Heat-induced maternal effects shapes avian eggshell traits and embryo response to high temperatures

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Abstract

Populations without a sufficient rate of genetic adaptation may risk extinction in the face of rapid environmental change, however, phenotypic plasticity can facilitate their persistence. For example, mothers can prepare offspring for the thermal environment young will experience through transgenerational plasticity. In oviparous species, whether mothers can prepare offspring to cope with thermal stress experienced as embryos is largely unknown. We demonstrate that when zebra finch mothers are exposed to a heat stress, their offspring show altered heart rates as embryos in response to high incubation temperatures, as well as an increase in eggshell pore density that was positively correlated with survival. These results are the first to show that temperature induced transgenerational plasticity may promote embryonic survival in an oviparous species.

One Sentence Summary

Embryos from heat stressed zebra finch mothers have more eggshell pores and lower heart rates at high incubation temperatures.

Main Text

Thermal biology drives geographical range limits of a species, and those that cannot rapidly adapt or migrate may experience severely diminished populations or extinction in the face of climate change (*1*). Populations with slower growth rates such as those with long generation times risk extinction before genetic adaptation to the new environment can occur (*2*). However, phenotypic plasticity (the ability of one genotype to produce multiple phenotypes) may allow organisms to persist in rapidly changing climates much quicker than genetic adaptation. The environmental conditions experienced by parents can also affect the phenotype of offspring without alteration of DNA. This is known as transgenerational phenotypic plasticity, or maternal effects when mediated by the mother, which can optimize the fitness of offspring for the prevailing environmental conditions (*3*). Most vertebrate studies examining maternal effects observe offspring performance postnatally, thus whether mothers can prepare their offspring to cope with thermal conditions as embryos is unknown.

Although avian embryos develop outside of the mother in an egg, providing a virtually closed environment, prenatal maternal effects play an important role in many species (*4*). Avian embryos are functionally ectotherms, and rely mostly on parental incubation for thermoregulation. Thus, incubation behavior of parents heavily affects incubation temperatures and influences development and survival of embryos (*5*). Embryos of many avian species are able to withstand low ambient temperatures for relatively prolonged periods, as parents must leave the nest to forage, leading to temporary cooling of the egg (*5, 6, 7*). In contrast, most embryos cannot withstand hyperthermic conditions for very long (*6, 7*). Rising incubation temperatures cause an increase in embryonic metabolic demands, resulting in higher oxygen consumption, water loss, and CO_2 build up, eventually leading to desiccation, respiratory or cardiac failure, and death (*8*). A high eggshell gas conductance can ameliorate some of these effects by

increasing the rate by which oxygen diffuses in and CO_2 diffuses out. However, this comes with the trade-off of increasing water loss to potentially fatal levels (*8)*. Small birds such as the zebra finch (*Taeniopygia guttata*) that live in arid regions experiencing high ambient temperatures are particularly vulnerable to such lethal hyperthermia and dehydration (*9, 10*). Currently, whether avian mothers can modify embryonic physiology and/or eggshell traits to allow them to better cope with environmental conditions during incubation is unknown.

Here we used zebra finches to investigate if mothers exposed to heat stress as juveniles and/or adults will adaptively mediate their embryo's physiological responses to high incubation temperatures through maternal effects. As juveniles, half of the birds underwent a mild heat treatment of 38°C over a 28-day period, while the other half was exposed to a constant control temperature of 21–23°C (*11)*. As adults, birds from each juvenile treatment group were exposed to either a high heat stress of 42°C or control temperature (21–23°C) for 3 consecutive days, resulting in four distinct maternal treatment groups (named Control-Control, Control-Heat, Heat-Control, and Heat-Heat) following a 2x2 factorial design. Approximately 3 weeks after the end of the adult treatment, females were again briefly exposed to their respective adult treatment temperature and afterword paired with non-experimental males to breed. Eggs laid by these females were collected and then incubated for the entire duration of embryonic development at either a control temperature of 37.2°C, or a high temperature of 38.5°C, which has been shown to decrease hatching success in this species (*12*).

