Embryonic Developmental Patterns and Energy Expenditure Are Affected by Incubation Temperature in Wood Ducks (*Aix sponsa*)

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ABSTRACT

Recent research in birds has demonstrated that incubation temperature influences a suite of traits important for hatchling development and survival. We explored a possible mechanism for the effects on hatchling quality by determining whether incubation temperature influences embryonic energy expenditure of wood ducks (*Aix sponsa*). Because avian embryos are ectothermic, we hypothesized that eggs incubated at higher temperatures would have greater energy expenditure at any given day of incubation. However, because eggs incubated at lower temperatures take longer to hatch than embryos incubated at higher temperatures, we hypothesized that the former would expend more energy during incubation. We incubated eggs at three temperatures (35.0°, 35.9°, and 37.0°C) that fall within the range of temperatures of naturally incubated wood duck nests. We then measured the respiration of embryos every 3 d during incubation, immediately after ducks externally pipped, and immediately after hatching. As predicted, embryos incubated at the highest temperature had the highest metabolic rates on most days of incubation, and they exhibited faster rates of development. Yet, because of greater energy expended during the hatching process, embryos incubated at the lowest temperature expended 20%–37% more energy during incubation than did embryos incubated at the higher temperatures. Slower developmental rates and greater embryonic energy expenditure of embryos incubated at the lowest temperature could contribute to their poor physiological performance as ducklings compared with ducklings that hatch from eggs incubated at higher temperatures.

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Introduction

Incubation conditions can have profound effects on offspring phenotype in reptiles, yet this phenomenon has rarely been explored in birds. However, recent research has demonstrated that subtle changes in incubation temperature can greatly influence avian hatchling characteristics (Goth and Booth 2005; Hepp et al. 2006; Olson et al. 2008; DuRant et al. 2010, 2011; Hopkins et al. 2011; S. E. DuRant, W. A. Hopkins, A. F. Wilson, and G. R. Hepp, unpublished manuscript). For example, Hepp and colleagues (2006) investigated the effects of incubation temperature on the duration of embryonic development and on hatchling size and body composition in wood ducks (*Aix sponsa*, Linneaus) by artificially incubating eggs at three temperatures (34.6°, 36.0°, and 37.6°C) that approximated the range of temperatures found in naturally incubated nests (range, 34.8°–37.7°C; Manlove and Hepp 2000; Folk and Hepp 2003; Hepp et al. 2006). They found that incubation at lower temperatures significantly extended embryonic development, with eggs incubated at 34.6°C pipping nearly 10 d later than eggs incubated at 37.6°C. Further, proximate analysis revealed that duckling embryos at the pipping stage that came from from eggs incubated at lower temperatures had significantly lower wet mass, dry mass, and lower protein content than ducklings incubated at the higher temperatures (Hepp et al. 2006). Several other studies on wood ducks have revealed that incubation temperature affects duckling phenotype after hatching as well. These studies have found that ducklings from the low incubation temperature group have slower growth, reduced thermoregulatory performance, reduced immune responses, higher corticosterone concentrations, and reduced locomotor performance (DuRant et al. 2010, 2011; Hopkins et al. 2011; S. E. DuRant, W. A. Hopkins, A. F. Wilson, and G. R. Hepp, unpublished manuscript).

Evaluation of embryo bioenergetics could provide an important mechanistic basis for the variation in hatchling phenotype noted in the previously mentioned studies on wood ducks. For instance, in the crocodilian *Crocodylus johnstoni* (Krefft), eggs incubated at lower temperatures have longer incubation periods and thus exhibit greater overall embryonic energy expenditure (Whitehead 1987). The greater energy expenditure during incubation presumably gives rise to reductions in hatchling residual yolk (Webb et al. 1987). However, in other reptilian species, total energy expended during incubation has been shown to be relatively insensitive to incubation temperature (Booth and Thompson 1991; Booth 1998; Oufiero and Angilletta 2010). Although many studies have examined the energetic cost of development in birds (Vleck and Bucher 1998), surprisingly few studies have examined the influence of

incubation temperature on avian embryonic metabolic rate and/or the total energetic cost of avian development (but see Booth 1987; Olson et al. 2006).

