development.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Wet Mass</th>
<th>Dry Mass</th>
<th>% H₂O composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryo</td>
<td>Yolk</td>
<td>Embryo</td>
</tr>
<tr>
<td>Constant 37.5°C</td>
<td>0.448 ± 0.085</td>
<td>0.195 ± 0.036</td>
<td>0.050 ± 0.010</td>
</tr>
<tr>
<td>Cooled to 30°C</td>
<td>0.407 ± 0.065</td>
<td>0.166 ± 0.042</td>
<td>0.047 ± 0.012</td>
</tr>
<tr>
<td>Cooled to 20°C</td>
<td>0.317 ± 0.065</td>
<td>0.167 ± 0.038</td>
<td>0.035 ± 0.010</td>
</tr>
</tbody>
</table>
not significantly different from the other two (ANOVA $F_{2,39} = 7.43$, $p = 0.0018$). $Y_d$ of periodically cooled eggs did not significantly differ from that of the control eggs after 12 days of incubation (ANOVA $F_{2,39} = 2.66$, $p = 0.08$), but $Y_d$ of the treatment eggs averaged smaller than that of the controls.

**Growth and yolk consumption**

The $Y_d$ in control eggs incubated for 8-13 days decreased as $E_d$ increased (Figure 4a; $p = 0.0057$, $r^2 = 0.16$, $n = 45$), as described by the equation:

$$Y_d = -0.32 \cdot E_d + 0.094.$$  

In treatment eggs, periodic cooling reduced the $Y_d$ for a given $E_d$ compared to control eggs (ANCOVA, $F_{2,67} = 5.67$, $p = 0.0053$). A post hoc examination (Tukey’s HSD) showed that embryos periodically cooled to 20°C had significantly smaller $Y_d$ at any given $E_d$ than those incubated at a constant temperature (Figure 4a; $p < 0.05$), whereas periodic cooling to 30°C resulted in a $Y_d$ that was intermediate between the constant temperature and 20°C treatments, but not statistically different from either.

$E_d$ of control eggs incubated at a constant temperature increased with thermal dose, described by the power equation:

$$E_d = 6.33 \cdot 10^{-13} \cdot TD^{4.09}$$

(Figure 4b; $F_{1,36} = 32.9$, $r^2 = 0.85$, $p < 0.001$), similar to the conventional descriptions of embryo growth as a function of development time. I used this relationship to compare $E_d$ of periodically cooled eggs with the expected $E_d$ based on the TD they had received. There were no detectable differences in $E_d$ for either the 30°C treatment (Wilcoxon ranked sign test, $p = 0.34$, $n = 12$) or the 20°C treatment ($p = 0.22$, $n = 14$) from the control eggs. That is, periodic cooling had no effect on the relationship between TD and embryo mass (Figure 4b).
Figure 4. A. Dry, residual yolk mass as a function of dry, yolk-free embryo mass. Periodic cooling to 20°C (thick solid line) reduced the amount of $Y_d$ relative to $E_d$ measured after 12 days of incubation compared to control eggs, resulting in both smaller embryos and reduced residual yolk ($p = 0.0035$). Regressions for control eggs (thin line) and eggs that periodically cool to 30°C (dashed line) are also drawn. B. Log of embryo mass as a function of thermal dose. Periodic cooling did not alter the relationship between embryo mass and thermal dose. C. Log of yolk mass as a function of thermal dose. Eggs that experienced periodic cooling had significantly less yolk that those held at constant temperatures for the thermal dose received (30°C treatment: $p = 0.029$; 20°C treatment: $p = 0.005$).
Y_d of control eggs incubated 8-13 days declined exponentially as TD increased (Figure 5c; F_{5, 44} = 5.0, r^2 = 0.10, p = 0.03), as described by the equation:

\[ Y_d = 0.12 e^{-0.00098 \cdot TD}. \]

In contrast to the results for E_d, Y_d of periodically cooled eggs was significantly lower for a given TD than that of control eggs, both for eggs cooled to 30°C (Wilcoxon ranked sign test, \( p = 0.052, n = 12 \)) and 20°C (\( p = 0.030, n = 14 \)).

**Ontogeny of metabolism**

Metabolic rates of embryos measured between 7-12 days of incubation increased exponentially with days of incubation (Figure 5a; \( p < 0.001 \)), but eggs that experienced periodic cooling had reduced \( \dot{V}_{O_2} \) compared to the control eggs (ANCOVA, \( F_{2, 29} = 16.88, p < 0.001 \)), described by the following equations:

- **Constant temperature:** \( \dot{V}_{O_2} = 0.0188 e^{0.2886 \cdot \text{time}}, \) \( (r^2 = 0.97) \),
- **Periodically cool to 30°C:** \( \dot{V}_{O_2} = 0.0202 e^{0.2625 \cdot \text{time}}, \) \( (r^2 = 0.87) \), and
- **Periodically cool to 20°C:** \( \dot{V}_{O_2} = 0.0201 e^{0.2381 \cdot \text{time}}, \) \( (r^2 = 0.90) \).

The lack of an interaction between time and treatment group (\( F_{2, 27} = 0.93, p = 0.41 \)) suggests that the ontogeny of metabolic rate among groups was fundamentally similar in shape, and statistical differences were due to a higher rate of increase of \( \dot{V}_{O_2} \) in the control eggs at any point in the growth period, compared to cooled eggs. *Post hoc* examination showed \( \dot{V}_{O_2} \) of control eggs > \( \dot{V}_{O_2} \) of 30°C treatment > \( \dot{V}_{O_2} \) of 20°C treatment (Tukey's HSD, \( p < 0.05 \)) at any given day of development.

\( \ln \dot{V}_{O_2} \) increased with TD (Figure 5b; ANCOVA, \( F_{1, 29} = 318, p < 0.001 \)) and, unlike the effect of TD on mass, metabolic rate also differed with cooling treatment (\( F_{2, 29} = 7.42, p = 0.0025 \)). Eggs incubated at a constant temperature had higher \( \dot{V}_{O_2} \) for a given thermal
Figure 5. A. Ontogeny of metabolism in reference to time for zebra finch eggs held at constant 37.5°C (thin line), and periodically cooled to 30°C (dashed line) and 20°C (thick solid line). B. Ontogeny of metabolism in reference to thermal dose. C. Metabolic rate (\(\dot{V}_{O_2}\)) as a function of dry embryo mass (Ed) in zebra finch eggs. Eggs that periodically cooled to 20°C (thick solid line) had \(\dot{V}_{O_2}\) elevated (\(p = 0.0371\)) over eggs periodically cooled to 30°C (dashed line) or held at constant temperature (thin solid line). All \(\dot{V}_{O_2}\) are measured at 37.5°C.
dose than eggs cooled to 20°C (t = -3.16, p = 0.004), but not 30°C (t = -0.19, p = 0.85). The following relationships describe the relationship between TD and $\dot{V}_O_2$:

Constant temperature: $\dot{V}_O_2 = 0.0188 \cdot e^{0.0077 \cdot TD}$, ($r^2 = 0.97$),

Periodically cool to 30°C: $\dot{V}_O_2 = 0.0202 \cdot e^{0.0071 \cdot TD}$, ($r^2 = 0.87$), and

Periodically cool to 20°C: $\dot{V}_O_2 = 0.0201 \cdot e^{0.0067 \cdot TD}$, ($r^2 = 0.90$).

**Oxygen consumption and body mass**

The metabolic rate of reference eggs incubated at constant temperature increased with $E_d$, as described by the equation:

$$\dot{V}_O_2 = 3.45 \cdot E_d^{0.67}$$

(n = 45, $r^2 = 0.80$, p < 0.001). $\dot{V}_O_2$ of eggs in the 20°C treatment was significantly higher for a given $E_d$ than that of eggs incubated at a constant temperature (Figure 5c; $F_{2,66} = 7.69$, p = 0.001), but eggs cooled to 30°C did not differ from that of the controls (p = 0.95). In eggs that periodically cooled to 20°C the relationship between $\dot{V}_O_2$ and $E_d$ was described by the equation:

$$\dot{V}_O_2 = 4.06 \cdot E_d^{0.68}$$

(n = 14, $r^2 = 0.92$, p < 0.0001).

**DISCUSSION**

Zebra finch eggs in these experimental treatments were exposed to periodic cooling patterns similar to those experienced by eggs of uniparentally incubating passerines in nature (White and Kinney 1974), which resulted in smaller embryos after 12 days of incubation than embryos incubated at a constant temperature. For their size, these embryos also had less remaining yolk and higher mass-specific metabolic rates than embryos from control eggs.
These results suggest periodic cooling imposes costs on zebra finch development, including decreased embryo mass, reduced residual yolk, and reduced efficiency of growth, in addition to the well known cost of an extended incubation period.

Eggs are essentially closed systems that receive no input of nutrients during development. Thermal responses of embryos should therefore reflect how temperature deviations affect embryonic ability to most efficiently use a fixed amount of resources during growth. The most important effect of periodic cooling on avian embryonic development may be the decrease in efficiency of development, resulting in a reduced hatchling size and reduced yolk reserves, compared to embryos that develop at constant temperatures. The effects of thermal conditions during incubation will have strong implications later in life if larger nestlings have higher fitness than smaller nestlings (Styrsky et al. 1999). Adverse conditions during growth may influence lifetime fitness parameters including immunocompetence, fecundity, and fat deposition (Lindström 1999). Decreased yolk reserves may impair post-hatch nutrition and the long-term development of neonates into adults (Metcalf and Monaghan 2001). In reptiles, differing thermal conditions during incubation also affect body size and yolk reserves (Rhen and Lang 1999, Shine 2004). It is not well understood how residual yolk influences post-hatch survival of altricial birds (Reed et al. 1999), but neonates of many oviparous organisms continue to rely on these energy stores for some time after hatch (Speake et al. 2003). Ultimately, embryo thermal tolerance should be defined in terms of its impact on the quality of the neonate rather than simply survival to hatching.

Reduced yolk reserves in eggs that periodically cool (Figures 4a, 4c) suggests cooling increases energy demand. This is unlikely to be due to costs of thermoregulation, because altricial embryos are ectothermic (Tazawa et al. 2001) and do not have strong
thermoregulatory capabilities (Vleck and Vleck 1996). I do not know how exposure to periodic cooling alters energy use in eggs, but can suggest several non-mutually exclusive possibilities: lipid movement out of yolk, increased usage of peroxisomal metabolism, increased production of isozymes, or increased basal metabolism.

In periodically cooled eggs, decreased yolk mass relative to embryo mass could result from variation in lipid movement, with more lipids being moved from the yolk into embryonic tissues. For example, acclimation of striped bass (*Morone saxatilis*) to 5°C increases the intracellular lipid content of slow oxidative muscle fibers 13-fold over that of bass living at 25°C (Eggington and Sidell 1989). I did not directly measure lipid content of these embryos, but lipid reallocation could only partially explain the change in yolk reserves produced by periodic cooling. First, both $E_d$ and $Y_d$ were reduced by periodic cooling. Second, the water content of embryos did not differ between treatments (Table 1), suggesting there were no significant differences in lipid content. Third, embryos cooled to 20°C would have had to reallocate lipids to intracellular stores by an additional 31% of their dry embryo mass to compensate fully for the missing yolk, but including lipid membranes and vacuoles, lipid makes up only about 20% of embryo dry mass (Romanoff 1967). Finally, cellular storage would require increased lipid absorption and transport from the yolk sac, but chicken embryos exposed to cold for 24-36 hours had reduced yolk absorption and less lipid stores in their livers than non-cooled embryos (Feast et al. 1998).

Non-mitochondrial pathways of lipid metabolism could also contribute to wasting of yolk reserves if they are upregulated by cooling. Peroxisomes specialize in the catabolism of lipids with carbon chains longer than 8 carbons, and are therefore able to oxidize many of the same substrates as mitochondria (Reddy and Hashimoto 2001), particularly the 18-carbon fatty acids that predominate in the yolk (Maldjian et al. 1996) and which are the embryo’s
primary energy source (Carey 1996). Oxidation of free fatty acids in peroxisomes produces heat that would easily dissipate from the egg, and fewer ATP molecules than are produced from mitochondrial metabolism, as well as potentially damaging H$_2$O$_2$ (Garrett and Grisham 1999). In birds, cellular increases in peroxisomes are induced by natural and synthetic proliferating agents, including fatty acids, hormones and cold exposure (Beck et al. 1992, Wahli et al. 1995, Diot and Douaire 2001, Reddy and Hashimoto 2001). The degree to which peroxisomes play a role in lipid metabolism in avian embryos is not yet known, but investigation into these alternative metabolic pathways in embryos may be fruitful.

Rapid changes in temperature affect a wide array of conditions at the sub-cellular level, including increased intracellular and extracellular pH, decreased fluidity, altered structure of the membrane phospholipid bi-layer, and activities and conformational states of transport proteins (Hochachka and Somero 2002). If avian embryos are capable of making cellular adaptations to cope with fluctuating temperatures, then these would come with an added cost. Periodic cooling may induce a more diverse array of metabolic isozymes that are active over a wide range of temperatures (Hochachka and Somero 2002). The activation energies of key metabolic enzymes, for example, are known to be highly temperature-dependent, and cooling could necessitate synthesis of alternative metabolic isozymes or increases in the concentrations of enzymes or their reactants (Clarke 2003). Changes in the population of a key enzyme could require concurrent changes by several enzymes in that same pathway (McNabb 2002). Repeated cooling may also increase synthesis of cold-shock proteins (Hochachka and Somero 2002). Such responses may be viewed as compensatory mechanisms that would ensure metabolic homeostasis as temperature changes, but would increase demands on yolk energy stores—nutrients that would otherwise go towards embryo growth if growth occurred at constant temperature.
By twelve days of development I observed a ~14% higher rate of metabolism in embryos that periodically cooled to 20°C, relative to eggs with a similar embryo mass incubated at constant temperature. This suggests that yolk reserves may be depleted by higher maintenance (basal) or increased growth costs. Extra-embryonic membrane metabolism is only 2-3% of whole egg metabolism late in incubation (Romanoff 1967, Ar et al. 1987), but are high relative to embryo metabolism only early in development. So it is unlikely that metabolism of the extra-embryonic membranes can account for differences between treatments.

Higher mass-specific metabolism and increased mitochondrial density results from cold acclimatization in some fish species (Eggington and Sidell 1989, Egginton et al. 2000). Additional mitochondria would increase total surface area through which proton leakage occurs, and maintaining mitochondria comprises a considerable proportion of somatic metabolism (McNabb 2002). Bird embryos with increased mitochondrial density in response to periodic cooling would therefore experience a necessary increase in \( \dot{V}_{O_2} \) at normal incubation temperatures. Under these conditions the higher metabolism would not contribute to higher rates of biosynthesis, but would increase maintenance costs.

The observed increase in mass-specific metabolism could also reflect higher growth rates at favorable temperatures to compensate for decreased growth during periodic cooling. If growth stops at lower temperatures, then speeds up when eggs are rewarmed to incubation temperatures, expected differences between cumulative thermal dose and accrued tissue mass would be obscured. Up-regulating growth rates at high temperatures would be an intriguing mechanism to partially compensate for increased development periods resulting from periodic cooling. This would help to ameliorate costs associated with extended incubation periods, such as increased exposure to predation (Martin 1995) or inappropriate rates of egg
water loss (Rahn and Ar 1974). Many organisms have the ability to increase their growth rates, particularly after periods of slow growth owing to environmental conditions that create a set-back (Arendt 1997) and we are just beginning to understand the costs and benefits of this catch-up growth (Gebhardt-Henrich and Richner 1998, Metcalfe and Monaghan 2001).

As egg temperature decreases the metabolic rates of eggs also decreases (Chapter 5). Periodic cooling is known to increase avian incubation periods (Lyon and Montgomerie 1985, Lyon and Montgomerie 1987), presumably by slowing tissue growth and increasing necessary development periods. The most parsimonious model to describe the relationship between growth and temperature would be that of a fixed thermal dose necessary to complete development (Gillooly and Dodson 2000), so long as the range of temperatures experienced did not exceed physiological limits. Under this model, as eggs cooled and rewarmed, embryo growth would slow and increase, respectively. The accumulated growth over the incubation period would therefore depend on the egg temperature at each instant summed over the entire incubation period. I find some support for this model because, although embryo mass was correlated with the cumulative thermal dose, it was independent of whether or not eggs cooled (Figure 4b). However, my results do not refute alternative growth models in which growth rates are a more complex function of temperature. A dosage-based growth model, for instance, would not necessarily predict an increase in yolk consumption in periodically cooled eggs or the up-regulation of metabolism that I observed. These results suggest that the temperature dependence of growth is complex in an environment of periodic cooling, compared to that predicted by a dosage-based model would.