For avian embryos, the demand for oxygen generally increases as temperatures rise, leading to an increased production of CO_2 as well as higher water loss through diffusion across the eggshell *(8*). We saw this expected pattern, where embryos from juvenile control mothers exhibited more water loss at a higher incubation temperature compared to the control incubation temperature (Fig. 1A) (Linear mixed model (LMM): $t = -2.30$, $P = 0.033$). However, mothers who were exposed to the mild heat treatment as juveniles produced embryos that were more resistant to heat-associated water loss. Embryos from these heat-conditioned mothers did not lose more mass at higher incubation temperatures compared to those at a control temperature. (Fig. 1B) (LMM: $t = -0.512$, $P = 0.616$). This effect cannot be attributed to differential egg size alone, as there was no differences in initial egg mass between the maternal treatment groups (LMM: juvenile: $t = 0.141$, $P = 0.887$; adult: $t = 0.248$, $P = 0.805$; incubation: $t = -0.125$, $P = 0.901$).

At standardized humidity, differences in embryonic water loss can be attributed in part to metabolic rate. Therefore, we investigated how maternal heat exposure shapes the metabolic rate of offspring as embryos. To do this, we measured embryonic heart rate, used as a proxy for metabolic rate, at ~ 30% and ~ 77% of embryonic development. As expected, across all treatment groups there was a negative correlation between the embryonic heart rate and change in egg mass during development (LMM: $t = -2.52$, $P = 0.018$). However, when incubated at the high temperature, embryos from mothers exposed to both heat treatments (Heat-Heat) had a significantly lower heart rates than embryos from all other maternal treatment groups at ~ 77% of embryonic development (Fig. 2) (LMM: all $P < 0.01$). Differences in incubation temperature can also result in changes of embryonic development time and/or differential

hatchling morphology (*12–15*). In such situations, embryos may prioritize development of essential organs such as the brain and heart at the expense of others (*8*). Here we saw no effect of maternal treatment or incubation temperature on embryonic development time (LMM: juvenile: $t = 0.963$, $P = 0.345$; adult: $t = 0.064$, $P = 0.950$; incubation: $t = -0.412$, $P = 0.682$). However, across treatment groups there was a significant negative correlation between development time and embryonic heart rate at ~ 77% of embryonic development (LMM: $t = -2.07$, $P = 0.044$), a pattern similar to that demonstrated in other species (*16, 17*). Interestingly, differential egg mass loss and heart rate patterns between treatment groups did not translate into morphology differences at hatching (*i.e.*, body, pectoralis, heart, or residual yolk mass) (LMM: all $P > 0.3$). This suggests that the lower heart rates of embryos from Heat-Heat mothers at high incubation temperatures were not at the expense of improper growth or morphology, but potentially an adaptive maternal effect.

Differential embryonic water loss may not only be attributed to metabolic rate, but also to eggshell traits. Lower pore density and thicker eggshells decrease gas conductance and therefore water loss. However, such morphology would also limit the rate of diffusion of oxygen and carbon dioxide across the eggshell, potentially decreasing the chance of survival at high temperatures. Therefore, we examined the role eggshell traits played in survival at different incubation temperatures. As expected, we found that successfully hatched individuals had higher number of eggshell pores compared to those that died (LMM: $t = -2.16$, $P = 0.034$) as well as thinner eggshells (LMM: $t = 2.10$, $P = 0.039$). We also saw that even among embryos that died, those with thinner eggshells and more pores tended to survive until further along in embryonic development (Fig. 3A) (LMM: $t = -2.42$, $P = 0.0182$). However, this positive correlation between the number of pores and survival was only found in embryos exposed to the high incubation temperature (Fig. 3B) (LMM: $t = 2.13$, $P = 0.037$), indicating the importance of eggshell characteristics in embryonic survival in this species at high ambient temperatures.

Differences in eggshell pore density and thickness across species ranges due to varying environmental conditions such as temperature, humidity, and altitude, is adaptive change that is thought to occur across many generations (*18, 19*). However, if these eggshell characteristics possessed some degree of plasticity, it could facilitate embryonic survival when faced with rapid change in ambient temperatures in a single generation. As such, we tested whether eggshell characteristics can change due to thermal conditions experienced by the mother. While we found no effect on eggshell thickness, we did find that mothers exposed to both heat treatments (Heat-Heat) laid eggs with higher pore density compared to Control-Control mothers (LMM: $t = 2.21$, $P = 0.035$). There was also an effect of maternal treatment on embryo survival, although not in the manner we anticipated. Among embryos exposed to the high incubation temperature, maternal treatment had no effect on embryo survival. However, at the control incubation temperature, embryos from mothers that experienced the mild heat as juveniles (Heat-Control) had a greater chance of survival than those from mothers with “matching” treatments (e.g. Control-Control, Heat-Heat) (Fig. 4) (COXME: HC-CC: $z = -2.88$, $P = 0.004$; HC-HH: $z = 2.81$, $P = 0.005$). These finding show that mothers have the capacity to modify the embryonic environment through eggshell characteristics in response to the thermal conditions they experience, producing embryos that have a better chance of survival.