In this study, we explored a possible mechanism for the above-referenced effects of incubation temperature on duckling phenotype by quantifying metabolic rates of embryos throughout incubation and estimating the total energetic cost of development in wood duck embryos incubated at three temperatures that fall within the range of naturally incubated nests (35.0°, 35.9°, and 37.0°C). We hypothesized that embryonic metabolic rate at any given point in development would increase with increases in incubation temperature. However, because the incubation period is extended at cooler temperatures, the total energetic cost of developing at cooler temperatures would be higher than the cost experienced at higher incubation temperatures (Angilletta et al. 2000). Thus, we predicted that embryos developing at cooler temperatures would expend more energy to support maintenance costs associated with protracted development.

Material and Methods

Egg Collection and Incubation

We collected wood duck eggs from nest boxes ($n = 201$) located near two large reservoirs and in several isolated wetlands at the U.S. Department of Energy's Savannah River site in west-central South Carolina. We checked nest boxes every 4 d until nests were initiated, and then we visited active nests daily, collected and marked all new eggs, and stored them at 20°C and 55%–60% humidity. After 4 d of collection, we transported eggs to Virginia Tech (Blacksburg, VA) and artificially incubated them in Grumbach incubators (BSS 160) at one of three temperatures (35.0°, 35.9°, and 37.0°C) and consistent 60%–65% humidity. This produced three incubation durations (37.2 ± 0.24, 34.4 ± 0.18, and 32.1 ± 0.18 d, respectively). Incubators were not maintained at constant temperatures but were programmed to allow two cool-down periods each day (~3°C reduction in mean temperature for 75 min at 0815 and 1830 hours) to simulate natural daily feeding recesses taken by mothers during incubation (Manlove and Hepp 2000). Eggs were randomly assigned to an incubation temperature group, ensuring that lay sequence and the number of days eggs were held were evenly distributed across treatments. All experimental procedures were conducted in accordance with Institutional Animal Care and Use Committees standards (protocol 10-015FIW).

Energetic Measurements

We determined embryonic metabolic rates throughout development and the total energetic cost of development in wood duck embryos incubated at different temperatures, using closed- and open-flow respirometry (Microoxymax, Columbus Instruments, Columbus, OH). This instrument is computer controlled, allows simultaneous monitoring of multiple ($n = 10$) independent respiratory chambers, and is interfaced with a large environmental chamber that allows control of temperature. Respirometry procedures generally follow those outlined in recent publications (Dorcas et al. 2004; Hopkins et al. 2004; DuRant et al. 2008). To avoid pseudoreplication—and thus female effects on embryonic respiration—we measured the metabolic rates of only one egg per clutch per incubation temperature treatment at each time point.

We measured respiration of embryos at days 1, 4, 10, 13, 16, 19, 22, 25, 28, 31, and 34 of incubation and at pipping ($n = 7$–9 embryos per day of incubation per incubation temperature). We also measured respiration rates of hatchling wood ducks within the first 2–20 h after hatching (at 35.0°C, $n = 21$; at 35.9°C, $n = 17$; at 37.0°C, $n = 11$). At each sampling interval we placed eggs in individual respiratory chambers (either 500-mL or 1-L mason jars, depending on embryonic stage) that contained a small platform to secure the egg. Because air is dried by the respirometer before sampling, a moist paper towel was placed in the chamber to maintain high chamber humidity during the measurement period. Respiratory chambers were placed inside the environmental chamber set at the assigned incubation temperature and kept in complete darkness. We measured the oxygen consumption rate (mL h^{-1}; $\dot{V}O_2$) of embryos every 1.7 h for 12 h, resulting in 8–12 $\dot{V}O_2$ measurements per embryo collected over 3-min sampling intervals. Following the 12-h respirometry trial, we returned eggs to their respective incubator. We measured the respiration of each individual embryo at only one sampling interval. Because respiration rates increased dramatically over the period of incubation, we used both open- and closed-flow respirometry methods to determine the respiration rates of embryos, depending on their developmental stage. We used closed-flow respirometry on embryos at up to day 13 of incubation and open-flow respirometry at all later sampling intervals.

