Incubation Temperature Affects Growth and Energy Metabolism in Blue Tit Nestlings

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abstract: Because the maintenance of proper developmental temperatures during avian incubation is costly to parents, embryos of many species experience pronounced variation in incubation temperature. However, the effects of such temperature variation on nestling development remain relatively unexplored. To investigate this, we artificially incubated wild blue tit (*Cyanistes caeruleus* L.) clutches at 35.0°, 36.5°, or 38.0°C for two-thirds of the incubation period. We returned clutches to their original nests before hatching and subsequently recorded nestling growth and resting metabolic rate. The length of the incubation period decreased with temperature, whereas hatching success increased. Nestlings from the lowest incubation temperature group had shorter tarsus lengths at 2 weeks of age, but body mass and wing length were not affected by temperature. In addition, nestlings from the lowest temperature group had a significantly higher resting metabolic rate compared with mid- and high-temperature nestlings, which may partly explain observed size differences between the groups. These findings suggest that nest microclimate can influence nestling phenotype, but whether observed differences carry over to later life-history stages remains unknown.

Keywords: RMR, egg temperature, embryonic development, epigenetic temperature adaptation, growth trajectories.

Introduction

Avian incubation normally involves a substantial increase in parental effort (Williams 1996; Tinbergen and Williams 2002), which varies depending on the physical attributes of the incubation environment (Williams 1996; Thomson et al. 1998). Parents use more energy when incubating enlarged clutches or at low ambient temperatures (Biebach 1979, 1981, 1984; Vleck 1981; Haftorn and Reinertsen 1985; Weathers 1985; de Heij et al. 2007), and they invest less energy in incubation when costs are relieved (Bryan and Bryant 1999; Cresswell et al. 2004; Pérez et al. 2008; Ardia et al. 2009; D'Alba et al. 2009). Consequently, ambient conditions are predicted to interact with intrinsic properties of the nest and/or clutch in determining the amount of energy required for incubation (e.g., Moreno and Sanz 1994; but see Engstrand et al. 2002; de Heij et al. 2008 for evidence of no such effects).

In its simplest form, incubation is little but a transfer of heat from parents to eggs (Deeming 2008). However, since the maintenance of incubation temperature can be energetically costly (Niizuma et al. 2005; Ardia and Clotfelter 2007; Ardia et al. 2009), parents may need to trade off investment in egg heating for self-maintenance. This is sometimes reflected as a reduction in incubation temperature when environmental conditions deteriorate (Haftorn 1983; Nord et al. 2010). Such variation in incubation investment may be directly harmful for developing young, because high and stable incubation temperatures are prerequisites for normal embryonic growth and maturation (Webb 1987; Nilsson 2006). Yet, surprisingly little is known about how avian development is affected by variation in embryonic environment. The data at hand originate largely from studies on poultry, where low incubation temperatures have long been known to result in abnormal embryonic growth and increased in ovo mortality (Lundy 1969). Likewise, suboptimal incubation temperatures reduce neonatal body mass and produce chicks with lower weight gain potential in early life (Joseph et al. 2006) and may also alter the relative timing of onset of physiological regulatory systems (Black and Burggren 2004*a*, 2004*b*). Considerably less is known about temperature effects on embryonic development in nonpoultry species. Periodic cooling of zebra finch (*Taeniopygia guttata*) eggs reduced the efficiency of embryonic tissue synthesis (Olson et al. 2006) and also resulted in a decreased embryonic body condition before hatching (Olson et al. 2008). These findings are largely corroborated by work on wild species. In wood ducks (*Aix sponsa*), eggs incubated in low temperatures took longer to hatch and produced hatchlings with reduced protein mass (Hepp et al. 2006). Similarly, experimentally reduced developmental temperatures in the Australian brush turkey (*Alectura lathami*) prolonged the incubation period but also increased the amount of energy needed for development (Booth 1987), which resulted in

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lower residual yolk reserves at hatch (Eiby and Booth 2008). However, since no studies on wild species have extended beyond the actual hatching event, it remains unclear whether observed phenotypic consequences of suboptimal developmental conditions persist into the posthatching period and even into adult life, thereby potentially impinging on individual fitness.

We experimentally manipulated incubation temperature within the natural range of variation in a free-ranging blue tit (*Cyanistes caeruleus* L.) population in southern Sweden. By applying a novel experimental design where field-collected clutches were artificially incubated in the lab and then returned to their respective nests of origin shortly before hatching, we were able to unambiguously assess whether variation in posthatching nestling phenotypes are best explained by embryonic developmental conditions or by parental behaviors after hatching. Our overall aim was to quantify the pre- and posthatching developmental consequences of variation in egg temperature with respect to both longitudinal growth and physiological maturation. In addition, developmental temperature can affect embryonic energy use (Booth 1987; Eiby and Booth 2008) and metabolic rate (Booth 1987; Olson et al. 2006). We were thus interested to see whether this effect, if present, persisted during the nestling stage, which could indicate that variation in incubation intensity may permanently modify the metabolic phenotype. This would offer important insights into the causes of interindividual variation in metabolic rate and its subsequent fitness consequences. We hypothesized that the length of the incubation period and embryonic mortality would vary inversely with egg temperature and that nestlings hatching from eggs incubated at lower temperatures would be smaller and grow less efficiently than nestlings from higher temperatures. To the best of our knowledge, this is the first attempt to relate a quantitative measure of the embryonic environmental conditions to subsequent posthatching growth and development in birds.

