Constant and Cycling Incubation Temperatures Have Long-Term Effects on the Morphology and Metabolic Rate of Japanese Quail*

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ABSTRACT

Incubation temperature can have profound effects on growth and development of embryos and young birds. However, few studies have examined the role that cycling incubation temperature may play in phenotypic variation and whether these effects persist to adulthood. We incubated Japanese quail eggs at control temperatures (37.5°C), at low temperatures (36.0°C), and under a cyclical treatment that maintained the same average temperature as the low treatment (36.0°C) with high temperatures that were the same as the control (37.5°C) and low temperatures that still allowed for development of the embryo (28.0°C). Individuals in the low treatment group were smaller in mass and size than individuals in the control group but had an increased basal metabolic rate relative to individuals in the cyclical treatment group. Temperature cycling offset the effects of low incubation temperatures on metabolic rate and embryonic development but not the effects on adult mass and size. Although Japanese quail are sexually size dimorphic, with females larger than males, we could detect no evidence of sex-specific sensitivity to suboptimal incubation temperatures. These results highlight the importance of incubation temperature and pattern as sources of morphological and physiological variation of adult birds.

Keywords: growth, sexual size dimorphism, epigenetics, development, avian, metabolism.

Introduction

Egg incubation temperature is highly regulated in oviporous species (e.g., birds, reptiles) and must be maintained within a certain range to ensure proper embryonic development (DuRant et al. 2013; McClintock et al. 2014). Deviations from optimal temperatures have been shown experimentally to have wide-ranging effects on subsequent morphological and physiological traits (Hepp et al. 2015). For example, in both precocial and altricial bird species, suboptimal incubation temperatures result in decreased offspring mass and/or size (wood ducks, *Aix sponsa* [Hepp and Kennamer 2012; DuRant et al. 2013]; tree swallows, *Tachycineta bicolor* [Ardia et al. 2010]; blue tits, *Cyanistes caeruleus* [Nord and Nilsson 2011]). Suboptimal incubation temperatures can also affect growth rates (DuRant et al. 2010, 2013) and locomotor performance (Hopkins et al. 2011). Although suboptimal incubation temperatures may have negative implications for postfledging survival (Berntsen and Bech 2015) and fitness (Hepp and Kennamer 2012; Hepp et al. 2015), effects of incubation temperature have, for the most part, rarely been examined beyond the juvenile stage (but see Wada et al. 2015) yet may be an important source of adult phenotypic variation.

Suboptimal incubation temperatures can also have negative metabolic effects on offspring. When Zebra finch (*Taeniopygia guttata*) eggs were periodically cooled, 12-d-old embryos displayed reduced mass and an increased metabolic rate, suggesting less efficient development (Olson et al. 2006). Additionally, low incubation temperatures have been shown to increase resting metabolic rate in 14–15-d-old blue tit fledglings (Nord and Nilsson 2011) and transiently in 25-d-old female zebra finch juveniles (Wada et al. 2015). This negative effect on metabolic rate may be more pronounced—and long-lasting—in precocial species that undergo a greater amount of development in the egg.

Studies investigating the influence of incubation temperature on posthatching development have largely focused on effects of constant temperatures. However, in many natural systems cyclical incubation temperatures are common, as the brooding parent leaves the nest periodically to either feed or avoid depredation (Weathers and Sullivan 1989). Since the cessation of incubation will cause a drop in egg temperature, cyclical temperature change may have a similar effect on developing embryos as constant low incubation temperatures (Olson et al. 2006). For example, when Japanese quail (*Coturnix japonica*) eggs were exposed to 8-h interruptions to incubation, the length of incubation increased by ~30% (Callebaut 1990). In wood ducks, extending interruptions to incubation has also been shown to increase the length of incubation (Carter et al. 2014). Whether any of these presumed negative effects on embryos persist into adulthood is not well known, and to date no study of which we are aware has examined whether cyclical incubation contributes to phenotype variation independently of reduced temperature. Previous studies of cyclical incubation (e.g., Olson et al. 2006,

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2008) compared treatments that were both cycling and at a reduced average temperature with constant high-temperature treatments. As such, the potential effects of cyclical incubation have not been examined in isolation. For example, it is possible that an incubation pattern that more closely mimics natural fluctuations may provide developmental benefits to embryos, as reported previously in some reptile species (reviewed in Bowden et al. 2014). Whether these potential benefits exist in birds, however, is not known. It is therefore important to consider temperature cycling when exploring the effects of incubation temperature on an individual's phenotype.

In some species, males and females differ predictably in body size (sexual size dimorphism), and during development the two sexes may respond differently to environmental stressors. The directionality of this effect (if any) may result from a combination of factors, including nutritional requirements, hormonal differences, genotype, and clutch size (Jones et al. 2009). In most avian species, males are larger than females, so it is often difficult to disentangle the effects of being male from that of the necessity of attaining a larger adult size (e.g., Chin et al. 2013). In zebra finches, however, males and females are similarly sized, yet when incubated at suboptimal temperatures males displayed reduced growth relative to females (Wada et al. 2015). This suggests that males may be more sensitive to incubation temperature than females, independent of body size. Japanese quail display reverse sexual size dimorphism, with females larger than males. This species thus presents an opportunity to further examine sex-specific effects of incubation temperature by uncoupling the effects of being male from the necessity of reaching a larger adult size.

