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RESEARCH ARTICLE

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 or 38.4°C for the entire incubation period. The post-hatch environment was identical among the treatment groups. We found that hatching success was lowest in the 38.4°C group, while post-hatch survival was lowest in the 36.2°C group. Incubation temperature had sex-specific effects on offspring phenotype: incubation temperatures affected body mass (M_b) but not physiological parameters of males and conversely, the physiological parameters but not M_b of females. Specifically, males from the 38.4°C group weighed significantly less than males from the 36.2°C group from the nestling period to adulthood, whereas females from different incubation temperature groups did not differ in M_b. In contrast, females incubated at 36.2°C had transient but significantly elevated basal metabolic rate and adrenocortical responses during the nestling and fledgling periods, whereas no treatment effect was observed in males. Innate immunity was not affected by incubation temperature in either sex. These results suggest that a 1°C deviation from what is considered an optimal incubation temperature can lower offspring performance and offspring survival.

KEY WORDS: Development, Corticosterone, Pre-hatch, Stress, Altricial, Birds

INTRODUCTION

Environmental heterogeneity induces phenotypic variance among individuals as animals adjust their phenotype to match the environment. Environmental cues during development are particularly important because 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 3°C or more. For example, daytime incubation temperature of biparental zebra finches in the wild ranges from 34.9 to 38.5°C (Zann and Rossetto, 1991). An optimal incubation temperature is suggested to be around 37°C 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 parents 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): altricial blue tit (*Cyanistes caeruleus*) nestlings that hatched from eggs incubated at 35.0°C had significantly higher resting metabolic rates at ∼14 days post-hatch (dph; hatch day is 0 dph) compared with nestlings hatched from eggs incubated at 36.5 or 38.0°C (Nord and Nilsson, 2011). Similarly, precocial wood duck (*Aix sponsa*) embryos that were incubated at 35.0°C expended significantly more energy during the hatching process compared with embryos incubated at 35.9 or 37.0°C (DuRant et al., 2011). The young hatched from eggs incubated at 35.0°C also had significantly higher baseline and stress-induced corticosterone levels compared with ducklings in the 35.9 or 37.0°C 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 similar 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 endure. 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

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List of symbols and abbreviations

ACTH	adrenocorticotropic hormone
BMR	basal metabolic rate
Dex	dexamethasone
dph	days post-hatch
HPA	hypothalamic–pituitary–adrenal
M_b	body mass
PMR	peak metabolic rate

phenotype. Towards this end, we artificially incubated zebra finch eggs at low (36.2°C), control (37.4°C) and high (38.4°C) 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, leading to reduced parental fitness.

RESULTS

Loss of egg mass

During the first 12 days of incubation, eggs incubated at 36.2, 37.4, and 38.4°C 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 38.4°C lost significantly more mass compared with controls at 37.4°C. On average, eggs incubated at 37.4°C lost 9.1±0.41 mg of their mass per day during the first 12 days of incubation compared with 7.8±0.24 mg in the 36.2°C group and 11.3±0.41 mg in the 38.4°C 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 36.2°C took 14.9 days to hatch, which was significantly longer than eggs incubated at 37.4°C with an average of 13.6 days, which was, in turn, significantly longer than eggs incubated at 38.4°C 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 means of 537.9±1.9, 507.6±2.87, 491.3±2.52 degree-days for the 36.2, 37.4 and 38.4°C groups, respectively. This difference in degree-days among the three incubation temperatures suggests that additional factors, such as embryonic metabolic rates, differ among the treatment groups.

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

Survival

Eggs incubated at the control temperature of 37.4°C had 84.6% hatching success (determined as percentage 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 36.2°C group ($\chi^2_{(1)}$=0.443, P=0.506). In contrast, the 38.4°C group only had 59.1% hatching success ($\chi^2_{(1)}$=12.04, P=0.001 compared with controls). Most of the mortality occurred during embryonic development (39.4% died during embryonic development versus 1.5% during the process of hatching).