Material and Methods

The experiment was conducted in a nest box breeding population of blue tits from April to June, 2008 to 2010, in the Revinge area, ~20 km east of the city of Lund in south-central Sweden (55°42′N, 13°28′E). The study area, which consists of small deciduous woodlots and groves interspersed among pastures and arable fields, contains ~500 nest boxes, scattered over 64 km^2, that have been monitored yearly since 1982.

Egg Collection and Incubation

We visited nests at least once weekly during nest building and egg laying to determine clutch initiation date; from the tenth egg onward (assuming 1 egg was laid per day), we visited every other day to determine clutch size. Incubation was arbitrarily defined to start at the day of clutch completion (incubation day 0). Two days later (i.e., on incubation day 2), we substituted the entire clutch with warm (~35°C) clay dummy eggs that were similar in size and color to the original eggs. Incubating females were temporarily removed from the nest and were held in the hand while egg substitution proceeded. The whole operation took <1 min to perform, after which females were put back on the eggs. Clutches were uniquely marked with a permanent, nontoxic, felt-tipped pen and transported to a nearby field station (transportation time, ≤40 min) for incubation in artificial incubators (Ruvmax, Ödskölt, Sweden). We randomly assigned clutches to one of three incubation temperatures: (1) 35.0°C ("low temperature"; $n_{2008} = 19$, $n_{2009} = 19$, $n_{2010} = 20$), (2) 36.5°C ("mid temperature"; $n_{2008} = 21$, $n_{2009} = 19$, $n_{2010} = 21$), and (3) 38.0°C ("high temperature"; $n_{2008} = 20$, $n_{2009} = 19$, $n_{2010} = 21$). These temperatures are within the natural range of variation of closely related species of similar size for which incubation temperature has been measured in the wild (Haftorn 1988). Clutch size did not differ between treatments (35.0°C: 11.3 ± 0.19 eggs; 36.5°C: 11.1 ± 0.17 eggs; 38.0°C: 11.0 ± 0.16; $P = .4$). One incubator was used for each treatment, and we used the same three incubators during the course of the study. We changed the incubator/treatment combination between years so that treatment was not replicated within an incubator. The incubators were kept indoors in a completely dark room with stable temperature. We monitored relative humidity (RH) inside the incubators using a standard hygrometer (Clas Ohlson, Insjön, Sweden) and maintained RH at a constant level of 70% throughout in all treatments.

We installed incubators approximately 1 week before collection of the first clutch for temperature calibration. We measured temperature at the position of the eggs in 24-h cycles, using a small temperature data logger (iButton DS1922-L, Maxim Integrated Products, Sunnyvale, CA; accuracy, ±0.5°C) with a sampling interval of 1 min and a resolution of 0.0625°C. We then calculated the temperature difference between the temperature logger and the desired treatment temperature (to the closest 0.1°C) and adjusted the incubator settings accordingly until no temperature deviance was recorded. The data loggers were also left in place when egg collection had begun, and temperatures were evaluated once daily and adjusted when necessary. However, temperature remained relatively constant after the initial calibration (mean temperature deviance ±

SE, for 35°C: 0.020° ± 0.017°C; for 36.5°C: 0.030° ± 0.017°C; for 38.0°C: 0.030° ± 0.018°C), thus necessitating only minor adjustments.

On day 10 of incubation, clutches were transferred back to their original nests. In cases where nests had been deserted or predated during the incubation period (in 2008, 5 nests; in 2009, 14 nests; in 2010, 12 nests), eggs were fostered to a replacement nest at the same stage (±1 day) and with the same clutch size (±2 eggs) as the original nest.

Sampling of Nestlings and Adults

Starting on day 11, we checked nests daily for hatched eggs. On nestling day 2 (day of hatching = 0), we measured brood mass (to the closest 0.1 g) using a Pesola spring scale (Pesola, Baar Switzerland). We returned to record nestling body mass and tarsus length (to the closest 0.1 mm) on day 6. We also banded nestlings with a uniquely numbered aluminum ring and collected any unhatched eggs. On day 14 we measured nestling mass (g) and tarsus and wing length (to the closest 0.1 and 0.5 mm, respectively). Throughout the study, all measurements on nestlings were made by the same person.

To subsequently monitor nest provisioning, in 2009 and 2010 we caught, measured (mass, tarsus and wing lengths), and equipped parents with a unique passive integrated transponder (PIT) tag glued to two plastic rings on day 6. All provisioning parents were caught in 2009, but two nests were left undisturbed in 2010 because of inclement weather. In all but four cases (two in each year), parents resumed normal provisioning behaviors immediately after capture. On day 8 we attached a circular antenna connected to a data logger (Trovan, AEG ID, Ulm, Germany) powered by a 12-V 72-Ah marine battery (Biltema, Helsingborg, Sweden) around the nest box entrance hole. The logger automatically stored the unique PIT tag number together with the time of entry each time a parent entered the nest until day 10, when the measuring period was finished and all equipment was removed.

Metabolic Measurements

We measured nestling resting metabolic rate (RMR) by means of flow-through respirometry during the night between days 14 and 15. Between one and four nestlings (depending on the number of synchronous nests [for 38.0°C, 62 nestlings from 34 broods; for 36.5°C, 67 nestlings from 37 broods; for 35.0°C, 69 nestlings from 37 broods]) were randomly collected from their nest box after 2000 hours. Parental nest provisioning is negligible after this time (A. Nord, personal observation). Nestlings were weighed and placed singly into a 0.6-L sealed metabolic chamber and placed in a dark, temperature-controlled cabinet (Heraeus Vötsch BK600, Vötsch Industrietechnik, Balingen, Germany) at 25°C, that is, within their thermoneutral zone (Gavrilov and Dolnik 1985). A measurement session ended between 0600 and 0700 hours the ensuing morning, at which point nestlings were weighed and transported back to their original nests. We used the nestlings' morning mass values in all analyses, as these were gathered closest to the time when actual RMR was recorded (A. Nord, personal observation).