Our results suggest that thermal conditions experienced by the mother can act as an anticipatory maternal effect, with the potential to increase embryonic survival when exposed to an otherwise detrimental environment. Such transgenerational plasticity could play a key role in species that live in regions experiencing temperatures close to their upper thermal tolerance, which are thought to be at greater risk of diminished populations as a result of climate change due. Our results also indicate that temperature may elicit an adaptive phenotypic change in offspring prenatally, allowing birds to cope with changes in temperature during a vulnerable period of development, much faster than through changes in allele frequency. Understanding physiological and morphological adjustments of embryos due to maternal effects could improve our ability to predict population responses to climate change and pinpoint species that will be most at risk.

Materials And Methods

Animal diet and husbandry

Experimental females used in this experiment were obtained by breeding zebra finches (*Taeniopygia guttata*) from our captive colony at Auburn University. The 32 pairs were housed in individual cages (38.10 cm Width x 45.72 cm Depth x 45.72 cm Height) where they received ad libitum mixed seeds (Kaytee Supreme (finch), Chilton, WI), water, grit, and cuttlefish bone. A nest box (19.5x14.5x14.5 cm) containing shredded paper covered with irradiated hay (SunSations® Timothy Hay, Vitakraft Sun Seed, Weston, OH) was attached to each individual cage, and pairs were sprayed with water twice per day in order to encourage the onset of breeding behavior. On the day eggs hatched, the down feathers of the birds were trimmed into recognizable patterns to allow for individual identification. Every day each pair received one tablespoon of egg mixture (hard-boiled chicken egg, white bread, and cornmeal) to supplement their diet until the oldest nestling reached 5 days post-hatch (DPH). From that point on, pairs were then given two tablespoons per day until the youngest nestling was 35 DPH and had reached nutritional independence. Pairs were also given a vitamin supplement (Wild Harvest, Blacksburg, VA) and chopped spinach once per week. Upon reaching 10 DPH, nestlings were temporarily removed from the nest to be weighed and given unique ID bands. Juveniles were separated from their parents at 39–43 DPH and kept with other juveniles until the start of treatment.

Juvenile treatment

A total of 48 females underwent the juvenile conditioning treatment, following a protocol that has been previously established (*11*) and used in our laboratory (*20*). Females were semi-randomly assigned to either the control or mild heat treatment group, balancing within nest and hatch order. At 42–45 DPH, half of the juvenile females began the mild heat treatment and were exposed to a temperature of 38°C on every other day for 28 days as previously described (*20*). This temperature of 38°C can be considered a mild heat stressor because although it is still within the thermoneutral zone for the species, it is near the upper critical temperature (*21*). For this species, body temperature begins to increase at ambient temperatures higher than 30°C, leading to mild hyperthermia (*21*). This increase in body temperature

limits water loss by creating a heat gradient allowing for better heat dissipation, and may initiate stress responses such as glucocorticoid secretion and heat shock protein synthesis (*22, 23*) without increasing oxidative damage (*11*). The other half of the females were used as a control group, and were placed into brooders in the same manner as the mild heat group, but instead set to room temperature at 21–23° C.

Adult treatment

At 170–180 DPH, half of the females from each juvenile stage treatment were exposed to a high heat stress treatment of 42° C for 3 hours a day, for 3 consecutive days as previously described (*11, 20*). This temperature is above the upper critical temperature of the thermoneutral zone and has been shown to cause oxidative damage in this species (*11, 21*). The remaining females from each of the juvenile stage treatment groups served as the adult-stage control group, and were exposed to room temperature (21–23° C) in the same manner as above. This 2 x 2 factorial design resulted in four maternal treatment groups that were the different combinations of juvenile and adult treatment (Control-Control, Control-Heat, Heat-Control, and Heat-Heat). The females were also weighed the day before, and on the final day of the adult treatment following the last treatment bout.

Female pairing

Females were paired with non-experimental males and on the day of pairing, females first underwent a single 3-hour bout of their respective adult-stage treatment (control or high heat) as described above. Pairs were housed in the same breeding conditions and received the same diet as previously described.

