

Periodic Cooling of Bird Eggs Reduces Embryonic Growth Efficiency

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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, we 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 d of incubation, embryo mass and yolk reserves were less in eggs that experienced periodic cooling than in 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 rate of development. This reduction in 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, 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 posthatch 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 covary (Ricklefs and Starck 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, posthatching 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 before 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-d incubation period is 156 kJ, while similar-sized chicken eggs that hatch after only 21 d 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 obtain-

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ing 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 d 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_c drops. To decipher the significance of periodic cooling to avian embryos, we measured growth and metabolism of zebra finch (*Taeniopygia guttata*) embryos over the first 12 d of development under either constant or fluctuating artificial incubation. We 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 g and readily breed in laboratory conditions. Their normal incubation period is ~14 d (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 nest box and nest material, so egg production was synchronized by supplying boxes and nesting material.

Eggs were collected 0–2 h after they were laid and replaced with nonfertile 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 nest boxes, all of which were used. We labeled eggs by the nest box from which they were collected and order of appearance with a nontoxic felt-tip pen. Eggs were weighed to the nearest 0.001 g, and their length and width were measured to the nearest 0.1 mm 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 nest box 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

We 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 zebra finch eggs in each chamber, and these eggs were placed on opposite ends of the rocking platform, closest to both the cold- and warm-air intakes, 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 effect 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 1981a; 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 h each day, followed by a 9-h "night" period at a constant temperature of ~37.5°C (Fig. 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 min. Mean egg temperatures (\pm SD) calculated over the entire incubation period were 37.4° \pm 0.04°C, 37.0° \pm 1.5°C, and 35.4° \pm 4.3°C 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, 30°C, and 20°C eggs, respectively. The largest differences occurred during times of cooling or rewarming, and differences declined when eggs were held at a constant temperature. This temperature gradient should increase variation within a treatment, but its effect was minimized because we 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-d 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 and weighed to the nearest 0.001 g, and its metabolic rate at 37.5°C was measured. Eggs were then dissected, and the shell, residual yolk, and yolk-free embryo were weighed to the nearest 0.001 g. We dried shell and residual yolk to a constant mass in a 60°C oven overnight to obtain dry mass. We froze embryos at –80°C, later thawed and photographed them for a morphometric analysis (C. R. Olson and C. M. Vleck, un-

