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Transient and permanent effects of suboptimal incubation temperatures on growth, metabolic rate, immune function, and adrenocortical responses in zebra finches

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Abstract

In birds, incubation temperature can vary by several degrees Celsius among nests of a given species. Parents may alter incubation temperature to cope with environmental conditions and/or to manipulate embryonic development, and such changes in incubation behavior could have long-lasting effects on offspring phenotype. To investigate short- and long-term effects of suboptimal incubation temperatures on survival and physiological functions in zebra finches, eggs were incubated at 36.2, 37.4, and 38.4ºC for the entire incubation period. Post-hatch environment was identical among the treatment groups. We found that hatching success was lowest in the 38.4ºC group, while post-hatch survival was lowest in the 36.2ºC group. Incubation temperature had sex-specific effects on offspring phenotype, where incubation temperatures affected body mass but not physiological parameters of males, while temperatures affected physiological parameters but not body mass of females. Specifically, males from the 38.4ºC group weighed significantly less than males from the 36.2ºC group from the nestling period to adulthood, while females from different incubation temperature groups did not differ in body mass. In contrast, females incubated at 36.2ºC had transient but significantly elevated basal metabolic rate and adrenocortical responses during the nestling and fledgling periods, while no treatment effect was observed in males. Innate immunity was not affected by incubation temperature in either sex. These results suggest that a 1ºC deviation from what is considered an optimal incubation temperature can lower offspring performance and offspring survival.

Keywords: development, corticosterone, pre-hatch, stress, altricial, birds

1. Introduction

Environmental heterogeneity induces phenotypic variance among individuals as animals adjust their phenotype to match the environment. Environmental cues during development are particularly important as developing young typically display the greatest phenotypic plasticity. For oviparous species, incubation temperature greatly influences offspring phenotype (DuRant et al., 2013). In the few avian species with known incubation temperatures, the difference in incubation temperature between nests can be 3ºC or more. For example, daytime incubation temperature of biparental zebra finches in the wild ranges from 34.9 to 38.5ºC (Zann and Rossetto, 1991). An optimal incubation temperature is suggested to be around 37ºC for zebra finches; it is the average incubation temperature in captive zebra finches (Vleck, 1981; Zann and Rossetto, 1991) with high embryo mass, residual yolk, and growth efficiency (Olson et al., 2006). Thus the observed variation indicates that not all parents incubate eggs at the optimal temperature for embryonic development. This is particularly true when environmental condition deteriorates; an artificial drop in the ambient temperature resulted in increased metabolic rate of the incubating zebra finches and a decline in incubation temperature (Nord et al., 2010). In such environmental conditions, parents face a trade-off between energetic needs of the incubation and of the developing embryos and can result in incubation temperatures that are not optimal for embryonic development.

Resulting changes in incubation temperature can have transient or long-term consequences on the offspring phenotype. Previous studies show that incubation temperature affects metabolic rates and the hypothalamic-pituitary-adrenal (HPA) axis function in birds for the first 2 weeks of life (DuRant et al., 2010; Nord and Nilsson, 2011). That is, altricial blue tit (*Cyanistes caeruleus*) nestlings that hatched from eggs incubated at 35.0ºC had significantly higher resting metabolic rates on ~14 days post-hatch (dph; hatch day is 0 dph) compared to nestlings hatched from eggs incubated at 36.5 or 38.0ºC (Nord and Nilsson, 2011). Similarly, precocial wood duck (*Aix sponsa*) embryos that were incubated at 35.0ºC expended significantly more energy during the hatching process compared to embryos incubated at 35.9 or 37.0ºC (DuRant et al., 2011). The young hatched from eggs incubated at 35.0ºC also had significantly higher baseline and stress-induced corticosterone levels compared to ducklings in the 35.9 or 37.0ºC groups during the first 9 days of their lives (DuRant et al., 2010). These studies illustrate that incubation temperature has the potential to shape offspring phenotype in birds similarly to maternal programming in mammals by licking and grooming behavior (Meaney, 2001) or through lactation (Fodor and Zelena, 2014).

One aspect of maternal programming in mammals is that changes to offspring physiology are often long-lasting or permanent (Fowden et al., 2006). If incubation temperature indeed shapes offspring phenotype in birds as maternal programming does in mammals, then impacts of incubation temperature should also be enduring. Surprisingly, this prediction has never been addressed. Therefore, we sought to shed light on this issue by

empirically assessing the long-term consequences of variation in incubation temperature on offspring phenotype. Towards this end, we artificially incubated zebra finch eggs at low, control, and high temperatures. We then measured pre- and post-hatch survival, growth, and vital physiological functions including metabolic rates, immune and endocrine function. We hypothesized that when incubation temperature deviates from optimal, as occurs in cases of egg neglect or inefficient incubation, it would lower offspring performance and survival, in turn reducing parental fitness.

2. Results

Egg mass loss

During the first 12 days of incubation, eggs incubated at 36.2, 37.4, and 38.4ºC lost 8.20%, 9.62%, 11.86% of mass, respectively. Both incubation temperature ($F_{2, 144} = 3.46$; $p = 0.034$) and incubation duration (covariate; $p < 0.001$) had a significant effect on egg mass loss (Table 1). The post-hoc analysis showed that eggs incubated at 38.4ºC lost significantly more mass compared to controls at 37.4ºC. On average, eggs incubated at 37.4ºC lost 9.1 ± 0.41 mg of its mass per day during the first 12 days of incubation compared to 7.8 ± 0.24 mg in the 36.2ºC group and 11.3 ± 0.41 mg in the 38.4ºC group.

Incubation duration and hatchling mass

Incubation duration increased at lower temperatures ($F_{2, 148} = 239.5$; $p < 0.001$; Table 1). Specifically, eggs incubated at 36.2ºC took 14.9 days to hatch, which was significantly longer than eggs incubated at 37.4ºC with an average of 13.6 days, which was significantly longer than eggs incubated at 38.4ºC with an average of 12.8 days. Since embryos are ectotherms, we calculated degree-days by multiplying incubation duration by the incubation temperature to more appropriately describe the timing of hatching. If the difference in hatching duration was solely due to incubation temperature, degree-days among the treatment groups would be similar. However, they were not; time to hatching still increased with decreasing incubation temperature with averages of 537.9 ± 1.9, 507.6 ± 2.87, 491.3 ± 2.52 degree-days for the 36.2, 37.4, and 38.4ºC groups, respectively. This difference in degree-days among the three incubation temperatures suggests that additional factors, such as embryonic metabolic rates, exist among the treatment groups.

Hatchlings weighed an average of 0.823, 0.816, and 0.824 g in the 36.2, 37.4, and 38.4ºC groups, respectively. When initial egg mass was accounted for (covariate; $p < 0.001$), hatchling mass did not differ among the treatment groups ($F_{2, 147} = 0.03$; $p = 0.97$; Table 1). Thus, incubation temperature did not affect hatchling mass.

Survival

Eggs incubated at the control temperature of 37.4ºC had 84.6% hatching success (determined as percent of fertilized eggs that hatched in each treatment) where 6.2% died during embryonic development and 9.2% died during the process of hatching (Fig. 1). Similar hatching success of 80.3% (8.5% and 11.3% died during embryonic development and during the process of hatching, respectively) was observed in the 36.2ºC group (χ^2 (1) = 0.443, p = 0.506). In contrast, the 38.4ºC group only had 59.1% hatching success (χ^2 (1) = 12.04, p = 0.001 compared to controls). Most of the mortality occurred during embryonic development (39.4% died during embryonic development vs. 1.5% died during the process of hatching, respectively).