Bird eggs require a narrow range of temperature for development to succeed, yet are able to survive cooling to near-freezing temperatures. Species vary in their mean incubation temperatures (Webb 1987) as well as patterns of egg neglect during incubation (Boersma and
Wheelwright 1979, Vleck 1981, Zerba and Morton 1983, Davis et al. 1984, Morton and Pereyra 1985, Weathers and Sullivan 1989, Conway and Martin 2000). In zebra finches, both the male and female take part in incubation and the periodic cooling documented in this biparental species is not likely to be a large part of their thermal biology, except in cases of egg neglect due to predators, loss of a mate or severe weather conditions (Zann and Rossetto 1991). Zebra finch eggs do remain viable after surviving repeated interruptions of incubation in the laboratory, suggesting these results are generalizable to those species whose eggs experience regular periodic cooling. It would be beneficial, however, to examine interspecific differences in embryo metabolism and growth rate with temperature. Embryos of uniparentally incubating species, which must develop in the face of periodic cooling, may have greater cold tolerance and higher growth efficiency during periodic cooling than a species like the zebra finch, which is less likely to experience periodic cooling (Zann and Rossetto 1991) and has a relatively long incubation period for its egg size (Rahn and Ar 1974). Further comparative study of the physiology and biochemistry of avian embryos will bring a new perspective to established patterns of behavioral ecology of nesting birds (Reid et al. 1999, Conway and Martin 2000) that in the past have focused primarily on predation (Martin 1995).

ACKNOWLEDGMENTS

R. Ackerman, D. Adams, D. Beitz and C. Drewes loaned equipment and/or provided helpful discussions. A. Bronikowski, M. Haussmann, M. Palacios, A. Reid, N. Scott and A. Sparkmann and two anonymous reviewers critically read drafts of this paper. This work was supported in part by a Sigma Xi Grant in Aid of Research, an American Ornithologists’ Union Research Grant, and the National Science Foundation under Grant No. IBN-0309371.
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ABSTRACT

Temperature affects growth and development, and morphometry provides a quantitative description of how temperature changes affect phenotype. I performed a morphometric analysis on zebra finch (*Taeniopygia guttata*, Vieillot) embryos that were either exposed to periodic cooling to 20°C or 30°C throughout incubation over a background temperature of 37.5°C, or were incubated at a constant temperature of 37.5°C. Using a principle components analysis (PCA) I found that the relationship between the linear size (first principle component) and dry embryo mass depended upon the thermal treatment to which the developing embryos were exposed. Periodic cooling resulted in a smaller embryo mass, but had no effect on the multivariate size of the embryo. This suggests that the growth of phenotypic traits such as the length of long bones and the skull are affected less by temperature than by the time spent in development. The development of muscle and organ systems, however, may be slowed during periodic cooling in order to maintain sufficient growth of those body components that are important for the post-natal survival in an environment of sibling competition and nest predation.

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1 Keywords: Zebra finch, *Taeniopygia guttata*, morphology, embryo, temperature effects, incubation
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3 Primary researcher, main author and corresponding author.
INTRODUCTION

For many oviparous organisms, developing embryos experience temperature variation that occurs seasonally, daily, or more frequently because of adult behavior patterns (Hipfner et al. 2001, Flatt et al. 2001, Shine 2004). Birds provide parental care to their offspring in the form of heat-provisioning to the eggs (Deeming 2002), and for many species, eggs are left exposed periodically when the incubating adult leaves the nest during obligatory foraging bouts (Zerba and Morton 1983, Morton and Pereyra 1985, Weathers and Sullivan 1989, Hainsworth et al. 1998, Conway and Martin 2000). This results in egg temperatures that fluctuate throughout the day and a reduction in the overall mean egg temperature during incubation. High temperatures accelerate development and shorten incubation periods (Deeming and Ferguson 1991), and this is widely seen as beneficial for bird eggs which are at a high risk of predation (Martin 1995, Bosque and Bosque 1995). Maintenance of high temperatures is also seen as beneficial because many have claimed that low temperatures result in abnormal embryonic growth, deformities and increased mortality (Lundy 1969, Drent 1975, Haftorn 1988, Cooper et al. 2005). Available data come mostly from studies of domestic poultry, however; and the effects of temperature variation on development of passerines is largely unknown (Reid 2002).

In a wide range of taxa, the effects of temperature on embryonic phenotype are striking, particularly in reptiles with long development periods relative to birds. In American alligator (Alligator mississippiensis) embryos, growth (increases in embryo mass) and development (differentiation of tissue) show different temperature sensitivities—a 3°C difference in temperature results in slower development than growth at the cooler temperature. Therefore cooler temperatures result in longer development times, but embryos are larger in terms of mass and length for a given stage of development (Deeming and
Incubation at low temperatures has been shown to alter other aspects of reptilian growth and development, including neonate behavior, running speed, morphology and residual yolk reserves (Rhen and Lang 1999, Booth et al. 2000, Shine 2004).

Compared to reptiles, birds exhibit rapid embryonic growth rates and require higher incubation temperatures (Deeming and Ferguson 1991), yet it is unclear how changes in incubation temperature affect avian embryonic development. Early experimental work on thermal effects on chicken development showed that embryos incubated at constant temperatures ranging from ~20°C to 30°C resulted in “disproportionate development” and showed an increased rate of deformities (Edwards 1902, Lundy 1969). These classic studies identified developmental consequences during the earliest stages of chicken development under conditions of artificial incubation where eggs were held at static temperatures. If these results are generalizable to dynamic thermal states that are more characteristic of natural incubation, phenotypes may be commonly influenced by incubation temperature during development, thus creating variation among individuals on which natural selection can act (Badyaev and Martin 2000, Kaplan and Phillips 2006). If temperature differentially affects the progression of growth and development, consequences such as altered timing of development relative to embryo mass may be apparent. This is important because the effects of altered phenotypes that occur during development may carry over to fitness and survival consequences later in life (Gebhardt-Henrich and Richner 1998, Bateson et al. 2004, Gluckman et al. 2005, Kaplan and Phillips 2006).

Across many avian species that range from having altricial to precocial modes of development, the developmental timing of tissue and organ differentiation is very conservative (Stark 1998). Despite this, variation occurs in the degree of tissue maturity and rates of cellular proliferation of certain organ systems (Stark 1998). Developmental variation
and covariation among traits changes through ontogeny (Zelditch and Carmichael 1989), resulting in different patterns of allometry (trait size versus overall size) throughout development. For example, nestling house finches (Carpodacus mexicanus) show heterochrony (changes in the developmental timetable) between body and bill growth with body size increasing early and bill growth increasing late in development (Badyaev and Martin 2000). If temperature variation influences the maturity of traits relative to one another, this would be reflected by differences in the allometry of these traits at a given body size.

Earlier I examined the consequences of periodic cooling on embryonic growth efficiency and the energetic physiology of zebra finch embryos (Chapter 2) and found periodic cooling reduced growth rate and growth efficiency (conversion of yolk solids to tissue). Both of these potentially affect hatchling size, and there may be long-term consequences to small size early in life. In this paper I analyze linear measurements and developmental maturity of zebra finch embryos under different thermal regimes to test how temperature variation affects their morphometric phenotypes.

MATERIAL AND METHODS

Study organism

Zebra finches are small passeriform birds that lay eggs that weigh between 0.8 and 1.2 g. This species has an incubation period of about 14 days (Zann and Rossetto 1991), after which their altricial chicks hatch, featherless and unable to thermoregulate. Adults generally form breeding pairs and both parents participate in incubating. This results in a relatively consistent temperature over the length of the incubation period. However, eggs in the wild are occasionally left exposed (Zann and Rossetto 1991) and in the lab are able to
withstand exposure to cool temperatures for several hours and hatch after incubation at variable temperatures (Chapter 2).

Artificial incubation and morphometric measurements

I supplied nest boxes to 12 female zebra finches in a captive population and collected fresh eggs within 2 hours after they were laid. These eggs were part of a larger study to measure the survival, growth efficiency and metabolic rate of embryos exposed to periodic cooling, and detailed methods for how eggs were maintained in incubators are described in Chapter 2. In brief, I collected at least 3 eggs from each breeding female and assigned them to one of three incubation treatments: hourly periodic cooling to (a) 20° or (b) 30° 15 times a day, then returning to 37.5°C, or (c) constant incubation at 37.5°C through out the entire incubation period. These treatments exposed eggs over the length of the experiment to mean temperatures of 37.4°C ±0.04 SD, 37.0°C ±1.5 and 35.4°C ±4.3 for control eggs and those cooled to 30°C and 20°C, respectively. These eggs were incubated for 12 days before embryos were measured. I also incubated a group of eggs for 8-13 days at a constant temperature (37.5°C) to generate a baseline growth curve for this species to which I can compare the eggs that were periodically cooled.

Development was terminated by placing eggs in a freezer, and a short time later they were thawed and dissected into components of eggshell, albumin and extra-embryonic tissue, remaining yolk, and embryo tissue. Embryos were re-frozen at -80°C for later measurement. I thawed the embryos and photographed each from dorsal and left lateral perspectives (Figure 1). I used a Nikon DXM 1200 digital camera mounted on a Nikon SMZ 1500 stereomicroscope. A mm scale was included in each digital photograph.
I used tpsDig software (Rohlf, 2004) to make linear measurements on specimens. Nine linear measurements were collected in triplicate from each specimen and the mean values of each measurement were used for the analysis. Traits measured are shown in Figure 1, and include (a) the tarsometatarsus (tarsus), (b) tibiotarsus (thigh), (c) distal length of wing that includes the carpometacarpus and the phalanges (wingtip), (d) the length of the wing which includes the ulna and radius (wingarm), (e) the diameter of the eye (eye dia.), (f) upper culmen distance (culmen), (g) the gape from the tip of the maxillary to the corner of the mouth (gape), (h) the head width from the top, and (i) the length of the body from the pygostyle (tail) to the insertion of the neck into the body (body length). At this stage of development, bones are incompletely ossified and tissues are malleable, so the shape of the embryo encased within the eggshell is probably different than those I measured. Embryos younger than 9 days did not clearly exhibit all traits and were very fragile, so I did not include them in the dataset. There were 12 embryos in the 20°C treatment, 9 embryos in the 30°C treatment, and 31 embryos that developed at constant 37.5°C, 13 of which developed for 12 days, while the remainder were measured at incubation ages of 8-13 d.

The developmental normal stage of each embryo was assigned using combined staging criteria based on Hamburger and Hamilton (1951) for chickens (*Gallus gallus*), and Yamasaki and Tonosaki (1988) for the society finch (*Lonchura striata*). Traits used as staging criteria (Appendix A) were easily visible in the digital photographs from which the linear measurements were taken and two persons scored them without knowledge of the embryos’ treatments or age. Although the relationship between staging criteria and incubation time is non-linear for chickens and society finches in the earliest stages of incubation (Yamasaki and Tonosaki 1988, Ricklefs and Stark 1998), in the range of stages 36 to 46, where individuals from this study were sampled, the relationship is linear. Visual
Figure 1. Sample specimen of a single zebra finch (*Taeniopygia guttata*) embryo from (A) the side and (B) the top with yellow bars denoting the linear measurements. Linear traits are described in the text.
inspection of embryos revealed variation in the development of the *Musculus complexus* hatching muscles located dorsally on the neck and inserting in the rear of the skull. These muscles are typically hypertrophied in avian embryos to enable the chick to break the shell at hatch (Brooks and Garrett 1970). I scored the development of this muscle independently of normal stage, from 1 (absent) to 4 (well developed), while blind to treatment group and age.

*Data analysis*

The primary design of this experiment was to let embryos grow under conditions of periodic cooling and compare them with embryos that grew at constant temperature for the same development time, thermal dose (degree-days, Chapter 2) and body mass. This allows the effects of development time and temperature to be separated among the cooling treatments. To understand the effect of thermal treatment on ontogenetic growth I first analyzed the growth trajectory over 9-13 days of development at constant temperature, and examined the effects of the three temperature treatments on embryo morphology at 12 days of age.

Linear body measurements were log₁₀ transformed before analysis. To partition variation due to embryo size from that of shape I executed principle components analyses (PCA) on log₁₀ transformed linear measurements based on the covariance matrix (Ricklefs and Miles 1994, Klingenberg 1996). Directions in growth trajectories among cooling treatments were compared by running a separate PCA for each temperature treatment, then calculating the angles between the PC 1 vectors. The angle $\phi$ between two PC 1 vectors, $a$ and $b$, was computed as $\phi = \arccos(a'b)$ (Klingenberg 1996). Differences in $\phi$ between the three groups were tested against a Monte Carlo randomization of PC 1 coefficients with 999 iterations. I then ran a PCA of all embryos together and examined how linear size (PC 1)
changed with dry embryo mass and normal developmental stage. Lastly, the remaining shape variations in PC 2-9 were analyzed in a MANOVA to test the effects of embryo dry mass and treatment on ontogenetic variation in shape.

RESULTS

Multivariate size

Among eggs incubated at constant temperature from 9 to 13 days, the first principle component represented 72.1% of the variation in the dataset, while the second and third contained an additional 9.6% and 5.7% of the variation, respectively (Table 1). Multivariate isometry, defined by $1/\sqrt{p}$ (Klingenberg 1996, Weston 2003), where $p$ is the number of traits examined, is 0.33 for an analysis of 9 traits. Linear measurements of limbs and the body exhibited positive allometry (>0.33) and traits associated with the head showed negative allometry (<0.33), although a bootstrap analysis showed few traits by themselves differed statistically from isometry (Figure 2a). The hind legs of these embryos showed the greatest positive allometry, while the diameter of the eye increased in size the slowest relative to overall body size.

Linear size (PC1) increased with development time ($p < 0.0001$), thermal dose ($p < 0.0001$), and dry embryo mass (Table 2; $p < 0.0001$). For eggs that developed at a constant temperature, thermal dose is strictly dependent on the time that eggs developed. I therefore used a model that examined linear growth at constant temperature in terms of dry embryo mass and development time, but without the thermal dose term. This model explained 86% of the variation in PC 1 ($F_{3, 27} = 60.13$, $p < 0.0001$), and there was a significant time by mass interaction ($F_{1, 27} = 4.85$, $p = 0.036$) indicating that the effect of mass on PC 1 changes with development time. Dry mass was the primary determinant of PC 1 ($F_{1, 27} = 44.7$, $p < 0.0001$),
Table 1. Scaling relationships for 9 linear morphometric measurements of zebra finch (*Taeniopygia guttata*) embryos incubated at constant temperature over a range of 9-13 days.

<table>
<thead>
<tr>
<th>Eigenvalue</th>
<th>0.0207</th>
<th>0.0028</th>
<th>0.0016</th>
<th>0.0012</th>
<th>0.0009</th>
<th>0.0007</th>
<th>0.0004</th>
<th>0.0003</th>
<th>0.0002</th>
</tr>
</thead>
<tbody>
<tr>
<td>% variation</td>
<td>72.09</td>
<td>9.59</td>
<td>5.69</td>
<td>4.24</td>
<td>3.05</td>
<td>2.33</td>
<td>1.45</td>
<td>0.91</td>
<td>0.65</td>
</tr>
<tr>
<td>Eigenvectors</td>
<td>PC1</td>
<td>PC2</td>
<td>PC3</td>
<td>PC4</td>
<td>PC5</td>
<td>PC6</td>
<td>PC7</td>
<td>PC8</td>
<td>PC9</td>
</tr>
<tr>
<td>tarsus</td>
<td>0.40</td>
<td>-0.33</td>
<td>-0.29</td>
<td>0.24</td>
<td>0.38</td>
<td>-0.09</td>
<td>-0.48</td>
<td>-0.42</td>
<td>0.17</td>
</tr>
<tr>
<td>thigh</td>
<td>0.43</td>
<td>-0.28</td>
<td>-0.10</td>
<td>-0.58</td>
<td>0.04</td>
<td>-0.35</td>
<td>-0.05</td>
<td>0.46</td>
<td>-0.20</td>
</tr>
<tr>
<td>wingtip</td>
<td>0.36</td>
<td>0.34</td>
<td>-0.33</td>
<td>-0.15</td>
<td>-0.45</td>
<td>-0.27</td>
<td>0.33</td>
<td>-0.46</td>
<td>0.13</td>
</tr>
<tr>
<td>wingarm</td>
<td>0.34</td>
<td>-0.19</td>
<td>-0.22</td>
<td>0.44</td>
<td>-0.42</td>
<td>0.32</td>
<td>0.05</td>
<td>0.49</td>
<td>0.28</td>
</tr>
<tr>
<td>eye dia.</td>
<td>0.11</td>
<td>0.18</td>
<td>-0.24</td>
<td>0.18</td>
<td>0.66</td>
<td>-0.05</td>
<td>0.61</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td>culmen</td>
<td>0.32</td>
<td>-0.02</td>
<td>0.69</td>
<td>0.41</td>
<td>-0.04</td>
<td>-0.47</td>
<td>0.12</td>
<td>-0.01</td>
<td>-0.05</td>
</tr>
<tr>
<td>gape</td>
<td>0.33</td>
<td>0.77</td>
<td>0.08</td>
<td>0.00</td>
<td>0.14</td>
<td>0.18</td>
<td>-0.43</td>
<td>0.20</td>
<td>-0.09</td>
</tr>
<tr>
<td>head width</td>
<td>0.30</td>
<td>-0.14</td>
<td>0.01</td>
<td>0.09</td>
<td>0.00</td>
<td>0.45</td>
<td>0.22</td>
<td>-0.22</td>
<td>-0.76</td>
</tr>
<tr>
<td>body length</td>
<td>0.29</td>
<td>-0.11</td>
<td>0.45</td>
<td>-0.41</td>
<td>0.11</td>
<td>0.49</td>
<td>0.17</td>
<td>-0.18</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Figure 2. Comparisons of first principle component coefficients for zebra finch (*Taeniopygia guttata*) embryos grown (A) at constant temperature over the ontogenetic sequence of 9 – 13 days, and (B) under different regimes of periodic cooling for 12 days. Plots show allometric relationships of different traits relative to an isometric growth coefficient of 0.33. Error bars are shown for embryos incubated under constant conditions and represent 95% confidence intervals calculated from bootstrap simulations resampled with replacement 999 times. Note the reordering of traits between the two analyses.
while incubation time alone had no significant effect \((F_{1, 27} = 1.69, p = 0.20)\) in the model.