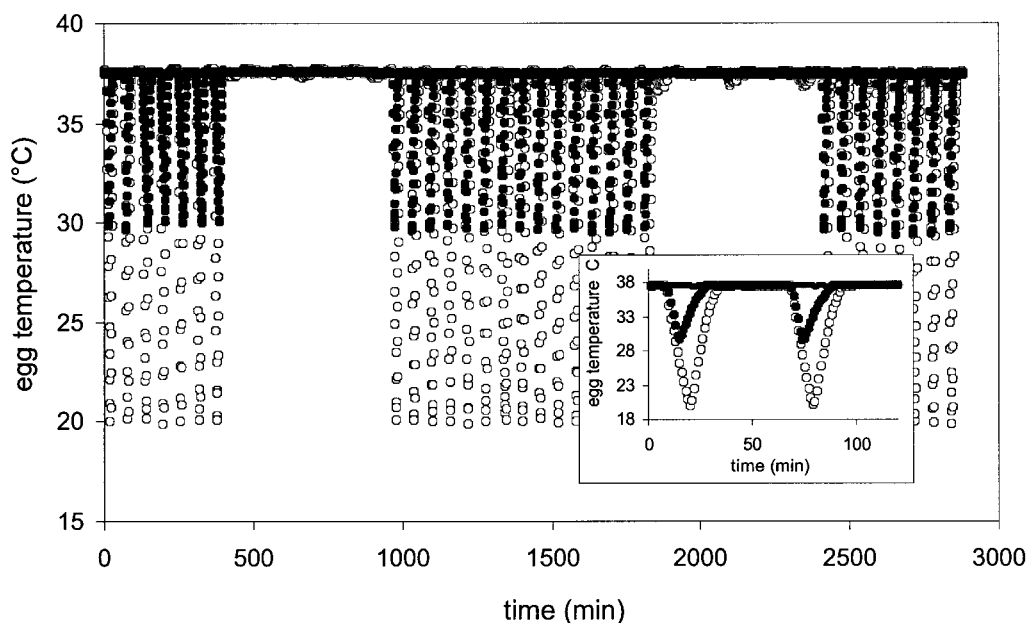


Figure 1. Thermal profiles experienced by eggs over 2 d of incubation. Periodic cooling occurred on an hourly basis 15 times a day, followed by a “night” time of constant temperature held at $\sim 37.5^{\circ}\text{C}$. Open circles represent eggs periodically cooled to 20°C , and filled circles represent eggs cooled to 30°C . Eggs in the constant-temperature treatment remained at $37.4^{\circ} \pm 0.04^{\circ}\text{C}$. The inset illustrates two cooling bouts over 2 h of incubation.

published manuscript), and then dried them to obtain dry embryo mass, as described above.

By day 12 of incubation, periodic cooling had resulted in developmentally delayed embryos that resembled younger embryos from the constant-temperature control treatment. We therefore measured dry, yolk-free embryo mass (E_d), dry residual yolk mass (Y_d), and the rate of oxygen consumption ($\dot{V}\text{O}_2$) at 37.5°C for a reference set of 29 eggs (in addition to the 16 control eggs that were allowed to develop for 12 d) incubated under constant-temperature conditions and then sacrificed at 8–13 d. We 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

We always measured egg $\dot{V}\text{O}_2$ (mL h^{-1}) at 37.5°C immediately before sacrificing eggs. To monitor general egg health in each group and develop an ontogeny-of-metabolism curve for the treatment and control eggs, we also measured $\dot{V}\text{O}_2$ of a subset of eggs ($n = 9$ eggs; three 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 (Fig. 1). $\dot{V}\text{O}_2$ was measured in a closed system (Vleck 1987), using 60-cm^3 plastic syringes as metabolism chambers. We controlled temperature during measurement periods by submerg-

ing 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, and dry (STPD). We left eggs in the chambers for enough time to reduce the concentration of oxygen available by no more than 2% (20–210 min), with older eggs requiring less time in the chambers than younger eggs. Although variation due to embryo activity would affect measurements of $\dot{V}\text{O}_2$, we assume that the recording intervals used adequately represent “long-term” metabolic activity for that stage of development. At the end of the sampling interval, we 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 CO_2 , 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 and equal variance and log-transformed as appropriate. Percent water composition of embryos and yolks was arcsine-transformed before analysis. We combined data from the control eggs incubated at constant tem-

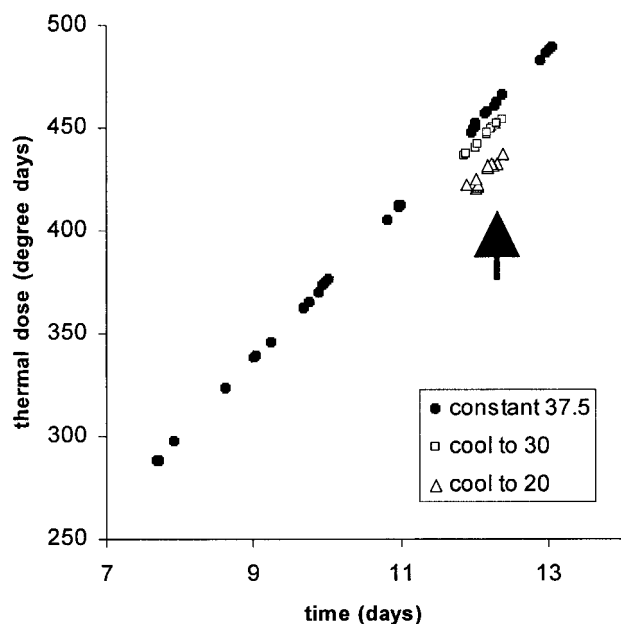


Figure 2. Accumulated thermal dose (TD) of control and treatment eggs. TD is determined by the time incubated and the cooling treatment. Periodic cooling reduced the TD accumulated at any given incubation time. The arrow indicates the cluster of treatment eggs incubated for ~ 12 d for which we measured tissue masses, yolk masses, and metabolic rates. Circles represent control eggs, and squares and triangles represent the 30°C and 20°C treatments, respectively.