The respirometer consisted of one block with eight parallel channels with identical setups, one of which was left empty for baselining. We were thus able to measure seven birds per night. Each chamber was connected to a PP2 bench pump (type UNMP830 KVDC-B; Sable Systems International, Las Vegas, NV) that was positioned downstream of the birds and consequently pulled air from the chambers. Channels were measured sequentially for 13 min each and were separated temporally by 2 min of flushing, during which time no data were collected. A baseline was recorded at the start and the end of each measurement cycle. Switching was maintained by a RM8 Intelligent Multiplexer (Sable Systems).

Oxygen and carbon dioxide concentrations of effluent sample air scrubbed on Drierite (W.A. Hammond Drierite Company, Xenia, OH) were analyzed sequentially by a CA-10A carbon dioxide analyzer (Sable Systems) and a FC-10A oxygen analyzer (Sable Systems) and automatically registered on a computer connected to the machinery via a UI2 Data Acquisition Interface (Sable Systems) every second during a measurement cycle. Oxygen concentration was calibrated against outside air to 20.95% O_2 before each measurement series. Flow rate was set at 166 mL min^{-1} (10 L h^{-1}) and was recorded continuously by a FlowBar8 Multichannel Mass Flow Meter (Sable Systems). It should be noted that the system did not have a loop that regulated flow, and this caused a slight drift in flow rate with as night progressed ($\leq$10 mL $min^{\leq 1}$ during a full measurement series). However, this variation did not affect the estimated metabolic rates, as the close monitoring of flow rate allowed calculations to be made on the actual flow at the time of sampling. Calculations of oxygen consumption were performed using ExpeData 1.1.9 for Windows. We checked all channels manually for drift before analyses were performed, and this confirmed that the oxygen consumption was stable for the full length of the cycle in all cases. Oxygen consumption (mL O_2 min^{-1}) was defined as the difference in oxygen concentration between effluent sample air and reference air from the empty metabolic chamber according to equation C in an article by Hill (1972). Because of the possible malfunction of the carbon dioxide analyzer, we conservatively considered the fraction of CO_2 in reference air to be 0.0005 during all calculations,

which is a reasonable assumption for indoor conditions (Lighton 2008). The value of oxygen consumption used in analyses was taken as the single lowest value from 7-min running averages for a full measurement session. Oxygen consumption was converted to metabolic rates assuming an energy equivalence of 20 J $(\text{mL } O_2)^{-1}$.

Statistical Analyses

All statistical tests were made using R, version 2.12.0 for Windows. Broods that were predated, deserted, or provisioned by one parent only (as determined by PIT tag records and repeated observations at the nest) were excluded from the data set. Because no adults bred in experimental nests in more than 1 year, and because only 13 nest boxes were included more than once during the study, we did not account for potential variance inflation due to nest box identity in the analyses. The length of the incubation period (from onset to the first signs of hatching) and hatching success (the proportion of the clutch that had hatched by nestling day 6) were analyzed in identical linear models, with treatment and year as factors and clutch size as a covariate. Data for hatching success were arcsine–square root transformed before analyses to meet assumptions of normality (Sokal and Rohlf 1995). We included data from 60 (20 in each year) unmanipulated nests (hereafter referred to as control nests) as a reference point in models for length of the incubation period and hatching success. Thus, the treatment factor had four levels in these models, compared with three in all other analyses. Mean nestling mass ($m_{\text{brood}}/n_{\text{brood}}$) on day 2 was analyzed with a general linear model with experimental treatment and year as factors and laying date (i.e., the day on which the first egg was laid) and (laying date)^2 as covariates. We analyzed variation in nestling biometrics (mass and tarsus and wing lengths) on days 6 and 14, respectively, using linear mixed-effects models fitted with restricted maximum-likelihood methods (using the lme function in the nlme package), with treatment and year as fixed factors, laying date and (laying date)^2 as covariates, and nesting attempt (defined as the specific nest by year combination) as a random factor. Nestling RMR was analyzed in a linear mixed model with the main effects of treatment and year as fixed effects and mass, laying date, and (laying date)^2 as covariates. Nesting attempt and respirometer channel were included as random effects. We analyzed variation in nest provisioning rates in terms of feedings per nestling and unit time for each parent, using a linear mixed model with treatment, year, and sex as fixed factors, laying date and (laying date)^2 as covariates, and nesting attempt as a random factor. The full model also included the two-way interactions between the experimental treatment and sex. Models were reduced by backward elimination of nonsignificant terms ($P > .05$; Seber and Lee 2003) until only significant variables remained. Differences between groups were compared following the Tukey method, with P values adjusted for unbalanced multiple comparisons, using the glht function in the multcomp package. Significances of random factors were assessed by comparing the restricted log-likelihood ratio of the reduced and saturated models to a χ^2 distribution with one degree of freedom (Sokal and Rohlf 1995). All means are reported with their standard errors, and all significances except for the restricted log-likelihood ratio tests are two tailed. For simplicity, only final models are presented in "Results."