Unlike hatching success, the 36.2°C temperature group had the lowest post-hatch survival compared with both 37.4°C and 38.4°C temperature groups ($\chi^2_{(1)}$=5.22, P=0.022 in comparison with 37.4°C; $\chi^2_{(1)}$=7.33, P=0.007 in comparison with 38.4°C; Fig. 2). In the 36.2°C temperature group, 17.5% died during the nestling period (21 dph or younger) compared with 5.2% and 0%, respectively, in the 37.4 and 38.4°C temperature groups. Most of these deaths occurred before nestlings were 3 days old. Another peak occurred between 32 and 38 dph (around the time of nutritional independence), when 7.5% of the 36.2°C temperature group died whereas none of the 37.4 or 38.4°C groups died. In total, 11 birds died from the 36.2°C group after hatching whereas 3 and 1 bird died from the 37.4 or 38.4°C groups, respectively. When averaged across pre- and post-hatch period, overall survival for 36.2, 37.4 and 38.4°C groups was 76.4, 88.4, and 77.9%, respectively.

Nestling growth

For both males and females, M_b of the genetic parents, measured just prior to breeding, had a significant effect on the offspring M_b (covariate; females: P<0.001; males: P=0.002). Incubation temperature alone affected M_b in males ($F_{2,50}$=4.30; P=0.019) but not in females ($F_{2,36}$=0.14; P=0.87; Fig. 3A,C). *Post hoc* analysis showed that male nestlings from the 38.4°C temperature weighed less than males from the 36.2°C temperature group (Fig. 3C,D). 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×treatment, females: $F_{16,288}$=0.59; P=0.89; males: $F_{16,400}$=0.89; P=0.59). Interestingly, nestlings from both 36.2°C and 38.4°C weighed less than controls at 2 and 5 dph for both sexes (Fig. 3B,D). Female nestlings from 36.2°C dropped to 86% of mean control mass at 2 dph whereas female nestlings from 38.4°C dropped to 89% of mean control mass at 5 dph. Females from both temperature groups recovered to nearly 100% of mean control mass by 10 dph. Similarly, male nestlings from 36.2°C dropped to

Table 1. Egg measures and survival in offspring incubated at 36.2°C, 37.4°C or 38.4°C

	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

Values are mean±s.e.m. (*N*).

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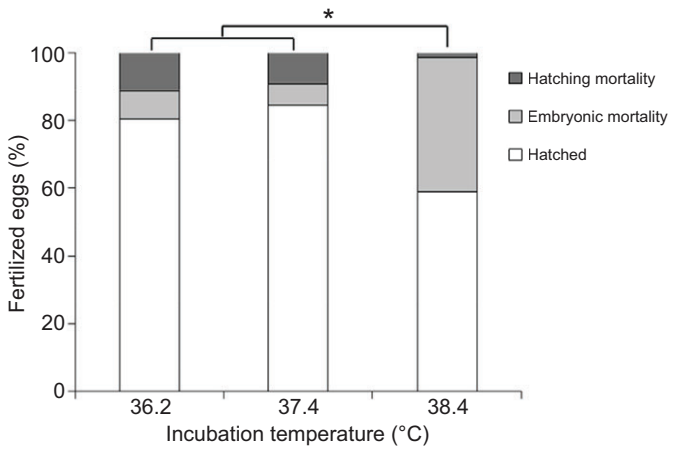

Fig. 1. Proportion of fertilized eggs that successfully hatched in zebra finch eggs incubated at 36.2, 37.4 or 38.4°C. The asterisk indicates a statistical significance (P<0.05).

91% while male nestlings from 38.4°C dropped to 83% of mean control mass at 2 dph. Although M_b of male nestlings from 36.2°C recovered by 5 dph, male nestlings from 38.4°C recovered slowly to only 90% of mean control mass at 10 dph and continued to be ~96% of mean control mass throughout adulthood.