In the current study, we tested the effect that constant incubation temperature and temperature cycling has on the eventual size and metabolic rate of adult Japanese quail. We hypothesized that the temperature experienced by an individual as an embryo would have short- and long-term effects on its phenotype. Specifically, we predicted that (1) individuals incubated at low temperatures would be lighter and smaller at hatch and at adulthood, (2) low incubation temperatures would result in adults with increased mass-adjusted basal metabolic rates (BMRs) relative to the controls, (3) cyclical incubation would offset some of the negative effects of low incubation temperature, and (4) there would be sex-specific effects of incubation temperature, although we could not predict directionality a priori.

Material and Methods

Husbandry and Treatments

Fertilized Japanese quail eggs were transported from Cro Quail Farms in St. Anns, Ontario, Canada, to Trent University by car at room temperature (~3 h). Eggs were then weighed, numbered using a nontoxic marker, and placed randomly into each of the three digital incubators (R-com 20; MX-20). Although artificial convection air incubation may have different implications for embryo development than parental contact incubation, it is commonly used in incubation temperature studies. The incubators were maintained at different temperatures: control (37.5°C, 48 eggs), low (36.0°C, 70 eggs), and cyclical (mean = 36.0°C, low = 28.0°C, high = 37.5°C [see below], 56 eggs). Sample sizes were unequal to account for predicted differences in hatching success in the second trial (see below). We chose 37.5°C for the control because this is the most commonly used temperature in commercial/recreational quail farming, while 36.0°C is at the lower end of the range that is used (Callebaut 1990). The cyclical treatment was designed to maintain an average temperature of 36.0°C, but, unlike the low-temperature treatment, the temperature cycled over 3-h periods. For 127 min of each 3-h period the incubator was maintained at 37.5°C. The incubator was then shut off by a digital timer, and the temperature decreased toward ambient for the remaining 53 min, eventually reaching 28.0°C before the timer turned the incubator back on and the temperature rose back to 37.5°C. It should be noted that we measured air temperature in the incubators, not internal egg temperature. While this is commonly done (e.g., Nord and Nilsson 2011; Wada et al. 2015), eggs in the cyclical treatment may therefore have experienced slightly different temperatures than were recorded due to thermal inertia. The cooling/warming sequence was established via preliminary trials to ensure that the incubation temperature was on average the same as that of the low-temperature treatment while maintaining the same maximum temperature as the control (i.e., 37.5°C). Preliminary temperature trials to establish appropriate cycling periods were conducted using empty incubators. Because of later concern that the presence of eggs may have influenced the cooling/warming sequence, we retested the cycling temperatures with and without eggs. We estimate that the presence of eggs increased the average temperature of the cycling incubator by up to 0.3°C. As a result, the average temperature of our cycling treatment may not have perfectly mimicked the low incubation temperature. Although we argue that the effect was likely minimal (see "Discussion"), our results are interpreted in light of this.

The humidity of incubators was maintained at 60% ± 5%, and all temperatures were measured ±0.1°C using a digital thermometer (BAT-12; Physiotemp). Because egg turning occurred via an automatic turning plate once every 3 h, each treatment had similar egg-turning bouts. Egg turning was stopped on incubation day 13 (onset of incubation = incubation day 0). Starting on incubation day 14, we increased the humidity to 75% ± 5% and started checking eggs for movement and pipping (breaking through the shell). In the cyclical treatment, any potential change in humidity during off-bouts was within the range of error of the humidity control.

Once pipping began, eggs were checked at least every 7 h around the clock, and hatch time was recorded. Because of the degree of wetness of the chicks and our previous experience with hatchling Japanese quail, it was possible to estimate age (in hours posthatch) to within 2 h. When discovered, hatchlings were marked on the leg(s) with a nontoxic colored marker for identification and moved to a different incubator to dry until 10 h after estimated hatch time (37° ± 1°C and 60% ± 10% humidity; Hovabator model 1583). At 10 h of age (based on the estimated hatch time, above), individuals were weighed and marked with acrylic paint on the head to indicate hatch day.

Posthatch husbandry followed Chin et al. (2013). Briefly, at 10 h of age hatchlings were moved to a metal brooder (35° ± 2°C, 40% ± 10% humidity). When chicks were 5 d old (hatch day = day 0), they were removed from the brooder and moved in groups of five or six to wire cages (45 cm × 45 cm × 95 cm) lined with paper towels and randomized between two environmental chambers. The environmental chambers were maintained at 30° ± 1°C for 2 wk, at which point the temperature was decreased by 3.5°C every 6–7 d until reaching a final temperature of 24°C. On day 10, they were separated into groups of three or four, and on day 25 they were housed individually. Males and females, sexed visually by plumage on day 25, were kept at separate ends of the environmental chamber to minimize aggression between males in adjacent cages. As individuals were sexed visually, sex ratios were not obtained at hatch. Individuals were fed MasterFeed Turkey Starter (24% protein) until day 24, and Mazuri Adult Breeder Diet (21% protein) was blended in after day 24 until given exclusively on day 37 ± 2 d. A light cycle of 14L:10D was used throughout the experiment. Water and food was supplied ad lib. from when chicks were moved to the brooder until the end of the study. To ensure adequate sample sizes, the experiment was replicated, separated by a 1-mo period. A total of 97 chicks hatched across the entire study (control = 29, low = 28, cyclical = 40), and a total of 88 birds survived to adulthood (control = 26, low = 25, cyclical = 37). Individuals were considered to be adults by day 55 (Chin et al. 2013). All animal use was approved by the Animal Care Committee at Trent University, in accordance with the Canadian Council on Animal Care (protocol 13027-23094).

Morphological Measurements

Starting at hatch (day 0) and then on days 3, 5, 10, 20, 30, 40, and 55 posthatch, we measured body mass using a digital pan balance (±0.01 g), and as an index of structural size we measured the distance from the back of the head to the front of the bill using digital calipers (±0.01 mm). This metric is commonly used as an index of body size in birds (e.g., Ardia et al. 2010). Head-bill measurements were taken in triplicate and averaged.