Results

Incubation Period and Hatching Success

Experimental manipulation of incubation temperature affected the length of the incubation period in the predicted direction (table 1; fig. 1). Low-temperature clutches required 1.7 and 1.2 more days to hatch than did high- and mid-temperature clutches, respectively ($P < .001$ in both cases), and high-temperature clutches hatched about 0.6 days earlier than mid-temperature clutches ($P = .023$). In addition, the high- and mid-temperature clutches hatched as fast as the unmanipulated nests in the field (i.e., control nests), but the low-temperature nests lagged behind the controls by 1.3 days ($P < .001$). The incubation period also varied between study years, with nests hatching later in 2010 compared with both previous years (table 1).

Patterns in hatching success followed those for incubation period. Hatching success was reduced by 30.7% and 29.2% in the low-temperature group compared with clutches exposed to the high and mid incubation temperatures ($P < .001$ in both cases; table 1; fig. 2), but there was no difference in hatching success between groups exposed to the two highest temperatures. Clutches in control nests hatched significantly better than did those in all other treatments (38.0°C: 8.2%, $P = .014$; 36.5°C: 9.5%, $P = .0043$; 35.0°C: 41.3%, $P < .001$). However, the difference in absolute values between the controls and the mid- and high-temperature groups, respectively, was relatively small (fig. 2).

Nestling Morphology

The experimental treatment did not have a significant effect on nestling mass at any of the sampling occasions (days 2, 6, and 14; table 1). However, nestlings were significantly heavier at all ages in the first 2 years of the study (table 1), and mass at days 6 and 14 also increased slightly with laying date (table 1).

Tarsus length did not differ between temperature treat-

ments at day 6; however, the experimental treatment significantly affected tarsus length at day 14 in the predicted direction (table 1, fig. 3). Specifically, mean tarsus length in chicks from the low-temperature group was reduced by 0.55 and 0.36 mm compared with that of high-temperature ($P < .001$) and mid-temperature chicks ($P = .0020$), respectively. Tarsus length did not differ between the latter treatments. In contrast, wing length at the same age was not affected by the experimental treatment but followed similar patterns as nestling mass. Thus, when controlling for the effect of laying date, wings were shorter in 2010 than in both of the previous years (table 1).

Resting Metabolic Rate

Variation in incubation temperature significantly affected nestling resting metabolic rate at 2 weeks of age (i.e., 16–18 days after manipulation; table 1; fig. 4). When controlling for mass, chicks from the lowest temperature group experienced an increase in resting metabolic rate of 8.1% ($P = .0069$) and 7.5% ($P = .016$) over that of high- and mid-temperature chicks, respectively. However, as above, we could not detect any differences between the two higher temperatures.

Nest Provisioning Rate

All nests, regardless of their respective temperature treatments, were provisioned at the same rate in both years. However, nest provisioning across treatments was higher in 2010 than in 2009, and it also increased slightly with (laying date)^2 (table 1).

Discussion

By manipulating incubation temperature within the natural range of variation, we have shown that early developmental conditions affected both pre- and postnatal growth and physiology in blue tits. This was manifested as a prolonged incubation period and lower hatching success at low incubation temperatures and reduced nestling growth and increased resting metabolic rate in broods from low incubation temperatures (i.e., 35.0°C), compared with nestlings from both the mid (i.e., 36.5°C) and the high (i.e., 38.0°C) incubation temperature groups. These observations corroborate recent experimental findings that support a potentially causal role of incubation temperature in determining nestling condition (Reid et al. 2002; Ardia and Clotfelter 2007; Nilsson et al. 2008; Pérez et al. 2008; Ardia et al. 2010). Some results also suggest that parents on the nest, via their direct influence on incubation temperature (Ardia and Clotfelter 2007; Ardia et al. 2009; Nord et al. 2010), can influence neonatal performance by altering embryonic investment. This is paralleled in some reptiles, which by nest site selection can alter the thermal environment of embryos, with carryover effects on the hatchling phenotype (e.g., Blouin-Demers et al. 2004). At this point it should be noted that our experiment differs from natural incubation, because constant incubation temperatures generally do not occur in nature (Deeming 2002), and it is likely that temperature variation as such may have developmental consequences. Nonetheless, our results are qualitatively similar to those of previous field studies (see above), and we feel confident that this work adequately reflects some of the possible developmental consequences of a suboptimal embryonic environment.

Effects of Egg Temperature on Incubation Period and Hatching Success

We found that incubation period was shorter for clutches incubated at higher temperatures. Evidence for a direct effect of incubation temperature on incubation period from natural populations is scarce and largely restricted to correlative studies. For example, wood duck eggs naturally incubated at higher temperatures hatched faster (Hepp et al. 2006), and biparentally incubated starling (*Sturnus vulgaris*) clutches spent more time at higher temperatures and had shorter incubation periods than did clutches in which the female incubated alone (Reid et al. 2002). Similarly, Martin (2002) showed that incubation periods vary predictably with embryonic temperatures across species (but see Tieleman et al. 2004 for evidence of no such effects). Lengthy incubation periods can be ecologically costly in terms of an increased predation risk with age of the nest (Tombre and Erikstad 1996; Remes and Martin 2002). Such risks might be further exacerbated by physiological costs, because the amount of energy required for embryonic maintenance processes increases rapidly with time in the egg (Booth 1987; Booth and Jones 2002). As a result, the residual yolk mass at hatching is often reduced in chicks that have experienced suboptimal embryonic conditions (Olson et al. 2006; Eiby and Booth 2009). The maintenance of proper incubation temperatures is thus presumably adaptive, since this generally appears to decrease the incubation period.