We express respiratory rates as functions of day of incubation and, for qualitative purposes, percentage of total incubation period (Angilletta et al. 2000). To quantify the total energetic cost of embryonic development, we calculated the integral of $\dot{V}O_2$ to external pipping, from external pipping to hatching, and during the entire incubation period. Then we converted total respiration values to energy equivalents, assuming $1\,L\,O_2 = 19.6\,kJ$ (Vleck et al. 1980; similar to procedures outlined in Angilletta et al. 2000; Hopkins et al. 2004). Because we did not use a repeated-measures design, we used a randomized sampling of data to allow statistical comparisons among the three incubation treatments. To calculate integrals, we randomly chose respiration profiles of one embryo or hatchling at each sampling point per incubation temperature and generated a time sequence and resulting integral for that curve. We repeated this procedure seven times for each incubation temperature. Each egg was used only once in developmental simulations.

Statistical Analyses

All statistical analyses were run in SAS, version 9.1 (SAS Institute, Cary, NC), or Microsoft Excel, and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested

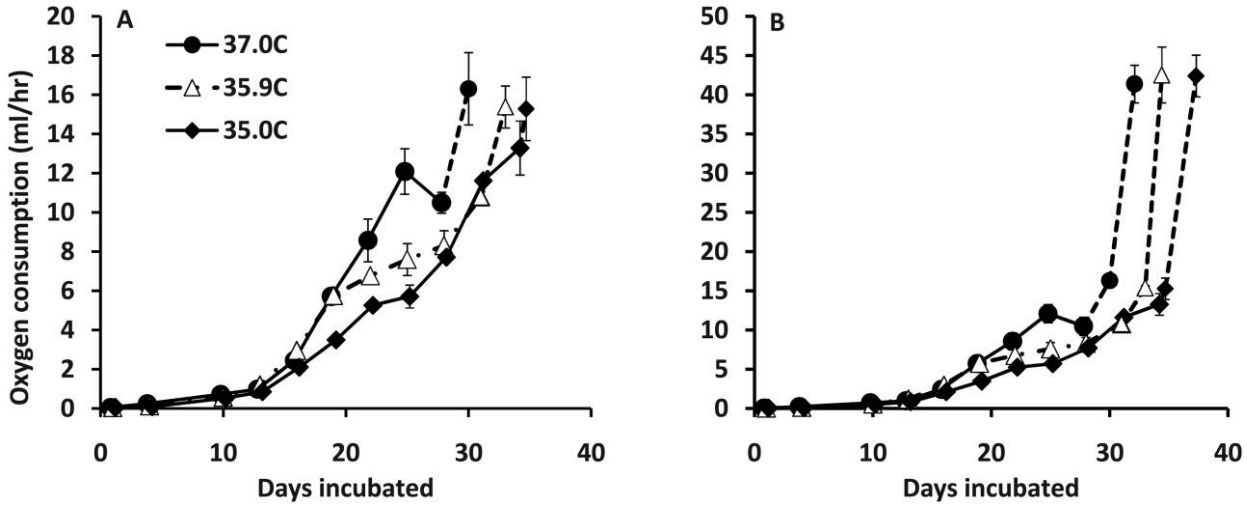

Figure 1. Oxygen consumption rates (mL h^{-1}) of wood duck (*Aix sponsa*) embryos during incubation to external pipping (*A*) and to hatching (*B*). Embryos were incubated at one of three temperatures (35.0°, 35.9°, or 37.0°C). Dashed lines connect respiration points associated with pipped eggs and ducklings after hatching. Error bars indicate ±1 SE of the mean. Statistical analyses were performed on $\log_{10}$-transformed O_2 consumption values.

for normal distribution of the data and homoscedasticity using Ryan-Joiner's and Bartlett's tests, respectively. Unless otherwise noted, raw data values were used in statistical analyses.

To confirm that fresh egg mass (preincubation) did not differ among incubation temperatures, we used an ANOVA. Incubation temperature was the main effect in the model. We also tested for differences in egg mass at each respiration time point to confirm that egg mass did not differ among treatments at any given day during incubation (ANOVA, SAS Proc GLM). The model included incubation temperature, day of incubation, and their interaction as independent variables. In addition, we tested for differences in mass of hatchlings among incubation temperatures used in the respiratory trial, using an ANOVA.

To determine whether respiration profiles differed throughout incubation, we used an analysis of covariance (ANCOVA; SAS proc mixed). In the model, incubation temperature, day of incubation, and their interaction were included as independent variables, and egg or duckling mass was included as a covariate. We $\log_{10}$-transformed $\dot{V}O_2$ values to better meet model assumptions. For qualitative purposes, we also present $\dot{V}O_2$ as a function of percentage of the total incubation period.

