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Effects of the temperature during embryonic development on adult reproduction and the phenotype of the second generation in zebra finches

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ABSTRACT

Across taxa, the temperature experienced by individuals early in life can have large effects on their development. However, comparatively little is known about whether the effects of this thermal developmental environment can be long-lasting or transgenerational. In birds, one important aspect of the developmental environment is incubation and, in general, eggs incubated at low temperatures produce offspring with smaller morphology, suboptimal physiology, and even lower long-term survival. Yet, little is known about whether incubation temperature may affect avian reproduction in adulthood, and nothing is known about whether the effects of avian incubation temperature may be transgenerational. To investigate this, we incubated zebra finch (*Taeniopygia guttata*) eggs at two different temperatures: 37.5 °C ('control') and 36.3 °C ('low'), raised nestlings until adulthood, and allowed same-temperature treatment pairs to reproduce. We found that F_1 individuals incubated at the low temperature had shorter beaks at the start of reproduction than those incubated at the control temperature. Further, compared to those from control parents, F_2 offspring from parents incubated at the low temperature had lighter body masses at 5 days-old and had shorter beaks at 30 days-old. However, we found little evidence that incubation temperature affected other aspects of reproduction, with no effect on latency to lay, clutch size, egg mass, incubation period, hatching success/asynchrony, fledging, or the number of offspring that ultimately survived until independence. Overall, we found some evidence that a difference in the early thermal developmental environment can have lasting morphological effects into the next generation. However, future work is needed to determine whether the incubation temperature that birds experience as embryos may influence parental care behaviors or lifetime reproductive success.

1. Introduction

Across taxa, subtle modifications of the developmental environment can have large consequences on offspring morphology, physiology, behavior and, more generally, fitness (Dixon et al., 2016; Du and Shine, 2022; Lindström, 1999; Monaghan, 2008; Mousseau and Fox, 1998; Williams, 1994). However, although many studies have investigated the direct effects of developmental conditions on early-life phenotypes, comparably fewer have investigated the long-term effects on these offspring during adulthood (Moore et al., 2019). Even less is known about whether and how these effects may translate into the next generation by, for example, affecting the ability of these offspring to survive and reproduce, with subsequent consequences for their offspring (Yin et al., 2019).

In birds, one crucial aspect of the developmental environment is incubation temperature (Clutton-Brock, 1991). Incubation temperature is regulated by parental behavior and parents must maintain their eggs within a small temperature range in order to ensure proper development and hatching (Deeming and Ferguson, 1991). However, incubation is energetically costly and time consuming (Nord and Williams, 2015; Tinbergen and Williams, 2002; Vleck, 1981) and varies among parents due to intrinsic factors (e.g., age, clutch size, parental experience, body condition) and extrinsic factors (e.g., ambient temperature and anthropogenic disturbances; Aldrich and Raveling, 1983; Coe et al., 2015; Conway and Martin, 2000; Haftorn and Reinertsen, 1985; Hope et al., 2020a, 2022a; Verhulst et al., 2001; Williams et al., 2021). This causes incubation temperature to vary both among and within nests (Boulton and Cassey, 2012; Coe et al., 2015; Hope et al., 2021). This is important because even small decreases (<1 °C) in average avian incubation temperature may result in offspring with suboptimal post-hatch phenotypes (reviewed in DuRant et al., 2013; Hepp et al., 2015; Hope et al., 2021). For example, lower incubation temperatures

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produce offspring with smaller body masses, weaker thermoregulatory abilities, weaker immune function, higher basal metabolic rates, reduced locomotor performance, and even lower long-term survival (Berntsen and Bech, 2016; DuRant et al., 2012a, 2012b, 2010; Hepp and Kennamer, 2012; Hopkins et al., 2011; Nord and Giroud, 2020; Nord and Nilsson, 2011; Olson et al., 2006; Ospina et al., 2018; Wada et al., 2015; but see Nord and Nilsson, 2016; Vedder et al., 2022, where no long-term effect on survival was found and Berntsen and Bech, 2021; Wada et al., 2015, where no or mixed effects on body mass and metabolic rate were found).