Reactivity of the HPA axis

At 16 dph, M_b 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 36.2°C group had significantly higher adrenocortical responses compared with females from the 37.4°C or 38.4°C 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). M_b at 266 dph was marginally correlated with integrated Dex response in males (F=3.87, P=0.055).

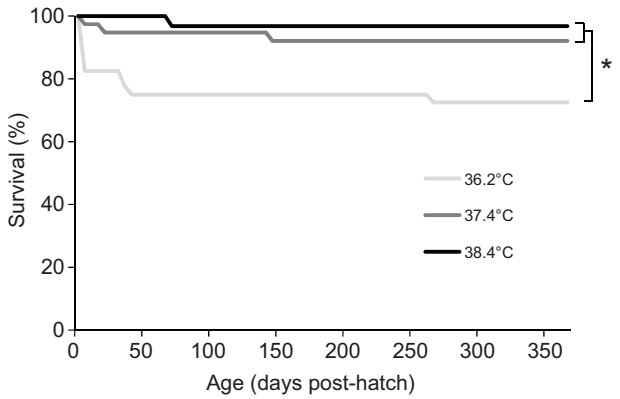

Fig. 2. Post-hatch survival of zebra finches hatched from eggs incubated at 36.2, 37.4 or 38.4°C. Zebra finch nestlings fledge and reach nutritional independence around post-hatch day 21 and 35, respectively, and reach sexual maturity around 90 days after hatching. *P<0.05.

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 the basal metabolic rate (BMR) at 25 dph where the 36.2°C females had a significantly higher BMR compared with 37.4°C females (Fig. 5, Table 2). The effect of incubation temperature on BMR was not observed at 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 (PMRs) in either sex.

DISCUSSION

The present study aimed to determine whether avian incubation temperature programs offspring vital physiological functions and influences offspring survival. In summary, we found that hatching success was lowest in the 38.4°C group while post-hatch survival was lowest in the 36.2°C 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 38.4°C group weighed less compared with males from the 36.2°C group throughout development and as adults, but no treatment effect was observed in regards to functioning of the HPA axis, innate immunity or metabolic rate. In contrast, females had similar M_b regardless of the temperature treatment but females from the 36.2°C group had a significantly higher adrenocortical response and BMR compared with females at 37.4 and 38.4°C 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 reduces hatching success whereas low incubation temperature reduces 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 and Martin, 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 above 40°C, 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 38.4°C 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 activity (Sulik et al., 1988), production of ROS or the risk for malformation during embryogenesis, leading to the 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

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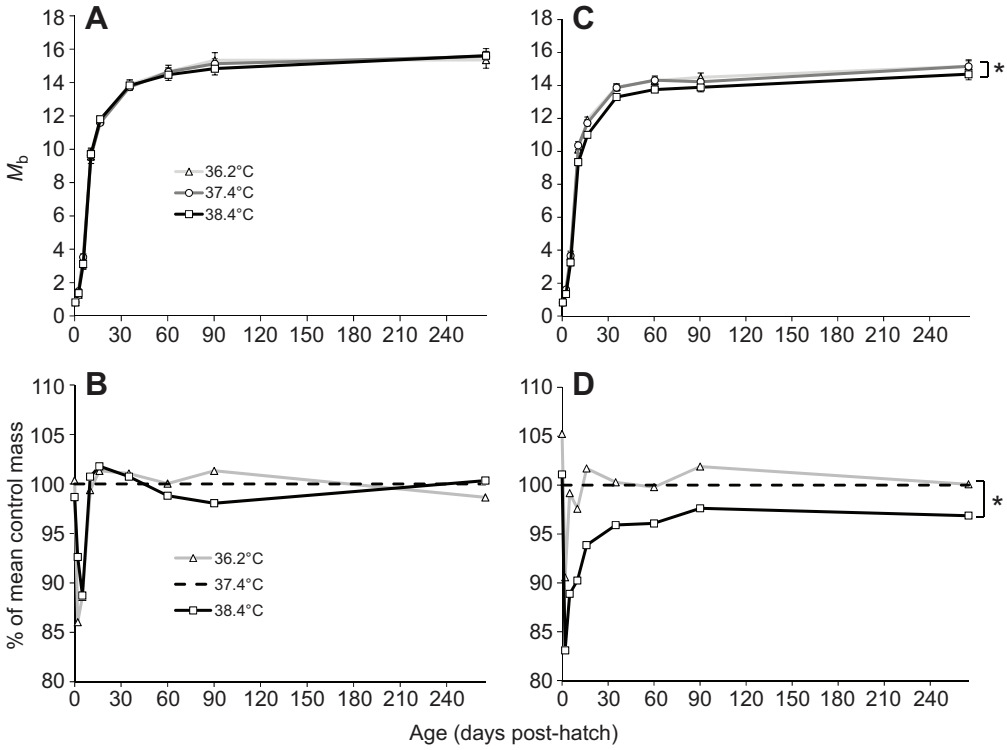