perature for ~ 12 d (± 0.15 d) with the data from eggs incubated over a range of 8–13 d (measured to the nearest 0.1 d) at the same constant temperature (hereafter all called “control” eggs). To address how periodic cooling affected E_d and Y_d , we compared our treatment eggs that had developed for ~ 12 d to control eggs. We plotted E_d and Y_d 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 d accumulated a TD that was highly dependent on the cooling treatment they experienced (one-way ANOVA: $F_{2,36} = 79.98$, $P < 0.0001$). Eggs that experienced periodic cooling for ~ 12 d had TDs comparable to those of eggs incubated at constant temperature but for fewer days (Fig. 2), thus making it possible to tease apart effects of both cumulative TD and periodic cooling on growth, yolk use, and metabolism. We examined these differences with nonparametric Wilcoxon ranked-sign tests.

Results

Survival

Survival of eggs to 12 d did not differ among control and cooling treatments ($\chi^2 = 0.06$, $P > 0.5$) and was 70%, 67%, and 67% for the constant-temperature, 30°C, and 20°C eggs,

respectively. Eggs did not survive to 12 d 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 seven eggs in this group lacked evidence of development. There were 15 (out of an original 18) fertile eggs in the 30°C treatment, but three died before day 12, resulting in 12 eggs that survived to 12 d. 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 d of age. Eggs that were infertile or died before 12 d of age were removed before subsequent analyses.

Wet and Dry Mass of Egg Components

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

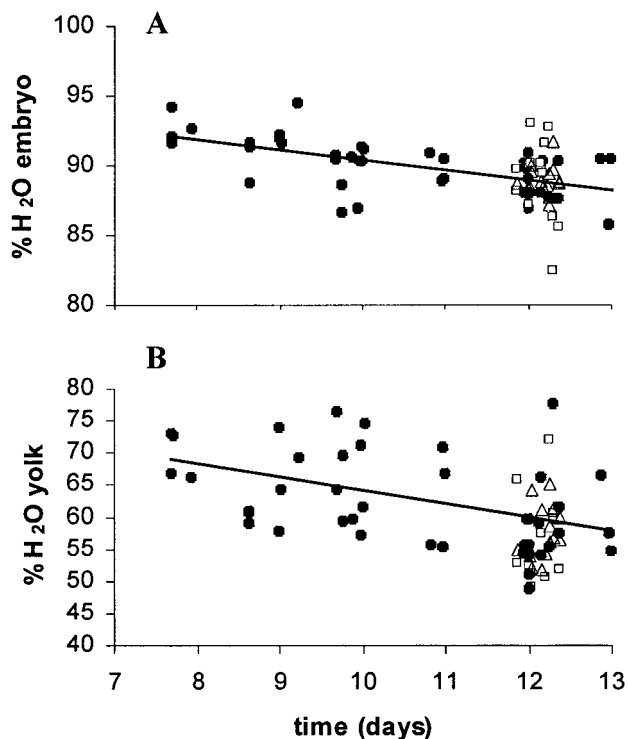


Figure 3. Changes in water content of zebra finch embryos (A) and residual yolk (B) with time. Regression lines for control eggs are $\%H_2O$ embryo = $-0.7216 \times \text{time} + 97.578$ ($r^2 = 0.37$), and $\%H_2O$ yolk = $-2.0675 \times \text{time} + 84.665$ ($r^2 = 0.19$). Symbols are as in Figure 2.