Unlike hatching success, the 36.2ºC temperature group had the lowest post-hatch survival compared to both 37.4ºC and 38.4ºC temperature groups (χ^2 (1) = 5.22, p = 0.022 in comparison with 37.4ºC; χ^2 (1) = 7.33, p = 0.007 in comparison with 38.4ºC; Fig. 2). In the 36.2ºC temperature group, 17.5% died during the nestling period (21 dph or younger) compared to 5.2% and 0% in the 37.4 and 38.4ºC temperature groups. Among those, most occurred before nestlings were 3 days old. Another peak occurred between 32 to 38 dph, around nutritional independence when 7.5% of 36.2ºC temperature group died while none of the 37.4 or 38.4ºC groups died. In total, 11 birds died from the 36.2ºC group post-hatch while 3 and 1 bird died from the 37.4 or 38.4ºC groups, respectively. When averaged across pre- and post-hatch period, overall survival for 36.2, 37.4, and 38.4ºC groups were 76.4, 88.4, and 77.9%, respectively.

Nestling growth

For both males and females, body mass of the genetic parents, measured just prior to breeding, had a significant effect on the offspring body mass (covariate; females: $p < 0.001$; males: $p = 0.002$). Incubation temperature alone affected body mass in males ($F_{2, 50} = 4.30$; $p = 0.019$) but not in females ($F_{2, 36} = 0.14$; $p = 0.87$; Fig. 3 AC). Post-hoc analysis showed that male nestlings from the 38.4ºC temperature weighed less than males from 36.2ºC temperature group (Fig. 3 CD). There was no interaction between age and incubation temperature treatment for either sex (age – females: $F_{8,288} = 0.77$; $p = 0.63$; males: $F_{8,400} = 4.53$; $p < 0.001$; age x treatment – females: $F_{16,288} = 0.59$; $p = 0.89$; males: $F_{16,400} = 0.89$; $p = 0.59$). Interestingly, nestlings from both 36.2ºC and 38.4ºC weighed less than controls on 2 and 5 dph for both sexes (Fig. 3 BD). Female nestlings from 36.2ºC dropped to 86% of mean control mass on 2 dph while female nestlings from 38.4ºC dropped to 89% of mean control mass on 5 dph. Females from both temperature groups recovered to nearly 100% of mean control mass by 10 dph. Similarly, male nestlings from 36.2ºC dropped to 91% while male nestlings from 38.4ºC dropped to 83% of mean control mass on 2 dph. Although body mass of male nestlings from 36.2ºC recovered by 5 dph, male nestlings from 38.4ºC recovered slowly

to only 90% of mean control mass on 10 dph and continued to be ~96% of mean control mass throughout adulthood.

Reactivity of the HPA axis

At 16 dph, body mass of the individual did not affect baseline corticosterone or integrated adrenocortical response in either sex (females: baseline: F = 0.56, p = 0.46, integrated response: F = 1.00, p = 0.32; males: baseline: F = 0.28, p = 0.60, integrated response: F = 0.005, p = 0.95). In males, incubation temperature did not affect nestling baseline ($F_{2,51}$ = 0.28; p = 0.76) or integrated adrenocortical response ($F_{2,51}$ = 0.06; p = 0.94; Table 2, Fig. 4). In contrast, females from the 36.2ºC group had significantly higher adrenocortical responses compared to females from the 37.4ºC or 38.4ºC groups (baseline – $F_{2,36}$ = 0.97; p = 0.39; integrated adrenocortical responses – $F_{2,36}$ = 3.67; p = 0.036).

When birds reached adulthood, the treatment effect on the adrenocortical response disappeared. In both sexes, there was no effect of incubation temperature on baseline or integrated adrenocortical response (Table 2). Furthermore, incubation temperature did not have a significant effect on the integrated response against administration of adrenocorticotropic hormone (ACTH) or dexamethasone (Dex). Body mass on 266 dph was marginally correlated with integrated Dex response in males (F = 3.87, p = 0.055).

Immune function

Incubation temperature did not affect microbicidal capacity against *Candida albicans* or *Escherichia coli* in either sex (Table 2). No effect of incubation temperature was observed either during nestling period or in adulthood.

Metabolic rates

In females, incubation temperature significantly affected basal metabolic rates (BMR) at 25 dph where the 36.2ºC females had a significantly higher BMR compared to 37.4ºC females (Fig. 5, Table 2). The effect of incubation temperature on BMR was not observed on 177 dph, suggesting that the effect on BMR was short-lived. In males, there was no significant effect of the incubation temperatures on the BMR on either age. Similarly, incubation temperature did not affect peak metabolic rates (PMR) in either sex.

3. Discussion

The present study aimed to determine whether avian incubation temperature programs offspring vital physiological functions and influence offspring survival. In summary, we found that hatching success was lowest in the 38.4ºC group while post-hatch survival was lowest in the 36.2ºC group, ultimately leading to comparable survival rates in the low and high temperature groups by the end of the experiment (which were both lower than that of the control group). We also found that suboptimal incubation temperature affected males and

females differently. Males from the 38.4ºC group weighed less compared to males from the 36.2ºC group throughout development and as adults, but no treatment effect was observed in regards to the HPA axis functioning, innate immunity, or metabolic rate. In contrast, females had similar body mass regardless of the temperature treatment but females from the 36.2ºC group had a significantly higher adrenocortical response and BMR compared to 37.4 and 38.4ºC females during nestling and fledgling stages, respectively. However, these treatment effects disappeared in adulthood. Immune function in females was not affected by incubation temperature.

High incubation temperature reduced hatching success while low incubation temperature reduced post-hatch survival

It is thought that a shorter developmental period is favored when predation pressure is high (Case, 1978; Bosque and Bosque, 1995; Remeš et al., 2002; Martin and Briskie, 2009). Thus, parents may increase incubation temperature in an attempt to accelerate embryonic development. At the same time, markedly high incubation temperatures, typically higher than 40ºC, can cause malformations in the central nervous system (Peterka et al., 1996; Krausova and Peterka, 2007), increase production of reactive oxygen species (ROS) (Sakatani et al., 2004), and are considered lethal (Lundy, 1969; Conway and Martin, 2000). Even though 38.4ºC is within the range of incubation temperature found in captive and free-living zebra finches (Zann and Rossetto, 1991), it is possible that this temperature increases abnormal mitotic activities (Sulik et al., 1988), production of ROS, or the risk for malformation during embryogenesis leading to higher embryonic mortality seen in this study.