Egg collection and incubation treatment

After pairing, nests were checked for eggs daily at approximately 1000 hrs. Once laid, eggs were removed from the nest and marked with a unique ID number using a fine tip permanent marker. The first egg laid in the clutch was replaced with a false egg made of modeling clay (Crayola Model Magic (white), Easton, PA) after removal, while any subsequent eggs removed were not replaced. The first overall egg laid was randomly assigned to an incubation treatment (a control or high incubation temperature), and following that the first laid egg of each nest was assigned a treatment group in an alternating manner to balance treatment allocation among the first eggs of the clutch. After the first egg, treatment group assignments alternated within each clutch to balance laying order and nest of origin between incubation temperature treatments.

Newly laid eggs were transported to the lab where they were weighed and then placed into one of two incubator treatments, representing a control or high incubation temperature. Those assigned to the control group went into an incubator (Brinsea Octagon 20 Advance EX incubator, Titusville, FL) set to 37.2° C for the entirety of embryonic development. This temperature was chosen as it is very close to the average incubation temperature of captive zebra finches (*24*) and was found to be optimal when considering both hatching success and post-hatch survival (*12*). Individuals assigned to the high group were placed into an incubator set to a temperature of 38.5° C, which is slightly above the range of incubation temperatures for wild and laboratory kept zebra finches (*24*), for the entire incubation period.

In this species, an incubation temperature of 38.4° C can result in lower hatching success overall and in lower overall and lean body mass in male offspring during their postnatal growth period (*12, 13)* and therefore considered outside the thermal optimum. Both incubators were set to a relative humidity of 55%. Incubators were calibrated to the desired temperature using a reference thermometer placed level with the eggs. Approximately three times per week, incubators were taken off of their rockers and allowed to sit level for at least one hour before reading the thermometer to determine temperature accuracy. The average measured temperature in control and high temperature incubators were 37.13°C ± 0.512 and 38.45°C ± 0.419, respectively.

Heart rate measurements

Embryonic heart rates were measured using the Buddy® digital heart rate monitor (Avian Biotech, Vetronic Services, Abbotskerswell, Devon, UK). This is a non-invasive method which quantifies heart rate by the amount of infrared light absorbed by the pulsing embryonic blood. The Buddy® monitor has been used in a variety of studies on oviparous embryos, often examining the effects of incubation temperature on reptilian embryos (*25, 26, 27*). It has also been used in thermal manipulation studies in poultry and turkey embryos as well as studies on other avian species using a variety of stimuli (*see 28*). More recently, a thorough investigation using the Buddy® system has been done on wild zebra finch embryos (*28*).

Heart rate measurements were recorded at two time points during an individual's embryonic development, between 1400 and 1700 during the day to account for any circadian variation. The first measurement was taken at embryonic day (ED) 4, which is at ~ 28.9% of development for embryos in the control temperature incubator and ~ 29.4% of development in the high temperature incubator. The second measurements were taken at ED 11 for embryos in the control incubator and at ED 10 for embryos in the high incubator, which is ~ 79.6% and ~ 73.5% of development respectively.

When measuring heart rates, eggs were individually removed and placed horizontally (pointed tip facing digital display) on the measuring platform of the monitor before closing the lid. A timer was started as soon as the egg was removed from the incubator, and the amount of time (s) that the embryo had been out of the incubator at each heart rate (bpm) measurement was recorded. This value was used in the analysis to account for the decrease in heart rate when eggs were removed from the incubator and began to cool. Heart rates were recorded every 10 seconds after the first recording until the egg had been outside of the incubator for ~ 70 seconds. If reliable heart rates could not be picked up by the monitor by the time the egg had been out of the incubator for ~ 40 seconds, the egg was flipped 180° on the platform. Measurements were considered reliable when the output values were consistent and continuous, with the "heart icon" on the digital display blinking. Eggs did not remain inside the monitor for longer than ~ 90 seconds. For day 4 measurements, eggs were immediately placed back into their respective incubator after heart rates had been recorded. For day 10/11 measurements, each egg was weighed after recording the heart rate before being returned to incubators. Egg did not remain outside of the incubators for longer than ~ 240 seconds total while recording heart rate measurements and egg mass. The heart rate measurement taken between the 50 and 60 second mark was used in statistical analyses. The order in

which eggs were removed from the incubator for measurements was also recorded to be taken into account in the analysis. When no heart rate signal could be found using the monitor, eggs were candled using a flashlight to determine infertility or mortality. If an egg was found to have a crack due to handling or collisions inside the incubator, the heart rate was not measured and that individual was not used in survival analysis. When an embryo was found to be dead and had no cracks in the shell, the developmental stage (very early, early, mid, late, very late, pipping) at which mortality occurred was estimated and recorded for later use.