Table 1: Mean (\pm SD) dry mass, wet mass, and percent water composition of zebra finch embryos and residual yolk at 12 d of development

	Wet Mass (g)		Dry Mass (g)		H ₂ O Composition (%)		<i>n</i>
	Embryo	Yolk	Embryo	Yolk	Embryo	Yolk	
Constant 37.5°C	.448 \pm .085	.195 \pm .036	.050 \pm .010	.081 \pm .013	88.82 \pm 1.26	57.74 \pm 6.68	16
Cooled to 30°C	.407 \pm .065	.166 \pm .042	.047 \pm .012	.070 \pm .013	88.48 \pm 3.01	56.71 \pm 6.85	12
Cooled to 20°C	.317 \pm .065	.167 \pm .038	.035 \pm .010	.071 \pm .017	88.98 \pm 1.10	57.64 \pm 4.25	14

Compared to the constant-temperature treatment, periodic cooling resulted in smaller embryos that consumed more yolk over the same development time. At 12 d, E_d for the 20°C treatment was less than that for the constant temperature treatment, while that for the 30°C treatment was intermediate but not significantly different from the other two (ANOVA: $F_{2,39} = 7.43$, $P = 0.0018$). The Y_d of periodically cooled eggs did not significantly differ from that of the control eggs after 12 d of incubation (ANOVA: $F_{2,39} = 2.66$, $P = 0.08$), but the Y_d of the treatment eggs was, on average, smaller than that of the controls.

Growth and Yolk Consumption

The Y_d in control eggs incubated for 8–13 d decreased as E_d increased (Fig. 4A; $P = 0.0057$, $r^2 = 0.16$, $n = 45$), as described by the equation

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

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 ($P < 0.05$; Fig. 4A), whereas periodic cooling to 30°C resulted in a Y_d that was intermediate between those in the constant-temperature and 20°C treatments but not statistically different from either.

The E_d of control eggs incubated at a constant temperature increased with TD, described by the power equation

$$E_d = (6.33 \times 10^{-13})TD^{4.09}$$

($F_{1,36} = 32.9$, $r^2 = 0.85$, $P < 0.001$; Fig. 4B), similar to the conventional descriptions of embryo growth as a function of development time. We used this relationship to compare the 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 (Fig. 4B).

The Y_d of control eggs incubated 8–13 d declined exponentially as TD increased ($F_{5,44} = 5.0$, $r^2 = 0.10$, $P = 0.03$; Fig. 4C), as described by the equation

$$Y_d = 0.12e^{-0.00098TD}.$$

In contrast to the results for E_d , the Y_d of periodically cooled eggs was significantly lower for a given TD than that of control eggs, for eggs cooled to both 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 days 7 and 12 of incubation increased exponentially with days of incubation ($P < 0.001$; Fig. 5A), 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: For the constant-temperature treatment, $\dot{V}_{O_2} = 0.0188e^{0.2886 \times \text{time}}$, ($r^2 = 0.97$); for periodic cooling to 30°C, $\dot{V}_{O_2} = 0.0202e^{0.2625 \times \text{time}}$, ($r^2 = 0.87$); and for periodic cooling to 20°C, $\dot{V}_{O_2} = 0.0201e^{0.2381 \times \text{time}}$, ($r^2 = 0.90$).