In contrast to the effects of high incubation temperature, reduced post-hatch survival in the low temperature group may be due to low residual energy at hatching. Higher temperature and shorter incubation period are linked to reduced yolk utilization in pine snakes (Burger et al., 1987), Australian brush turkeys (Eiby and Booth, 2009), and zebra finches (Olson et al., 2006). Conversely, low incubation temperature raises energy expenditure during development. In wood ducks for instance, total energy expenditure was similar among low, medium, and high incubation temperature groups before the start of pipping; however, between pipping and hatching, embryos from the low temperature group consumed more energy than those from the high and medium groups (DuRant et al., 2011). Olson et al (2006) found that periodic cooling during the incubation period resulted in lighter embryos and less yolk reserve on embryonic day 12. Moreover, the mass-specific metabolic rates at embryonic day 12 were higher in the embryos that experienced periodic cooling compared to controls that were incubated at a constant temperature of 37.5ºC. These results suggest that cooler temperature increases energetic demand of the embryos during development and requires yolk consumption beyond that of optimal incubation temperature (Olson et al., 2006). Since we measured % egg mass loss at embryonic day 12 for all the temperature groups but did not measure residual yolk mass at hatching, it is difficult to compare the residual energy available

at hatching. However, based on the data in reptiles and birds mentioned above, it is likely that embryos and hatchlings from the 36.2ºC group used more yolk and hatched with lower residual yolk reserve compared to 37.4 or 38.4ºC groups. In fact, young from the 36.2ºC group suffered the highest mortality particularly when the nestlings were between 1 and 3 days old. As all foster nests contained nestlings from all three treatment groups, the observed mortality in the early nestling stage may be due to a disadvantage to compete for food caused by low energy stores in the first days of life after hatching.

High temperature males weighed less throughout their lives

Previous studies showed that hatchling mass was positively correlated with incubation temperature. In precocial wood ducks, hatchling mass decreased with decreasing incubation temperature ((Hepp et al., 2006) but also see (DuRant et al., 2010)). This is due to the increased energy expenditure during embryonic development at low incubation temperature (DuRant et al., 2011). In altricial zebra finches, periodic cooling to 20ºC as opposed to constant incubation temperature of 37.5ºC reduced embryonic mass on embryonic day 12 without an impact on body size (Olson et al., 2006; Olson et al., 2008). In contrast to the studies above, we did not observe any effect of incubation temperature on hatchling mass. However, body mass in males from the 38.4ºC group was lower after hatching compared to males from the 36.2ºC and the effect persisted into their adulthood. This mass difference among treatment groups was largely due to the treatment difference in lean mass rather than combined lean and fat mass (Wada et al., unpublished data). As the quantitative magnetic resonance instrument we used to measure lean and fat mass does not detect skeleton or keratin-based structures (Guglielmo et al., 2011), the higher than optimal incubation temperature likely reduced muscle and organ mass in male offspring. Since BMR did not differ in males during fledgling or adult stages, metabolic rates do not contribute to the reduced muscle and/or organ mass. It is possible that males hatched from 38.4ºC had suppressed begging, appetite, or competitive ability reducing food intake. Whether the reduced body mass is an adaptation to the suboptimal temperature is not known. Further study is needed to test whether the low body mass in high temperature group enhances survival, reproductive performance, and competitively ability relative to individuals with high body mass in a warm environment. It is worth noting that nestlings from both 36.2 and 38.4ºC weighed less in the early nestling period compared to 37.4ºC nestlings regardless of sex (Fig. 3). In the statistical analysis, all the birds that died before the juvenile period were excluded. Thus, this reduction in body mass at an early nestling period reflects a tangible and common mass difference between optimal and suboptimal incubation temperature.

Low temperature females showed transient increase in adrenocortical response and BMR

In altricial birds, lower than optimal incubation temperature increases metabolic rates during embryonic development as well as in the nestling period (Olson et al., 2006; Nord and Nilsson, 2011). We observed that female fledglings from the 36.2ºC group had a relatively high BMR compared to fledglings hatched from 37.4ºC. Our results indicate that the incubation temperature can affect BMR beyond the nestling stage. Lower than optimal incubation temperature also elevated adrenocortical responses in female nestlings. Similar patterns in the adrenocortical responses are reported in wood ducks, where ducklings from low incubation temperature exhibited elevated baseline and adrenocortical response compared to intermediate (with highest hatching success and post-hatch survival) and high incubation temperature on 2 and 9 dph (DuRant et al., 2010). In our study, these effects on the HPA axis function and metabolism were observed for 16 and 25 dph, respectively. Offspring from the 36.2ºC group suffered mortality at nutritional independence, approximately around 35 dph. Although we did not have a large enough sample size to test the relationship between treatment-related mortality and BMR or HPA axis function, further investigation into this relationship is warranted.

Conclusions

In summary, a 1ºC increase in incubation temperature reduced hatching success while a 1.2 ºC decrease in incubation temperature reduced post-hatch survival. Suboptimal incubation temperatures also transiently, but significantly, elevated metabolic rate and adrenocortical responses and permanently reduced body mass in a sex-specific manner. These results therefore demonstrate that deviations as small as 1ºC from optimal incubation temperature can have both short-term and permanent effects on offspring phenotypes in zebra finches. Since incubation temperature serves as an important source of offspring's phenotypic variability, it is imperative to assess the effects of environment, e.g., climate change, on incubation temperature and offspring phenotype in oviparous species. Whether or not the resulting phenotype is an adaptation to the forecasted environment or developmental constraint merits further investigation.

4. Materials and Method

Animal husbandry

Zebra finches used in this experiment were obtained from our breeding colony at the Advanced Facility for Avian Research, University of Western Ontario, London, Canada. Animal husbandry and experimental protocols were approved by the Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. From October 2010 to July 2011, animals were kept in 36 x 43 x 42 cm cages with an external nest box (20 x 13.5 x 13.5 cm) attached to each cage. The room was kept at 22ºC on 14 h:10 h

light:dark cycle. All pairs had access to seed (Living World premium finch seed, Mansfield, MA; 11.0% protein, 5.9% lipid), water, grit, and cuttlebone *ad libitum* from pairing to rearing. In addition, protein-rich egg mixture (hardboiled chicken eggs, cornmeal, and white bread) was provided daily to the pair from pairing to when the oldest nestling reached 35 dph.

Manipulation of incubation temperature

Based on variation observed in captive zebra finches, we chose three incubation temperatures: high, control, and low. To manipulate incubation temperature, we incubated zebra finch eggs in three Brinsea Octagon 20 Advance EX incubators (Brinsea Products Inc. Titusville, FL). Prior to the start of the experiment, all incubators were calibrated to a single reference thermometer (VWR ASTM thermometer, 34/42ºC, ± 0.1ºC accuracy, cat. No. 61126-943). Throughout the experiment, the temperature of each incubator at two positions within the row containing eggs was measured for 90 minutes weekly using the reference thermometer. Mean (± SEM) temperatures in low, control, and high incubators were 36.20 ± 0.07 (n = 7), 37.42 ± 0.08 (n = 7), and 38.40 ± 0.07ºC (n = 7), respectively, with 0.48% mean coefficient variation between the two positions. We thus refer to treatments as 36.2, 37.4, and 38.4ºC hereafter. Relative humidity was set to 55% in all three incubators.

A total of 202 fertilized eggs from 26 breeding pairs were used in this experiment. Each nest box was checked daily and newly laid eggs were distributed to one of the three incubation temperatures. The first laid eggs were randomized and consecutive eggs were systematically allocated among the incubators so that an equal number of eggs from each nest were distributed among the three treatments and laying order was balanced among the treatments. Incubators were checked multiple times a day for new hatchlings. Hatchlings were placed into a nest with foster parents until each nest contained 5 or 6 nestlings comprised of nestlings from all three treatment groups. To minimize mortality unrelated to the temperature treatment, we minimized the age difference among foster siblings; all nests had nestlings that were no more than 1 day apart in hatch day except for 2 out of 22 nests that had a 2-day age difference. Any nests that had less than 5 nestlings in a nest at the beginning of the nestling period or did not have nestlings from all three treatment groups were excluded from further analysis.