Apart from requiring a longer time to hatch, eggs incubated at low temperatures showed a higher incidence of embryonic mortality. It is possible that the sustained hypometabolism at low incubation temperatures (Vleck and Vleck 1996) reduced the efficiency of nutrient uptake (cf. Feast et al. 1998; Olson et al. 2006), thereby resulting in a chronic nutritional stress that in the end may have been incompatible with embryonic survival. However,

Table 1: Test statistics, degrees of freedom, and the corresponding *P* values derived from marginal ANOVA tables for final models, and parameter estimates for significant terms ($P < .05$)

Parameter	Estimate (SE)	df	F or Λ^a	P
Incubation period:				
Treatment:				
Control [AB]	12.95 (.16)	3, 207	25.28	<.001
38.0°C [A]	12.52 (.12)			
36.5°C [B]	13.13 (.15)			
35.0°C [C]	14.30 (.16)			
Year:				
2008 [A]	12.91 (.13)	2, 207	8.89	<.001
2009 [A]	13.07 (.16)			
2010 [B]	13.58 (.15)			
Hatching success:				
Treatment:				
Control [A]	1.30 (.035)	3, 210	30.74	<.001
38.0°C [B]	1.13 (.045)			
36.5°C [B]	1.11 (.037)			
35.0°C [C]	.75 (.045)			
Mean mass, day 2:				
Year:				
2008 [A]	1.98 (.048)	2, 147	7.16	.0011
2009 [A]	1.98 (.036)			
2010 [B]	1.78 (.042)			
Mass, day 6:				
Year:				
2008 [A]	6.00 (.095)	2, 136	9.49	<.001
2009 [A]	5.87 (.10)			
2010 [B]	5.40 (.10)			
Laying date	.053 (.014)	1, 136	12.98	<.001
Nesting attempt (random)		1	120.63	<.001
Mass, day 14:				
Year:				
2008 [A]	11.60 (.099)	2, 111	10.92	<.001
2009 [A]	11.84 (.11)			
2010 [B]	11.13 (.11)			
Laying date	.065 (.0196)	1, 110	16.13	<.001
Nesting attempt (random)		1	281.50	<.001
Tarsus length, day 6:				
Laying date	.056 (.016)	1, 138	11.38	<.001
Nesting attempt (random)		1	174.79	<.001
Tarsus length, day 14:				
Treatment:				
38.0°C [A]	18.58 (.071)	2, 110	11.15	<.001
36.5°C [A]	18.41 (.070)			
35.0°C [B]	18.10 (.074)			
Laying date	.032 (.010)	1, 110	9.62	.0076
Nesting attempt (random)		1	193.01	<.001
Wing length, day 14:				
Year:				
2008 [A]	42.83 (.29)	2, 110	1,278	<.001
2009 [A]	42.34 (.33)			
2010 [B]	40.42 (.32)			
Laying date	.17 (.048)	1, 110	16.63	<.001
Nesting attempt (random)		1	121.88	<.001

Table 1 (*Continued*)

Parameter	Estimate (SE)	df	F or Λ[a]	P
Resting metabolic rate:				
Treatment:				
38.0°C [A]	32.023 (1.38)	2, 194	5.65	.0041
36.5°C [A]	32.30 (1.37)			
35.0°C [B]	34.62 (1.37)			
Mass	2.00 (.36)	1, 194	30.59	<.001
Nesting attempt (random)		1	119.16	<.001
Respirometer channel (random)		1	45.40	<.001
Nestling provisioning:				
Year:				
2009 [A]	1.62 (.070)	1, 71	7.95	.0062
2010 [B]	1.91 (.075)			
(Laying date)^2	−.00080 (.00029)	1, 71	7.97	.0080
Nesting attempt (random)		1	1.28	.26

Note: For factors, estimates represent the fitted values (and their SEs) corrected for variation in the random factor (when applicable), with covariates fixed at their respective mean values. For continuous variables, estimates are the slope of the regression between the dependent variable and the covariate, with SE for the fit of the regression. Nonsignificant terms were removed from the original models by backward elimination, as described in the main text. Shared letters within brackets indicate nonsignificant ($P > .05$) differences between treatment categories and years, respectively.

[a] For fixed effects, the test statistic is F; for random effects it is Λ.

since exposure to low incubation temperatures can obstruct the development of muscle tissue (including that of the hatching muscle; Olson et al. 2008), it is perhaps also possible that embryos exposed to the lowest incubation temperature were physically incapable of hatching.

Our results indicate that the effect of incubation temperature on embryonic mortality was not necessarily linear, because hatching success did not differ between high- and mid-temperature clutches (see also Eiby and Booth 2009). It thus seems plausible that embryonic development was relatively robust to temperature deviation within a given interval delimited at the lower end by a threshold temperature of between 36.5° and 35.0°C. Sustained exposure to temperatures below this threshold seems to adversely affect embryonic development. High incubation temperatures may be equally detrimental for embryonic survival (Strausberger 1998; Moraes et al. 2004), but because we did not sample above 38.0°C (where embryonic survival was not negatively affected) we do not have enough information to speculate about the upper limit of temperature tolerance of blue tit embryos.

Effects of Egg Temperature on Nestling Morphology

Contrary to the results of previous studies (Hepp et al. 2006; Mortola 2006), we found no effect of incubation temperature on nestling body mass shortly after hatching (i.e., when nestlings were 2 days old). Because hatching seems to occur at a relative rather than an absolute age (Black and Burggren 2004*a*), this could potentially be explained if the effect of developmental temperature on embryonic growth delayed physical maturation (thereby extending the incubation period) without affecting neonate size or mass. However, periodic cooling of zebra finch eggs has previously been shown to reduce yolk assimilation efficiency (Olson et al. 2006), which suggests that this might not be the case. Because we did not assess nestling body composition, it is therefore possible that differences in residual yolk mass (e.g., Eiby and Booth 2009) or protein content (e.g., Hepp et al. 2006) at hatching could explain the absence of temperature effects on nestling mass. It should also be mentioned that, because of the rather imprecise measurements of nestling mass at day 2 (see "Material and Methods"), we might have been unable to detect phenotypic differences between treatments.