Adult BMR

As an index of adult BMR (day 60+), we measured the nocturnal oxygen consumption rate ($\dot{V}O_2$) of 77 (control = 25, low = 29, cyclical = 23) of 88 individuals surviving to adulthood using flowthrough respirometry. Food was removed at 0730–0830 hours on the day of measurement. At 1600–1800 hours, two or three quail were removed from their cages, transported to the respirometry room (~30 m) in a breathable cloth bag, weighed (± 0.01 g), and placed in one of three 4-L paint-can respirometry chambers, painted flat gray inside. The chambers were placed in an incubator (Thermo Low Temperature Incubator model 915; Fisher Scientific) set at 24°C (within the Japanese quail thermoneutral zone; Ben-Hamo et al. 2010). At 0800–0900 hours the following morning, individuals were removed from the chambers, reweighed, and returned to their cages. Mass during respirometry was calculated as the average of the two masses. Due to a scale malfunction, three birds from the second trial were missing prerespirometry masses; these masses were estimated using a linear regression ($r^2 = 0.97$, $F_{1,72} = 2{,}656.14$, $P < 0.001$, $n = 74$) based on the reduction in mass of all other birds during respirometry. On average, individuals lost 2.5% of their initial mass during a respirometry trial.

The respirometry protocol followed Chin et al. (2013). Briefly, ambient air was scrubbed of CO_2 and water using three columns of Drierite, one column of soda lime, and one column of Ascarite II, connected in series with Bev-A-Line tubing (Cole-Palmer). The column of Ascarite II also contained layers of Drierite at the top and bottom of the column to absorb any additional water produced during the absorption of CO_2. Air then flowed through the multiplexor (model TR-RM; Sable Systems) into the respirometry chambers or a piece of Bev-A-Line tubing when measuring baselines. Flow rate was determined by mass flow controllers (model 840; Sierra Instruments) and was set to 1,800 mL/min. On exiting the chamber, air was scrubbed of water vapor using magnesium perchlorate. A subsampler pump (TR-SS3; Sable Systems) drew in air at 150 mL/min into the carbon dioxide analyzer (CA-10; Sable Systems) followed by the oxygen analyzer (FC-10a; Sable Systems). Before each trial, we calibrated the CO_2 analyzer by spanning it to 0% and 1.00% using dry ambient air and compressed gas, respectively. We calibrated the O_2 analyzer to 20.95% using ambient air. ExpeData software (ver. 1.7.30) was used to record and analyze the data. To calculate $\dot{V}O_2$, the lowest continuous 5-min period of oxygen consumption was selected for each individual, and the average carbon dioxide emission and oxygen consumption from this period were recorded. $\dot{V}O_2$ was calculated using equation (10.6) in Lighton (2008) and multiplied by 20.08 to convert $\dot{V}O_2$ (mL O_2) to metabolic rate (J; Schmidt-Nielson 1990). Raw data are available in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.76qt1; Ben-Ezra and Burness 2016).

Statistics

All statistical analyses were performed in JMP 11.0 (SAS), and statistical significance was claimed at $P < 0.05$. No data were transformed.

To analyze initial egg mass, we used a general linear model (GLM) with trial number, treatment, and the treatment × trial number interaction. Eggs in the first trial were ~5% lighter than eggs in the second trial (least squares mean ± 1 SEM: trial 1, 13.24 ± 0.12 g; trial 2, 13.91 ± 0.12 g; trial number: $F_{1,168} = 14.69$, $P = 0.002$) but did not differ between treatments (treatment: $F_{2,168} = 0.98$, $P = 0.379$). By chance, because eggs were distributed randomly, there was a treatment × trial number interaction ($F_{2,168} = 3.41$, $P = 0.035$), with eggs allocated to the low temperature incubator of trial 2 being heavier than eggs in all treatments of trial 1 (Tukey HSD, $P < 0.05$) but not differing from those of the other treatments in trial 2 (Tukey HSD, NS). When split by trial number, we confirmed that egg mass did not differ among treatments in trial 1 (treatment: $F_{2,83} = 0.33$, $P = 0.717$).

In trial 2, egg mass differed among treatments ($F_{2,85} = 4.49$, $P = 0.014$), with eggs in the low treatment group being heavier than those in the control treatment group (least squares mean ± 1 SEM: control, 13.57 ± 0.22 g; low, 14.38 ± 0.18 g; Tukey HSD, $P < 0.05$); there were not other significant differences (Tukey HSD, NS). Trial number and the interaction between trial number and treatment were included in all subsequent analyses to examine the influence of egg distribution.

To analyze the mass and size of hatchlings and adults, we used GLMs. We started with a full model, including sex, treatment (incubation temperature), trial number, the interaction between sex and treatment, and the interaction between trial number and treatment. Sex, treatment, and the sex × treatment interaction were retained in all tests regardless of significance due to a priori interest. All other main effects and interactions were excluded using backward stepwise elimination if P values were >0.10, starting with the least significant interactions followed by main effects. To include the main effect of sex, individuals that hatched but died before day 25 (when sexing became possible; total, $n = 9$; each treatment, $n = 3$) were excluded from all posthatch analyses.

To analyze hatching success and sex ratio, we used a GLM with a binomial distribution and logit function using treatment as the main effect. Trial number was initially included in these models but was removed as it was nonsignificant ($P > 0.1$). To test for an effect of treatment and trial number on the length of incubation, we used ANOVA, with a Welch's ANOVA for analysis of treatment due to lack of homogeneity of variance. Dunn-Šidák-adjusted P values were used for post hoc analyses of length of incubation and hatching success. Because it was not always possible to match a hatchling to the egg from which it came, we could not compare these two metrics.