All treatment groups appeared to share a common allometric growth trajectory \((p = 0.235)\) with limbs tending to show positive allometry and head measurements showing negative allometry (Figure 2b). However, considerable variation between temperature treatment groups in the PC 1 coefficients also indicates that this dataset of non-rigid, soft embryonic structures are inherently noisy and/or that sample sizes are too small to overcome temperature effects. The angles of linear growth trajectory (PC 1 coefficients) of embryos that developed for 12 days in the three temperature treatment groups varied from \(\theta = 24^\circ\) to \(31^\circ\), but these angles did not differ from \(\theta = 0\) in pair-wise comparisons among the three treatments (Table 3).

PC 1 scores (linear size) of individual embryos were compared among the cooling treatments and the controls at the same thermal dose, incubation time and dry embryo mass. I employed Wilcoxon ranked-sign tests to compare the PC 1 scores of cooled embryos to the expected values obtained from a linear regression of constant temperature PC 1 scores against the explanatory variable (Table 3) and Bonferroni-corrected for 6 sequential comparisons. Periodic cooling did not affect the relationship between linear size and incubation time \((p > 0.5)\) or linear size and thermal dose \((p > 0.5)\). There was a significant treatment effect on the relationship between linear size and embryo dry mass between the 20°C and constant temperature embryos (Figure 3a; \(p < 0.0006\)), but not between the 30°C and the constant temperature embryos \((p = 0.66)\). Analysis of covariance also revealed that PC 1 scores of embryos cooled to 20°C differed from those of constant-temperature eggs \((F_{1, 40} = 6.58, p = 0.014)\). Those eggs that were periodically cooled to 20°C had longer linear body measurements for their body masses compared to the controls (Figure 3a).
Table 2. Linear regressions of PC 1 against incubation time, thermal dose (TD) and dry embryo mass for zebra finch (*Taeniopygia guttata*) embryos. Wilcoxon ranked sign tests of PC1 scores obtained from cooling treatments of 20°C and 30°C treatments were compared to the estimated values based on the linear relationships at constant temperature.

<table>
<thead>
<tr>
<th>x</th>
<th>R²</th>
<th>p</th>
<th>Wilcoxon 30°C treatment</th>
<th>Wilcoxon 20°C treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>z</td>
</tr>
<tr>
<td>Time</td>
<td>0.088x-1.017</td>
<td>0.63</td>
<td>&gt;0.0001</td>
<td>0.5</td>
</tr>
<tr>
<td>TD</td>
<td>0.0024x-1.015</td>
<td>0.63</td>
<td>&gt;0.0001</td>
<td>-0.5</td>
</tr>
<tr>
<td>Mass</td>
<td>7.71x-0.32</td>
<td>0.84</td>
<td>&lt;0.0001</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

Table 3. Pair-wise comparisons of the angles (ο) between growth trajectories (PC 1 coefficients) for zebra finch (*Taeniopygia guttata*) eggs that experienced thermal regimes of periodic cooling to 20°C, 30°C, and constant 37.5°C for 12 days of incubation.

<table>
<thead>
<tr>
<th>Angle between vectors ο</th>
<th>20°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>30.91</td>
<td></td>
</tr>
<tr>
<td>p = 0.235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>24.13</td>
<td>26.16</td>
</tr>
<tr>
<td>p = 0.383</td>
<td>p = 0.395</td>
<td></td>
</tr>
</tbody>
</table>
The hypertrophy of neck muscles (*Musculus complexus*) increased with embryo age (linear regression, \( p < 0.02 \)) and embryo mass (\( p < 0.05 \)). I therefore calculated the residual of the neck score based on embryo age and mass and tested differences between embryos in the different cooling treatments. Embryos cooled to 20°C had less-developed neck muscles compared to the constant-temperature embryos (Welch’s \( t = 5.40, p = 0.016 \)), indicating that cooling negatively affects the growth of muscle tissue.

**Normal Development Stages**

By 12 days of incubation embryos had grown to a range of normal stages (38-45) that varied significantly with treatment (ANOVA \( F_{2, 30} = 8.9, p < 0.001 \)). Post hoc examination showed embryos cooled to 20°C were at a lower stage compared to the constant and 30°C treatments (pairwise student’s \( t \), \( p = 0.0005 \) and 0.003, respectively). Embryos in the 30°C treatment were at the same developmental stage as embryos that grew at constant temperature (\( p = 0.86 \)). For all embryos, including those that developed for 9 – 13 days at constant temperature, normal stages were positively correlated with development time (\( R = 0.67, p < 0.0001 \)).

Normal stages were positively correlated with dry embryo mass (figure 3b; \( R = 0.75, p < 0.0001 \)) and linear size (Figure 3c; \( PC1; R = 0.80, p < 0.0001 \)) for all eggs that developed from 9-13 days. Residuals of normal stage accounting for dry embryo mass did not differ between treatments (ANOVA, \( F_{2, 48} = 0.63, p = 0.54 \)); cooled embryos did not suffer a setback at least in terms of the traits used to stage the embryos. However, residuals of stage accounting for PC1 did differ between treatments (\( F_{2, 48} = 3.54, p < 0.04 \)), with embryos from the 20°C treatment at a lower developmental stage than embryos from either the constant or 30°C treatment. That is, embryos periodically cooled to 20°C had a larger linear size for a
Figure 3. Scatterplot matrix of developmental traits for zebra finch (*Taeniopygia guttata*) embryos incubated from 9-13 days, including (A) dry embryo mass and principle component 1, (B) normal stage normal stage and principle component 1, and (C) dry embryo mass and normal stage. Black symbols are embryos incubated at a constant 37.5°C, grey symbols are embryos cooled to 30°C, and open symbols are embryos periodically cooled to 20°C. Least square regressions are shown for the constant temperature (dashed line) and 20°C embryos (solid line) when these two treatments differ.
given normal stage than embryos from the control treatment (Figure 3b; ANCOVA $F_{1, 40} = 6.06, p < 0.02$).

Multivariate shape

In the dataset including only embryos which developed at constant temperature for ~9-13 days there was no difference in body shape (PC 2-9) with incubation time and dry embryo mass (Wilks’ $\lambda_{16, 42} = 0.52, p = 0.47$). By day 9 of incubation the body plan of zebra finch embryos was well-defined in terms of shape and did not subsequently change with ontogenetic growth.

A biplot of PC 2 (11.2% of the variation) and PC 3 (9.2% of the variation) showed no separation among the treatment groups (Figure 4). I used a model that incorporated the effects of embryo dry mass and the three temperature treatments to test shape variation among embryos at day 12 of incubation. The whole model showed shape variation was present in this dataset (MANOVA, Wilks’ $\lambda_{24, 70.2} = 0.23, p = 0.02$), while treatment (Wilks’ $\lambda_{16, 48} = 0.42, p = 0.09$) and dry mass ($F_{8, 24} = 0.64, p = 0.10$) alone were marginally non-significant. There were no significant interactions between treatment and dry embryo mass.

Pairwise examination in principle component scores showed a treatment effect in PC7 ($F_{2, 31} = 5.90, p = 0.0068$), composed primarily of differences in eye diameter, and PC 9 ($F_{2, 31} = 3.85, p = 0.0321$), composed primarily of head width.

DISCUSSION

The greater linear body sizes (PC 1 scores) for their body mass and normal developmental stage in the 20°C treatment suggests that periodically cooled zebra finch embryos either enhance the growth of linear traits compared to embryos that developed at a
Figure 4. Biplot of shape variables, PC 2 and PC 3, among zebra finch (*Taeniopygia guttata*) embryos grown for 12 days of age under conditions of periodic cooling to 20°C, 30°C, and at constant 37.5°C.
constant temperature, or reduce the growth of traits that are not reflected by the linear length of the skeleton. Temperature treatments did not affect the trajectory of linear growth, as seen by the similarity of PC 1 vectors between temperature treatments. Rather, the longer body sizes for a given embryo mass in the periodically cooled embryos may be the result of reapportioning nutrients to maintain growth along the longitudinal axis, but at a cost to the growth of muscles and other organs that have an effect on body mass and developmental stage. There was a detectable effect of incubation temperature on embryo shape, but its effect may be subtle as differences between the temperature treatments were not seen in the shape axes that carried the most remaining variation (PC 2 and 3). It is, however, important to point out that differences among smaller principle components may be ecologically significant (Ricklefs and Miles 1994). Cross-fostering of passerine eggs into different nests, which may have had natural variation in microclimate or parental care behavior, has little effect on the heritability of morphology (e.g. similarity of tarsus length), presumably because these traits are primarily genetically determined (Smith and Dhondt 1980, Dhondt 1982). However, these experiments were not explicitly designed to test how the thermal environments of foster nests affect embryonic growth. My results are similar to studies on reptile embryos that grow at different temperatures—linear traits increase relative to body mass at lower incubation temperature (Deeming and Ferguson 1989, Booth et al. 2000, Flatt et al. 2001).

Periodically cooled embryos experienced conspicuously reduced development of the large muscle complex on the back of the neck that is necessary for hatching to occur. In growing organisms muscle growth may occur through either hyperplasia—the increase in fiber number, or hypertrophy—the increase in fiber size. Cold exposure in chicken and quail embryos slows the growth rate of muscle tissues (Hohtola and Visser 1998), and decreases in mean incubation temperature by as little as 2°C reduced the number of muscle fibers in
breast muscle of turkey (*Meleagris gallopavo*) poults (Maltby et al. 2004). It is thought that adult organisms can only increase muscle size through hypertrophy, so compensation for reduced muscle growth in embryos might be restricted to hypertrophy of muscle cells in adults because at some point in development the number of muscle fibers becomes fixed. It is not clear how a small *Musculus complexus* would affect hatchability of the finches in this study because they were not allowed to hatch, but zebra finch eggs exposed to hourly periodic cooling as low as 20°C can hatch (C. R. Olson, unpublished). It is possible that reduced muscular development in the neck and other regions of the body would negatively affect the chick’s ability to hatch, and subsequently, to beg for food as a neonate. Fewer muscle fibers resulting from cold temperatures during development are likely to impair the ability of adults to compete with conspecifics and escape predators.

Interest in how environmental conditions influence embryonic growth and physiology has increased with the realization that environmental insults to the embryo may have long lasting effects on organismal fitness (Bateson et al. 2004). The *Thrifty Phenotype Hypothesis* was proposed to explain the response of growing embryos to poor growth conditions (Hales and Barker 1992, Petry et al. 2001). If periodic cooling alters avian embryonic development by causing disproportionate growth and increased incidence of deformities (Edwards 1902, Lundy 1969, Drent, 1975), the thrifty phenotype hypothesis may provide a conceptual foundation for how we view the avian embryonic response to a poor thermal environment. A central tenant of this theory is that during periods of poor growth conditions, nutrients are redistributed from the growth of less essential organs (muscle, liver, and pancreas) to the growth of organs that are critical to the short-term survival of the organism (brain). Because certain critical functional traits are defined during development, reductions in development of these less critical tissues may have strong physiological impacts later in life. A developing
embryo facing a thermal challenge therefore may alter its growth phenotype to enhance its short term survival (e.g maintain skeletal growth), but the altered phenotype is expected to have consequences later in life.

Periodic cooling of bird eggs results in body masses that are smaller overall than control eggs incubated for the same amount of time, while linear body size (PC 1) was not affected by the cooling treatment. The growth of long bones and structures along the longitudinal axis of the body plan are less affected by reductions in temperature than is deposition of soft tissue. The different relationships I see between linear size and mass between the treatments may be highly influenced by canalization of linear growth over the developmental time period (Arendt 1997, Boersma and Wit 1997), resulting in a trade-off between growth of different structures in the body that may vary in importance to the short-term survival of the embryo. This would result in hatchlings being able to achieve a sufficient linear size at hatch, but at a cost to the development of other systems that may be less important for immediate survival. Future studies of the effects of periodic cooling on relative organ size, function and neuronal development would be useful to test this hypothesis.

Cold exposure may affect embryonic development in a similar way to that of food restriction on embryo growth of placental mammals (Hales and Barker 1992, Petry et al., 2001), where organ systems that are essential for short-term survival are favored over those that are less-essential. Chicken embryos exposed to cold have reduced uptake of yolk solids (Feast et al. 1998), and over time this may result in chronic nutritional stress of the growing embryo due to disrupted delivery of nutrients to the tissues. Altered growth with periodic cooling may resemble dietary restriction because cold disrupts the absorption and transport of nutrients from the yolk into the growing embryo. Mammals may show placental insufficiency where delivery of nutrients is restricted due to a small placenta or restricted
flow in the uterine arteries (Sadiq et al. 1999, Petry et al. 2001). In ectotherms that experience cooling, barriers to nutrient transport may exist at multiple levels due to the temperature effects at all levels of organization, including reduced diffusion rates across biological membranes and decreased membrane permeability at lower temperatures (Hochachka and Somero 1998).

During growth, if low temperature disproportionately favors elongation of the skeleton relative to growth of muscles and other tissues, this would then set the stage for “catch-up” growth to occur once environmental conditions improve (Metcalfe and Monaghan 2001). For altricial birds, this may not come until after they hatch, become endothermic, or leave the nest and start foraging on their own. Although the adaptive significance of catch-up growth is difficult to study (Gebhardt-Henrich and Richner 1998), I suggest that high predator-induced mortality at the nest during the incubation and nestling stages would favor linear growth and maturation over accumulation of tissue mass. This strategy would reduce the time that chicks remain in the nest and vulnerable to predators. Nest predation is thought to have had a profound influence on the evolution of the rapid growth rates typically seen in birds (Bosque and Bosque 1995, Angelletta and Sears 2003) resulting in the most rapid growth rates among the vertebrates (Case 1978). Selection to avoid nest predation may be the ultimate cause of the dissociation between linear growth and mass that we see in this study, if it allows chicks that grew under poor thermal conditions to leave the nest in a timely way. Sibling competition may also select for large linear size and rapid maturation (Ricklefs 1993, Lloyd and Martin 2003), if it allows chicks to succeed in the competitive environment of the nest.

A consequence of the measures undertaken to survive unfavorable conditions is that once the poor conditions subside, the resulting phenotype may be poorly suited for the
environment in which the organism is normally found (Hales and Barker 1992). The embryonic response in the short-term of favoring the growth of some systems over that of others may have long-term consequences to fecundity and life-span due to catch-up growth costs (Lindstrom 1999, Metcalfe and Monaghan 2001, Yearsley et al. 2004). There are numerous examples of how nutritional stress in fetal rodent models predisposes individuals to obesity, and metabolic disorders later in life (Breier et al. 2001, Gluckman et al. 2005, McMillen and Robinson 2005), yet little is known about how this would affect a wild bird. Considering that birds rely on a high degree of dietary efficiency to maintain their high metabolic scope during activities such as migration and over wintering (Karasov and McWilliams 2005), disruptions in the development of normal physiological function during ontogeny may impose an increased risk to survival and fitness in the long-term.

ACKNOWLEDGEMENTS

All work reported here was approved by the Iowa State University IACUC, log number 6-2-5145-Q. D. Adams provided the use of the Nikon Scope and image collecting software. R. Ackerman provided use of the Campbell Scientific dataloggers that operated the environmental chambers where eggs were incubated. This work was supported in part by a Sigma Xi Grant in Aid of Research, an American Ornithologists’ Union Research Grant, and the National Science Foundation under Grant No. IBN-0309371.