Fig. 3. Changes in body mass of zebra finches hatched from eggs incubated at 36.2, 37.4 or 38.4°C over the course of their lifetime. (A,B) Female birds. (C,D) Male birds. B and D show percentage M_b in the 36.2 and 38.4°C groups compared with the mean M_b in the 37.4°C group. Error bars indicate ±s.e.m. around the mean. Sample size at the end of the experiment (265 dph) was 21, 14, 19 for males and 8, 21, 11 for females incubated at 36.2, 37.4 and 38.4°C, respectively. *P<0.05.

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 with controls that were incubated at a constant temperature of 37.5°C. These results suggest that a cooler temperature increases the energetic demand of embryos during development and requires yolk consumption beyond that of optimal incubation temperature (Olson et al., 2006). Since we measured percentage loss of egg mass 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 36.2°C group used more yolk and hatched with lower residual yolk reserve compared with 37.4 or 38.4°C groups. In fact, young from the 36.2°C 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 in competing for food caused by low energy stores in the first days of life after hatching.

High-temperature males weigh less throughout their lives

Previous studies showed that hatchling M_b is positively correlated with incubation temperature. In precocial wood ducks, hatchling M_b 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 20°C as opposed to constant incubation temperature of 37.5°C reduced M_b on embryonic day 12 without an impact on body size (Olson et al., 2006, 2008). In contrast to the studies above, we did not observe any effect of incubation temperature on hatchling M_b. However, M_b in males from the 38.4°C group was lower after hatching compared with males from the 36.2°C and the effect persisted into their adulthood. This difference in M_b among treatment groups was largely due to the treatment difference in lean mass rather than lean and fat mass combined (H.W., B.K. and S.A.M.-S., unpublished data). As the quantitative magnetic resonance instrument we used to measure lean and fat mass does not detect skeletal or keratin-based structures (Guglielmo et al., 2011), the higher than optimal incubation temperature probably 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 38.4°C had suppressed begging, appetite or competitive ability, which reduced food intake. Whether the reduced M_b is an adaptation to the suboptimal temperature is not known. Further study is needed to test whether the low M_b in the high-temperature group enhances survival, reproductive performance and competitive ability relative to individuals with high M_b in a warm environment. It is worth noting that nestlings from both 36.2 and 38.4°C weighed less in the early nestling period compared with 37.4°C nestlings, regardless of sex (Fig. 3). In the statistical analysis, all birds that died before the juvenile period were excluded. Thus, this reduction in M_b at an early nestling period reflects a tangible and common difference in M_b between the optimal and suboptimal incubation temperature.