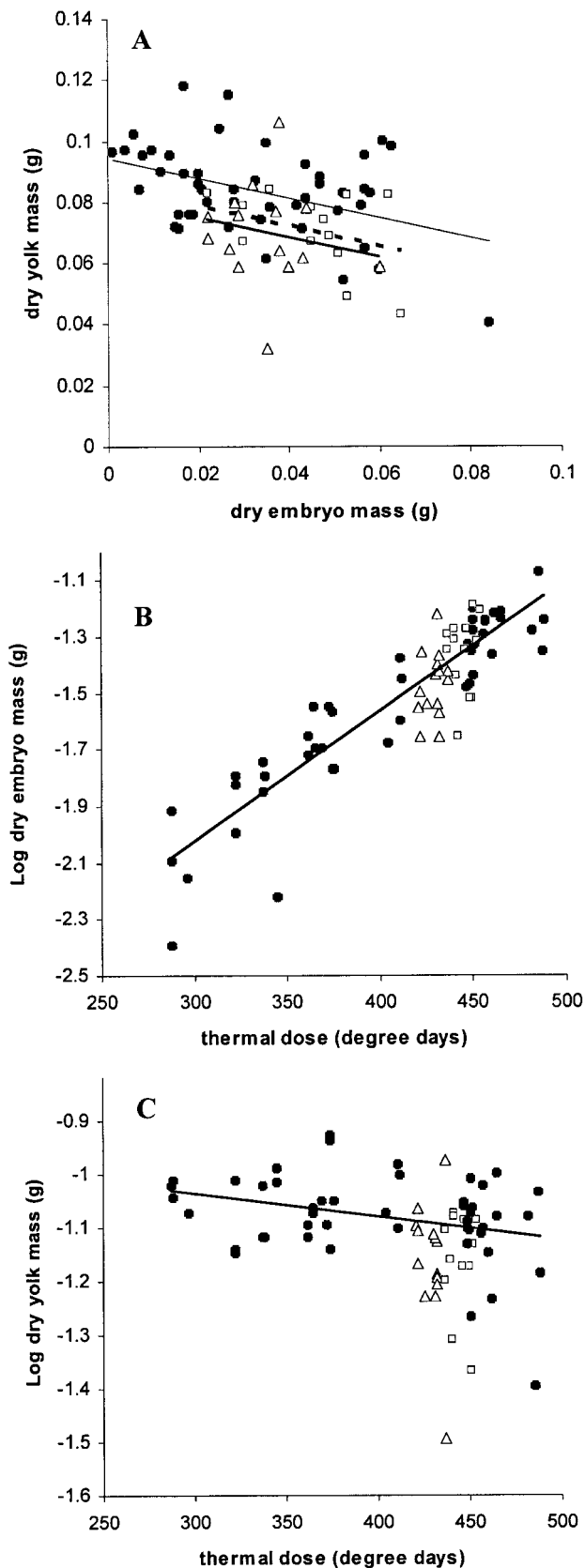
The lack of an interaction between time and treatment group ($F_{2,27} = 0.93$, $P = 0.41$) suggests that the ontogeny of metabolic rate among groups was fundamentally similar in shape and that statistical differences were due to a higher rate of increase of \dot{V}_{O_2} in the control eggs at any point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

Oxygen Consumption and Body Mass

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

$$\dot{V}_{O_2} = 3.45E_d^{0.67}$$

($n = 45$, $r^2 = 0.80$, $P < 0.001$). The \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 ($F_{2,66} = 7.69$, $P = 0.001$; Fig. 5B), but that of eggs cooled to 30°C did not



differ from that of the controls ($P = 0.95$). In eggs that were periodically cooled to 20°C, the relationship between $\dot{V}O_2$ and E_d was described by the equation

$$\dot{V}O_2 = 4.06E_d^{0.68}$$

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

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 embryos smaller after 12 d 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. Our results suggest that 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 posthatch nutrition and the long-term development of neonates into adults (Metcalf and Monaghan 2001). In reptiles, differing thermal conditions dur-

Figure 4. A, Dry residual yolk mass (Y_d) as a function of dry, yolk-free embryo mass (E_d). Periodic cooling to 20°C (thick solid line) reduced the amount of Y_d relative to E_d measured after 12 d of incubation compared to that in control eggs, resulting in both smaller embryos and reduced residual yolk ($P = 0.0035$). Regressions for control eggs (thin solid line) and eggs that were periodically cooled 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 than those held at constant temperatures for the thermal dose received (30°C treatment: $P = 0.029$; 20°C treatment: $P = 0.005$). Symbols are as in Figure 2.