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Hamburger, V. and H. L. Hamilton. 1951. A series of normal stages in the development of


APPENDIX

Appendix A. Normal staging criteria used to describe developmental maturity for late-term zebra finch embryos (*Taeniopygia guttata*) based on Hamburger and Hamilton (1951) and Yamasaki and Tonosaki (1988).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>Beak: lower beak begins to elongate, but does not reach length of upper beak; no cornification evident.</td>
</tr>
<tr>
<td>36</td>
<td>Beak: Lower and upper beak equal in length; egg tooth begins to be cornified.</td>
</tr>
<tr>
<td>37</td>
<td>Tail: Extends straight out from body.</td>
</tr>
<tr>
<td>38</td>
<td>Tail: Tail curves ventrally.</td>
</tr>
<tr>
<td>39</td>
<td>Beak: egg tooth cornified at base; cornification not evident on rest of bill.</td>
</tr>
<tr>
<td>40</td>
<td>Beak: upper beak begins to be cornified at the top; lower bill not cornified.</td>
</tr>
<tr>
<td>41</td>
<td>Beak: upper cornified in the anterior 1/3, continuous to egg tooth; top of lower bill begins to be cornified. Feet: claws are pink</td>
</tr>
<tr>
<td>42</td>
<td>Beak: Cornification spreads proximally to anterior 50%; lower bill 50% cornified. Feet: claws cornified and white.</td>
</tr>
<tr>
<td>43</td>
<td>Beak: Cornified to the base in upper and lower bill; Beginnings of gape visible.</td>
</tr>
<tr>
<td>44</td>
<td>Beak: Cornification complete; gape characterized by a prominent zone of pigmentation. Skin: thin.</td>
</tr>
<tr>
<td>45</td>
<td>Beak: gape pronounced with distinct downward bend. Skin: wrinkled and thickened.</td>
</tr>
<tr>
<td>46</td>
<td>Hatch</td>
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ABSTRACT

Temperature regulation is an important element in avian reproductive success. It has been suggested that bird embryos exposed to suboptimal temperatures incur direct fitness costs through developmental consequences and reduced embryo health. Yet the importance of these costs to the developing embryo and to the evolution of adult incubation behaviors is poorly defined, and may be particularly important for passerines that regularly experience variable egg temperatures as part of their incubation biology. I experimentally reduced egg temperature using a heat pump arranged to draw heat out of the clutch during development in natural nests of house wrens (Troglodytes aedon). I measured how the resulting reduced egg temperature affected adult incubation behaviors and embryo growth and metabolism. Adult wrens altered their incubation behavior to ameliorate the poor thermal state of their eggs by increasing the overall amount of time they sat on the eggs, decreasing the length of off-bout lengths and increasing length of on-bouts. However, the birds were not able to fully compensate for the cooling treatment, and egg temperatures were ~3°C lower than eggs in
control nests. Low egg temperatures increased incubation periods and reduced embryo growth rates, but did not decrease embryo survival. However, embryo growth efficiency (embryo tissue per g of yolk utilized) was lower at low egg temperatures. Relatively high egg temperatures, typical of avian incubation, permit high embryo growth efficiency in addition to short incubation periods, which should select for increased adult attentiveness in cool environments. These results draw attention to the importance of embryonic temperature sensitivity in the tradeoff between offspring fitness and adult maintenance and highlight an important selective force that underlies the evolution of avian incubation behavior.

INTRODUCTION

The most prominent feature of avian incubation is the adult attentiveness required to regulate egg temperature within the limits required for successful embryonic development. Most birds employ behavioral strategies that maintain relatively constant, high egg temperature (Kendeigh 1952, White and Kinney 1974). Commonly, both members of the pair alternate on the nest to provide heat to the eggs, or less commonly the male assists by feeding the female on the nest who incubates the eggs full time (Drent 1975). Eggs of these species are expected to experience relatively constant temperatures throughout incubation (Cresswell et al. 2003). However, many small passerines have adopted a strategy of female-only, intermittent incubation with minimal or no help from the male (Drent 1975). Because females of these species are unassisted by males in the direct care of their eggs, the time available to forage may be constrained by the need to incubate the eggs, at the same time the need to rewarm their clutch following a foraging bout increases adult energy requirements (Williams 1996). In addition, eggs of female-only, intermittent incubators undergo periodic cooling, which delays embryonic development and prolongs incubation periods (Vleck
Female-only, intermittent incubation represents a classic parent-offspring conflict (Trivers 1974), because the incubation strategy that yields the highest survival to the incubating female is not necessarily the same as that which maximizes fitness of the developing embryos (White and Kinney 1974, Webb and King 1983). Incubation behavior should therefore represent a trade-off to promote efficient and rapid embryonic development, while at the same time optimizing the current and future reproductive prospects of the incubating female. Changing environmental conditions that affect either the parents or the developing embryos are expected to shift the allocation of parental care, yet the extent to which females alter their incubation behavior when thermal conditions change remains poorly understood (Reid Monaghan and Nager 2002).

Thermal effects have direct and indirect effects on the adults and eggs (Saino et al. 2004). Many birds that breed seasonally in temperate North America encounter low ambient temperatures during incubation because earlier reproduction conveys a fitness advantage to the young due to higher arthropod abundance and increased time for maturity prior to autumn migration, relative to later seasonal reproduction (Haywood and Perrins 1992, Young 1994). Yet warm ambient temperatures, usually found later in the reproductive season, are directly beneficial to the incubating adult through reduced costs of thermoregulation (Haftorn and Reinertsen 1985) and lower demands on their energy reserves (Mertens 1987), or indirectly through reduced costs to provide heat to cold eggs (Vleck 1981b, Biebach 1986). Lower energy demands as a result of warmer temperatures are expected to reduce the food intake required for incubation and would thereby reduce the number and duration of foraging bouts to and from the nest. Adult activity around the nest attracts the attention of diurnal nest predators (Skutch 1949, Fontaine and Martin 2006), therefore warmer temperatures would also allow for fewer foraging bouts during the day (Martin and Ghalambor 1999, Conway
and Martin 2000a). Embryos benefit directly from warm temperatures by more rapid and efficient growth (Chapter 3, Booth 1987), resulting in short incubation periods (Lloyd 2004). Numerous studies on the effects of temperature variation on non-avian oviparous organisms have revealed hatchling phenotypes are affected by the temperatures they experience during development (Booth 1987, Rhen and Lang 1999, Booth et al. 2000, Flatt et al. 2001, Shine 2004), but birds differ from most of these other egg-layers in that adults behaviorally control the temperature of their eggs throughout development.

Avian incubation behavior is therefore a strategy (1) that balances the energetic needs of the incubating female required for self maintenance and (2) provides adequate heat to the eggs for the development of viable offspring, while (3) minimizing the attention of nest predators. When environmental conditions change, incubating birds may alter this balance by altering the duration and frequency of the bouts they take on and off the nest, as well as by increasing or decreasing their overall nest attentiveness (White and Kinney 1974, Vleck 1981b). If adult maintenance costs increase with colder temperatures, high nest attentiveness should become increasingly difficult as ambient temperatures decline, yet numerous studies have revealed a negative relationship between nest attentiveness and air temperature in cold environments (reviewed in Conway and Martin 2000b), suggesting that the strategy used by parents is to increase the amount of care to the current brood, possibly at a cost to the energy balance of the adult and possibly future reproduction.

Several lines of evidence suggest that birds perceive the thermal state of their eggs through their brood patches (Lea and Klandorf 2002). Experimentally warming or cooling eggs decreases or increases attentiveness, respectively, independent of air temperature (Drent et al. 1970, Davis et al. 1984). Anesthetizing the brood patch increased attentiveness in village weavers (*Ploceus cucullatus*, White and Kinney 1974), suggesting a neuroethological
feedback mechanism in which the timing of off-bouts are triggered by egg temperature. The above studies indicate incubating adults respond directly to the temperature of their eggs, possibly at increased cost to themselves, however these behavioral responses should only be adaptive if a reduction in offspring fitness due to unfavorable temperatures occurs. These fitness consequences to embryos have not been examined in a wild population despite a long-held belief that exposure to cold temperatures is harmful. In addition, the few studies that have modified egg temperature and examined adult behaviors have done so only over short time scales (one day or less). Due to the low available fat reserves held by breeding birds, incubation behaviors may be very different when thermal conditions are experimentally altered over the long-term. Incubating adults may also habituate to the thermal condition of their clutches over the long-term, while abruptly changing the thermal state of the nest in a short-term experiment may yield exaggerated responses by incubating birds.

I examined how experimentally cooled egg temperatures affected house wren (*Troglodytes aedon*) incubation behavior concomitant with embryonic development. House wrens are extremely tolerant of human activity at the nest (Baldwin and Kendeigh 1932), making them an ideal study organism to study the thermal effects on avian incubation biology. They are small migratory passerines that lay clutches of 6-8 eggs in a secondary cavity and have two broods a season (Johnson 1998). Second broods are laid mid-summer when ambient temperatures are relatively high. House wrens are uniparental incubators and take 40 to 80 foraging bouts a day (Johnson 1998). I used late-season wren nests that typically experience seasonally warm air temperatures and experimentally reduced the egg temperature of clutches over their entire incubation periods. By overriding the effect of air temperature on egg temperature I was able to test the effects of egg temperature independent of ambient temperature in models that described adult incubation behaviors. In addition, I
examined how low incubation temperature impacted embryonic development and growth efficiency in these same nests.

MATERIAL AND METHODS

I conducted a field study at Iowa State University’s Hind’s Research Farm, located 42° 3' N, 93° 37' W. The site is composed of mixed woodland and agricultural fields of maize and soy crops. Wrens that settled on territories at the study site were exposed to human activity typical of farms in Iowa (tractors, herbicide applications), as well as the frequent presence of human workers conducting crop research. Work reported here was approved by ISU-IACUC protocol #4-04-5638-Q.

I set out an array of ~150 nest boxes in wooded areas, near out-buildings and along the Skunk River. I artificially cooled six late-season house wren clutches initiated between 12 of June and the 14 of July and compared them with six control nests that initiated egg laying on the same dates in order to separate the effects of seasonal temperature variation and artificial nest cooling treatment on adult nest attentiveness and embryo growth parameters. I checked nest boxes daily and recorded the appearance of new eggs in nests. To determine laying order I marked each egg on the day it was laid with an indelible felt-tip marker. I calculated the incubation period of clutches from the day the last egg was laid, to the day the first egg hatched (Nice 1954). This method does not account for hatching asynchrony that may occur due to the female beginning incubation when the penultimate egg is laid (Chapter 1, Johnson 1998), but it is practical because identifying chicks from individual eggs after they have hatched is difficult.
**Egg temperature manipulation**

I cooled eggs of house wrens over their entire incubation period with heat pumps consisting of 70 watt Peltier thermoelectric coolers that removed heat from the nestbox from a ~20 cm$^2$ focal area beneath the eggs. Peltier thermoelectric devices function to transfer heat across their surface from one thermal mass into another when an electrical current is applied. This makes it possible to reduce the temperature of a heatsink (thermal mass) well below ambient temperature. A shallow, cup-shaped, aluminum heatsink (mass ~100 g), and a larger heatsink (660 g) with a large surface area to dissipate the heat into the environment, were coupled to each side of a Peltier thermoelectric device with ThermoSet Interface Pads (Melcor Corporation), which are thin, adhesive films with high thermal conductivity. This unit was then built into the floor of a nest box and nest materials (consisting mostly of small sticks, 1-2 mm dia., and grass) taken from an unused house wren nest were arranged to closely resemble a wren nest (Figure 1). The entire clutch of eggs rested on a layer of burlap that was glued with epoxy cement into the cup-shaped heatsink so that eggs did not come into direct contact with metal. When ambient temperature exceeded 30°C, a small CPU fan placed below the nestboxes was activated by a datalogger to increase the efficiency of the cooling units. Heat pumps and fans were powered with 12-volt deep-cycle batteries that were recharged at 2-day intervals.

I recorded temperature with type-T thermocouples in three locations in nest boxes fitted with heat pumps: (1) in the center of a house wren egg implanted with a small thermocouple wire that was threaded through a hole in the side of the cup-shaped heatsink, (2) in the center of the large heat sink, via a hole bored through its side, and (3) in the nest lining of the nest box, 3-4 cm from the edge of the cup-shaped heat sink (Figure 1) using a Campbell Scientific CR10 datalogger. All datalogger input channels were calibrated against a
Figure 1. A Peltier cooling device used to cool eggs in a nest box. Two heat sinks (dark grey) sandwiched a Peltier module that moved heat across its surface, thus creating a focal cold spot beneath the eggs. A Campbell CR10 data logger (located on the ground beneath the nestbox) controlled the temperature of the heatsink apparatus by opening and closing a relay switch as needed, and recorded temperature data at locations in (1) an egg, (2) in the lining of the nest and (3) inside the larger heat sink. The heat sink apparatus was powered by a 12V deep cycle battery.
standard Hg thermometer to the nearest 0.1°C. The heat pump functioned most efficiently by regulating the internal temperature of the large heatsink (thermocouple 2) at 2-3°C above the temperature in the nest box (thermocouple 3). When the temperature of the large heatsink was less than 2°C above air temperature, a datalogger control port activated a reed relay that would close the circuit to the Peltier thermoelectric device, cooling the eggs and warming the large heatsink (Figure 1). This allowed the relatively smaller heatsink to be quickly drained of heat when the apparatus was turned on, and the small temperature gradient between the large heat sink and the rest of the system avoided the back-flow of heat into the nest when the unit was deactivated by the datalogger. The datalogger took readings at 10 second intervals and activated or deactivated the Peltier cooler, as necessary.

I used thermocouple 1 to calculate the mean egg temperature measured over the entire incubation period as well as event-specific egg temperatures (Te), defined as the mean temperature over a ±1 minute interval (n = 3 measurements) at the time a behavioral event occurred. Ambient air temperature generally increased through the morning hours and a polynomial function that described air temperature as a function of the time of the day was used to calculate the ambient temperature at the time of each behavioral event. Hourly air temperatures available from the National Oceanic and Atmospheric Administration (http://www.noaa.gov/) were recorded at the Ames Municipal Airport weather station located 41° 59' N, 93° 37' W, and were used to estimate ambient thermal conditions (Ta) at the study area.

Experimentally cooled nests with heatsink devices were paired with control nests without heat sinks. Nest boxes were checked daily and eggs in each nest were pre-assigned experimental fates based on their laying order. In both experimental and control nests, the first, third and fourth eggs were allowed to hatch. The second egg was collected on the day it
was laid and dissected to estimate component masses of yolk and albumin in fresh eggs. The fifth egg was collected 10-12 days after the initiation of incubation, the sixth egg was swapped with the sixth egg from the control clutch at the time it was laid, and each was collected after 10-12 days of incubation in the opposite nest. Two of the fifth eggs and two of the sixth eggs did not survive to 10 d, but there was no effect of this swap on embryo survival (p = 0.93). The 7th egg, if present, was allowed to hatch. If an egg that was assigned to be collected died during the incubation period (detectable by the lack of oxygen consumption — see below), I instead collected egg 3 or 4, but not the first egg, from that clutch.

On day 2 of the laying period, when I collected egg number 2, I installed the heat pump in experimental nests by switching the original nest box with a new box containing a preinstalled heat pump, and placing the clutch from the original box in the basin of the small heatsink. For both experimental and control nests I used a fresh egg collected from an unrelated wren nest to measure egg temperature of the clutch (thermocouple 1), which replaced the second egg in the clutch that I collected, above. The incubating female usually returned to the box shortly after the switch of its nestbox with a heat pump box. I did not activate the heat pump until one day after the clutch was completed. Control clutches remained in their natal nests and had two thermocouples installed—one into the second egg of the clutch and the second into the nest lining.

Measurement of metabolic rate

I measured oxygen consumption ($\dot{V}_{\text{O}_2}$) in the field of each egg individually at 2-day intervals throughout the incubation period. I used 60cc plastic syringes as egg metabolism chambers (Vleck and Vleck 1987, Chapter 3) that were submerged in a circulating water bath at 37.5°C. I left eggs in the chambers long enough to reduce the [O2] by no more than 2%
(16-190 minutes). At the end of the sampling interval, I transferred 40cc of chamber gas into another syringe which was transported under positive pressure to the laboratory for gas analysis. In the lab, gas samples were pushed through a column containing silica gel and soda lime to absorb water vapor and CO₂, respectively. The [O₂] was measured to the nearest 0.01% with an Applied Electrochemistry S3-A oxygen analyzer and $\dot{V}_{O_2}$ was corrected to standard temperature (0°C), 1 atm pressure, dry (STPD).