Pre-hatch measures on eggs

Eggs were weighed to the nearest mg on the day they were laid and on the 12th day of incubation to determine egg mass loss during incubation. All eggs were candled to assess whether the egg was fertilized. Only fertilized eggs were used in this study. For those fertilized eggs that failed to hatch, eggs were candled again to determine whether mortality occurred during embryonic development or during hatching process. The number of days for eggs to hatch was recorded as incubation duration.

Nestling growth

On the day of hatch (0 dph), hatchlings were weighed to the nearest mg. Nestlings were weighed to the nearest 0.1 g on 2, 5, 10, 16, 35, 60, and 90 dph between 0930 h and 1230 h before egg food was provided (see Fig. 6 for the timeline). The final body mass was measured on one day when birds were, on average, 266 ± 6.7 dph (standard deviation) between 0915 h and 1415 h.

Functioning of the hypothalamic-pituitary-adrenal axis

We measured the functioning of the HPA axis in 4 ways: baseline corticosterone levels (ng/mL) as well as adrenocortical response to standardized capture and handling stress, ACTH challenge, and Dex suppression test (integrated response, ng/mL*min, see the statistical analysis section for details). We collected blood samples from individual birds by puncturing the brachial vein with a 26-gauge needle and collecting blood into heparinized microhematocrit tubes. All blood samples were immediately refrigerated. After centrifugation, plasma was extracted and frozen within 6 h of sample collection. Baseline blood samples for corticosterone when birds were 16, 142 ± 6.8, and 198 ± 5.9 dph were obtained between 0900 h and 1105 h, within 3 minutes of entering the animal room. We measured adrenocortical responses to a stress using a standardized capture and handling protocol (Wingfield, 1994). After the baseline sample was collected, birds were restrained in an opaque bag, and a second blood sample was collected 30 minutes after the time of initial disturbance. We collected measures of adrenocortical responses to this standardized stress protocol twice for each bird, once when at 16 dph and again at 198 ± 5.9 dph. No blood samples were pooled for analyses.

To assess the adrenal responsiveness and negative feedback in the HPA axis, we administered ACTH challenges and Dex suppression tests on a separate day. The ACTH challenge measured the adrenals' capacity to secrete corticosterone in response to exogenous ACTH. The appropriate injection volume for each bird was calculated based on the body mass of the individual one day before the ACTH challenge. On the day of the ACTH challenge, baseline blood samples were collected, followed by an intramuscular injection of 25 IU/kg ACTH (Sigma-Aldrich, cat no. A6303) delivered via a 300 µL-insulin syringe. After the injection, birds were held in an opaque bag and then blood sampled again 30 min post-injection. Dex is a synthetic glucocorticoid that suppresses the endogenous secretion of glucocorticoids via negative feedback and has a low cross-reactivity to the anti-corticosterone antibody used in radioimmunoassay. Consequently, Dex suppression tests measure the strength of negative feedback exerted on the HPA axis. After the capture and handling protocol, each bird was injected intramuscularly with 1000 µg/kg Dex (Sandoz Canada, Dexamethasone sodium phosphate injection USP, 2302; adjusted to body mass taken a day before). Following injection, birds were released into a cage and an additional blood sample was collected 30 min post-injection. ACTH challenges and Dex suppression test were conducted when birds were 142 ± 6.8 and 198 ± 5.9 dph, respectively.

Plasma corticosterone was quantified using a ^{125}I radioimmunoassay (MP Biomedical) previously validated for zebra finch plasma (Schmidt and Soma, 2008). The standards ranged from 1.25 to 250 pg/tube. The interassay variations for high (125 pg/tube) and low (12.5 pg/tube) controls were 6.36% and 9.32%, respectively. The intra-assay variations for high and low controls were 4.78% and 11.53%, respectively.

Innate constitutive immunity

To assess how incubation temperature affected innate constitutive immunity, we measured the antimicrobial capacity of whole blood against *C. albicans* (ATTC #10231; Epower Microorganisms, catalog no. 0443E7, MicroBiologics) and *E. coli* (ATTC #8739; Epower Microorganisms, catalog no. 0483E7, MicroBiologics) when the birds were, on average, 16 and 142 ± 6.8 dph. All protocols were optimized for this species and age prior to analyses.

Antimicrobial activity against *C. albicans* is dependent on both plasma components and phagocytosis while killing of *E. coli* is dependent on plasma proteins only (Matson et al., 2006; Millet et al., 2007). We followed the method of Liebl and Martin (2009) with some modifications. Details of the assay are described in Kriengwatana et al. (Kriengwatana et al., 2013). Briefly, sterile blood samples were collected within 5 minutes of entering the animal room. Antimicrobial assay for *C. albicans* was done immediately after blood collection while the assay for *E. coli* was done within 10 days of blood collection. The samples for the latter were kept on ice until frozen at -80ºC and assayed antimicrobial activity against *E.coli*. For the *C. albicans* killing assay, 1:48 dilution of whole blood to cell media was used for 16 dph samples while 1:96 dilution for adults 142 dph samples. The primary incubation of whole blood, cell media, and *C. albicans* ($1x10^5$ colony forming units (CFU)/mL) was 15 minutes at 30ºC. Sample duplicates, sample blank, positive and negative controls were incubated with tryptic soy broth for the second incubation at 30ºC. We used a nanodrop spectrophotometer (Nanodrop 2000c, Thermoscientific) to determine the absorbance at 300 nm after 24 to 48 h of secondary incubation. The time it took for the positive controls to reach an optimal absorbance varied between assays. Thus we calculated the antimicrobial capacity as the average absorbance of sample duplicates/average absorbance of positive controls, both adjusted to its blank (% microbial survival).

Antimicrobial activity against *E. coli* was assessed using the same protocol as for *C. albicans*, except that we used $1x10^4$ CFU/mL working solution, 1:6 dilution of blood to cell media for both 16 and ~142 dph, and 30-minute primary incubation and 12 h secondary incubation at 37ºC.

Metabolic rates

We measured BMR in a subset of birds twice, when they were 25 ± 1.7 and 177 ± 5.0 dph. BMR was measured using a flow through respirometry system similar to that described in Gerson & Guglielmo (2011). The analyzer was calibrated daily with standard air containing 20.9% O_2 and 1.02% CO_2. Starting at 2000 h, birds were weighed in a paper bag, put into an airtight stainless-steel chamber inside an incubator. The incubator maintained the ambient temperature at 35ºC which is within a thermoneutral zone for this species (Calder, 1964; Bech et al., 2004). Birds fasted for three hours. Then the post-absorptive oxygen consumption from the remaining 9 hours during the night cycle was used to analyze BMR. Incoming air was scrubbed of CO_2 and water vapor and entered each chamber at constant rate of ~350 mL/min. Excurrent air was scrubbed of water vapor and subsampled for CO_2 at a rate of 150 mL/min, then subsampled for O_2 after scrubbing of CO_2 (Sable Systems, Las Vegas NV). Each chamber containing birds and a chamber sampling room air as a baseline were sampled for 10 min by a multiplexer every 70 minutes. After the overnight measuring period, birds were weighed again then returned to the home cage. Vo_2 (mL/min) was calculated from a 10-min interval with the lowest mean oxygen consumption among the measuring period after correcting for lab time using Expedata software (Sable Systems, Las Vegas, NV). The equation we used to calculate Vo_2 uses both fractional concentrations of O_2 and CO_2 (equations 10.6 and 10.7 in Lighton (2008)). Here we analyzed mass-corrected Vo_2 where Vo_2 was divided by the body mass of the individuals after the respirometry measurement (Vo_2 (mL/min)/g).