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Table 2. Effects of incubation temperature on physiological functions

			Females				Males			
Response	Age	Factor	36.2°C	37.4°C	38.4°C	*P*	36.2°C	37.4°C	38.4°C	*P*
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 rate	Nestling	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
		PMR	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

HPA axis function was analyzed in four ways: baseline corticosterone levels (ng ml^{-1}), integrated adrenocortical stress response to 30 min of handling and restraint (ng ml^{-1} min), and integrated response to exogenous adrenocorticotropic hormone (ACTH) or dexamethasone (Dex) (ng ml^{-1} min). Innate immunity is presented as percentage microbial survival and metabolic rates are presented as $\dot{V}_{O_2}$ (ml min^{-1}) g^{-1}.
[a,b]Different letters for a variable within sex represent significant difference. Values are means±s.e.m. (*N*).

Low-temperature females show a transient increase in adrenocortical response and BMR

In altricial birds, a 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 36.2°C group had a relatively high BMR compared with fledglings hatched from 37.4°C. 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 with intermediate (with highest hatching success and post-hatch survival) and high incubation temperature at 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 36.2°C 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.

Conclusion

In summary, a 1°C increase in incubation temperature reduced hatching success, whereas a 1.2°C decrease in incubation temperature reduced post-hatch survival. Suboptimal incubation temperatures also transiently, but significantly, elevated metabolic rate and adrenocortical responses and permanently reduced M_b in a sex-specific manner. These results therefore demonstrate that deviations as small as 1°C 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 phenotypic variability of offspring, 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.

MATERIALS AND METHODS

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 36×43×42 cm cages with an external nest box (20×13.5×13.5 cm) attached to each cage. The room was kept at 22°C 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, USA). Prior to the start of the experiment, all incubators were calibrated to a single reference thermometer (VWR ASTM thermometer, 34/42°C, ±0.1°C accuracy; cat. no. 61126-943). Throughout the

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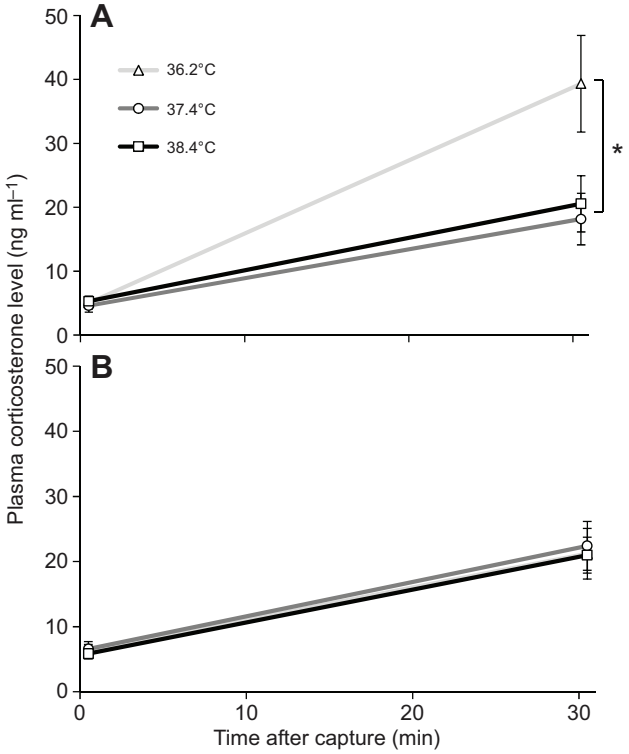

Fig. 4. Adrenocortical responses of zebra finches at 16 days post-hatch. (A) Female birds. (B) Male birds. Error bars indicate ±s.e.m. around the mean. *P<0.05.

experiment, the temperature of each incubator at two positions within the row containing eggs was measured for 90 min weekly using the reference thermometer. Mean (±s.e.m.) temperatures in low, control, and high incubators were 36.20±0.07 (N=7), 37.42±0.08 (N=7) and 38.40±0.07°C (N=7), respectively, with 0.48% mean coefficient variation between the two positions. Relative humidity was set to 55% in all three incubators.