Embryo mass and yolk use

Two eggs from each nest were collected after 10-12 days of development and dissected to separate residual yolk, yolk-free embryo, shell and albumin plus extra-embryonic membranes. Yolk-free embryo and intact yolk sac were blotted dry with pre-weighed filter paper, and then dried at 60°C to constant mass prior to weighing to the nearest 0.001g.

Adult incubation behavior

Small video cameras with infrared illumination were installed in the tops of nest boxes to record adult behavior inside the nest boxes. Video data were recorded on the same day for each pair of nests (control and cooled) on two non-consecutive days on which I did not measure egg $\dot{V}_{O_2}$, ~1/3 and ~2/3 of the way through incubation. Later, from the video tapes I determined the following behavioral events: when and for how long the incubating female sat on the eggs (on-bout), was sitting in the nestbox but not on eggs (door), and when the bird was not in the nest box (off-bout). Attentiveness was quantified separately for the % time the bird was in the door and the % time on eggs. Behaviors were recorded between the
hours of 07:00 and 11:00, and for each behavioral event the ambient air and egg temperatures from the temperature datasets were linked to the behavioral observation on the video.

Data analysis

Data were tested for normality and were log-transformed, when necessary, or in the case of attentiveness data (% time) were arc-sine transformed. I used t-tests to test for differences between treatments, and regression analyses were performed with cooling and control treatments combined. Survival analysis with right censoring was done with all eggs in the experiment to compare the effects of the treatments on egg survival. In control nests, egg temperature and ambient temperature were expected to be correlated, and the degree to which these two sources of temperature variation contribute to female incubation behaviors is difficult to disentangle. ANCOVAs tested the effects of mean incubation temperature and temperature treatment on the lengths of (1) on-bouts, (2) off-bouts and (3) bouts sitting in the door, with Bonferroni correction to the p-value for testing three sequential ANCOVAs.

I used an information-theoretic approach (Burnham and Anderson 2002) to evaluate the relative contribution to individual bout lengths of $T_a$ and $T_c$. Two parallel analyses using a linear mixed-effects models fit by maximum-likelihood (LME) to explain off-bout length and on-bout length were performed using the stepAIC procedure with the MASS library loaded in the statistics package, R (R Development Team 2005). Treatment (control or cooled) and nest ID were coded as factors, while nest ID was treated as a random factor in the fitting of models. Date, time of day (t) and the length of the previous bout (prev.on when response variable is off-bout length; prev.off when response variable is on-bout length) were also evaluated, along with all possible interactions. I used the lowest Akaike’s Information Criterion (AIC) to evaluate the best-fit model. Values of differences in
competing models (ΔAIC) were calculated as the difference between the best-fit AIC and the competing model’s AIC. Values <2 indicate that competing models are equally good at describing the data, whereas values >2 indicate that the best-fit model is better at describing the data (Burnham and Anderson 2002).

RESULTS

House wren incubation periods declined by 2-3 days over the course of the breeding season (Figure 2; p < 0.001). Among the 6 control nests measured between 12 of June and 14 of July, the average egg temperature measured over the entire incubation period increased as the breeding season progressed (p < 0.05), while cooled nests showed no seasonal increase in mean egg temperature over the course of the season (p > 0.5). The cooling treatment reduced mean egg temperature measured over the length of the incubation period by 3.1°C ±1.2 s.d., compared to control nests. In general, mean egg temperature in nest boxes with Peltier coolers was both shifted down from the incubation temperature of the control clutch (Figure 3) and the distribution of temperatures experienced by eggs increased (Figure 4). Of the 12 nests in the experiment, 2 failed near the end of the incubation period. One failure was due to nest predation of a control nest, while in the other case the eggs were thrown out of the box by an intruding male wren (recorded on the video). For the latter nest, I retrieved the eggs below the nest shortly after the event and used them in the analysis of embryo growth efficiency.

Egg survival, metabolic rate and incubation period

Egg survival among nests in this experiment (excluding accidents and predation) was 78%; similar to that documented in other house wren populations (Johnson 1998). Hatching
Figure 2. House wren (*Troglodytes aedon*) incubation periods decline from 13-14 days early in the season to 11-12 days late in the season (p < 0.001). Nests were checked at 1-2 day intervals and incubation periods were defined as the time interval from when the final egg was laid to when chicks were first recorded in nests.
Figure 3. Simultaneous temperature recordings of house wren (*Troglodytes aedon*) eggs from a representative control nest (nest 62, red) and a representative nest that was cooled with a Peltier heat sink apparatus (nest 73, blue). Ambient temperature is shown in green and shows daily cycles in temperature at the study site. Data for eight days are shown. Vertical lines represent midnight of each day.
Figure 4. Frequency distributions of house wren (*Troglodytes aedon*) egg temperatures recorded from two nests (data from Figure 3) over the entire incubation period. Counts represent the frequency of recordings that were made by the data logger at 1-minute time intervals.
success was not affected by cooling treatment ($X^2 = 0.35$, $p = 0.55$), and eggs whose mean temperature was as low as 31°C hatched. The incubation period was inversely correlated with mean egg temperature recorded over the incubation period (Figure 5; $F_{1,8} = 18.37$, $p = 0.0027$). Lowering mean egg temperature increased incubation periods by up to 3 days (27%) over the modal value of 11 days for incubation periods of control nests.

Egg metabolic rate was used as a proxy for embryo size. The metabolic rate of treatment eggs was consistently lower than that of the controls (Figure 6; ANCOVA, $F_{1,194} = 42.91$, $p < 0.0001$), while metabolic rate increased with development time in both groups ($F_{1,194} = 644.31$, $p < 0.0001$). I used multiple regression to describe the effects of development time and incubation temperature on egg metabolic rate. There was no interaction effect ($p > 0.05$) and both mean egg temperature ($F_{1,194} = 55.01$, $p < 0.0001$) and development time ($F_{1,194} = 705.63$, $p < 0.0001$) were significant:

$$\ln(\dot{V}_{O_2}) = 0.098\bar{T_e} + 0.26d - 6.35,$$

where $\bar{T_e}$ is the mean egg temperature over the incubation period and $d$ is time in days. However, unexplained variation remained in the model ($R^2 = 0.78$).

**Efficiency of embryo growth**

For the 22 embryos collected after 10-12 days of development, dry yolk ($Y_d$; unassimilated yolk) was lower in eggs incubated at cooler temperatures than at higher temperatures (multiple regression, $F_{1,18} = 4.53$, $p < 0.05$), when fresh egg mass ($F_{1,18} = 0.0065$, $p = 0.94$) and dry embryo mass ($F_{1,18} = 3.44$, $p = 0.08$) were included in the model. An index of growth efficiency can be defined as the ratio of mass deposited in the embryo relative to mass consumed from the yolk (Ricklefs and Cullen 1973). I estimated the initial amount of yolk available to each embryo based on the fresh eggs collected from each clutch.
Figure 5. Length of house wren (*Troglodytes aedon*) incubation periods versus their mean egg temperature for artificially cooled clutches (open symbols) and control clutches (closed symbols). Incubation periods are calculated based on the time between when the last egg was laid to when the first nestling was recorded.
Figure 6. The relationship between embryonic house wren (*Troglodytes aedon*) oxygen consumption and time (days) incubated by adult wrens. Solid symbols represent data from control nests ($\ln(\dot{V}_O_2) = 0.245 - 2.798t$, where $t$ is time in days). Open symbols represent data from experimental nests where eggs were artificially cooled by a Peltier heat sink device in the nest ($\ln(\dot{V}_O_2) = 0.245 - 3.126t$).
I measured the dried, fresh yolk mass of one egg from each clutch (2nd egg in laying sequence) and used these to generate an equation to estimate initial dry yolk mass (Y₀, in g) based on fresh egg mass:  

\[ Y₀ = 0.096 \cdot E + 0.0095 \]  

(p < 0.001; R² = 0.72), where E is the fresh egg mass in g. The quantity of yolk consumed after 10-12 days of incubation was then determined as the difference, Y₀ – Yₐ. I then examined Eₐ as a function of Y₀ – Yₐ, forcing the equation through the origin since at the beginning of incubation both Eₐ and yolk consumed (Y₀–Yₐ) are presumed to be near zero. Embryos from nests subjected to cooling treatments were ~30% smaller for the amount of yolk they had consumed than were control embryos (Figure 7a; F₁, 22 = 6.9, p = 0.016). The index of embryo growth efficiency was lower when eggs were incubated at lower mean incubation temperatures (Figure 7b; p < 0.02).

If temperature conditions affect growth rate and growth efficiency, there should be a difference in the mass of the chicks at the time of hatch based on incubation temperature. However, it was not possible to obtain accurate measures of new hatchling mass because parents fed chicks as soon as they hatched. Chick mass when first measured ranged from 1.03 to 1.71 g (mean 1.29 g), which contrasts with masses obtained from 8 chicks I removed from their egg shells of 0.99 ±0.16 g. Chicks in all nests appeared healthy and no obvious deformities were observed.

*Adult behavior*

Behavior data were analyzed relative to the mean egg temperature measured over the entire incubation period, as well as the egg temperature measured during the behavioral event (Tₑ). Among the 12 nests there were 199 observations of wrens sitting in the nestbox door, 300 observations of off-bouts and 404 observations of on-bouts. I determined mean percent
Figure 7. (a) Dry house wren (*Troglodytes aedon*) embryo mass ($E_d$) versus the dry yolk consumed ($Y_0 - Y_d$) from cooled (open symbols) and control (solid symbols) nests. (b) An index of embryo growth efficiency (GE) as a function of mean egg temperature recorded over the length of the incubation period. Symbols as in (a). Growth efficiency is defined as the dry mass of the embryo divided by the yolk consumed to that point in development.
attentiveness and average lengths of off-bouts and on-bouts for each nest. There was no difference in the overall attentiveness (determined from behaviors measured over two days) between treatment and control groups (ANCOVA, $F_{1,9} = 0.39, p = 0.56$) when accounting for the effect of mean egg temperature. Thus my treatment affected egg temperature, and birds in both control and experimental nests responded to egg temperature in the same way. Nest attentiveness increased with lower mean egg temperatures (Figure 8a; $F_{1,10} = 15.08, p = 0.003$). The percent time spent guarding (in the door of the nest, but not actively incubating eggs) was not affected by mean egg temperature (Figure 8b; $F_{1,10} = 2.64, p = 0.14$). Two nests (1 control and 1 cooled) did not have guarding behavior of the females during the time I collected video data. When examining individual behavior bouts, the amount of time spent in the doorway was not affected by either mean egg temperature over the incubation period (Figure 9a; $F_{1,196} = 0.44, p > 0.5$) or the treatment ($F_{1,196} = 0.98, p > 0.5$). Off-bout lengths were shorter at cooler mean egg temperatures (Figure 9b; $F_{1,297} = 17.22, p < 0.0003$), but there was no treatment effect on off-bout length ($F_{1,297} = 0.98, p > 0.05$). On-bouts were longer at cooler mean egg temperatures (Figure 9c; $F_{1,401} = 20.20, p < 0.0003$), but there was no treatment effect ($F_{1,401} = 4.05, p > 0.05$). Interactions between treatment and mean egg temperature were not significant for any of the three behaviors.

In the subsequent analyses on-bouts are redefined as the combined time spent at the nest, either in the box, in the door, or on the eggs. Although this resulted in fewer “on-bouts” that were of greater length, it also allowed me to examine the effects of the previous bout length on subsequent on-bout or off-bout lengths. I used behavior-specific measures of egg temperature ($T_e$) and ambient temperature ($T_a$; see Methods), as well as date and time of day ($t$) to model individual off-bout and on-bout lengths. $T_a$ and $T_e$ were strongly correlated among control nests for off-bouts ($r = 0.76, p < 0.0001$) and on-bouts ($r = 0.75, p < 0.0001$),
Figure 8. (a) House wren (*Troglodytes aedon*) nest attentiveness (% time on eggs) versus mean egg temperature of the eggs recorded over the incubation period. (b) The relationship between the time the adult spent in the door of the nest not incubating eggs (% time in door) and mean egg temperature measured over the incubation period. Symbols are as in figure 5.
Figure 9. House wren (*Troglodytes aedon*) bout lengths versus mean egg temperature over the incubation period for (a) door bouts, (b) off-bouts and (c) on-bouts. Lines, when present, are significant least-square regressions. Symbols are as in figure 5.
whereas the cooling treatment removed the correlations between $T_a$ and $T_e$ for off-bouts ($r = 0.15, p = 0.08$) and for on-bouts the correlation was much reduced compared to control nests ($r = 0.32, p < 0.002$).

A single model describing off-bout lengths stood alone from the other best-fit models ($\Delta AIC > 2$) and described 38% of the variation in the dataset. All main terms with the exception of date were present in the 5 best models selected (Table 1). In the best model, previous on-bout length had the largest (Table 1) and most significant effect on off-bout length ($p < 0.001$). Time of day was positively related to off-bout length ($p = 0.04$), and the effect of treatment was significant ($p = 0.05$) in this model. $T_e$ ($p = 0.005$) and $T_a$ ($p = 0.02$) were both positively related to off-bout length with $T_e$ having the stronger effect (Table 1). There were seven significant interaction terms in the best model, including a three-way interaction between the previous on-bout length, $T_e$ and $T_a$ (Table 1). Interpretation of the interaction effects to understand incubation behavior in this model is challenging. The treatment x time (trmt x t) interaction may be driven by the observation that in cooled nests, the first off-bout of the morning was often exceptionally long and $T_e$ was sometimes allowed to drop to near ambient temperature. To understand how behaviors change with temperature in the context of the other main effects and the interactions, I plotted off-bout length residuals (accounting for the main effects and all interactions in the best-fit model except for $T_a$) against $T_a$ (Figure 10a) and $T_e$ (Figure 10b). Locally weighted regression (LOWESS, $\lambda = 100$) was then used to identify non-linear relationships between bout length and temperature. Residuals of off-bout lengths showed a slight S-shaped relationship with $T_a$, but were relatively constant over the range of air temperatures that the incubating birds encountered (Figure 10a). Off-bout lengths increased gradually with $T_e$ (Figure 10b), consistent with the parameter estimates in Table 1.
Table 1. Model selection using Akaike’s information Criterion (AIC) to explain house wren (*Troglodytes aedon*) off-bout length during incubation. Model parameters tested include the treatment effect (trmt), length of the previous on-bout (prev.on), egg temperature (T_e), ambient temperature (T_a), time of day (t), and date. The lower portion of the table shows parameter estimates and significance tests of the best-fit model from the analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Model off-bout ~</th>
<th>AIC</th>
<th>∆AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trmt, prev.on, T_e, T_a, t, prev.on x T_a, prev.on x t, prev.on x T_e, trmt x t, trmt x prev.on, T_e x T_a, prev.on x T_e x T_a</td>
<td>541.06</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
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<td>17.6</td>
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Main effects

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<th>SE</th>
<th>t</th>
<th>p</th>
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<tbody>
<tr>
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<td>9.54</td>
<td>-3.03</td>
<td>0.0027  **</td>
</tr>
<tr>
<td>trmt</td>
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<td>-2.29</td>
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<tr>
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<td>4.12</td>
<td>4.20</td>
<td>0.0000  ***</td>
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<tr>
<td>T_e</td>
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</tr>
<tr>
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<td>0.30</td>
<td>2.33</td>
<td>0.0205  *</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>prev.on x T_e x T_a</td>
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<td>0.004</td>
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</table>
Analysis using AIC of on-bout lengths differed from the previous analysis of off-bout lengths in having fewer significant main effects and interactions (Table 2), as well as describing a greater proportion of the variation in the dataset (42%). Two models were similar in their ability to describe on-bout lengths ($\Delta\text{AIC} < 2$) and differed by including treatment in the model with the higher AIC. Previous off-bout length had the most significant (positive) effect on-bout length ($p < 0.001$). Neither date nor time of day appeared in the two best-fit models. $T_a$, $T_e$ and the $T_a \times T_e$ interaction were in the best-fit models, but main effects of $T_e$ and $T_a$ were not significant (Table 2). Residuals of on-bout lengths (accounting for previous off-bout length, $T_e$ and $T_e \times T_a$) did not change with changes $T_a$ (Figure 10c).

However, plotting on-bout residuals (accounting for previous off-bout length, $T_a$ and $T_e \times T_a$) against $T_e$ revealed a shift in behavior from stable or slightly increasing on-bout lengths with increases in $T_e$ below $\sim 34^\circ\text{C}$, to a sharp decrease in on-bout lengths with increasing $T_e$ above $\sim 34^\circ\text{C}$ (Figure 10d).