Although there is evidence that incubation temperature affects fitness-related offspring phenotypic traits, and even survival, almost nothing is known about how the avian embryonic thermal environment may have subsequent long-term effects on another central aspect of fitness—reproduction in adulthood. To date, only two studies have investigated how incubation temperature affects reproductive success in birds. The first study found that wood ducks (*Aix sponsa*) that had been artificially incubated at a lower temperature and then released into the wild were less likely to be recruited into the breeding population and less likely to have a successful nest (i.e., at least one duckling hatched) than those that had been incubated at a warmer temperature (Hepp and Kennamer, 2012). However, few birds were recruited into the breeding population in this study (i.e., only one individual from the low temperature treatment). The second study found that the temperature at which Japanese quail (*Coturnix japonica*) were incubated as eggs had no effect on any aspect of reproduction measured (onset of reproduction, egg laying rate, egg mass; Vedder et al., 2022). However, no study has investigated potential effects of developmental temperature on reproductive success in altricial birds and no study to date has investigated whether avian incubation temperature may have effects that last until the F_2 generation. Understanding the long-term and transgenerational effects of incubation temperature in birds is crucial for understanding the implications of environmental changes and parental care decisions on population dynamics, life history strategies, and evolution (Badyaev and Uller, 2009).

There are mulitple lines of evidence that suggest that incubation temperature might affect F_1 reproduction and F_2 phenotype. First, in multiple avian species, incubation temperature affects long-term survival (Berntsen and Bech, 2016; Hepp and Kennamer, 2012; but see Nord and Nilsson, 2016; Vedder et al., 2022) and telomere length (Hope et al., 2023; Stier et al., 2020), which can be an indicator of longevity (Wilbourn et al., 2018). Although the directionalities of the effects in these studies vary, any difference in anticipated survival could theoretically lead to altered investment in the current reproductive event (Clutton-Brock, 1984; Stearns, 1992). Second, there is evidence that incubation temperature can have long-term effects on adult body mass (Nord and Nilsson, 2016; Wada et al., 2015; but see Berntsen and Bech, 2021; Vedder et al., 2022, where no effect was found), which could influence the energy available for reproduction. Third, one study found that zebra finches incubated at a lower temperature had a greater accumulation of oxidative damage in adulthood compared to those incubated at higher temperatures (Berntsen and Bech, 2021). Because it has been experimentally shown in birds that increased oxidative stress alters reproductive investment (i.e., longer latency to lay, reduced clutch size; Costantini et al., 2015), incubation temperature may also alter reproductive investment. In turn, any direct effects that incubation temperature has on the F_1 generation may then lead to differences in F_2 phenotype. For example, if incubation temperature affects reproductive investment, either physiologically (e.g., egg yolk composition) or behaviorally (e.g., nestling food provisioning), this altered quality of care could impact the development and growth of the F_2 generation. Further, if the mechanism by which incubation temperature affects F_1 phenotype is through epigenetic changes, this could then be inherited by offspring (Dunislawska et al., 2022).

In this study, we investigated whether incubation temperature affected avian reproductive success and offspring phenotype of the next generation. To do this, we incubated zebra finch (*Taeniopygia guttata*) eggs at two different temperatures: 37.5 °C (i.e., 'control'), which is optimal for zebra finches (Berntsen and Bech, 2016; Wada et al., 2015) and 36.3 °C (i.e., 'low'), which is within the natural range of zebra finch incubation temperatures, but is likely suboptimal (Berntsen and Bech, 2016; Wada et al., 2015). When these individuals reached adulthood, we created same-incubation temperature treatment pairs and allowed pairs to reproduce. To comprehensively monitor reproduction, we measured the latency to lay the first egg, clutch size, egg mass, incubation period, hatching success and asynchrony, the latency to fledge, and the number of offspring that survived until Day 30. Further, we measured body mass and size (tarsus, beak, and wing length) of both F_1 and F_2 birds throughout development to determine any potential effects of incubation temperature on morphology.

2. Methods

2.1. Study species and study subjects

Zebra finches (*T. guttata*) are passerines that breed opportunistically and can reproduce at ~3 months of age (White, 2007). Both sexes build the nest, and the female lays one egg per day, with clutch sizes ranging from 1 to 9 eggs, although most clutch sizes are between 4 and 6 eggs (Haywood, 1993). At clutch completion, both the male and female alternate to incubate the eggs, and the incubation period lasts 11–15 days (Zann, 1996). Both sexes feed nestlings, which fledge from the nest after ~20 days (White, 2007), and offspring reach nutritional independence at ~30 days of age (White, 2007). Birds are sexually dimorphic in their plumage coloration but not in body size or mass (White, 2007).