For the analysis of BMR (as indexed by $\dot{V}O_2$), we used a GLM with body mass at respirometry included as a covariate. Sex, treatment, and sex × treatment were again retained regardless of statistical significance. Interaction terms with respirometry mass (respirometry mass × sex, respirometry mass × treatment) as well as trial number, trial number × treatment, bird age at respirometry, and chamber number were initially included. Bird age (range: 60–94 d posthatch) and chamber number did not affect BMR ($P > 0.1$ for both) and were removed from the final model.

Results

Length of Incubation Was Affected by Temperature and Cycling

Incubation temperature significantly affected how long it took for individuals to hatch (Welch's ANOVA, $F_{2,42} = 109.70$, $P < 0.001$; fig. 1). Individuals in the low treatment group took significantly longer than individuals in the cyclical treatment group (Welch's ANOVA, $F_{1,31} = 102.53$, Dunn-Šidák-adjusted $P = 0.003$), which in turn took significantly longer than individuals in the control treatment group (Welch's ANOVA, $F_{1,33} = 104.74$, Dunn-Šidák-adjusted $P = 0.002$). When split by trial, the effect of treatment remained the same, as did all subsequent comparisons described above ($P < 0.01$ for all), so trials were kept pooled. Variance in hatch day also differed among treatment groups (Levene, $F_{2,94} = 11.06$, $P < 0.001$). The cyclical treatment showed significantly less variance than the control (Levene, $F_{1,67} = 20.48$, Dunn-Šidák-adjusted $P = 0.003$) and the low-temperature treatment (Levene, $F_{1,66} = 20.32$, Dunn-Šidák-adjusted $P = 0.002$). The control and low-temperature treatments did not differ from each other (Levene, $F_{1,55} = 0.04$, $P = 0.851$).

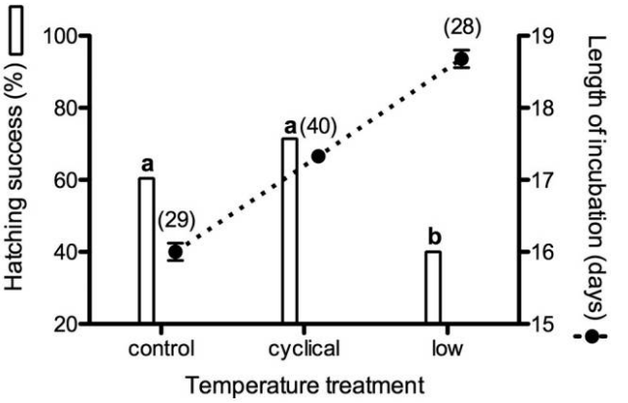

Figure 1. Hatching success and length of incubation (least squares mean ± 1 SEM) for Japanese quail eggs in control (37.5°C), cyclical (mean: 36.0°C; range: 28.0°–37.5°C), and low (36.0°C) temperature treatment groups. Sample sizes for length of incubation are in parentheses; error bars for the cyclical treatment are shown but are hidden behind the symbol. Number of eggs incubated: control, $n = 48$; cyclical, $n = 56$; low, $n = 70$. Differing letters denote $P < 0.05$ for a Tukey HSD.

Hatching Success Was Affected by Temperature and Cycling

Incubation temperature significantly affected hatching success (GLM, $\chi^2 = 13.24$, $P = 0.001$, $n = 174$; fig. 1). Hatching success was significantly higher in the cyclical treatment group (71%) than in the low treatment group (40%; Dunn-Šidák-adjusted $P = 0.001$). The control treatment (60%) did not differ from the low-temperature treatment (Dunn-Šidák-adjusted $P = 0.057$) or the cyclical treatment (Dunn-Šidák-adjusted $P = 0.236$). Mortality rates were similar across treatments (table 1).

Incubation Temperature and Cycling Had Short-Term Effects on Hatchling Size but Not Mass

Incubation temperature did not affect hatchling body mass (least squares mean ± 1 SEM: control, 9.35 ± 0.21 g; cyclical, 9.38 ± 0.18 g; low, 9.57 ± 0.22 g; treatment: $F_{2,79} = 0.32$, $P = 0.731$), but hatchlings in the second trial were heavier than those in the first (least squares mean ± 1 SEM: trial 1, 9.10 ± 0.16 g; trial 2, 9.76 ± 0.17 g; trial number: $F_{1,79} = 7.91$, $P = 0.006$), consistent with their heavier egg mass. The interaction between trial number and treatment was not significant ($F_{2,79} = 3.00$, $P = 0.055$) but approached significance due to the initial difference in egg mass (see "Material and Methods").

Table 1: Hatching success of Japanese quail eggs ($n = 174$) and survival of hatchlings

	Temperature treatment		
Variable	Control	Cyclical	Low
Eggs incubated	48	56	70
Eggs hatched	29	40	28
Individuals surviving to sexing (day 25)	26	37	25
Posthatch survival (%)	89.7	92.5	89.3

Note. Survival was 100% between day 25 and the end of the study.

Incubation temperature significantly affected hatchling head-bill length (least squares mean ± 1 SEM: control, 22.98 ± 0.14 mm; cyclical, 22.45 ± 0.12 mm; low, 23.01 ± 0.15 mm; treatment: $F_{2,82} = 5.80$, $P = 0.044$). Hatchlings in the control and low treatment groups had greater head-bill lengths than those in the cyclical treatment group (Tukey HSD, $P < 0.05$ for both) but did not differ from each other (Tukey HSD, NS). At hatch, males and females did not differ in body masses or head-bill lengths, nor was there a sex-specific effect of incubation temperature (sex × treatment, $P > 0.10$).