Organ measurements

All incubators were checked a minimum of three times daily for potential hatchlings. When a hatchling was found, it was removed from the incubator, weighed, and then euthanized via isoflurane inhalation as per approved protocol. Dissected tissues included the brain, liver, residual yolk sac, heart, and pectoralis muscle, with the latter four being weighed prior to freezing. After removal, all dissected tissues were then flash frozen in liquid nitrogen before being placed in a -80° C freezer, while the carcass was placed in a freezer at -20° C for later use.

Eggshell measurements

Pore density was determined using a protocol refined for songbird eggs by Stein and Badyaev with some modification (*29*). Briefly, shell pieces of each egg were placed in a biopsy case. Shells were incubated in hot NaOH for 3 minutes to remove membrane. Shells were rinsed in ddH2O, then dried in an incubator set to 41°C. Once dry, thickness of the shell was measured using a micrometer (Mitutoyo, cat number 395-371-30, 0.001mm resolution) to the nearest 0.001mm. Shells were then incubated in 2.5% nitric acid for 12 seconds, followed by a rinse in ddH2O. The shells were dried once again in an incubator set to 41°C. Once dry, number of pores were counted using a Nikon Eclipse microscope. One researcher measured all the thickness and another researcher counted all the pores. Both were blind to the treatments of the eggshells.

Statistical analyses

Statistical analysis was done using RStudio (*30*) utilizing the packages “nlme” and “coxme”. Statistical significance was determined as $P \leq 0.05$, and all models included the maternal ID as a random effect to account for multiple eggs originating from the same mother. Predictors that were believed to have plausible biological effect on some aspect of the model were included regardless of significance.

Egg mass and heart rate

Linear mixed-effects models (*LMM*) were used to analyze effects on all egg mass, heart rate, and hatchling mass variables. To test the effect of maternal treatment on initial egg mass, we used a model with juvenile treatment, adult treatment, and lay order as fixed factors, as well as a juvenile*adult treatment interaction term. Incubation treatment was not included in this egg mass analysis, as the initial egg mass was taken before being placed in the incubator. Change in egg mass over the first ~ 77% of

embryonic incubation was analyzed using a model with a categorical “dummy coded” treatment variable representing the four combinations of juvenile and incubation treatment as a fixed factor, as well as the embryonic heart rates at both ~ 30% and ~ 77% of embryonic development included as fixed factors. The dummy coded variable represents the four distinct maternal treatment groups resulting from the 2x2 factorial design (CC, CH, HC, HH), and allows for easy pairwise comparisons. The relationship between the change in egg mass and heart rate at ~ 77% of embryonic development was also determined using a model which included juvenile, adult, and incubation treatment as fixed factors, as well as the two and three way interaction terms. The effect of maternal and incubation treatments on embryonic heart rates at ~ 77% development was analyzed using juvenile, adult, and incubation treatment, as well as the two and three way interaction terms, and mass change as fixed factors. The relationship between incubation time and heart rate at ~ 77% embryonic development was determined using a model which included juvenile, adult, and incubation treatment, as well as the two and three way interaction terms, and heart rate as a fixed factors.

Organ mass

The effect of maternal and incubation treatments on body, pectoralis, and heart mass of hatchlings was analyzed using *LMMs* which included juvenile, adult, and incubation treatment as fixed effects, their two and three way interaction terms, initial egg mass, and embryonic heart rates at both ~ 30% and ~ 77% of embryonic development as fixed factors. The model examining the effects of treatments on hatchling residual yolk mass included juvenile, adult, and incubation treatment, their two and three way interaction terms, as well as initial egg mass as a fixed factors.