Peak metabolic rates were measured ~26 h after the end of the BMR measurement on ~ 177 dph using the same respirometry system. Birds were fasted for approximately 2 hours, then placed in a closed flight wheel (16 cm width x 24 cm diameter) lined with rubber. The wheel was supplied with constant air of 3.5 L/min. The exiting air was subsampled at ~285 mL/min. Birds were left undisturbed for 5 min in the wheel covered with a cloth to acclimate. Beginning at 0945 h and no later than 1330 h, the wheel was spun manually by an experimenter who performed all the tests to encourage birds to hop and hover until the PMR was reached (generally occurred within 7 min). Four ping-pong balls were placed inside of the wheel to prevent birds from walking. After reaching PMR, the experimenter kept the wheel spinning at the maximum speed at which birds could maintain the exercise for 2 min. This method has been previously shown to estimate PMR in small passerines (Pierce et al., 2005; Price and Guglielmo, 2009). The PMR of each individual was calculated as a maximum average V_{O2} over a 15 second period corrected for body mass (Vo_2 (mL/min)/g).

Statistical analyses

All statistical analyses were performed using IBM SPSS 21. Data were transformed whenever necessary to meet the assumptions of normal distribution and homoscedasticity. All covariates whose p-value was higher than 0.1 were removed prior to the final analysis. Post-hoc analysis was performed using Sidak adjustment.

Egg measures: Egg measures were analyzed using one-way ANCOVA. Egg mass loss (inverse transformed), incubation duration, and hatchling mass were analyzed in 3 separate one-way ANCOVAs with incubation temperature as a main factor. For egg mass loss, incubation duration was used as a covariate and for hatchling mass, initial egg mass was also used as a covariate.

Survival: Kaplan-Meier estimate with a log rank test was used to compare survival among treatment groups. Survival due to temperature treatment during the incubation stage and survival after hatching (post-treatment) were analyzed separately.

Body mass: Body mass and physiological measures were analyzed using repeated measures ANCOVA and MANCOVA. Each sex was analyzed separately, excluding individuals that died before the juvenile stage. Growth, measured as increase in body mass (square-root transformed) over time, was analyzed using repeated measures ANCOVA with an average of genetic parents' body mass (square-root transformed) just prior to pairing, as a covariate.

Functioning of the hypothalamic-pituitary-adrenal axis: We analyzed 6 parameters that reflected individuals' HPA axis function: baseline corticosterone levels at 16 and 198 dph, integrated stress response at 16 and 198 dph, and integrated responses to ACTH and Dex administration. Integrated responses were calculated as an area under the response curve and represent total corticosterone released during the monitored period. Baseline and integrated stress response collected on 16 dph (log and square-root transformed, respectively) were analyzed in MANCOVA with 16 dph body mass as a covariate. Similarly, baseline and integrated stress response collected on 198 dph, integrated ACTH and Dex response on 140 and 198 dph (log and square-root transformed) were analyzed in MANCOVA with 266 dph body mass as a covariate.

Innate constitutive immunity: Pearson correlation of microbicidal capacity against *C. albicans* and *E. coli* on 16 and 142 dph revealed that only microbicidal capacity against *C. albicans* and *E. coli* on 142 dph were marginally correlated ($p = 0.093$). Thus data were analyzed together in MANOVA without data reduction. Arcsine square-root transformation was used for all the microbicidal capacity data.

Metabolic rates: Pearson correlation of BMR and PMR revealed that only BMR of 25 and 177 dph are marginally correlated ($p = 0.06$). Thus BMR on 25 and 177 dph as well as PMR on 177 dph were analyzed together in MANOVA with incubation temperature as a fixed factor, without data reduction. Mean ± SEM is presented in results section and figures.

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Figures

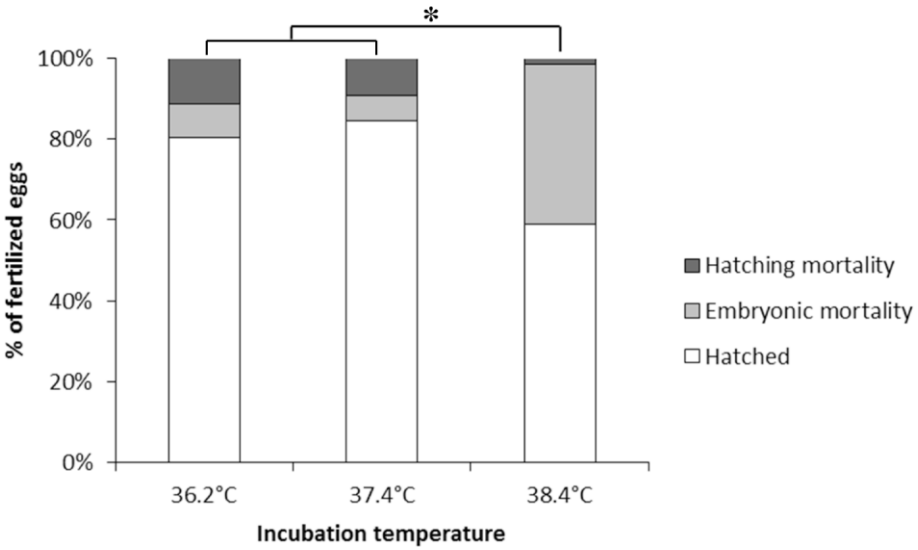


Figure 1. **Proportion of fertilized eggs that successfully hatched (white), died during embryonic development (embryonic mortality; light gray), or during pipping (hatching mortality; dark gray) in eggs incubated at 36.2, 37.4, and 38.4ºC.** The asterisk indicates a statistical significance ($p < 0.05$).