DISCUSSION

In the present study I examined the effects of artificially modified egg temperatures on house wren incubation behaviors, concomitant with changes in embryo growth parameters that can affect fitness. By removing heat from below the eggs, I was able to maintain mean egg temperature in the experimental nests $\sim 3^\circ\text{C}$ below that of control nests, yet have no measurable effect on hatching success. Adult wrens allocated more time to their eggs when they were artificially cooled, which they achieved through both shorter off-bouts and longer on-bouts at lower egg temperatures. Behavioral adjustments occurred in response to deviations in egg temperature, whereas air temperature that wrens in this study experienced
Table 2. Model selection using Akaike’s information Criterion (AIC) to explain house wren (*Troglodytes aedon*) on-bout length during incubation. Model parameters tested include the treatment effect (trmt), length of the previous off-bout (prev.off), egg temperature ($T_e$), ambient temperature ($T_a$), time of day (t), and date. The lower portion of the table shows parameter estimates and significance tests of the best-fit model from the analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Model on-bout ~</th>
<th>AIC</th>
<th>ΔAIC</th>
</tr>
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<tbody>
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<td>1</td>
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<td>0</td>
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<tr>
<td>2</td>
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<td>1.64</td>
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<tr>
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<td>Estimate</td>
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<tr>
<td>1</td>
<td>Intercept</td>
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<td>prev.off</td>
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<tr>
<td></td>
<td>$T_e$</td>
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<td>0.11</td>
</tr>
<tr>
<td></td>
<td>$T_a$</td>
<td>0.26</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>$T_e \times T_a$</td>
<td>-0.01</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Figure 10. House wren (*Troglodytes aedon*) off-bout (A, B) and on-bout (C, D) length residuals versus ambient (A, C) and egg (B, D) temperature recorded at the time of the behaviors. Residuals were calculated as natural log-transformed bout lengths that accounted for the main effects and interactions present in the best fit models in Tables 1 and 2, minus the main effects of $T_a$ and $T_e$ for (A, C) and (B, D), respectively. Locally-weighted regression (LOWESS, $\lambda = 1000$) curves were fit to the data to detect shifts in behavioral strategies with temperature. Symbols are as in figure 5.
had less of an effect on bout lengths. These results are consistent with other studies that have shown birds alter their incubation patterns to increase heat delivered to the eggs when the eggs’ thermal conditions are poor (Kendeigh 1952, White and Kinney 1974, Vleck 1981b, Davis et al. 1984). Cold eggs and reduced nest insulation generally increase the energetic costs of incubation (Williams 1996), and this should increase energetic demands on incubating female house wrens in the treatment group compared to those in the control group. House wrens in the control nests incubated for ~65% of the morning hours, which is relatively low for passerines (Conway and Martin 2000a). House wrens in the experimental nests incubated for up to 70-80% of morning hours when their eggs were cold, and this may represent the maximum time that incubating females are able to devote to their eggs in compensation for the cold treatment without compromising their own condition.

It is significant that female attentiveness and bout lengths did not vary strongly with treatment per se (weak treatment effect on off-bout length, but no effect on attentiveness or on-bout length), but did vary with egg temperature and previous bout length in both treatment and control nests. That is, the treatment affected egg temperature to achieve a wider range of egg temperature, yet not outside the range over which incubation can be successful. This allowed me to demonstrate that birds, even over the whole incubation period, alter their incubation behavior to favor the egg temperature at a cost to adult self maintenance. Because off-bout times did not increase significantly at cool egg temperatures (Figure 10b) females apparently were able to obtain the energy they needed without neglecting the eggs. That is, house wrens in this population and under these thermal conditions were capable of decreasing time spent away from the nest and increasing incubation in order to partially offset the effects of the Peltier cooling units on the temperature of their eggs. I do not know, however, whether females incurred additional costs
to themselves (e.g. reduced body condition, reduced adult survival) due to the extra time spent on the eggs.

Exposure of eggs to cold temperature is thought to result in longer incubation and reduced hatching success (Lundy 1969, Romanoff and Romanoff 1972). In the current study I increased incubation periods by decreasing mean egg temperature, but with no decrease in hatchability. These results are supported by artificial incubation experiments in the laboratory in which periodic cooling of house wren eggs did not affect embryo survival (Chapter 2). Studies of passerines in which clutch size is experimentally increased have shown that incubation periods increase (Moreno and Carlson 1989, Moreno et al. 1991, Monaghan and Nager 1997) and hatchability decreases (Moreno and Carlson 1989, Monaghan and Nager 1997, Reid et al. 2000), presumably in conjunction with lower egg temperatures. However, results from these studies may also reflect constraints on the number of eggs that birds can physically incubate in a nest, as opposed to the temperatures that the eggs experience. For instance, Starling (Sturnus vulgaris) nests with additional eggs have reduced egg cooling rates and increased mean egg temperatures, which should be thermally favorable to hatching success. Yet hatchability is reduced in these enlarged clutches (Reid et al. 2000), suggesting that eggs in enlarged clutches are more likely to experience long-term neglect or physical damage. My results suggest that lethal effects of cooling to passerine embryos may be less than was previously suspected. More significant costs of cold to embryos are instead due to altered embryo phenotypes that can affect subsequent growth and survival, as well as reduced embryo growth efficiency. In fact, house wren eggs in this study hatched at mean egg temperatures as low as 31°C, and a goal of future studies should be to define the consequences to post-hatch growth and survival of chicks that develop in cold eggs. Limits to the amount of cold that embryos can survive are probably still important in extreme
conditions. For example, removal of mate-feeding males of arctic-breeding snow buntings \textit{(Plectrophenax nivalis)} forced females to increase the time away from their nests (Lyon and Montgomerie 1985). This reduced attentiveness was enough to reduce snow bunting hatching success (Lyon and Montgomerie 1985), which suggests that the thermal environment of the nest sites that this species uses is on the edge of what their embryos can tolerate.

In this study, female house wrens adjusted their incubation behavior based on egg temperature more than on ambient temperature. Off-bout length decreased steadily as egg temperature declined, but changes in on-bout length with temperature were more complex. Below ~34°C the relationship between on-bout length and egg temperature remained relatively constant, but above 34°C on-bout lengths decreased (Figure 10d). White and Kinney (1974) provided strong evidence that incubating birds rewarm eggs to a threshold temperature and only begin off-bouts after this minimum threshold is reached. The pattern in house wrens can be explained by the threshold hypothesis if the short on-bouts are the result of birds getting on their nests when eggs are above the threshold temperature to begin with. This behavioral mechanism suggests that a requirement for maintaining embryo viability is to regularly bring the eggs to a high threshold temperature, while the mean temperature over the incubation period may not be an important determinant of hatching success.

The poor thermal conditions of the egg that increased parental investment in incubation also slowed embryonic growth rates and decreased the index of growth efficiency. Chicks presumably hatched with less residual yolk, representing a loss of nutrients that would otherwise have been available to the chick, post-hatch. Cooling eggs resulted in incubation periods of these mid- to late-season experimental nests comparable to those found in early season nests (see Figure 2) for this population. Seasonal increases in ambient temperature are associated with shortened incubation periods (Figure 2, Hipfner et al. 2001),
suggesting mean egg temperature is lower earlier in the season. This begs the question, is embryonic growth efficiency also lower in these early nests than it is later in the season when egg temperatures are higher? If so, this may in part explain the observation that the consequences of egg size variation on the fitness-related traits of altricial nestlings are stronger early in the nestling period than later, even though effects of egg size on chick performance frequently disappear by the time chicks have fledged (Magrath 1992, Smith and Wettermark 1995, Styrsky et al. 1999, Reed et al. 1999). Furthermore, egg size may not be an indication of quality (Ricklefs 1984), and adults could provision early-season eggs with additional nutrients or hormones that benefit the embryo to make up for low growth efficiency at low temperatures (Schwabl 1993, Schwabl 1996, Saino et al. 2004). Increased post-hatching care early in the year may also make up for disproportionate losses in residual yolk (Reed et al. 1999). It may also be that residual yolk is more important to late-season broods than early broods, as small house wren hatchlings are able to catch up in growth to larger hatchlings early in the season, but not late in the season (Styrsky et al. 1999). Further studies on how embryo and chick growth parameters differ seasonally would provide a better understanding of the importance of growth efficiency and incubation temperature to avian embryos, particularly in variable environments.

This study has demonstrated that egg temperatures play an important role in determining adult incubation behavior in house wrens. It supports the contention that breeding is constrained seasonally and across the breeding distribution by the bird’s ability to maintain high egg temperatures. Furthermore, it supports the idea that embryo thermal tolerance has been a selective force in the evolution of adult incubation behaviors, in addition to the better-understood effects of nest predation (Skutch 1949, Fontaine and Martin 2006) and constraints on adult breeding energetics (Vleck 1981a, Biebach 1986, Williams 1996).
The fact that the best possible AIC models explained only 38% and 42% of the variation in off-bout and on-bout length, respectively, suggests that incubation behavior is the result of several factors, including nest predation and adult energetic constraints (Conway and Martin 2000b, Ghalambor and Martin 2001). For example, cold ambient temperatures that are more characteristic of breeding conditions early in the season should increase the energetic requirements of incubating adults (Vleck 1981a, Biebach 1986), particularly below the bird’s lower critical temperature (Schmidt-Nielsen 1990). The relationship between adult breeding energetics is a function of a bird’s habitat as well as its evolutionary history (Weathers and Greene 1998), and the lower critical temperature varies among species (Yarbrough 1971, Haftorn and Reinertsen 1985). The lower critical temperature for breeding house wrens is not known, but probably lies close to 20°C. I tested behavioral responses of wrens when daytime ambient temperatures were generally between 26°C and 38°C. This range of temperatures excludes the colder temperatures that wrens are likely to experience early in the season and in different parts of their breeding range (e.g. mountainous or high latitude habitats). Cold ambient temperatures may therefore play a more important role in incubation behaviors in certain environmental conditions, and may over-ride the effect of egg temperature in these instances. Despite this caveat, it is clear that incubating house wrens will change their incubation strategy when the thermal conditions of the eggs are poor in an attempt to actively regulate the temperature of their eggs. Along with the effects of nest predation and the energetic costs of adult maintenance, the fitness consequences of cold to avian embryos should be considered as an important selective force in the evolution of adult incubation behaviors.
ACKNOWLEDGEMENTS

L. Anderson, M. Biancardi, and J. Bockenstedt assisted in the field and R. Ackerman provided equipment. W. King and the ISU Department of Agronomy allowed access to Hind’s Research Farm, and provided a work space in the field to conduct measurements of metabolic rate. D. Christensen, L. Conrad and M. Palacios provided comments on a previous draft of this manuscript. Work was funded by the Animal Behavior Society and the National Science Foundation under Grant No. IBN-0309371.

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CHAPTER 5: CHANGES IN AVIAN EMBRYONIC METABOLIC RATE WITH EMBRYO BODY TEMPERATURE

A paper to be submitted to Proceedings of the National Academy of Sciences

Christopher R. Olson\textsuperscript{2, 3}, Carol M. Vleck\textsuperscript{2} and David Vleck\textsuperscript{2}

ABSTRACT

The effect of body temperature on metabolic rate should have the greatest ecological significance to those organisms that regularly experience variable body temperatures in their natural environments. Passerine birds in general lay small eggs that regularly cool when the incubating adult leaves the nest to forage. I studied the metabolic responses of embryos to a range of temperatures in eggs of house wrens (Troglodytes aedon) and zebra finches (Taeniopygia guttata). A long-standing suggestion that tissue synthesis in bird eggs comes to a stop at low egg temperatures leads to the prediction that sensitivity of metabolism to temperature will increase at the same temperature where growth stops. For both species, Q\textsubscript{10}s were between 3 and 4 at temperatures from about 32°C down to 15°C, but above 32°C Q\textsubscript{10} was ~2. Changes in Q\textsubscript{10} occurred abruptly, and Arrhenius plots also revealed the presence of Arrhenius break points for both species. These shifts occurred well above the oft-cited physiological zero temperature of 25°-26°C. House wren embryos had a higher metabolic rate at a given temperature when rewarming compared to cooling. This hysteresis may be a physiological response to alter the rate of rewarming by increasing circulation. Alternatively, it may result from the energy costs of repairing departures from homeostasis produced by the

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effects of cooling on the kinetics of biochemical reactions. Despite the fact that house wrens are larger at hatching than zebra finches and have a shorter incubation period, mass-independent embryo oxygen consumption did not differ between the two species, suggesting that growth may be more efficient in house wrens than in zebra finches.

INTRODUCTION

Bird embryos exhibit some of the fastest growth rates among vertebrates (Case 1978). To achieve this rapid growth, bird eggs require relatively high temperatures over the length of the incubation period (Deeming 2002). Embryo temperature is maintained by adult attentiveness at the nest with contact incubation between the brood patch and the eggs (Zann and Rossetto 1991, Turner 1997, Lea and Klandorf 2002). Embryos lack the ability to generate the heat necessary for thermoregulation (Webb and King 1983, Tazawa et al. 2001), therefore the eggs experience cooling whenever the incubating adult leaves the eggs, and eggs cool repeatedly when incubating adults must forage for themselves (Zerba and Morton 1983, Davis et al. 1984, Morton and Pereyra 1985, Weathers and Sullivan 1989). Concomitant with periodic cooling, embryo metabolic rate declines (Ewert 1991). When the adult resumes incubation, egg temperatures increase and rates of metabolism presumably return to normal. The effect of egg temperature on embryo metabolic rates when temperatures are dynamic is hard to study. Periodic cooling and changes in growth and metabolic rate are important in nature, yet we know little about how embryo metabolism and growth are influenced by periodic cooling.

Avian embryos are ectothermic, and altricial embryos do not transition to endothermy until several days after they hatch (Whittow and Tazawa 1991, Hohtola and Visser 1998). The relationship between metabolism and body temperature in ectotherms generally
increases approximately exponentially with increasing temperature (Ege and Krogh 1914, Cameron 1989, McNabb 2002) and early work with chicken (Gallus gallus) eggs showed that as temperatures decline from normal incubation temperature, embryonic metabolism declines according to the characteristic ectothermic pattern (Freeman 1967). Embryo physiology should not be assumed to scale with body size and temperature like that of adult organisms (Spicer and Burggren 2003, Burggren 2005), and the data on the sensitivity of embryonic metabolism to changes in temperature suggests complexity. In Heermann’s Gulls (Larus heermanni), embryonic metabolic rate declines from 30° to 20°C as expected, yet does not change between 40° to 30°C (Bennett and Dawson 1979). The changing sensitivity of metabolism to temperature can be described by changes to Q_{10} over different intervals of temperature (see Methods for definition). Gulls and procellarid seabird embryos have Q_{10}s between 2.8 to 5.1 in the range of 15°-20°C, but Q_{10} decreases to 1.3-2.4 when temperatures are between 30°-35°C (Vleck and Kenagy 1980, Dawson 1984, Williams and Ricklefs 1984). Ostrich (Struthio camelus) embryos also show a decreased Q_{10} as temperature increases over the range of 32.5-37.5°C (Hoyt et al. 1978). Information on how embryo metabolism changes with temperature on a finer scale does not exist, and data on temperature sensitivity for passerine embryos are non-existent.

For avian embryos, energy metabolism provides the ATP necessary for maintenance activities (homeostasis and survival), as well as tissue growth (Vleck et al. 1980, Hoyt 1987). The relative energy costs of basal maintenance versus embryo growth are difficult to quantify, but available data from ostrich embryos show a considerable (~25%) decline from peak metabolic rate immediately prior to hatching that is thought to result from the decline of growth during the plateau phase of precocial development (Hoyt et al. 1978). Embryonic growth has been suggested to stop at a lower threshold of temperature, which has come to be
known as the physiological zero temperature (PZT), defined as the temperature below which tissue synthesis is suspended, typically 25°-26°C (Lundy 1969, White and Kinney 1974, Drent 1975, Haftorn 1988). If PZTs are an actual emergent property of the physiological limitations of embryo growth at lower temperatures, PZTs should be detectable by careful examination of the relationship between embryo metabolic rate and temperature.

Physiological hysteresis, in which the physiological state of an animal is dependent on its immediate history, has been noted in animals during periods of temperature flux (Bartholomew and Tucker 1963, Bartholomew and Vleck 1979, Bartholomew et al. 1981, Ricklefs and Williams 2003). For example, juvenile shorebirds show a metabolic hysteresis before they gain full thermoregulatory competency. Metabolic rates of chicks at a given body temperature are higher during cooling because they are attempting thermogenesis. When parents rewarm the chicks through brooding, the chicks’ metabolic rates are lower at the same body temperatures (Ricklefs and Williams 2003). Avian embryos could exhibit lower metabolic rates during rewarming than during cooling if processes that demand or supply energy are impaired by a cold shock and recovery of function is delayed upon rewarming. Alternatively, the opposite may occur if metabolic rates during rewarming is elevated due to cooling caused by metabolic damage, perhaps via differential effects on kinetics of different reactions, that requires energy-expensive repair. Metabolic hysteresis could also occur if embryos alter circulation differentially during warming vs. cooling (Bartholomew and Tucker 1963, Bartholomew et al. 1981). Altricial birds are not known to show endothermy until after they hatch (Tazawa et al. 2001), so elevation of metabolism for heat production is unlikely to contribute.