A total of 202 fertilized eggs from 26 breeding pairs was 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 comprising nestlings from all three treatment groups. To minimize mortality unrelated to the

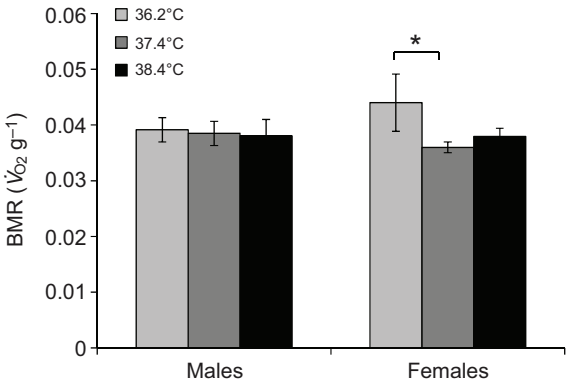

Fig. 5. Basal metabolic rates of zebra finches at 25 days post-hatch. Error bars indicate ±s.e.m. around the mean. *P<0.05.

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 fewer 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 09:30 h and 12:30 h before egg food was provided (see Fig. 6 for the timeline). The final M_b was measured on one day between 09:15 h and 14:15 h when birds were, on average, 266±6.7 dph (±s.d.).

Functioning of the hypothalamic–pituitary–adrenal axis

We measured the functioning of the HPA axis in 4 ways: baseline corticosterone levels (ng ml^{-1}) as well as adrenocortical response to standardized capture and handling stress, ACTH challenge and Dex suppression test (integrated response, ng ml^{-1} 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 09:00 h and 11:05 h, within 3 min 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 min 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 M_b of the individual 1 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^{-1} 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 after 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^{-1} Dex (Sandoz Canada, Dexamethasone sodium phosphate injection USP, 2302; adjusted to M_b taken a day before). Following injection, birds were released into a cage and an additional blood sample was collected 30 min after 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^{-1}. The

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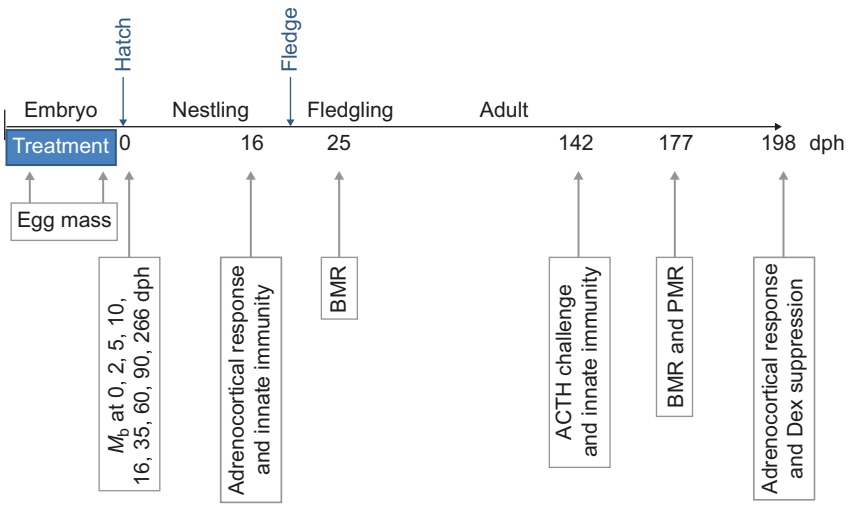

Fig. 6. Timeline of the experiment. Zebra finch eggs were incubated at 36.2, 37.4 or 38.4°C for the entire incubation period. Post-hatch environment was identical among the treatment groups.