We tested for effects of incubation temperature on the total energetic cost of embryonic development (the integral of $\dot{V}O_2$ during incubation), energy expended up to external pipping, and energy expended during the hatching process, using a MANOVA. We included incubation temperature as the main effect in the model. We were unable to include mass as a covariate in the model because 11–13 unique eggs and one hatchling were used for each simulation that produced integrals. To account for any influence of mass, we also ran a MANOVA on integrals derived using mass-corrected $\dot{V}O_2$ values ($\dot{V}O_2$ divided by embryo or hatchling mass). Analyses using mass-corrected estimates of total energetic cost (not presented) yielded similar results as analyses using raw (not mass-corrected) $\dot{V}O_2$ values.

Results

There was no difference in fresh egg mass among incubation temperatures (for egg mass: $F_{2,297} = 0.51$, $P = 0.600$). There was also no difference in egg mass among incubation temperatures at any day during incubation (for egg mass, incubation temperature: $P = 0.445$; incubation temperature × days of incubation: $P = 0.329$). However, egg mass did vary with the number of days incubated ($P = 0.030$), but not with any distinct pattern (e.g., decreasing or increasing slowly over time), because a different group of eggs was represented at each sampling day and each day incorporated a broad range of egg masses. There was no difference in the mass of hatchlings used in the respiration trial (for hatchling mass, incubation temperature: $P = 0.143$).

Oxygen consumption rates (mL h^{-1}) differed among incubation temperatures throughout the incubation period (for incubation temperature × day of incubation: $F_{21,263} = 2.68$, $P < 0.001$; Fig. 1). Embryos from the high incubation temperature treatment had slightly higher respiration rates at most days of incubation and hatched approximately 2–5 days sooner than did embryos incubated at the medium and low temperatures (for incubation periods: at 35.0°C, 37.2 ± 0.24 d; at 35.9°C, 34.4 ± 0.18 d; and at 37.0°C, 32.1 ± 0.18 d). Similarly, embryos incubated at the medium temperature had slightly higher respiration rates at most days of incubation than did low-temperature embryos. Mass also significantly influenced respiration rates (for mass, $F_{1,263} = 8.04$, $P = 0.005$), with larger eggs consuming more oxygen than smaller eggs.

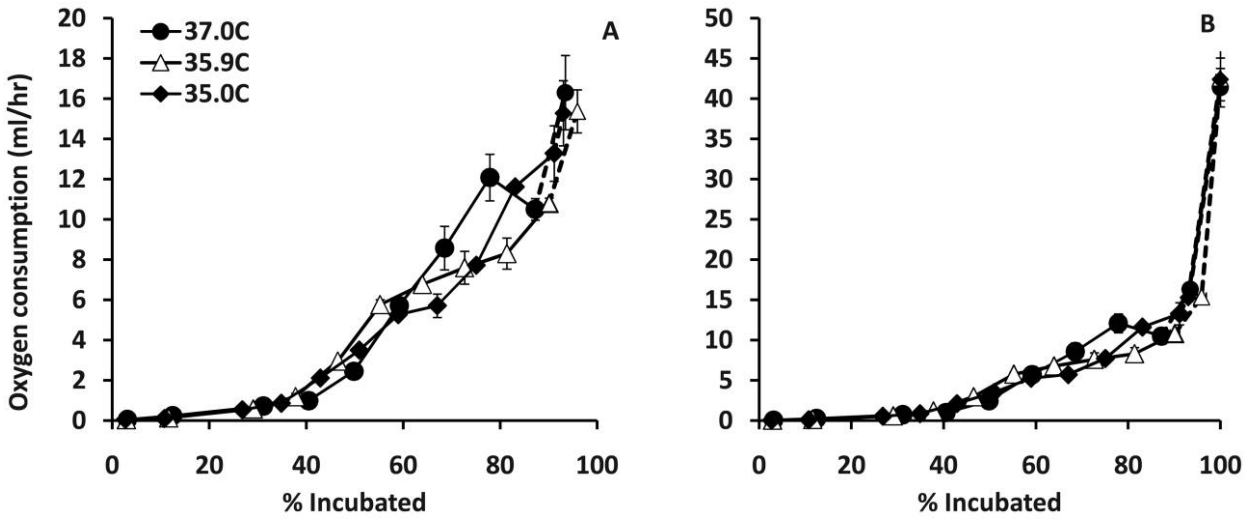

Figure 2. Oxygen consumption rates (mL h^{-1}) of wood duck (*Aix sponsa*) embryos during incubation to external pipping (*A*) and to hatching (*B*) plotted as a function of percent incubation. Embryos were incubated at one of three temperatures (35.0°, 35.9°, or 37.0°C). Dashed lines connect respiration points associated with pipped eggs and ducklings after hatching. Error bars represent ±1 SE of the mean.