Adult Mass and Size Were Affected by Incubation Temperature but Not Cycling

Incubation temperature had significant long-lasting effects on adult body mass (least squares mean ± 1 SEM: control, 254.96 ± 4.81 g; cyclical, 240.13 ± 4.10 g; low, 239.64 ± 4.94 g; treatment: $F_{2,82} = 3.42$, $P = 0.038$; fig. 2*A*), although a post hoc Tukey HSD test was unable to identify where this difference lay (control vs. low, $P = 0.055$; control vs. cyclical, $P = 0.073$; cyclical vs. low, $P = 0.99$). This likely was the result of the borderline significant sex-specific effect (sex × treatment: $F_{2,82} = 2.69$, $P = 0.074$). In fact, less conservative post hoc Student's *t*-tests showed that control birds were heavier than those in both the cyclical (Dunn-Šidák-adjusted $P = 0.031$) and low (Dunn-Šidák-adjusted $P = 0.029$) treatment groups, while birds in the low temperature and cyclical groups did not differ from each other (Dunn-Šidák-adjusted $P = 0.470$).

Incubation temperature also affected adult size (least squares mean ± 1 SEM: control, 43.55 ± 0.21 mm; cyclical, 42.53 ± 0.18 mm; low, 42.60 ± 0.21 mm; treatment: $F_{2,82} = 8.07$, $P = 0.001$; fig. 2*B*). Control birds were larger in size (as head-bill distance) than birds in the cyclical and low treatment groups (Tukey TSD, $P < 0.05$ for both), which did not differ from each other (Tukey HSD, NS). Females were heavier than males at adulthood (least squares mean ± 1 SEM: female, 268.86 ± 3.79 g; male, 220.96 ± 3.77 g; sex: $F_{1,82} = 80.26$, $P < 0.001$; fig. A2*A*) and had greater head-bill lengths (least squares mean ± 1 SEM: female, 43.16 ± 0.16 mm; male, 42.63 ± 0.16 mm; sex: $F_{1,82} = 5.33$, $P = 0.023$; fig. A2*B*). There was no sex-specific effect of treatment on adult size (sex × treatment: $F_{2,82} = 2.02$, $P = 0.1395$), meaning that males and females were affected similarly by incubation temperature.

Adult BMR Was Affected by Incubation Temperature and Cycling

Incubation temperature affected adult BMR (treatment: $F_{2,67} = 6.48$, $P = 0.003$; fig. 3). Birds in the low treatment group had a higher BMR than those in the cyclical treatment group (Tukey HSD, $P < 0.05$); control birds fell between those in the other treatment groups but did not attain a significant difference from either (Tukey HSD, NS). Individuals in the second trial had higher BMRs than individuals in the first (least squares mean ± 1 SEM: trial 1, 145.34 ± 4.81 kJ/day; trial 2, 163.60 ± 4.54 kJ/day; trial: $F_{1,67} = 7.89$, $P = 0.007$). As expected, there was a positive effect of body mass on BMR ($P < 0.05$; table 2). Adult mass-adjusted BMR did not differ between males and females, nor was there an interaction between sex and treatment ($P > 0.1$ for both). The interaction between respirometry mass and treatment approached significance ($P = 0.069$; table 2) but did not attain significance even when the nonsignificant sex × treatment interaction term was removed. Due to a priori interest (see "Material and Methods"),

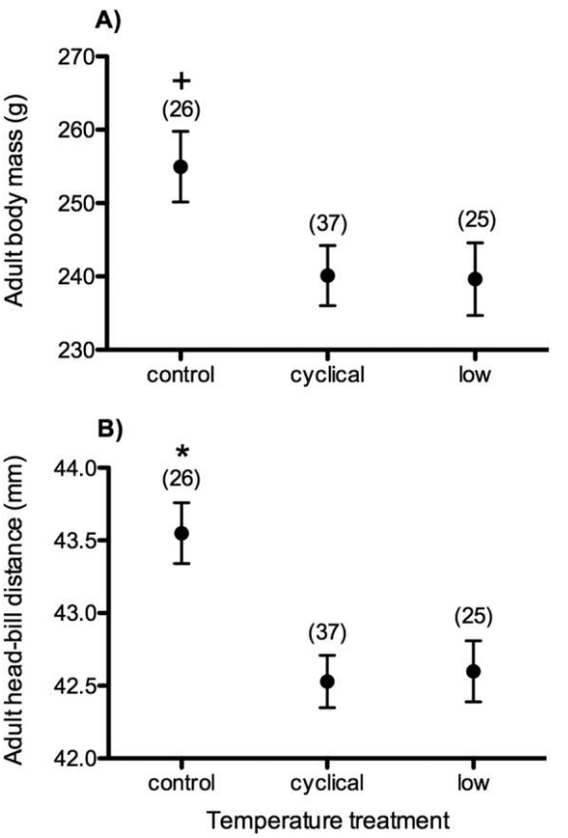

Figure 2. Japanese quail adult (day 55) measurements for body mass (*A*) and head-bill length (*B*) for individuals incubated as embryos at control (37.5°C), cyclical (mean: 36.0°C; range: 28.0°–37.5°C), and low (36.0°C) environmental temperatures, with sexes pooled. Plotted values are least squares means ± 1 SEM from a general linear model. Sample sizes are in parentheses; the plus sign in *A* denotes $P < 0.10$ for a Tukey HSD (see "Results" for details), and the asterisk in *B* denotes $P < 0.05$ for a Tukey HSD.