interassay variations for high (125 pg tube^{-1}) and low (12.5 pg tube^{-1}) 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, cat. no. 0443E7, MicroBiologics) and *E. coli* (ATTC #8739; Epower Microorganisms, cat. 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. (2013). Briefly, sterile blood samples were collected within 5 min 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 −80°C. For the *C. albicans* killing assay, 1:48 dilution of whole blood to cell medium was used for 16 dph samples while 1:96 dilution was used for adult 142 dph samples. The primary incubation of whole blood, cell medium and *C. albicans* [1×10^5 colony forming units (CFU) ml^{-1}] was 15 min at 30°C. Sample duplicates, sample blank and positive and negative controls were incubated with tryptic soy broth for the second incubation at 30°C. 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, each 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 1×10^4 CFU ml^{-1} working solution, 1:6 dilution of blood to cell medium for both 16 and ∼142 dph, and 30 min primary incubation and 12 h secondary incubation at 37°C.

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 and Guglielmo (2011). The analyzer was calibrated daily with standard air containing 20.9% O_2 and 1.02% CO_2. Starting at 20:00 h, birds were weighed in a paper bag and placed in an airtight stainless-steel chamber inside an incubator. The incubator maintained the ambient temperature at 35°C, which is within a thermoneutral zone for this species (Calder, 1964; Bech et al., 2004). Birds fasted for 3 h. Then the post-absorptive oxygen consumption from the remaining 9 h 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^{-1}. Excurrent air was scrubbed of water vapor and subsampled for CO_2 at a rate of 150 ml min^{-1}, 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 min. After the overnight measuring period, birds were weighed again then returned to the home cage. $\dot{V}_{O_2}$ (ml min^{-1}) 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). The equation we used to calculate $\dot{V}_{O_2}$ uses both fractional concentrations of O_2 and CO_2 [equations 10.6 and 10.7 in Lighton (2008)]. Here, we analyzed mass-corrected $\dot{V}_{O_2}$, where $\dot{V}_{O_2}$ was divided by the M_b of the individuals after the respirometry measurement [$\dot{V}_{O_2}$ (ml min^{-1}) g^{-1}].

PMR was measured ∼26 h after the end of the BMR measurement at ∼177 dph using the same respirometry system. Birds were fasted for approximately 2 h, then placed in a closed flight wheel (16 cm width×24 cm diameter) lined with rubber. The wheel was supplied with constant air of 3.5 liters min^{-1}. The exiting air was subsampled at ∼285 ml min^{-1}. Birds were left undisturbed for 5 min in the wheel covered with a cloth to acclimate. Beginning at 09:45 h and no later than 13:30 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 $\dot{V}_{O_2}$ over a 15 s period corrected for M_b [$\dot{V}_{O_2}$ (ml min^{-1}) g^{-1}].

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 Šidák 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) was analyzed separately.

Body mass

M_b 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 M_b (square-root transformed) over time, was analyzed using repeated-measures ANCOVA with an average of genetic parents' M_b (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 at 16 dph (log and square-root transformed, respectively) were analyzed in MANCOVA with 16 dph M_b as a covariate. Similarly, baseline and integrated stress response collected at 198 dph, integrated ACTH and Dex response at 140 and 198 dph (log and square-root transformed) were analyzed in MANCOVA with 266 dph M_b as a covariate.

Innate constitutive immunity

Pearson correlation of microbicidal capacity against *C. albicans* and *E. coli* at 16 and 142 dph revealed that only microbicidal capacity against *C. albicans* and *E. coli* at 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 at 25 and 177 dph were marginally correlated (P=0.06). Thus BMR on 25 and 177 dph as well as PMR at 177 dph were analyzed together in MANOVA with incubation temperature as a fixed factor, without data reduction. Mean±s.e.m. is presented in the Results section and figures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.W. and S.A.M. designed the study; H.W., B.K., N.A. and S.A.M. carried out the experiment; K.L.S. and K.K.S. analyzed hormone levels. H.W. and S.A.M. performed the statistical analyses. All authors contributed to manuscript preparation and approved the final version of the manuscript for publication.

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