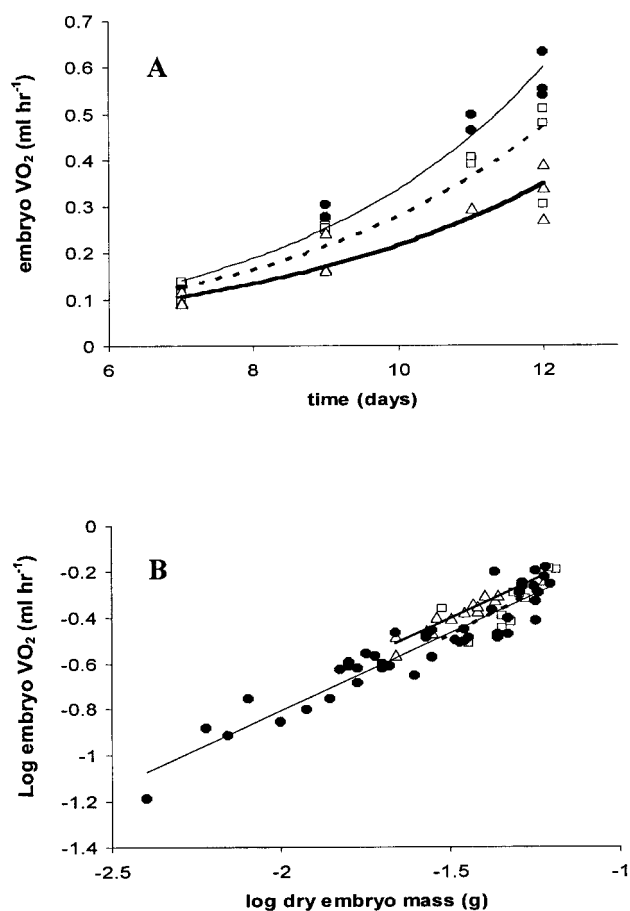


Figure 5. A, Ontogeny of metabolism for zebra finch eggs held at constant 37.5°C (*thin solid line*) or periodically cooled to 30°C (*dashed line*) or 20°C (*thick solid line*). B, Metabolic rate ($\dot{V}O_2$) as a function of dry embryo mass (E_d) in zebra finch eggs. Eggs that were periodically cooled to 20°C (*thick solid line*) had elevated $\dot{V}O_2$ ($P = 0.0371$) compared to 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. Symbols are as in Figure 2.

ing incubation also affect body size and yolk reserves (Rhen and Lang 1999; Shine 2004). It is not well understood how residual yolk influences posthatch 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 effect on the quality of the neonate rather than simply survival to hatching.

Reduced yolk reserves in eggs that periodically cool (Fig. 4A, 4C) suggests that 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). We do not know how exposure to periodic cooling alters energy use in eggs, but we can suggest several non-mutually exclusive

possibilities: lipid movement out of yolk, increased usage of peroxisomal metabolism, increased production of isozymes, and 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 (Egginton and Sidell 1989). We did not directly measure lipid content of our embryos, but lipid reallocation can 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 that 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 E_d to compensate fully for the missing yolk, but including lipid membranes and vacuoles, lipid makes up only about 20% of E_d (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 h had reduced yolk absorption and less lipid stores in their livers than noncooled embryos (Feast et al. 1998).

Nonmitochondrial 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 eight 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 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_2O_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 1999; 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 subcellular level, including increased intracellular and extracellular pH, decreased fluidity, altered structure of the membrane phospholipid bilayer, 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 the same pathway (McNab 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 toward embryo growth if growth occurred at constant temperature.

By day 12 of development, we 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 metabolism) 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 it is 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 result from cold acclimatization in some fish species (Egginton and Sidell 1989; Egginton et al. 2000). Additional mitochondria would increase total surface area through which proton leakage occurs, and maintaining mitochondria constitutes a considerable proportion of somatic metabolism (McNab 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 and then speeds up when eggs are rewarmed to incubation temperatures, expected differences between cumulative TD and accrued tissue mass would be obscured. Upregulating 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 setback (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 zebra finch eggs also decrease (C. R. Olson, C. M. Vleck, and D. Vleck, unpublished manuscript). Periodic cooling is known to increase avian incubation periods (Lyon and Montgomerie 1985, 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 TD 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. We find some support for this model because, although embryo mass was correlated with the cumulative TD, it was independent of whether eggs were cooled (Fig. 4B). However, our 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 upregulation of metabolism that we 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.

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 in patterns of egg neglect during incubation (Boersma and Wheelwright 1979; Vleck 1981*b*; 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 the 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 that our 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 those of a species like the zebra finch, which are less likely to experience periodic cooling (Zann and Rossetto 1991) and have a relatively long incubation period for their egg size (Rahn and Ar 1974). Further comparative study of the physiology and biochemistry of avian embryos will bring a new perspective to established pat-

terns 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).

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