Eggshell characteristics

We used *LMMs* for all models to determine the effect of maternal and incubation treatments on eggshell characteristics. The difference in eggshell pore density between embryos that lived and died was analyzed using a model which included juvenile, adult, and incubation treatment, as well as the two and three way interaction terms, survival, and egg mass as fixed factors. The difference in average eggshell thickness between embryos that lived and died was analyzed using a model with survival and lay order as fixed factors. To determine the relationship between eggshell thickness and stage of embryonic mortality, we again used a *LMM* with mortality time and egg mass as fixed factors. Pore density in relation to embryonic mortality time and incubation temperature was analyzed using a model with mortality time, incubation treatment, and egg mass as fixed factors, as well as a mortality time*incubation treatment interaction term. We analyzed the effect of maternal treatment on egg pore density using a model with treatment and egg mass as fixed factors.

Embryo survival

The effect of maternal and incubation treatment on embryo survival was analyzed using a Cox proportional hazard model, with the term full treatment – a variable that is the maternal juvenile, maternal adult, and incubation treatments “dummy coded” together – and egg mass as fixed factors. The dummy

coded variable represents the eight distinct treatment groups resulting from the combinations of maternal juvenile, maternal adult, and incubation treatments.

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Declarations

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

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Author contributions

AJH and HW designed the study, AJH, LD, and HW carried out the experiment and collected data, AJH and HW performed statistical analyses and prepared the manuscript.

Competing Interests

The authors declare no competing interests.

Data and materials availability

All data is available in the main text or the supplementary materials.

Supplementary Materials:

Figure S1

Figures

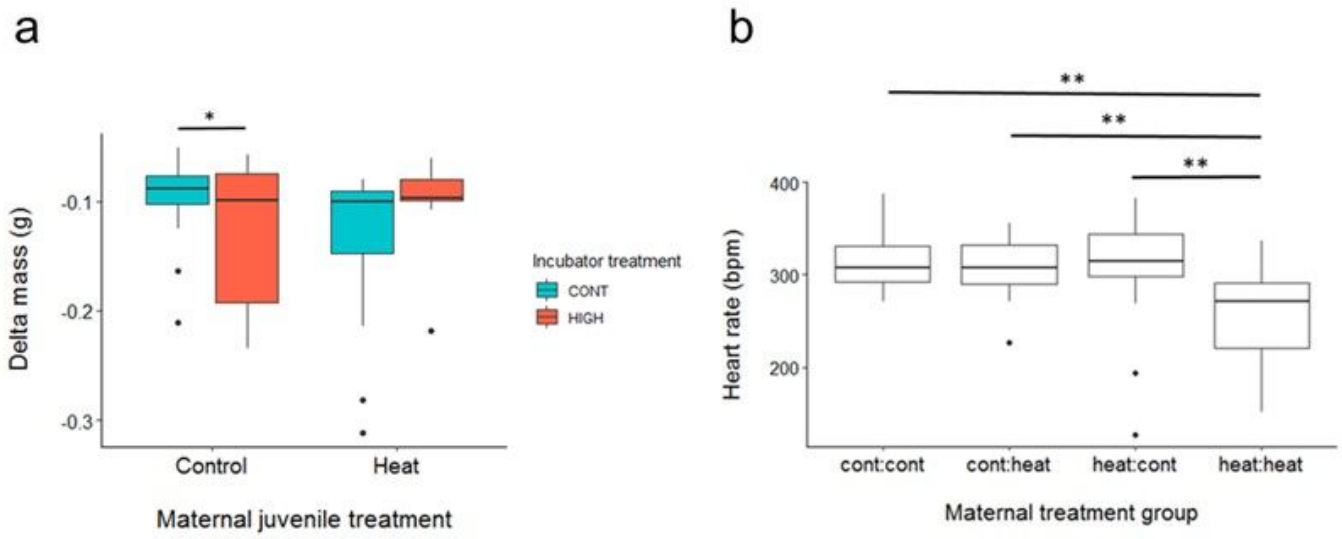

Figure 1

At high temperatures embryos showed differences in egg mass change and heart rate when produced by mothers exposed to a prior heat stress (significance indicated as follows for all graphs *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.). a, The change in egg mass (g) occurring from the day the egg was laid until approximately 77% of the incubation period. The x-axis denotes the treatment mothers received as juveniles, either a control (22°C) or mild heat (38°C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2°C) or high incubation temperature (38.5°C). b, The heart rates of embryos at the high incubation temperature (38.5°C) at approximately 77% of the incubation period. For each group on the x-axis, the first word denotes the treatment the mothers received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat).