Nestlings from the different incubation temperatures did not differ in any of the biometric measurements at 6 days of age (although treatment means varied in the predicted direction). However, nestlings from the lowest incubation temperature were structurally smaller than mid- and high-temperature nestlings at 2 weeks of age. Low temperature has been shown to reduce limb growth by constraining the efficiency of cartilage proliferation in laboratory-reared mice (Serrat et al. 2008). Similarly, Hammond et al. (2007) showed that appendage growth in ovo was positively affected by high incubation temperatures in domestic fowl, and they attributed this to the higher levels of embryonic activity at high temperatures. However, it is unlikely that the difference in structural size that we observed in this study can be explained solely by tempera-

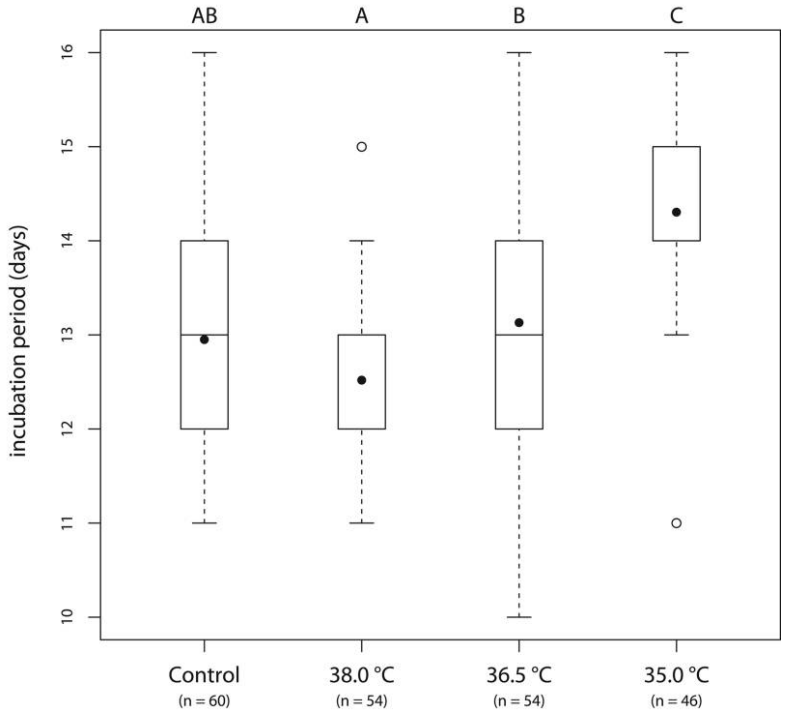

Figure 1: Length of the incubation period by experimental treatment for blue tit clutches exposed to different temperatures during artificial incubation in laboratory conditions. Clutches were returned to their original nests shortly before hatching. "Control" refers to randomly selected unmanipulated nests in the wild. Boxes show medians and first and third quartiles. Whiskers extend to the last observations within 1.5 times the interquartile range (IQR). Open circles denote observations outside 1.5 times the IQR; filled circles within boxes show means. *N* values correspond to the number of broods. Shared letters above the boxes indicate nonsignificant differences ($P > .05$) between treatment categories.

ture-related constraints on prenatal longitudinal growth, because differences between treatments were not present when nestlings were 6 days old. This suggests that the main differences between treatments became apparent during the period of peak nestling growth rather than during embryonic and early postnatal development. Therefore, it seems likely that incubation temperature constrained nestling growth trajectories indirectly by affecting intrinsic physiological properties such as energy turnover rates. In line with this, we found that incubating blue tit eggs in suboptimal temperatures produced nestlings with higher resting metabolic rates. As a result, low-temperature nestlings most likely had to trade off maintenance for growth to a larger extent than did nestlings from mid and high temperatures, thereby explaining the observed reduction in structural size.

Despite the fact that low-temperature nestlings had shorter tarsi, wing length and mass did not differ between treatments. This observation suggests that low-temperature nestlings traded off energy allocation to different growth compartments by investing more resources in body mass and feather growth. Predation risk is a major driving force in avian life-history evolution (Martin 1995) and, although it is sometimes dependent on nest site characteristics (Martin et al. 2000), we would expect predation risk to increase with nest age (Remes and Martin 2002). It is therefore possible that the reduced tarsus length in low-temperature nestlings can be explained if these nestlings traded off structural size for plumage development (reflected as a higher wing-length to tarsus-length ratio), thereby reducing predation risk by mediating earlier fledging (cf. Nilsson and Svensson 1996).