Respiration curves plotted as a percentage of incubation period also qualitatively differed among incubation temperatures during the latter portion of incubation (Fig. 2). Embryos incubated at the highest temperature had 11%–57% higher metabolic rates than did embryos incubated at the other two temperatures when embryos were ~70%–80% incubated.

We also detected a significant effect of incubation temperature on total energy expended during incubation (Wilks's lambda, 0.22; $F_{4,34} = 9.65$, $P < 0.001$; Fig. 3), where embryos incubated at the lowest temperature expended 37% and 20% more energy than did ducklings incubated at the medium and the high temperature, respectively. Post hoc comparisons of individual ANOVAs revealed that the disparity in total embryonic energy expended during development ($F_{2,18} = 17.94$, $P < 0.001$) arose from greater energy expenditure during the hatching process ($F_{2,18} = 31.45$, $P < 0.001$) and not from differences in energy expended before pipping ($P = 0.869$). During the hatching process, embryos incubated at the lowest temperature expended 173% and 73% more energy than did embryos incubated at the medium and the high temperature, respectively.

To approximate the amount of energy available in the egg at laying and the percentage of that energy used during incubation, we assumed 8.37 kJ g^{-1} of fresh egg mass (Hepp et al. 1987). Using this conversion factor, we determined that the average energy content of an egg used in this study was 334.7 ± 2.2 kJ. Our estimates of total energy expended during incubation were 120, 83, and 94 kJ for the low, medium, and high incubation temperatures, respectively (Fig. 4). Assuming that the energy content of ducklings is equivalent to the energy content of an egg minus the energy expended during incubation (Vleck and Bucher 1998), the energy content of ducklings from the low, medium, and high incubation temperature treatments is 214, 251, and 240 kJ, respectively (Fig. 4).

Discussion

Our study demonstrated that the total energy expended by wood duck embryos during the incubation period differs depending on incubation temperature, with energetic costs accruing primarily during the hatching process. At most time

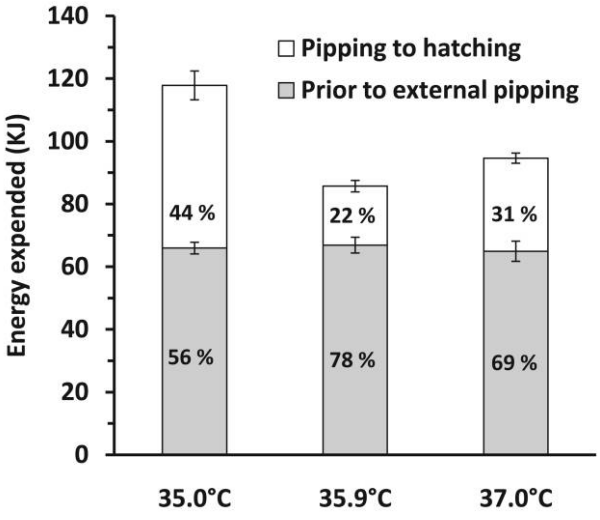

Figure 3. Total energy expended (kJ) during incubation (to hatching) in wood duck embryos (*Aix sponsa*) incubated at one of three temperatures (35.0°, 35.9°, or 37.0°C). The gray portion of each bar represents energy expended up to external pipping, whereas the white portion represents energy expended during the hatching process. Percentages within bars represent the percentage of total embryonic energy expenditure allocated to embryonic development up to pipping versus the energy needed for hatching. Error bars represent ±1 SE of the mean.