I measured metabolic rate and temperature simultaneously in house wren (*Troglodytes aedon*) and in zebra finch (*Taeniopygia guttata*) embryos at a range of
developmental stages to understand how embryonic metabolic rate responds to periodic cooling. In house wrens, I compared metabolic rates at the same body temperature during cooling and rewarming to test for metabolic hysteresis, and to evaluate hypotheses about alternate mechanisms that might produce such hysteresis.

MATERIAL AND METHODS

I used different methods to measure egg metabolic rate in the two species. For house wren eggs I used the down-up (DU) protocol, modified from (Ricklefs and Williams 2003) and employing the instantaneous oxygen consumption technique (Bartholomew et al. 1981). This provided concurrent measurements of metabolic rate and internal egg temperature while cycling down from favorable warm temperatures to cool temperatures, then back up again to warm temperatures. With the DU protocol I examined the metabolic response of embryos to dynamic temperature change and the direction of temperature change. Due to equipment difficulties with the zebra finch eggs I used closed chamber respirometry (Vleck 1987) to measure embryo metabolic rate at a series of fixed temperatures. Closed chamber respirometry yields reliable measurements of oxygen consumption averaged over the recording interval, but cannot describe how metabolic rate changes dynamically with time and over as many egg temperatures. In addition, closed circuit respirometry requires eggs to remain at low temperatures for prolonged periods, which may be detrimental to embryo health (Drent 1975).

House wrens: metabolic rate in an open system

House wrens are a ~12g passerine that lays eggs that weigh ~1.4g. I collected eggs (n = 5) from different house wren nests during incubation and immediately transported them to
the laboratory for metabolic rate measurements. Embryos ranged in dry mass from 0.02 to 0.11 g (mean 0.06 g ± 0.03 s.d.). Because temperature gradients may occur across an egg, particularly during cooling or warming (Turner 1997), actual embryo temperature throughout metabolic measurements was measured by placing a miniature type T thermocouple made from 0.002 in. diameter thermocouple wire (Omega Engineering, Inc.) inside the egg adjacent to the embryo. A small hole was drilled into the shell of the egg with a high-speed Dremel tool, exposing a 1-2 mm diameter section of the outer shell membrane. The thermocouple was then carefully inserted through the shell and allantoic membranes and placed next to the embryo. Thermocouples were sealed in place with Zapit dental cement (Dental Ventures, Inc.). In all but one case, the thermocouple was positioned in the amnion between the body of the embryo and the yolk, the exception being when the thermocouple pierced the yolk sac, but with no apparent adverse effects to the short-term health of the embryo. The thermocouple wire was fed through a small hole in the side of the chamber and sealed with dental cement. Temperature was recorded to the nearest 0.1°C from a calibrated Omega HH-25TC digital thermometer.

Whole-egg oxygen consumption was measured in an open flow system (Withers 1977) with an Applied Electrochemistry S3-A dual channel oxygen analyzer. Each egg was placed in a small metabolism chamber built from a 10cc syringe. Ports at opposite ends of the chamber were connected to Tygon tubing for incurrent and excurrent air. The flow rate of air, pushed through a column of silica gel and soda lime to remove water and CO₂, respectively, was controlled with a Brooks mass flow meter. Air flow through the metabolism chamber was then drawn through a second column of silica gel and soda lime, and finally through the oxygen sensor. A parallel circuit of dried CO₂-free air fed the second oxygen sensor to measure the difference in [O₂] between the two sensors to the nearest 0.001%.
At 30 second intervals I simultaneously recorded the embryonic body temperature and the depression in % O₂ caused by the egg’s metabolism. Embryonic body temperature was controlled by submerging the metabolism chamber into a 2-liter water bath and changing the temperature of the water by adding ice or warm water, as needed. Initially the egg temperature was measured at 37.5°C for several minutes to verify a stable consumption of O₂. The egg was then cooled to ~15°C, and then rewarmed back to 37.5°C. I used equation 3 from (Bartholomew et al. 1981) to calculate \( F_{E\text{eq}} \), which is the equilibrium value of oxygen concentration that would occur if rates of oxygen consumption were to remain constant at any point during a DU cycle:

\[
F_{E\text{eq}} = F_{E_{t+1}} + \left[ \frac{F_{E_t} - F_{E_{t+1}}}{\frac{V}{V} \cdot \Delta t} \right] \cdot \left( 1 - e^{-\frac{V}{V}} \right),
\]

[Eq. 1]

where \( F_E \) is the oxygen concentration in chamber excurrent air at a given time, \( t \). The denominator is the fraction of the interval to the new steady state that is reached in the interval of time between measurements \( \Delta t \) (30s). \( \dot{V} \) is the flow rate of air through the system, and \( V \) is the effective chamber volume defined by the washout characteristics of the system with a non-oxygen consuming mass (dead egg) inside. Prior to measurement of egg metabolic rate, I measured \( \dot{V} \) by saturating the system with water vapor and connecting it to a burette to measure the time required to move a soap bubble 5ml, then correcting to standard temperature and pressure, dry (STPD). I estimated \( V \) using three duplicate washout curves generated at a known flow rate by breathing into the system and closing it with a dead, non-metabolizing egg. I also measured the lag time from when air left the chamber to when it reached the oxygen analyzer by breathing into the system and closing the chamber. The flow
rate of the system was rechecked following each run to verify the same flow throughout the experiment.

The rate of oxygen consumption was calculated with Equation 4a of (Withers 1977):

\[
\dot{V}_{O_2} = \dot{V} \left[ \frac{F_I - F_{E_{res}}}{1 - F_I} \right],
\]

where \( F_I \) is the O\(_2\) concentration difference between the two cells of the oxygen analyzer.

Because the instantaneous technique is noisy (Bartholomew et al. 1981) I used a three-point running average to smooth the oxygen consumption data over one minute time intervals.

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**Zebra finches: metabolic rate in a closed system**

Zebra finches are a \(~9\)g passerine that lays eggs that weigh \(~1.0\)g. Eleven zebra finch eggs were collected from 4 nests and immediately brought into the laboratory and held in a forced-draft incubator at \(~37^\circ\)C prior to measurement of metabolic rate. I used a closed system to measure zebra finch egg metabolic rate (Vleck 1987; Chapter 2). Eggs were placed individually in 60cc syringes fitted with one-way stopcocks and held at each prescribed temperature for enough time to allow the egg to consume oxygen in the chamber at that temperature. At the end of a prescribed period, gas from the syringe was forced through a column of silica gel and soda lime to absorb water vapor and CO\(_2\), respectively. The fractional O\(_2\) concentration of each gas sample was then measured with an Applied Electrochemistry S3-A oxygen analyzer and the difference was subtracted from a reference sample of room air collected at the time the experimental syringe was filled. This fractional difference in O\(_2\) was then used to calculate the amount of O\(_2\) consumed over the sampling interval. I made the assumption that egg temperature came to waterbath temperature quickly.
enough that the effects of a higher initial metabolism would be negligible over the whole
interval (27-230 min). Barometric pressure and air temperature were measured at the
beginning of the experiment to correct $V_O$ to standard units (STPD).

The metabolic rate of each finch egg was measured 7 times at the following
temperatures in the following sequences: 25°, 37.5°, 35°, 30°, 15°, 37.5°, 20°C or
alternatively at 30°, 37.5°, 25°, 35°, 15°, 37.5°, 20°C. Movement of eggs through two
different sequences occurred because of limits to the number of eggs that could be placed in
the water bath at one time. However, in both sequences low temperatures were alternated
with relatively high temperatures to maintain embryo health through the experiment.

Between each temperature in the series, eggs were returned to the forced-flow incubator set
at ~37°C. Oxygen consumption at 37.5°C was measured twice, near the beginning and ends
of each sequence, to determine the metabolic health of the eggs after prolonged exposure to
cold temperatures. Two eggs had ~20% reduction in the rates of metabolism at the end of the
experiment, and reduced metabolic rates were also evident during the final 20°C
measurement in these same eggs. These measurements for these animals were removed from
subsequent analyses. For the other eggs, metabolic rates were repeatable from the beginning
to the end of the experiment (paired t test, t = 1.39, p = 0.10). To collect the 7 measurements
required ~14 hours.

Following metabolic measurements of finch and wren embryos, I dissected eggs to
determine embryo mass. Embryos were carefully separated from the eggs and the extra-
embryonic chorioallantoic membrane and the yolk sac were cut away at the point where they
attached to the abdomen. In all cases the yolk sac was not yet internalized into the abdomen
and removal of yolk contents from the embryo was complete. A section of filter paper was
used to blot albumin away from surface of the embryo and the surface of the yolk sac.
Tissues were dried to constant mass at 60°C and dry tissue was measured to the nearest 0.001 g.

The temperature coefficient for metabolism ($Q_{10}$) was calculated as:

$$Q_{10} = \left( \frac{\dot{V}_{O_2(T_2)}}{\dot{V}_{O_2(T_1)}} \right)^{10/(T_2-T_1)}.$$  \[\text{Eq. 3}\]

The continuous nature of the data for house wrens allowed me to make a calculation of $Q_{10}$ for every 1°C change in temperature, whereas for zebra finch eggs I made calculations over 5°C or 2.5°C intervals.

In addition to $Q_{10}$ as a measure of temperature sensitivity, I also plotted the relationship between metabolic rate and temperature in Arrhenius plots. The combined non-linear effects of temperature and body mass on metabolic rate have been incorporated into an Arrhenius relationship by Gillooly et al. (2001):

$$B = b_0 M^b e^{E/kT},$$  \[\text{Eq. 4}\]

where $B$ is the metabolic rate, $M =$ body mass, $b$ is the scaling coefficient for body mass (Lasiewski and Dawson 1967) and $b_0$ is a normalization coefficient that is independent of $M$ and $T$. The main effect of temperature is described by the Boltzmann Factor, $e^{E/kT}$, where $E$ is the rate-limiting combination of activation energies of all of the biochemical reactions that are involved in aerobic metabolism, $k =$ Boltzmann’s constant and $T$ is the thermodynamic temperature in Kelvin. Rearranging Equation 3 and taking the natural log of each yields:

$$\ln(\dot{V}_{O_2} \cdot M^b) = \ln(b_0) - \frac{E}{k} \cdot T^{-1}.$$  \[\text{Eq. 5}\]

In an Arrhenius plot, $\ln(\dot{V}_{O_2} \cdot M^b)$ plotted against $T^{-1}$, yields the slope, (-E/k), as a measure of an organism’s sensitivity to temperature. This mass-independent measure of metabolic rate
enables interspecific comparisons across a range of body mass, assuming a common scaling exponent, b (Gillooly et al. 2001).

Data Analysis

ANOVA, ANCOVA and t-tests were used to compare groups. Egg-ID was treated as a random variable. For analysis of \( \text{Q}_{10} \), and Arrhenius relationships, embryonic metabolic rate was categorized to the nearest whole °C. Significance was accepted at \( \alpha = 0.05 \).

RESULTS

House wren egg metabolic rate measured while eggs were held at a static temperature of 37.5°C, prior to the DU procedure, was positively related to dry embryo mass \((t = 4.82, p = 0.017)\), described by the function:

\[ \dot{V}_{O_2} = 3.57 \cdot M^{0.70}. \]  

[Eq. 6]

Finch egg metabolism increased with dry body mass (Chapter 2: \( n = 45, r^2 = 0.80, p < 0.001 \)), described by the function:

\[ \dot{V}_{O_2} = 3.45 \cdot M^{0.67}. \]  

[Eq. 7]

Exponent values were lower than the commonly used \( \frac{3}{4} \)-power scaler (Calder 1984), and closer to the value of 0.72 reported in new hatchlings (Vleck et al. 1980). Therefore in subsequent analyses I used the mean exponent of 0.685 to calculate dry mass-independent \( \dot{V}_{O_2} \) for both zebra finch and house wren embryos.

At constant temperature prior to the beginning of a DU-cycle, house wren embryo metabolic rate fluctuated by ~20% from peak metabolism (Figure 1). These events occurred at periods of 4-10 minutes and continued during the time that embryos were cooled. House wren embryonic metabolic rates decreased when body temperatures declined, then increased
Figure 1. Dual record of temperature (open symbols) and instantaneous $\dot{V}_O_2$ (closed circles) of a house wren (*Troglodytes aedon*) egg recorded at 30 second intervals. Data for $\dot{V}_O_2$ and egg temperature were collected at 30 s intervals and values of $\dot{V}_O_2$ was smoothed over three datapoints.
when body temperature returned to normal incubation temperature (Figure 1).

Mass independent embryo metabolic rate increased with temperature in an approximately exponential pattern between 15° and 32°C. The pattern appeared to change between 32°-38°C with metabolism increasing less rapidly with temperature than at the lower temperatures (Figure 2a). Temperature sensitivity of house wren eggs based on Q_{10} values calculated at 1°C intervals showed a negative relationship with temperature (Figure 2b, F_{1, 21} = 6.62, p = 0.02, R^2 = 0.24). A locally weighted regression (LOWESS, f = 0.7) fit to the data in Figure 2b detected a steep decline in Q_{10} at ~32°C.

Mass independent metabolic rate increased with temperature in the zebra finch embryos similarly to that of the house wren embryos (Figure 2c). Zebra finch Q_{10} values varied with temperature (Figure 2d, ANOVA, F_{1, 3} = 57.57, p = 0.005). Q_{10}s were ~3.4 between 15°-20°C, and were ~2 between 35°-37.5°C. The largest change in Q_{10} occurred between the intervals 25-30 °C and 30-35 °C (Figure 2d).

Arrhenius plots of ln-transformed mass-independent $\dot{V}_{O_2}$ versus the inverse of temperature revealed a characteristic negative relationship for both species (Clarke and Johnston 1999, Gillooly et al. 2001). However, above 35°C (T^{-1} x 1000 = 3.25) metabolic rates were lower than predicted for house wrens (t = 4.38, p = 0.01), based on an estimate calculated from linear regression of data between 15° and 30°C (R^2 = 0.995; Figure 3a). Zebra finches also showed lower than expected metabolic rates above at 35°C (T^{-1} x 1000 = 3.30, t = 9.73, p < 0.0001), but at 30°C (and below) embryo metabolism matched very closely to a linear relationship (R^2 = 0.999) based on temperature data between 15° and 30°C (Figure 3b, t = 1.27, p = 0.33).
Figure 2. Temperature sensitivity of metabolic rate in house wrens (*Troglodytes aedon*) (A, B) and zebra finch (*Taeniopygia guttata*) (C, D) embryos over a range of biologically relevant temperature. Oxygen consumption ($\dot{V}_{O_2}$, divided by dry mass$^{0.69}$) increases with temperature in an exponential-like pattern in (A) house wren and (C) zebra finch embryos. House wren (B) and zebra finch (D) $Q_{10}$ decreased with temperature with a steep decline at ~30°-32°C. Error bars are ±1 SD. Lines in B are a least-square regression (solid line, $Q_{10} = 6.59 - 0.12T_0$, $r^2 = 0.24$), and a locally weighted regression (LOWESS, smoothing parameter $f = 0.6$, dashed line).
Figure 3. Arrhenius relationships of $\ln(V_O)$ and the inverse of temperature in Kelvin for (A) house wrens (*Troglodytes aedon*) and (B) zebra finch (*Taeniopygia guttata*) embryos. Error bars represent one standard deviation and note that the direction of temperature is reversed in the independent axis. Lines represent least square regressions fit through data below 30°C.
I compared finch embryo metabolic rates to those of wrens in an ANCOVA using ln-transformed values of mass-independent $V_{O_2}$ and temperature ($°C$) as the independent variable. There was a significant interaction between species and temperature ($F_{1, 26} = 5.74, p = 0.02$; finches had metabolic rates 11-28% higher than wrens at the temperatures 15°-25°C. This higher apparent metabolic rate in finch eggs may be due to egg temperatures that are warmer than the water bath for a short period at the beginning of a trial, which could have led to an underestimate of $Q_{10}$s at these cooler temperatures. Excluding measurements of egg temperatures below 30°C in the analysis, the interaction effect was no longer present ($F_{1, 8} = 0.45, p = 0.5$) and no difference in metabolic rates between wrens and finches were evident ($F_{1, 8} = 0.03, p = 0.87$).