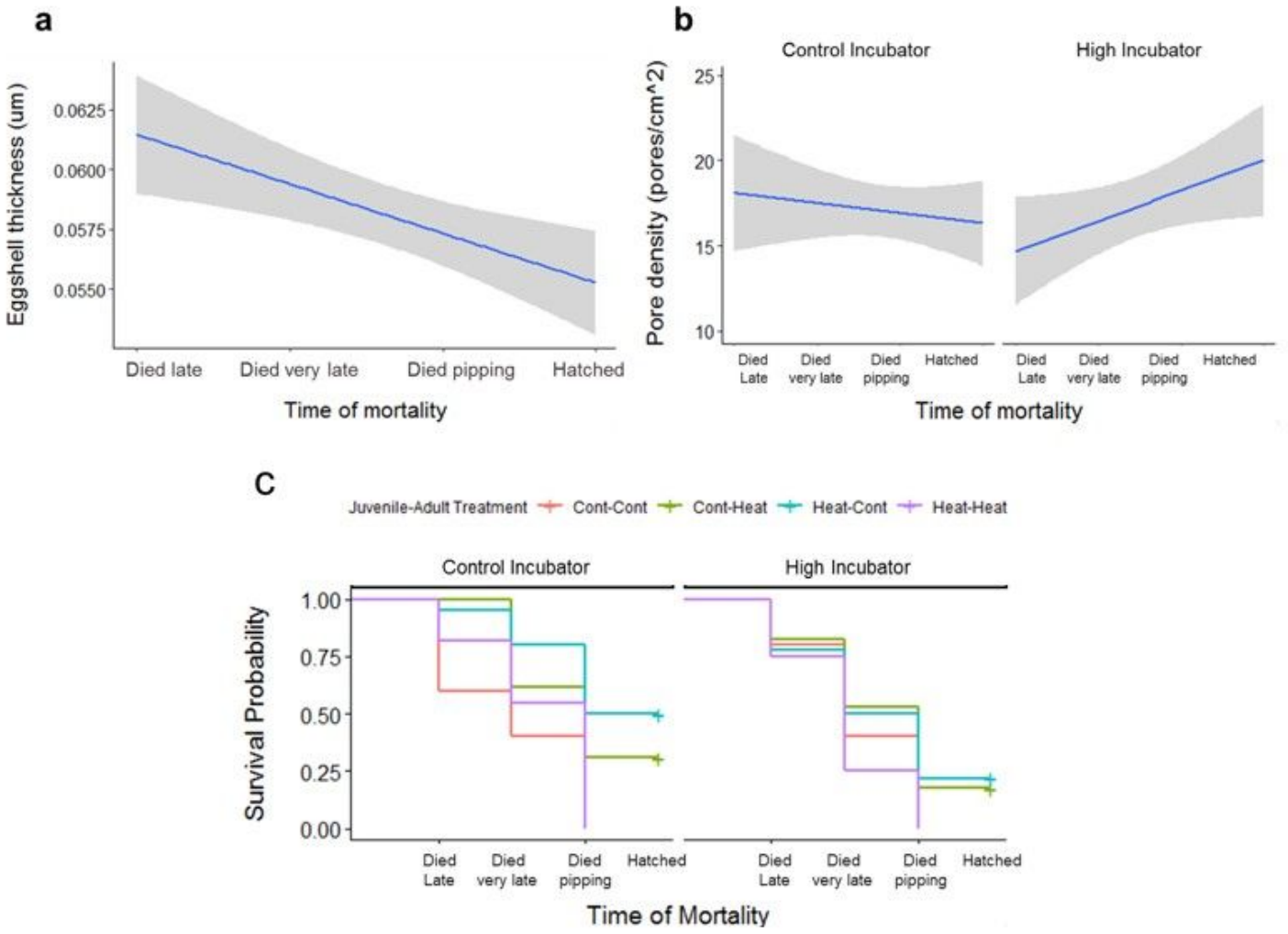

Figure 2

Embryonic survival in relation to eggshell thickness, pore density, and maternal treatment. a, The relationship between eggshell thickness and embryonic survival. b, The relationship between pore density and embryonic survival at the two incubation temperatures. The blue lines represent the regression line and shaded areas show 95% confidence intervals. c, Survival probabilities of embryos at different points in embryonic development, grouped by the respective treatments received by their mothers. The left side of the graph shows embryonic survival at the control incubation temperature (37.2°C) while the right shows survival at the high incubation temperature (38.5°C). In the legend, the first word denotes the treatment the mothers received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat).

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