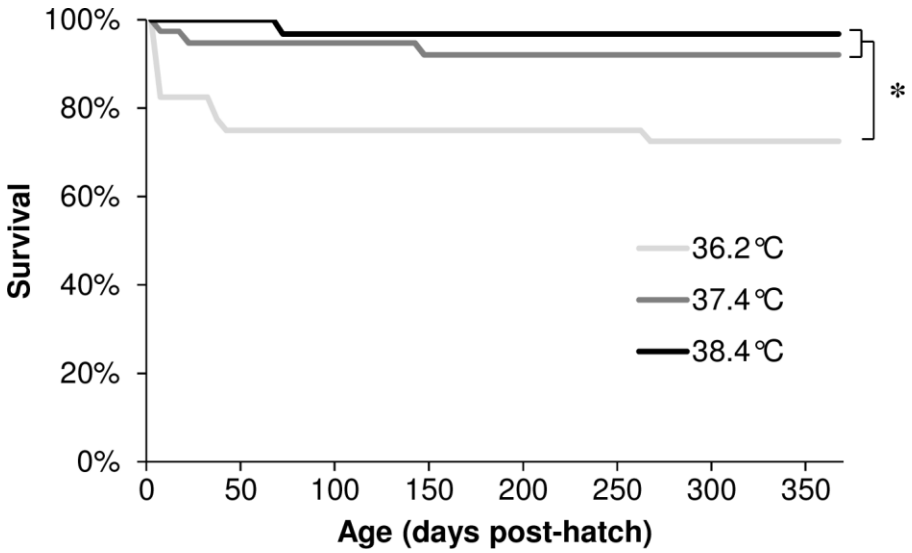
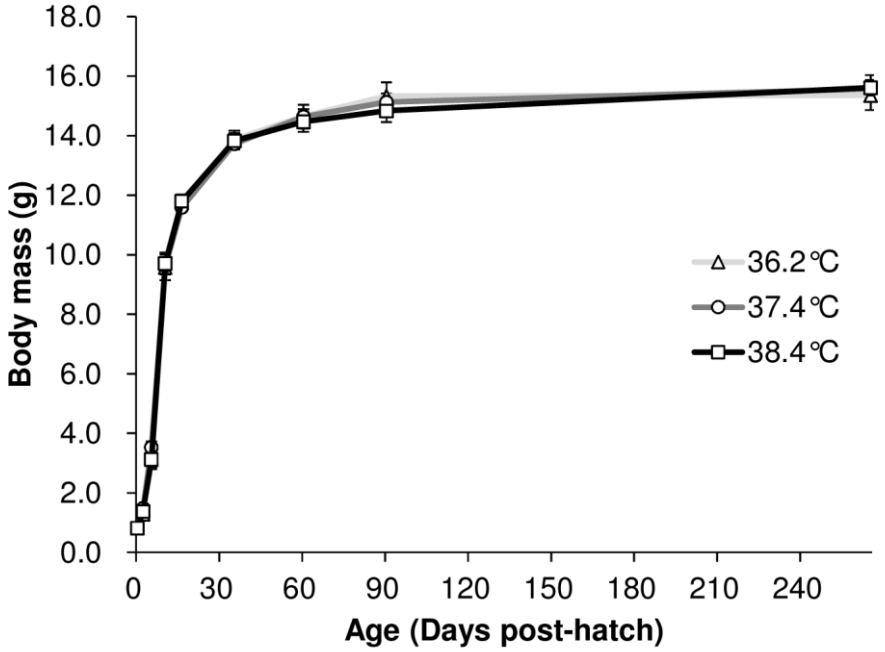
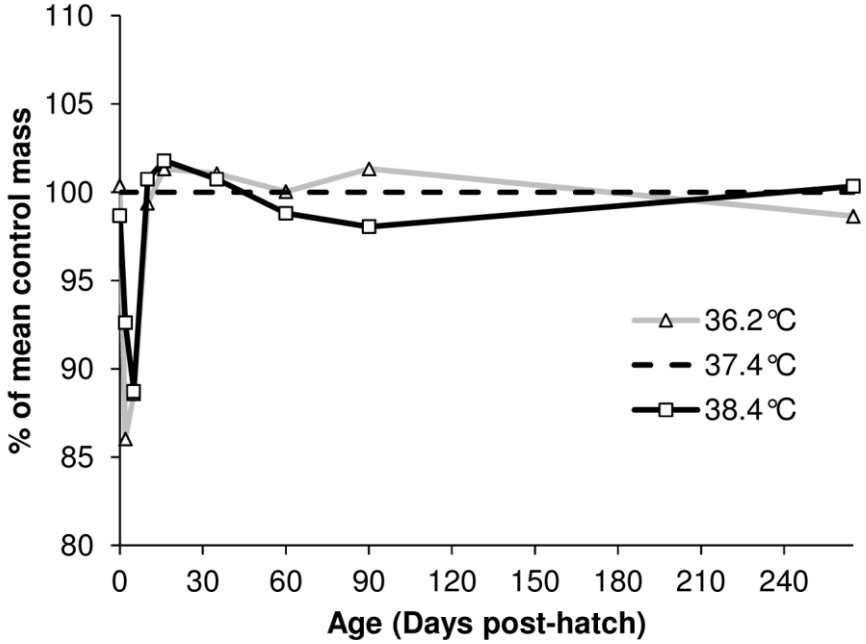


Figure 2. **Post-hatch survival of individuals hatched from eggs incubated at 36.2 (light gray line), 37.4 (dark gray line), and 38.4ºC (black line).** Zebra finch nestlings fledge and reach nutritional independence around post-hatch day 21 and 35, respectively, and reach sexual maturity around 90 days after hatching. The asterisk indicates a statistical significance ($p < 0.05$).