Effects of Egg Temperature on Resting Metabolic Rate

This experiment affected nestling resting metabolic rate in a nonlinear fashion, as nestlings from the low-temperature group had elevated resting metabolic rates compared with both mid- and high-temperature nestlings. Our experimental design does not allow us to separate the following two alternative hypotheses: (1) the effect of developmental temperature on metabolic rate is a physiological response to suboptimal embryonic conditions, without any positive

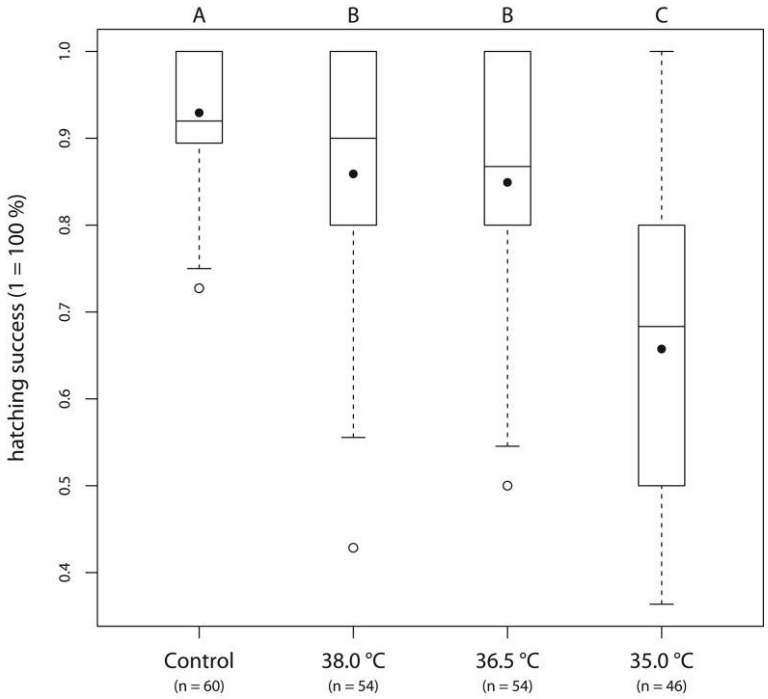

Figure 2: Hatching success (hatched divided by unhatched eggs) by experimental treatment for blue tit clutches that were incubated in laboratory conditions and subsequently returned to their original nests shortly before hatching. "Control" refers to randomly selected unmanipulated nests in the wild. Calculations were performed on arcsine-square-root-transformed proportions, but untransformed values are illustrated for simplicity. Boxes show medians and first and third quartiles. Whiskers extend to the last observations within 1.5 times the interquartile range (IQR). Open circles denote observations outside 1.5 times the IQR. Filled circles within boxes show means. *N* values correspond to the number of broods. Shared letters above the boxes indicate nonsignificant differences ($P > .05$) between treatment categories.

fitness consequences, and (2) the observed pattern is of adaptive significance.

In precocial species, a reduction in temperature toward the end of incubation (when the thermoregulatory system begins to form) increases metabolic rate and improves cold tolerance in embryos and neonates, whereas an increase in temperature at this time produces the opposite effects (Nichelmann and Tzschentke 1999, 2002; Tzschentke 2007, 2008). It has been speculated that this epigenetic perinatal temperature adaptation (terminology sensu Tzschentke 2007) might serve as a way for parents to preadapt hatchlings to prevailing ambient conditions, and the effect of cold exposure during incubation on thermoregulatory capacity may persist throughout adult life (Shinder et al. 2009). There are indications that low incubation temperatures elevate embryonic metabolic rates in other bird species (in mallee fowl *Leipoa ocellata*, Booth 1987; in zebra finch, Olson et al. 2006), and a similar thermal acclimation of metabolic rate was recently described for a diverse array of reptiles (Du et al. 2010). However, whether this is a consequence of a suboptimal embryonic thermal environment or whether it functions as a preadaptation to low ambient temperatures is not known, because in neither of the above cases were eggs allowed to hatch. Nonetheless, the existence of a similar mechanism in blue tits would provide a functional explanation for the higher resting metabolic rate observed in nestlings from the low incubation temperature. The energetic cost of keeping eggs warm varies with ambient temperature (Ardia et al. 2009; Nord et al. 2010) and results in a corresponding variation in incubation temperatures (Haftorn 1983). Thus, prenatal temperature adaptation could potentially also account for some of the variation in metabolic rate between conspecific populations native to different latitudes (e.g., Broggi et al. 2004).

Alternatively, differences in resting metabolic rate between treatments might have occurred if low-temperature nestlings upregulated their metabolism to improve the rate of tissue synthesis, thus compensating for a potentially bad start. However, growth rates (gain per day) in the traits we measured were independent of incubation temperature. Still, suboptimal developmental conditions may retard the maturation of physiological regulatory systems or visceral organs independently of the longitudinal growth axis