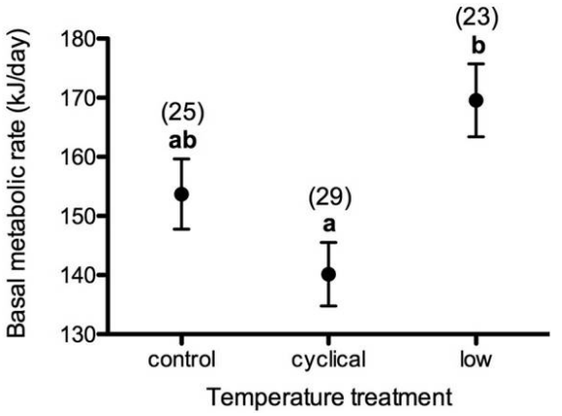

Figure 3. Basal metabolic rate of adult Japanese quail, incubated as embryos at control (37.5°C), cyclical (mean: 36.0°C; range: 28.0°–37.5°C), and low (36.0°C) environmental temperatures. Plotted values are least squares means ± 1 SEM from a general linear model, with body mass included as a covariate. Sample sizes are in parentheses; different letters denote $P < 0.05$ for a Tukey HSD.

we retained the nonsignificant sex × treatment interaction in the final model.

Discussion

Length of Incubation

Incubation was extended for the low treatment (~18.6 d) relative to the control (~16.0 d), with the cyclical treatment (~17.3 d) displaying an intermediate duration as well as a reduced variance in hatch time. This suggests that cyclical temperatures during incubation may counteract some of the negative impact of constant low incubation temperatures. A decrease in average temperature is widely known to increase incubation period (Eiby and Booth 2009; Ardia et al. 2010; Nord and Nilsson 2011; DuRant et al. 2012; Kolackova et al. 2015). Our results, however, demonstrate that fluctuating incubation temperatures can lead to more rapid hatching. Although we recognize that the temperature of our cycling treatment may have been up to 0.3°C above the low treatment temperature, we do not think this was the cause of differences in hatching time, as explained below. Instead, the decrease in length of incubation of the cyclical treatment compared with the low treatment more likely reflects accelerated embryonic development that may occur during the higher on-bout temperatures (Olson et al. 2006).

Because of the temperature sensitivity of development in birds, incubation period has been expressed previously in degree-days (e.g., Olson et al. 2006; Wada et al. 2015). This assumes that high temperatures linearly increase the rate of development, so that developmental stage can be predicted on the basis of incubation temperature (Olson et al. 2006; Koch 2015). We calculated degree-days as the mean incubation temperature above physiological zero (when development does not occur) multiplied by the number of days (e.g., Matsuzawa et al. 2002). Using 26.0°C as physiological zero for Japanese quail (Conway and Martin 2000; Lacin et al. 2008), hatching times of control, cyclical, and low treatments were 184.1 ± 1.1, 173.3 ± 1.0, and 187.4 ± 1.1 degree-days, respectively (mean ± SE). Although birds in the control and low treatment groups hatched at similar degree-days, the development of the birds in the cyclical treatment group was accelerated. Even if the mean temperature of the cyclical treatment was elevated by up to 0.3°C (see "Material and Methods"), embryonic development was still faster than that for the other treatments (176.8 ± 0.9 degree-days). This suggests that the acceleration and deceleration of developmental rate that occurs when incubation temperature is fluctuating can alter the relationship between developmental rate and temperature, as reported previously for a variety of insect species (Wu et al. 2015).

Hatching Success

Hatching success was reduced by low temperature but not by cycling. In fact, the cyclical treatment had the highest hatching success at 71%, although this was not significantly different from that for the control treatment (60%), and both were similar to previously reported hatching successes in Japanese quail (Copur et al. 2010; Javurkova et al. 2015). This result mirrors what was found with even longer cycling periods (8 h off-bouts) of incubation in Japanese quail (Callebaut 1990). In that study, however, average incubation temperature was not controlled for. Our results reveal that cycling can mitigate the reduced hatching success normally seen with constant low incubation temperature (e.g., blue tits [Nord and Nilsson 2011], wood ducks [Hepp et al. 2006]). This further suggests that high on-bout temperatures during cyclical incubation have more of an influence on hatching success than the average temperature. Eggs in the cyclical treatment group spent only 53 min per 3-h cycle at the control temperature (37.5°C), but hatching success was similarly high. The significantly reduced hatching success for the low temperature treatment also indicates that even with the same average temperature as the cyclical treatment, maintaining incubation at a constant 36.0°C, without 1.5°C higher on-bouts, has a dramatic negative influence on embryo development and egg hatchability.

Hatchling Morphology

Incubation temperature did not affect hatchling body mass, although cycling did affect size, as hatchlings in the cyclical treat-

Table 2: Results of a general linear model for factors influencing basal metabolic rate in adult Japanese quail

Main effect	df	*F*	*P*
Sex	1, 67	.43	.514
Treatment	2, 67	6.48	**.003**
Sex × treatment	2, 67	1.33	.270
Trial	1, 67	7.89	**.007**
Respirometry mass	1, 67	7.29	**.009**
Respirometry mass × treatment	2, 67	2.78	.069

Note. Numbers in boldface type indicate significant main effects.

ment group had shorter head-bill lengths than individuals in both the low and the control treatment group. In contrast, when zebra finch eggs were incubated with a temperature treatment that cycled (and was also cooler on average), embryos were structurally larger (in longitudinal measurements) relative to control embryos that were incubated at a warmer and constant temperature (Olson et al. 2008). Although this is opposite to our finding, zebra finches are an altricial species, while Japanese quail are precocial. These differing developmental strategies may therefore lead to an altered response to suboptimal growth conditions, especially if growth metrics are being differentially prioritized (Olson et al. 2008). It is also possible that the smaller structural size that we detected at hatch reflects a trade-off between development of adequate structural size and duration of incubation. Hatchlings from the cyclical incubation treatment were structurally the smallest and in terms of degree-days hatched the earliest, indicating an acceleration of development (Wu et al. 2015).