A) Females



B) Females



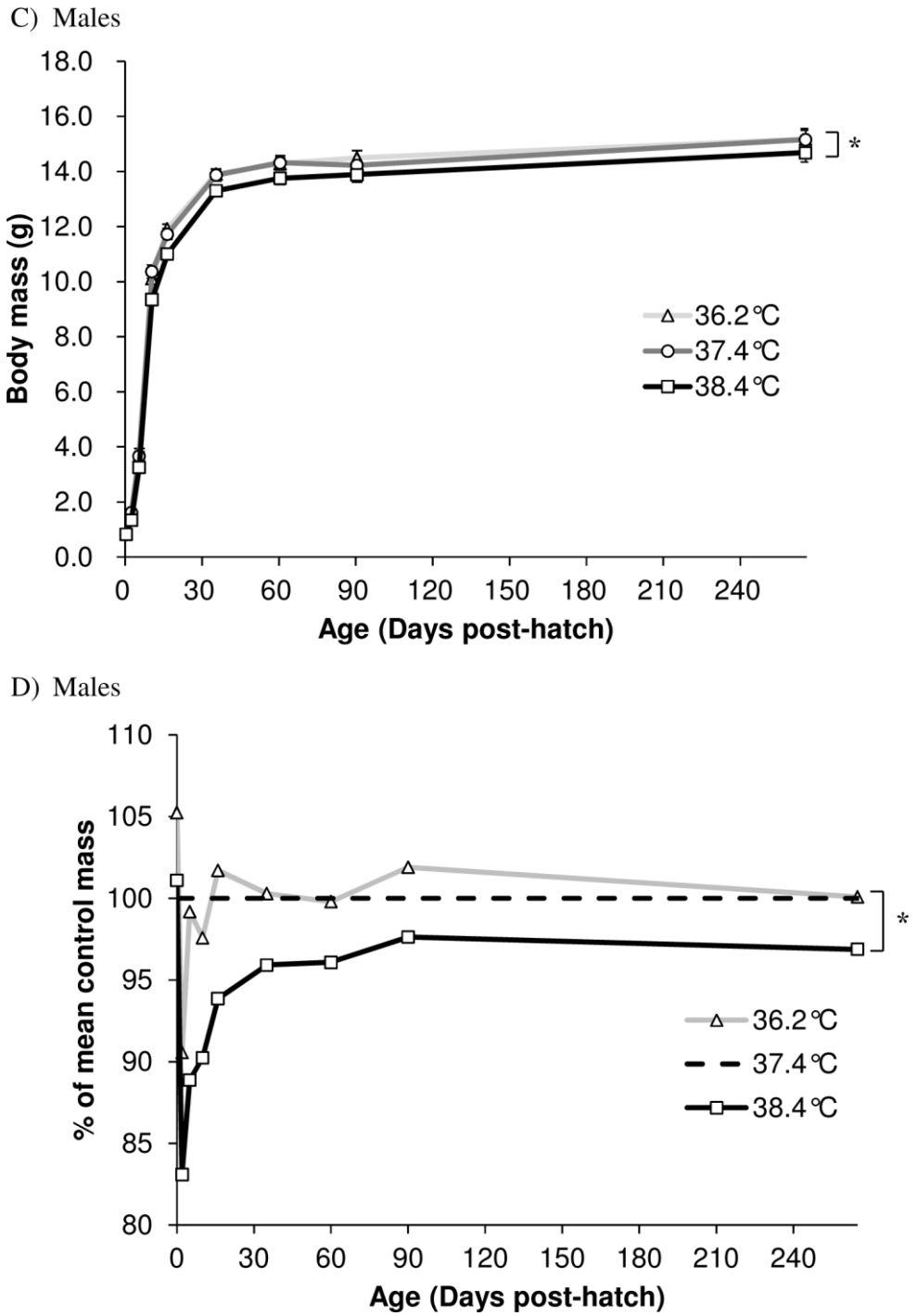


Figure 3. **Changes in body mass of female (A, B) and male (C, D) zebra finches hatched from eggs incubated at 36.2 (light gray line), 37.4 (dark gray line), and 38.4ºC (black line) over the course of their lifetime.** B and D show percent body mass in the 36.2 (light gray line) and 38.4ºC (black line) groups compared to the mean body mass in the 37.4ºC group. Error bars indicate ± SEM around the mean. Sample size at the end of the experiment (265 days post-hatch) was 21, 14, 19 for males incubated at 36.2, 37.4, and 38.4ºC and 8, 21, 11 for females incubated at 36.2, 37.4, and 38.4ºC. The asterisk indicates a statistical significance ($p < 0.05$).

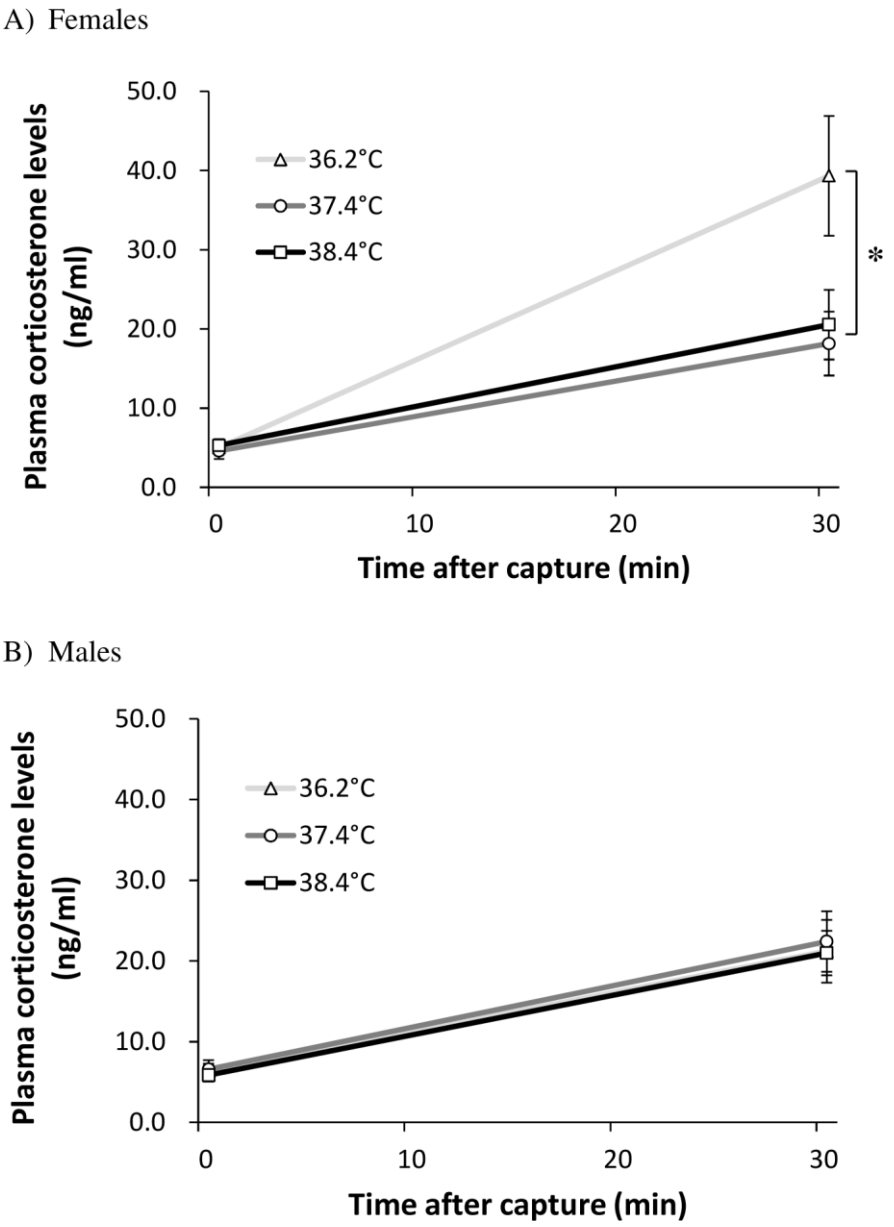


Figure 4. **Adrenocortical responses of female (A) and male (B) zebra finches on 16 days post-hatch**. Three lines indicate incubation temperature treatments of 36.2 (light gray line), 37.4 (dark gray line), and 38.4ºC (black line). Error bars indicate ± SEM around the mean. The asterisk indicates a statistical significance ($p < 0.05$).

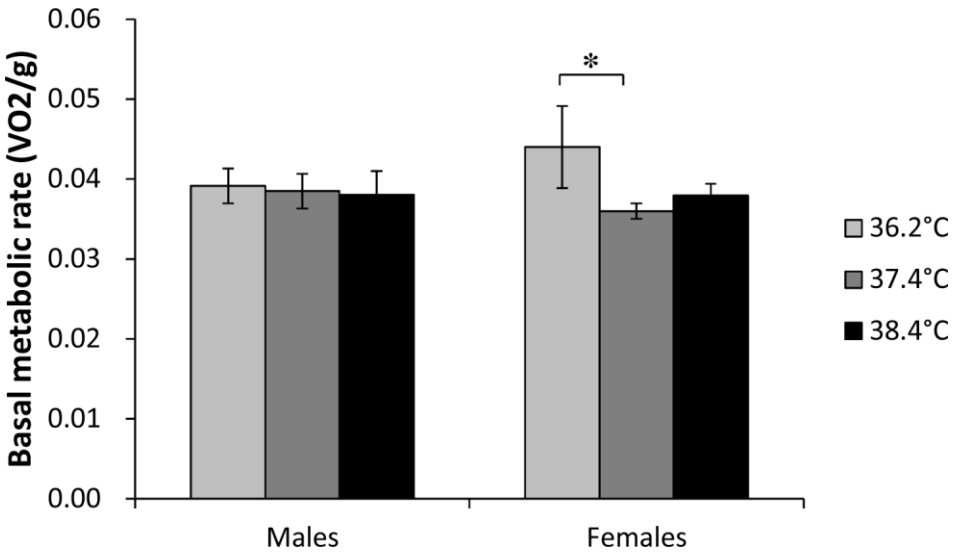


Figure 5. **Basal metabolic rates (BMR) of zebra finches on 25 days post-hatch.** Light gray, dark gray, and black bars indicate the incubation temperature treatments of 36.2, 37.4, and 38.4ºC, respectively. Error bars indicate ± SEM around the mean. The asterisk indicates a statistical significance ($p < 0.05$).