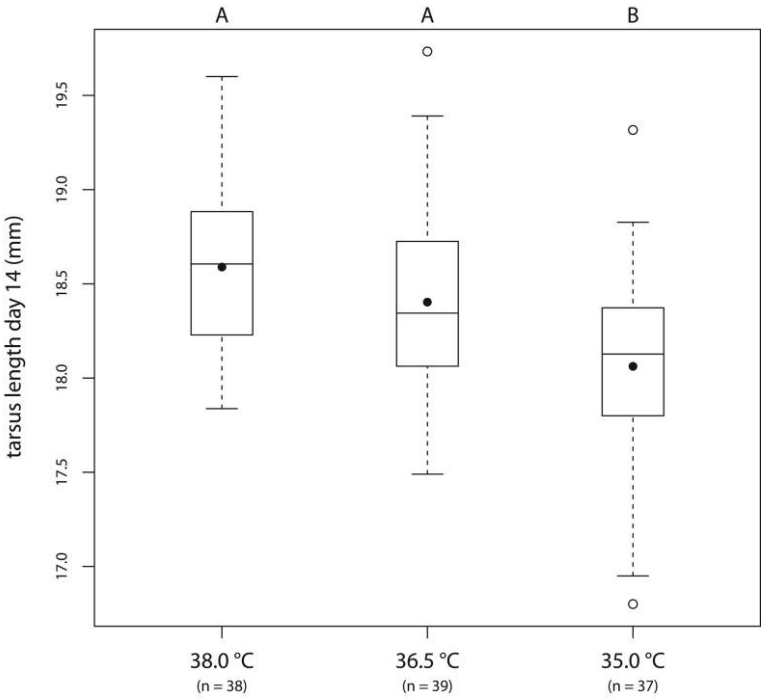

Figure 3: Tarsus length at 2 weeks of age by experimental treatment for blue tit nestlings originating from clutches incubated in different temperatures. Illustrations are based on mean brood values. Boxes show medians and first and third quartiles. Whiskers extend to the last observations within 1.5 times the interquartile range (IQR). Open circles denote observations outside 1.5 times the IQR; filled circles within boxes show means. *N* values correspond to number of broods. Shared letters above the boxes indicate nonsignificant differences ($P > .05$) between treatment categories.

(Deeming and Ferguson 1989; Black and Burggren 2004*a*; Mortola 2006). Any compensatory growth in such traits would not have been detected by us. Regardless, this strategy would necessitate an increased energy intake to fuel the higher metabolic demands. Because parental feeding effort did not differ between treatments, we thus consider the compensatory growth hypothesis to be unlikely.

It is also possible that a nonrandom subset of eggs survived incubation in the lowest temperature. Because hatching success was markedly lower in the 35.0°C group, only embryos with certain characteristics such as a higher metabolic rate may have been able to withstand these conditions. If there was higher survival of embryos with high metabolic rate in the low-temperature group, we predict that the within-brood metabolic phenotype would be less variable in this treatment. This was not the case. If anything, the repeatability (sensu Lessells and Boag 1987) of resting metabolic rate was lower in low-temperature chicks (35.0°C: 0.034, $P = .053$; 36.5°C: 0.063, $P = .013$; 38.0°C: 0.090, $P < .001$). This suggests that egg survival was random also with the lowest temperature. However, these ideas remain untested because we do not have empirical data on embryonic metabolic rate and embryonic growth.

Conclusions

We have provided evidence of the effect of the embryonic environment on incubation period, hatching success, nestling morphology, and metabolic rate. However, the long-term effects of variation in incubation temperature remain unknown. Unfavorable conditions during early development can decrease reproductive success in adulthood (Gorman and Nager 2004; Naguib and Gil 2005), and nestling size at the time of fledging is often positively related to a variety of fitness-related traits, such as survival and recruitment (McCarty 2001; Naef-Daenzer et al. 2001; Schwagmeyer and Mock 2008) Although nestlings can sometimes compensate for a bad start should conditions improve, such compensations can be energetically costly (Criscuolo et al. 2008) and result in reduced subsequent survival and reproductive output (Lindström 1999; Metcalfe and Monaghan 2001). Additionally, the extent of compensation need

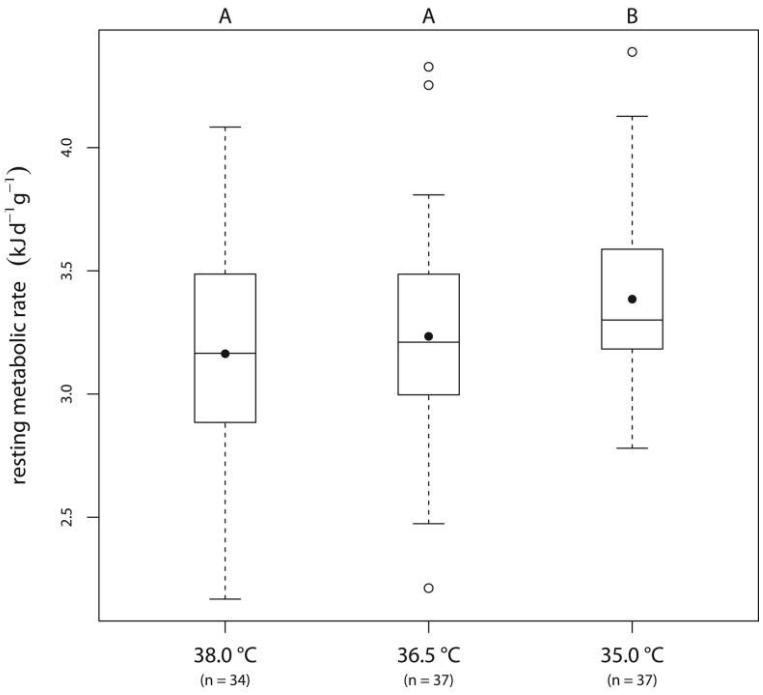

Figure 4: Mass-specific resting metabolic rate, measured at night by flow-through respirometry, of 14-day-old blue tit nestlings originating from clutches exposed to different incubation temperatures. Illustrations are based on mean brood values. Boxes show medians and first and third quartiles. Whiskers extend to the last observations within 1.5 times the interquartile range (IQR). Open circles denote observations outside 1.5 times the IQR; filled circles within boxes show means. *N* values correspond to number of broods. Shared letters above the boxes indicate nonsignificant differences ($P > .05$) between treatment categories.

not always be complete (e.g., Schew and Ricklefs 1998), suggesting that intrinsic constraints may prevent a complete recovery from a suboptimal developmental period. If that is the case, the offspring phenotype may be permanently altered by conditions experienced during early life. This remains speculative, as studies that relate the embryonic environment to neonatal phenotype and then assess the relationship between phenotype and fitness are largely absent. Thus, even though it seems likely that phenotypic consequences of a suboptimal incubation environment may extend far beyond the nestling phase, studies explicitly assessing this relationship are currently highly warranted.

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