Adult Morphology

Although a number of studies have looked at the effects of low temperature on hatchlings and subsequent development (reviewed in DuRant et al. 2013), seldom have individuals been measured through to adulthood (but see Berntsen and Bech 2015; Wada et al 2015). In the current study, we demonstrated that incubation temperature can have long-term effects on an individual's body mass and size. As predicted, adults in the control treatment group (37.5°C) were heavier and larger than individuals in the low treatment group (36.0°C). This implies that the effects of incubation temperature may be far reaching. It has previously been shown that low incubation temperature can lead to decreased offspring mass and survival (e.g., wood ducks; Hepp and Kennamer 2012) as well as decreased recruitment (e.g., wood ducks; Hepp and Kennamer 2012), while reduced mass alone can increase time to sexual maturity (e.g., lesser scaups, *Aythya affinis*; Dawson and Clark 2000). Incubation temperature may therefore play a significant role in the survival and reproduction of offspring.

Although cycling offset several of the negative effects of a low average incubation temperature, it appeared to have no influence on adult body mass and head-bill length. This finding is contrary to our prediction that cycling would offset the long-term negative effects of low incubation temperature on the size and mass of adults. These results show that regardless of whether incubation temperature was constant or cyclical, low mean incubation temperatures negatively affect adult morphology. This is consistent with data on the influence of incubation temperature on the growth rates of wood ducks from hatch to 9–12 d (DuRant et al. 2010, 2013).

Female quail grew at faster rates and were both heavier and structurally larger at adulthood than males, as shown previously (e.g., Chin et al. 2013). Contrary to our prediction, however, there was no observed sex specificity in how individuals responded to the treatments. It has been found that suboptimal incubation temperatures result in lower body masses of male zebra finches but not females (Wada et al. 2015). Chin et al. (2013) demonstrated that female Japanese quail, as the larger sex, were more negatively affected by early food restriction. Meanwhile, in a previous study we did not detect any sex-specific effects in Japanese quail when altering rearing temperature (Burness et al. 2013). In the current study, we found that both sexes responded equally to low incubation temperature, showing a reduction in size and body mass at adulthood. The difficulty in detecting a consistent pattern even within the same species likely reflects the complex suite of interactions that influences sex-specific effects (Jones et al. 2009). Our results suggest, however, that incubation temperatures do not appear to influence male and female Japanese quail differently.

Adult Metabolic Rate

As predicted, birds in the low treatment group had a higher BMR than birds in the cyclical treatment group. However, the BMR of the birds in the low and cyclical treatment groups did not differ from that of the birds in the control treatment group. Effects of incubation temperature on metabolism have been detected previously in altricial species. For example, experimentally reduced incubation temperatures resulted in an elevated metabolic rate in fledgling blue tits (Nord and Nilsson 2011). Similarly, there was also a transient increase in BMR in female zebra finches as a result of low incubation temperature, although this effect was no longer present by adulthood and was never seen in males (Wada et al. 2015). Our results suggest that depending on developmental strategy, there may be long-lasting metabolic effects of incubation temperature pattern. Japanese quail are precocial and undergo a greater proportion of their development within the egg than do altricial species, such as blue tits and zebra finches. As such, the effects we detected may differ among species coincident with life-history strategies and developmental patterns.

A plausible mechanistic explanation for the increased BMR in adults in the low incubation treatment group relative to the cyclical treatment group may be the efficiency of embryonic development (e.g., Nord and Nilsson 2011). When zebra finch embryos were exposed to periodic cooling, they showed elevated metabolic rates and decreased yolk reserves, indicating that developmental efficiency was being impaired (Olson et al. 2006). If such inefficiency is maintained during postnatal growth, it may contribute to the phenotypic variation we detected in BMR at adulthood. Furthermore, the results of the current study suggest that it is incubation temperature alone, not a cyclical pattern, that elevates BMR. This distinction was not made by Olson et al. (2006), as they compared a constant high temperature treatment with a cyclical low temperature treatment.

An alternative mechanism that could explain the variation we found in adult BMR is that embryonic survival differed between treatments. Hatching success was decreased for the low treatment (40%) relative to the cyclical treatment (71%). If embryos with high metabolic rates experienced increased survival at low temperatures and their relatively high metabolic rates persisted to adulthood, this could explain the difference we found. Because

we did not measure embryonic metabolic rate, however, we do not know whether this may have occurred.

Cyclical Temperature Treatment

As previously mentioned (see "Material and Methods"), the mean temperature of the cyclical treatment may have been underestimated by as much as 0.3°C. Although individuals in this treatment group hatched at an intermediate time between those in the low (36.0°C) and control (37.5°C) treatment groups, there were no other measures for which they were an intermediate. Long-term morphology measurements showed that birds in the cyclical treatment group were just as negatively affected as those in the low treatment group, while their hatching success and BMR were similar to the control birds. These results are inconsistent with what would be expected of an intermediate temperature treatment. Furthermore, the small potential increase of 0.3°C (vs. 1.5°C between the low and control treatments) suggests that the marked differences found between the cyclical and control treatments (including length of incubation) were a result of cycling alone, as originally intended.