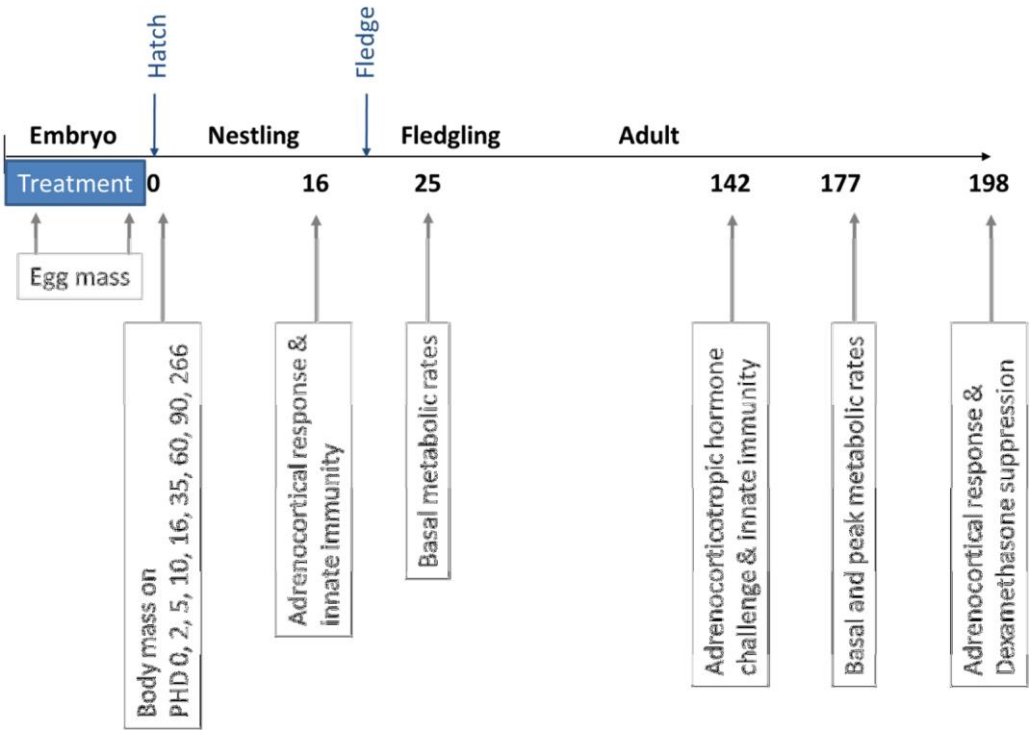


Figure 6. **Timeline of the experiment.** The numbers below the horizontal line indicate days post-hatch (dph). Zebra finch eggs were incubated at 36.2, 37.4, and 38.4ºC for the entire incubation period. Post-hatch environment was identical among the treatment groups.

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Tables

Table 1. Egg measures and survival in offspring incubated at 36.2ºC (low), 37.4ºC (control), and 38.4ºC (high) with statistical results. Values are mean ± SEM (n).

	Incubation temperature			
Variable	**36.2°C**	**37.4°C**	**38.4°C**	***P* value**
Egg mass loss (%)	8.20 ± 0.22 (56)	9.62 ± 0.43 (54)	11.86 ± 0.42 (36)	0.034
Incubation duration (days)	14.86 ± 0.05 (57)	13.56 ± 0.08 (55)	12.79 ± 0.07 (39)	<0.001
Hatch mass (g)	0.823 ± 0.013 (57)	0.816 ± 0.012 (55)	0.824 ± 0.011 (39)	0.968
Hatchling success (%)	80.28 (71)	84.62 (65)	59.09 (66)	see text
Post-hatch survival (%)	72.50 (40)	92.11 (38)	96.77 (31)	see text

Table 2. Summary of effects of incubation temperature on physiological functions. The hypothalamic-pituitary-adrenal (HPA) axis function was analyzed in four ways: baseline corticosterone levels (ng/mL), integrated adrenocortical stress response to 30 min of handling and restraint (ng/mL*min), and integrated response to exogenous adrenocorticotropic hormone (ACTH) or dexamethasone (Dex) (ng/mL*min). Innate immunity is presented as % microbial survival and metabolic rates are presented as VO_2 (mL/min)/g. Different letters for a variable within sex represent significant difference. Values are mean ± SEM (n).

			Females				Males			
Respons	**Age**	**Factors**	36.2°C	37.4°C	38.4°C	*P* value	36.2°C	37.4°C	38.4°C	*P* value
HPA axis function	**Nestling**	Baseline	5.04 ± 0.96 (8)	4.60 ± 0.99 (20)	5.29 ± 0.79 (11)	0.39	6.40 ± 0.84 (22)	6.57 ± 1.15 (14)	5.87 ± 0.80 (18)	0.76
		Stress response	614 ± 107 (8) a	321 ± 57 (20) b	365 ± 63 (11) b	0.036	392 ± 56 (22)	411 ± 61(14)	380 ± 39 (18)	0.94
	Adult	Baseline	6.54 ± 1.18 (8)	7.55 ± 0.74 (20)	7.85 ± 1.34 (11)	0.76	7.93 ± 1.18 (21)	10.22 ± 1.09 (13)	10.77 ± 1.47 (18)	0.19
		Stress response	353 ± 46 (8)	360 ± 55 (20)	362 ± 51 (11)	0.95	344 ± 40 (21)	425 ± 52 (13)	403 ± 43 (18)	0.25
		ACTH response	1022 ± 138 (8)	927 ± 73 (20)	1134 ± 148 (11)	0.43	993 ± 70 (21)	1047 ± 98 (13)	1003 ± 87 (18)	0.84
		Dex response	438 ± 104 (8)	354 ± 67 (20)	352 ± 50 (11)	0.65	320 ± 42 (21)	396 ± 70 (13)	343 ± 48 (18)	0.44
Innate immunity	**Nestling**	*C. albicans*	84.5 ± 6.4 (8)	85.1 ± 4.6 (19)	81.4 ± 6.0 (12)	0.97	83.3 ± 4.5 (18)	90.2 ± 5.0 (14)	79.0 ± 4.8 (19)	0.17
		E. coli	75.3 ± 10.0 (9)	88.8 ± 4.2 (15)	82.5 ± 5.8 (11)	0.22	73.8 ± 4.5(20)	73.8 ± 7.2 (13)	72.0 ± 5.2 (17)	0.84
	Adult	*C. albicans*	85.9 ± 2.9 (9)	84.7 ± 3.4 (21)	78.6 ± 5.1 (11)	0.44	83.9 ± 2.7 (21)	80.9 ± 3.8 (14)	77.3 ± 3.7 (19)	0.26
		E. coli	68.2 ± 11.9 (7)	70.4 ± 6.0 (16)	60.8 ± 8.8 (9)	0.92	76.1 ± 6.1 (15)	70.3 ± 10.0 (10)	72.8 ± 6.6 (13)	0.75
Metabolic rates	**Fledgling**	BMR	0.044 ± 0.005 (4) a	0.036 ± 0.001 (12) b	0.038 ± 0.002 (8) b	0.046	0.039 ± 0.002 (14)	0.039 ± 0.002 (10)	0.038 ± 0.003 (12)	0.84
	Adult	BMR	0.044 ± 0.002 (6)	0.045 ± 0.002 (13)	0.043 ± 0.003 (8)	0.83	0.048 ± 0.002 (15)	0.047 ± 0.001 (9)	0.045 ± 0.002 (14)	0.76
		Peak	0.351 ± 0.027 (7)	0.354 ± 0.019 (13)	0.357 ± 0.015 (8)	0.50	0.365 ± 0.015 (15)	0.361 ± 0.021 (11)	0.347 ± 0.017 (14)	0.47