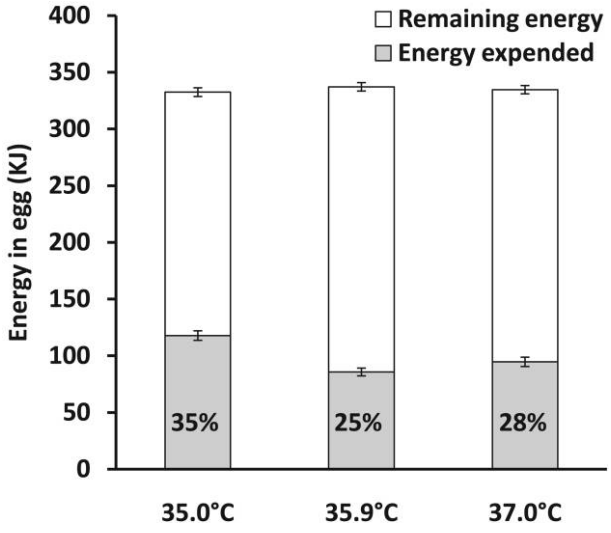

Figure 4. Energy consumed (kJ) during incubation (gray area) by wood duck embryos in relation to the energy content of freshly laid wood duck eggs (entire bar). Egg energy content was calculated by assuming 8.37 kJ g^{-1} of freshly laid egg mass (average egg mass: at 35.0°C, 39.7 ± 0.46 g; at 35.9°C, 40.3 ± 0.45 g; at 37.0°C, 40.0 ± 0.43 g). Embryos were incubated at one of three temperatures (35.0°, 35.9°, or 37.0°C). The percentages within the gray bars represent the percentage of energy in an egg that was consumed during incubation. Error bars indicate ±1 SE of the mean.

points, embryos incubated at the highest temperature had higher oxygen consumption rates than embryos incubated at the lower temperatures. However, because embryos incubated at the two higher temperatures had shorter incubation periods than the embryos incubated at the lowest temperature, they expended significantly less total energy during incubation. Our results are similar to a study on the effects of incubation temperature on embryonic metabolism in mallee fowl (*Leipoa ocellatas*, Gould), where eggs incubated at the lowest temperature took longer to develop and expended more energy during incubation than did eggs incubated at higher temperatures (Booth 1987). Contrary to much of the literature on reptiles, the total energy expenditures of wood duck and mallee fowl embryos appear to be sensitive to incubation temperature (Booth and Thompson 1991; Booth 1998; Oufiero and Angilletta 2010; but see Whitehead 1987; Angilletta et al. 2000).

Oxygen consumption rates of wood duck embryos and hatchlings at all incubation temperatures were similar to those of other similarly sized waterfowl embryos or hatchlings (Koskimies and Lahti 1964; Hoyt et al. 1979; Hoyt and Rahn 1980; Figs. 1, 2), but the patterns of oxygen consumption appear to differ among incubation temperatures. In the high incubation temperature group, embryonic metabolism increased exponentially during the first 80% of development, followed by a plateau in metabolic rates just before pipping (Figs. 1, 2) This plateau in metabolism is typical of precocial embryos (Hoyt et al. 1979; Hoyt and Rahn 1980) and is thought to occur because embryos transition from rapid and energetically costly tissue development to maturation of physiological systems important for precocial behavior (Vleck et al. 1980). The plateau phase was less evident at the medium and low temperatures; however, the detectability of the plateau phase at the medium and low incubation temperatures may have been obscured by our sampling interval, which was every 3 d during incubation. Alternatively, the differences in metabolic profiles may relate to differences in the timing of the development and maintenance of expensive physiological systems (e.g., the nervous system). A study on zebra finches (*Taeniopygia guttata*, Vieillot) incubated at varying temperatures demonstrated that incubation temperature affects the developmental rates of embryos, with the slowest development occurring in embryos incubated at the lowest temperature (Olson et al. 2006). If development of expensive systems is delayed in the medium- and low-temperature treatments, then these embryos may continue to both develop and mature new tissues until just before pipping. Conversely, embryos incubated at the highest temperature may develop these systems more rapidly and thus undergo only maturation during the days immediately before pipping.

Another line of evidence that incubation temperature may affect the relative timing of developmental processes and not just protract development comes from the metabolic profiles of embryos plotted as a function of the percentage of incubation period (Fig. 2). There appear to be differences in metabolic rate when embryos were 70%–90% incubated, suggesting that embryos are on different developmental trajectories. This finding contrasts with the findings of a study on lizards (Angiletta et al. 2000), wherein metabolic rates of embryos as a function of percentage of incubation period appeared to be temperature independent and total energy consumed was solely a product of the length of incubation. The authors of that study postulated that either lizard embryos are temperature insensitive or temperature acclimation occurred, such that at any given developmental stage, embryos incubated at cool temperatures upregulate metabolism and embryos incubated at warm temperatures downregulate metabolism, resulting in no difference in stage-specific metabolic rates.