Finally, I examined metabolic rates during cooling and rewarming for house wren eggs to determine if there was any hysteresis. Because considerable individual variation in metabolic rate was present among individual house wren eggs ($n = 5$ eggs, $F_{4, 816} = 52.0, p < 0.0001$) with temperature ($n = 822$ individual measurements, $F_{1, 816} = 15650, p < 0.00001$), I calculated mean-centered residuals based on the random variation that existed around individual eggs for each measurement at each temperature to the nearest degree. I then used ANCOVA to test for differences by plotting the residuals against temperature as a function of whether the eggs were in the cooling or warming part of their DU cycle ($n = 233$ observations). In this model, temperature had the strongest effect on metabolic rate ($F_{1, 230} = 2426, p < 0.0001$), and in all 5 eggs, metabolic rate was higher at a given temperature during the rewarming phase than during the cooling phase ($F_{1, 230} = 39.4, p < 0.0001$; Figure 4) by ~8%.
Figure 4. Residual $\ln(\dot{V}_{O_2})$ accounting for random variation of individual embryos versus egg temperature in house wren (Troglodytes aedon) embryos when cooling (solid circles, solid line) and rewarming (open circles, dashed line).
DISCUSSION

The metabolic rates of house wren and zebra finch embryos exhibited the lowest temperature sensitivity in the range of temperatures most commonly encountered during incubation (32°-38°C), but below ~32°C metabolism was more sensitive to changes in temperature. This is supported by sharp increases in Q₁₀ from ~2 to ~4 as temperature declined (Figure 2b, 2d), as well as Arrhenius break temperatures that occurred between 30°-35°C (Figure 3). House wren embryos displayed a metabolic hysteresis (Figure 4); metabolic rates during rewarming were higher than metabolic rates during cooling. Finally, both species showed similar metabolic rates at favorable incubation temperatures above ~32°C, despite clear differences in tissue growth rates and incubation periods.

The pattern of a declining Q₁₀ with increasing temperature is well-known (Schmidt-Nielsen 1990) and the data presented here are not unexpected. The temperature sensitivity of avian embryos, however, has not previously been determined at this fine scale, and the analysis presented here provides insight into how temperature effects may limit embryonic growth and development. The more precipitous decline of metabolic rate with declines in temperature when below ~32°C may indicate that costs of maintaining growth and homeostasis over the incubation period decline rapidly with temperature. However, if this were true we might expect birds to take advantage of a higher metabolic efficiency at lower incubation temperatures by incubating eggs at lower temperatures. Furthermore, the index of growth efficiency I measured in chapters 2 and 4 increases with declining temperatures. It is more likely that function is impaired below 32°C, and avian embryos are not able to maintain homeostasis. This reduced metabolic demand corresponds to compromised embryo survival when eggs are held at this range of cooler temperatures over the long term (Baldwin and Kendeigh 1932, Vleck and Kenagy 1980). It has recently been suggested (Clarke 2003) that
thermal limits on physiological function should be an evolved trait in all organisms. Yet it is not clear why birds have evolved embryonic thermal limits that are well above ambient temperatures which most birds breed. However, the presence of parental care (i.e. incubation) in this lineage was probably key to the evolution of these thermal limitations.

Embryonic metabolism includes basal maintenance costs, costs of growth (Hoyt et al. 1978, Vleck et al. 1979), specific dynamic action (Nobel 1987, Noble et al. 1990) and potential muscular movement in ovo (Tullis and Peterson 2004), unlike adult measurements of basal metabolic. These energy-consuming functions may change differentially with temperature, and could explain why growth efficiency declines with lower temperatures over the length of incubation (Chapters 2, 4). Costs for growth must ultimately be paid regardless of the time it takes to complete growth at a given temperature. However, low incubation temperatures that result in longer incubation periods may require higher overall energy costs over the incubation period to reach a certain stage of development. For this to be true, periodic cooling must negatively affect growth activity more than maintenance processes. In megapodes, total embryonic energy expenditure is 55-76% more when eggs are maintained at low temperatures (30°C-32°C) than at higher temperatures (34-36°C; Vleck et al. 1984, Booth 1987), which has been attributed to greater energy directed towards maintenance relative to growth at lower temperatures (Vleck and Vleck 1996, Booth and Jones 2002). If overall costs of development increase as incubation temperatures decline, future investigation of the underlying physiology should address how and why this occurs. Cooling may perturb homeostasis because of its differential effects on rates of different metabolic and synthetic reactions (Hochachka and Somero 2002). Alternatively, cooling may also affect transport of essential nutrients through reduced diffusion rates and decreased permeability of lipid membranes (Hochachka and Somero 2002).
The Arrhenius relationship provides additional support for the changing sensitivity to temperature at lower temperatures (higher Q10s) and also allows us to speculate about the underlying biochemistry. The Arrhenius relationship is predicated on how biochemical reaction kinetics vary with temperature according to the Boltzmann Factor, $e^{-E/kT}$. These models liken organisms to simple chemical systems with metabolic rate reflecting rates of chemical reactions that are themselves temperature dependent. For many organisms, carefully collected datasets of individual animals measured over a range of temperature (Lannig et al. 2004), do show an Arrhenius-like linear relationship between metabolic rate and the inverse of temperature (Clarke and Johnston 1999, Gillooly et al. 2001). House wren and zebra finch embryos, however, exhibit a relatively constant slope with inverse temperature only at temperatures that are considered unfavorable for hatching success (below 32°C), but deviated from linearity at temperatures approaching normal incubation temperatures (34°-35°C). That is, at temperatures near normal incubation temperatures, they do not match the Arrhenius model.

The function of oxygen consumption is to generate ATP required for internal and external work, and it is important to recognize that it is not temperature, but demand for ATP that drives ATP production and oxygen consumption (Pearson et al. 1991, Clarke and Fraser 2004). Thus, it appears that at a range of temperatures between 35°-38°C demand for ATP is relatively constant compared to at lower temperatures. It may be that embryo growth and oxygen demand in the range of 35°-38°C are temperature compensated (as evidenced by the drop from the rate predicted by the strict Arrhenius relationship). This difference from the predicted Arrhenius relationship may allow for regulatory control of growth rates to occur by up-regulating or down-regulating metabolism (i.e. increasing metabolism up to the Arrhenius line), and allow for selection to act on growth rate (Arendt 1997). Below ~34°C demand for
ATP may decrease at a rate that is determined by temperature, or possibly the ability to generate ATP is constrained via limitations of biochemical enzyme kinetics that temperature places on metabolic rates, despite a high demand for ATP for growth or maintenance.

House wrens had a statistically higher metabolic rate during rewarming than during cooling. It is notable that recovery of metabolic rate during rewarming was not temporally delayed. Therefore any physiological functions that declined during the cold shock were immediately able to recover. The increase in metabolic rate during rewarming may reflect the necessity to repair deleterious consequences of cooling, or could function to alter rates of temperature change. One mechanism that could create a metabolic hysteresis is thermoregulation, but my data show a hysteresis in the relationship of metabolic rate to body temperature opposite to that in neonate shorebirds that show active thermoregulation when foraging in the cold, but do not thermoregulate during brooding (Ricklefs and Williams 2003). Furthermore, altricial embryos are shown not to have the ability to thermoregulate (Tazawa et al. 2001). An increase in physical activity during rewarming may also increase metabolism (Tullis and Peterson 2004). In adult agamid lizards and turtles, heart rate is more rapid during warming than during cooling (Bartholomew and Tucker 1963, Galli et al. 2004). This cardiac hysteresis is thought to be an adaptation to increase heat flow into the center of the body from the body surface of basking reptiles. Increased heart rate when warming would increase demand for ATP and be reflected by increased $\dot{V}_{\text{O}_2}$. The same mechanism might enable bird embryos to deliver warm blood rapidly to the egg’s center from near the shell where the brood patch contacts the egg. Investigation of how rewarming affects blood perfusion to the chorioallantoic membrane may support this hypothesis.

Alternatively, the upregulated metabolic rate during rewarming may be a consequence of increased costs incurred in returning to homeostatic norms that are affected
by periodic cooling. Nutrient transport or protein synthesis costs may need to be higher during rewarming to recover nutrient levels or enzyme levels that may be depleted during the cold spell. If cold decreases the delivery of oxygen (Egginton and Sidell 1989) or nutrients (Feast et al. 1998) to tissues, a reasonable response will be to increase transport to tissues. Alternatively, if cold-induced conformational changes in key metabolic enzymes reduce enzyme activity, enzyme synthesis may be upregulated in response to the deficit during the cold period. Cold shock may activate synthesis of cold-shock proteins (Hochachka and Somero 2002) and increase proliferation of cellular organelles such as mitochondria (Egginton and Sidell 1989). Therefore, cooling would reduce the index of growth efficiency (Chapters 2, 4) by increasing metabolic costs during rewarming. This may also explain why embryos that have experienced cold have higher metabolic rates for a given mass than embryos that have not been exposed to cold temperatures (Chapter 2). Future studies should investigate how expression of metabolic enzymes, and the transport of nutrients are affected by sudden exposure to cold.

House wren and zebra finch embryos appear to have similar mass-independent rates of oxygen consumption when measured at incubation temperatures between 30°C and 38°C. This similarity in metabolic rate is striking and unexpected considering the relatively larger size to which wren embryos must grow (~ 1g vs. ~0.7 g for zebra finches), in combination with the shorter observed incubation periods of house wrens (12 days) compared to zebra finches (14 days). This result suggests that faster development of house wrens is less costly than in finches. Alternatively, there may be qualitative differences between house wrens and zebra finches that require slower growth and longer development in zebra finches, such as development of immune function or a longer life-span (Ricklefs 1992, 2006).
The intermittent incubation behavior of female house wrens exposes embryos to cool temperatures more frequently than does the bi-parental incubation behavior of the zebra finch parents. House wren eggs incubated in the wild are rarely kept at constant temperatures, but instead cool periodically throughout the day (Chapters 1, 4). However, exposure to temperatures as low as 15°C used in this study are probably rare, except as a result of a storm, unseasonably cold temperatures, or a prolonged absence by the incubating female. More commonly, egg temperatures of passerines fluctuate within the range of temperatures (30°-34°C; Chapter 1, Zerba and Morton 1983, Morton and Pereyra 1985, Weathers and Sullivan 1989) in which metabolic rates are less dependent on temperature. Prolonged cold exposure has negative consequences for embryo health, and some studies have suggested short-term cooling may also carry negative consequences for embryo survival (Lyon and Montgomerie 1985, Haftorn 1988). The reduction in oxygen consumption of two zebra finch eggs at the end of the prolonged measurement period in this experiment was due either to a decline in the health of the embryos while at low temperatures, or be a consequence of the implanted thermocouples for many hours. Maintaining egg temperature above 30°-34°C is important to the hatchability of avian eggs (Drent 1975, Vleck and Kenagy 1980), and the increased sensitivity of metabolic function below those temperatures may directly affect embryo mortality. However, behavioral responses to cold eggs that quickly return them to warm temperatures may serve to reduce embryo mortality, even when the degree of periodic cooling is severe. In Chapter 4, incubating house wrens always spent a long time on the eggs when mean egg temperature was below ~34°C, but frequently had shorter on-bouts when eggs were above 34°C, possibly because temperatures above this are sufficient for non-impaired embryo survival or fitness. This dynamic between egg temperature, embryo physiology and incubation behavior suggests that behaviors have evolved to keep eggs at or
above temperatures where metabolism is less temperature dependent in order to maintain high embryo growth efficiency and hatchability.

ACKNOWLEDGEMENTS

L. Anderson assisted in the monitoring of house wren nests and J. Muse cared for the zebra finches. R. Ackerman provided use of equipment. Funding was provided by a Sigma Xi Grant in Aid of Research, and the National Science Foundation under Grant No. IBN-0309371.

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CHAPTER 6: CONCLUSION

In this dissertation I examined how temperature variation and parental care during incubation affect avian embryo growth parameters. Careful regulation of egg temperatures has long been thought to be essential for proper embryo development (Lundy 1969, Drent 1975), and a survey of the literature showed that incubating adults maintain egg temperatures within a narrow range (Webb 1987). However, defining the consequences of egg temperature variation to developing embryos has not been undertaken (Reid et al. 2002). Understanding the thermal constraints on embryo development and fitness is especially important for species in which only the female incubates the eggs. Females of these uniparental incubators face a trade-off between providing care to the eggs to increase the value of the current clutch, versus allocating time for foraging and adult self-maintenance that would increase the possibility of future reproduction. Adult incubation behaviors therefore must balance adult energy requirements with optimizing the temperature of the eggs, while at the same time minimizing the attention of nest predators. Historically, more emphasis has been placed on the tradeoff between nest predation and adult energy requirements than on the temperature effects on the embryo (Skutch 1949, Martin 1995, Williams 1996). Therefore increased understanding of the effects of egg neglect to embryo fitness will increase our understanding of incubation behavior and clarify how embryo thermal requirements constrain the evolution of parental incubation strategies.

I have shown several direct effects of periodic cooling on avian development. These include reductions in the index of growth efficiency (Chapters 2, 4), reduced growth rates (Chapters 2, 4), increased mass-specific metabolic rates (Chapter 2), and altered embryo morphology (Chapter 3). These altered embryonic phenotypes arise during a relatively short
development period. However, the true costs of these altered phenotypes may not lie *in ovo*, but during subsequent stages of nestling growth, winter survival and life-long reproduction. Surprisingly, there was no consequence of periodic cooling to embryo survival up to 12 days of development (Chapter 2) or to hatchability (Chapter 4). I terminated incubation at 12 days to guarantee an adequate number of growing embryos for measurements, but future studies should address if and how the phenotypic changes that I have shown during development affect subsequent hatchling phenotypes, nestling growth and survival (Reed et al. 1999, Styrsky et al. 1999), and ultimately adult survival and life-time reproductive success (Lemon 1993, Ricklefs 2006).

At a range of temperatures between 32° and 38°C, metabolic rates of avian embryos change relatively little, compared to below 34°C, where metabolic rates decrease rapidly with falling temperatures (Chapter 5). It is probably not a coincidence that adult birds regulate egg temperatures above 32°C (Webb 1987), and prolonged exposure to temperatures below this results in poor embryo survival (Baldwin and Kendeigh 1932, Vleck and Kenagy 1980). The decrease in metabolic rate below 34°C undoubtedly reflects the inability of embryos to maintain homeostatic function at cold temperatures, and may either be the result of growth slowing faster than maintenance metabolism, or growth and maintenance metabolism both slowing precipitously at temperatures below 34°C. In house wren eggs that were cooled and rewarmed, metabolic rates were ~8% higher during rewarming than during cooling. This suggests there are costs incurred upon cooling that are must be paid when rewarming. Future studies should examine effects of cooling in embryos on protein synthesis (Hochachka and Somero 2002), nutrient transport (Hochachka and Somero 2002), and gene expression (Podrabsky and Somero 2004) that may explain increased embryo metabolic rate during rewarming.
I have shown here that temperature variation results in phenotypic variation of avian embryos. Natural selection can act upon this temperature-induced variation as it occurs in nature (Kaplan and P. C. Phillips 2006). If this variation is important to fitness, selection may have acted on adult incubation behaviors to favor egg temperature maintenance during periods of cold. Indeed, in response to artificially cooled eggs, incubating house wrens increased their nest attentiveness, decreased lengths of individual off-bouts, and increased the lengths of individual on-bouts (Chapter 4). However, future work should address the extent to which warm egg temperatures that result in short incubation periods are the result of selection pressure to reduce the window of opportunity for nest predation versus avoiding deleterious effects of cold to embryo phenotypes. Island species that are isolated from nest predators have longer incubation periods (Bosque and Bosque 1995), suggesting that selection for short incubation periods is to minimize predation. But a test to see whether island species would increase parental care in response to cold eggs would help to sort out the extent to which behaviors, as seen in this study, result from selection due to the thermal needs of embryos, or are due to selection from nest predation. Natural selection also may have acted on the growth and developmental physiology of avian embryos to allow them to cope with the nest climate that they experience (Clarke 2003). Although within species, egg temperatures are regulated within narrow limits, across species, mean egg temperatures vary more widely (Webb 1987). It is not clear the degree to which incubation periods in nature are determined by egg temperature versus innate rates of embryo growth. House wrens clearly have faster embryo growth rates than zebra finches (12 d versus 14 d). Yet incubation periods of closely related species also vary widely on a geographic scale (Martin 2002). If incubation periods vary mainly because of differences in egg neglect that are appropriate for different environments (e.g. tropical versus temperate; Martin 2002) these species may have also
evolved greater tolerance to cold in terms of higher embryo growth efficiency at lower temperatures, less dependence of metabolic rate on egg temperature, or be less affected morphologically by temperatures outside the normal range. Ultimately we would like to understand how the thermal tolerance of avian embryos has evolved in concert with adult incubation behavior, and such a study will require a careful comparative analysis. However this series of experiments establishes that the thermal state of the embryo is important to its physiological and morphometric development, as well as the behavioral strategy of the incubating adults.

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