Summary and Conclusions

In summary, our hypothesis that there would be short- and long-term effects of incubation temperature and cycling on offspring phenotype was supported. As predicted, low incubation temperature increased the time it took for individuals to hatch and decreased hatching success. It did not, however, have the predicted effect on hatchlings, with birds in the low and control treatment groups hatching at similar mass and size. At adulthood, birds in the low treatment group were lighter and smaller than birds in the control treatment group and had elevated mass-adjusted BMR, indicating long-term impacts of incubation temperature. Consistent with our prediction, the cyclical treatment ameliorated some of the negative effects of low incubation temperature. This was seen in improved hatching success for the cyclical treatment (same as control), duration of incubation (intermediate between low and control), and adult BMR, where birds in the cyclical treatment group had a lower BMR than birds in the low treatment group. Measures of body mass and size in the cyclical treatment group showed no improvement relative to birds in the low treatment group. Contrary to our prediction, males and females were equally affected by temperature treatment. Future studies using a similar technique of isolating cyclical incubation from a change in average temperature would provide valuable information regarding the potential benefits of cyclical incubation.

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APPENDIX

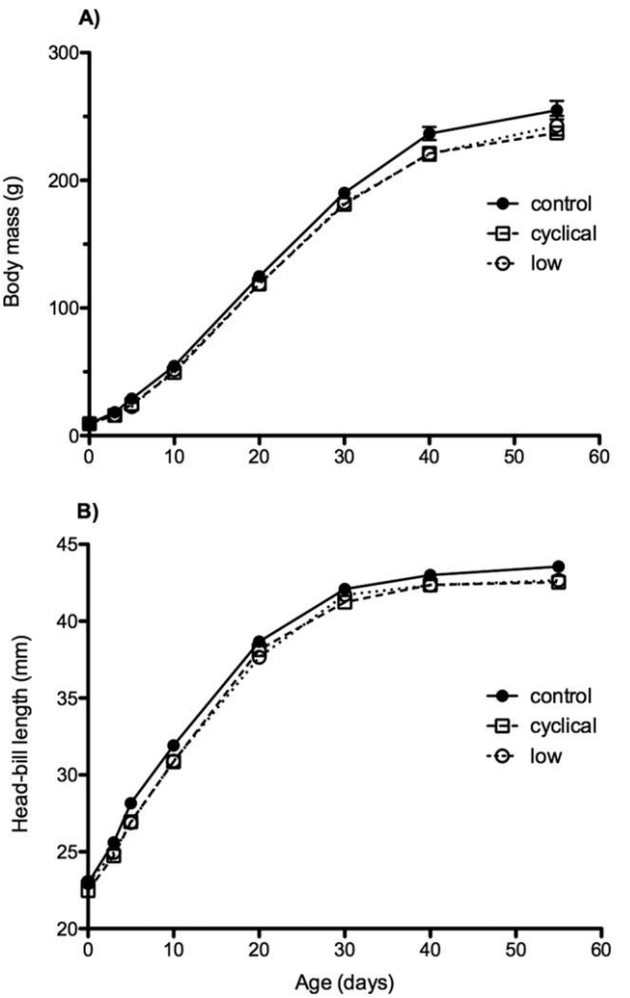

Figure A1. Japanese quail growth curves for body mass (*A*) and head-bill length (*B*) for control, cyclical, and low treatment groups, with sexes pooled. Plotted values are means ± 1 SEM; error bars are included, but some are hidden behind symbols.

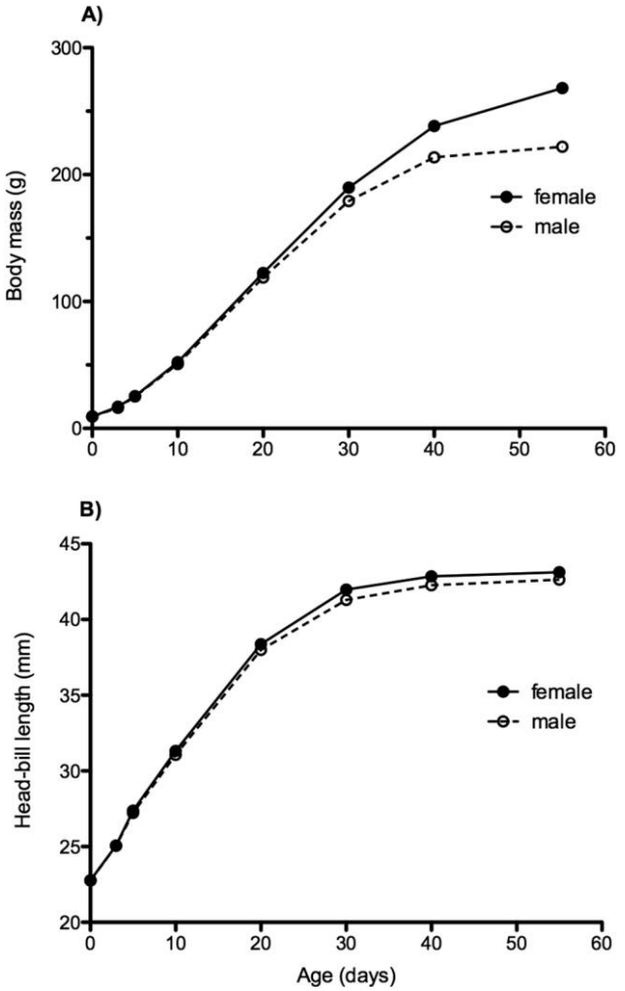

Figure A2. Japanese quail growth curves for body mass (*A*) and head-bill length (*B*) for males and females, with experimental treatments pooled. Plotted values are means ± 1 SEM; error bars are included but are hidden behind symbols.

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