The differences among treatments in total energy expended during incubation in our study was attributable to greater energy expenditure of low-temperature embryos during the hatching process, and not energy expended before external pipping (Fig. 3). Embryos incubated at the low temperature took 1.2–1.9 d longer to hatch once pipping occurred than did embryos incubated at the higher temperatures, which likely explains the difference in energy expended during hatching. The longer time needed for hatching in embryos incubated at the lowest temperature could result from differences in thyroid hormones and/or glucocorticoid (e.g., corticosterone) concentrations. These hormones increase in the late stages of incubation in precocial embryos and are responsible for tissue maturation and the preparation of organs (e.g., gut and lungs) for posthatch life (McNabb et al. 1998; Wada 2008). Our previous study on wood ducks supports this hypothesis, as we demonstrated that corticosterone concentrations differ at 2 d posthatch (dph) in ducklings incubated at the same three temper-

atures we used in this study (DuRant et al. 2010). It is surprising that while the time to pipping was 3–4.5 d shorter for embryos in the high incubation temperature treatment, energy expenditure up to pipping was nearly identical in embryos from all incubation temperatures. Again, this may result from differences in the timing of key developmental stages; expensive physiological systems may develop earlier at the highest temperature and, once developed, incur higher maintenance costs (Vleck and Vleck 1987).

During incubation, wood duck embryos consumed between 25% and 35% of the energy content of a freshly laid wood duck egg (Fig. 4). Regression equations relating egg energy content to the energetic cost of development up to hatching for precocial birds (Vleck and Vleck 1987) predict that wood ducks would expend between 112 and 123 kJ during development; however, these regression models do not incorporate variations in incubation temperature. Our estimates among the three incubation temperatures encapsulated this range (range, 83–120 kJ; Fig. 4). On the basis of our energy expenditure estimates, the energy content of ducklings from the low, medium, and high incubation temperature treatments were 214, 251, and 240 kJ, respectively. Previous studies in wood ducks have found that low incubation temperatures reduce duckling protein (Hepp et al. 2006) and lipid contents (G. R. Hepp, unpublished data) by 9%–12% and 20%–22%, respectively, which could account for the differences in energy content. A study on crocodiles noted that incubation temperature affected the size of yolk reserves but not hatchling wet mass, with lower incubation temperatures increasing incubation periods and subsequently decreasing yolk reserves (Webb et al. 1987). Olson et al. (2006) noted that at 12 d postincubation, zebra finch embryos regularly cooled to 20°C had both lower masses and smaller yolk reserves than did embryos maintained at a constant 37.5°C. We cannot currently determine whether the disparities in duckling composition and energy content are attributable to differences in hatchling tissues or the size or composition of yolk reserves. To better understand the implications of differences in embryonic energy expenditure induced by variable incubation temperature, future studies should quantify energy expended during the entire hatching process and energy content and composition of hatchling tissue and yolk reserves. In addition, the relative importance of humidity should be considered in future experiments because it will fluctuate with temperature, subsequently affecting vapor pressure (Vleck and Bucher 1998). However, humidity was held constant across temperature treatments in our study.

Finally, our results suggest that the effects of incubation temperature on embryonic energy expenditure and developmental trajectories may be contributing mechanisms for effects of incubation temperature on avian offspring phenotype. Our previous studies, using the same incubation temperature treatments as this study, have found that ducklings incubated at the lowest temperature have reduced physiological performance relative to ducklings incubated at the higher temperatures (Hepp et al. 2006; DuRant et al. 2010, 2011; Hopkins et al. 2011; S. E. DuRant, W. A. Hopkins, A. F. Wilson, and G. R. Hepp, unpublished manuscript). Because low-temperature ducklings expend significantly more energy during development than the higher temperature ducklings, they hatch with reduced energy reserves, subsequently inhibiting their ability to grow, develop, and fuel expensive physiological processes such as thermoregulation and mounting an immune response. Furthermore, because incubation temperature appears to affect the relative timing of developmental events, ducklings incubated at the lowest temperature may also be developmentally delayed relative to ducklings incubated at higher temperatures. Developmental delays could also contribute to the previously observed differences in duckling physiological performance.

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