Birds that bred in this study (F_1 generation: $N = 21$ males and 21 females) were adult offspring raised from a breeding colony of zebra finches (F_0 generation: $N = 23$ pairs which, at the time of breeding, were naïve to all forms of experimental intervention) housed at the Centre d'Etudes Biologiques de Chizé. To raise these individuals, we artificially incubated eggs at one of two temperatures, raised hatchlings using foster parents, and maintained individuals until they were at breeding age. This F_1 generation produced a total of 56 F_2 birds, 54 of which survived until they were at least 30 days-old. All procedures were approved by the French national ethics committee for animal experimentation under file number APAFIS#23727–2020011311559318.

2.2. F_1 egg incubation treatments

We checked nests of F_0 pairs for new eggs once per day. Once a new egg was found, we marked it with a unique ID and immediately placed it in an incubator. We randomly assigned the incubation treatment to the first laid egg of each breeding pair (F_0), and then systematically alternated among temperature treatments for each subsequent egg derived from that breeding pair. We replaced eggs taken from F_0 nests with clay eggs, so that pairs would continue incubating until they were given newly hatched nestlings.

We incubated eggs using Brinsea© (Weston-super-Mare, North Somerset, UK) Ovation 28 incubators and using similar methods to Wada et al. (2015) and Hope et al. (2022b). We used two incubation temperature treatments, which were within the natural range for wild zebra finches (i.e., 34.9–38.5°C; Zann and Rossetto, 1991): the 'control' temperature was set at a constant 37.5 °C and the 'low' temperature was set at a constant 36.3 °C. Both incubators were set at a humidity of 55%. We considered the control temperature of 37.5 °C to be optimal and the low temperature of 36.3 °C to be suboptimal based on previous studies that have used similar temperatures to investigate the effects of incubation temperature on zebra finches (Berntsen and Bech, 2016; Wada et al., 2015). We verified the temperature and humidity by placing iButton© (Hygrochron DS1923, Maxim Integrated™, San Jose, CA, USA) temperature loggers inside of each incubator (average

temperatures ± SD: control = 37.54 ± 0.13, low = 36.28 ± 0.11 °C; average humidity ± SD: control = 53.74 ± 3.34, low = 54.68 ± 4.27%).

Once an egg hatched, we noted hatch date in order to calculate incubation period per nestling (difference between hatch date and incubation start date) before placing the nestling in its foster nest (and replacing one clay egg). As much as possible, nestlings placed in a foster nest together did not have the same biological parents: of the birds in this study, 26 birds were raised in nests without any biological siblings; 10 birds were raised in a nest with one biological sibling; and 6 birds were raised in a nest with one biological sibling and one non-sibling. Foster parents were chosen based on which parents had been incubating clay eggs for a sufficient amount of time (i.e., at least 9 days) and, thus, would accept nestlings. Nestlings were never more than two days apart in age, which is within the range of hatching asynchrony in wild nests (Zann and Rossetto, 1991). Logistically, because the incubation period for F_1 control and low eggs differed (see *Section 3.1*), this means that similar-aged chicks in the same foster nest sometimes hatched from eggs that were laid, and thus placed in the incubators, on different days.

Eggs were incubated in two rounds of reproduction as part of other coinciding experiments (e.g., Hope et al., 2022b), and to achieve a larger sample size for the current study. In the first round of reproduction, in addition to the two temperature treatments described above, we also had a third 'variable temperature' treatment (S. Hope, unpublished data), which is not used in the current study. In the second round of reproduction, we only used the two temperature treatments described above; these methods are also described in Hope et al. (2022b). Each foster nest contained a maximum of one nestling from each treatment (i.e., up to 3 chicks in a nest for the first round and up to 2 chicks for the second round), except one nest that contained two low treatment individuals. Further, in the first round, chicks hatched in their respective incubators, while in the second round, chicks hatched in a hatcher (37.5 °C and 67% humidity) that they were placed in 1 day before their expected hatch date (Hope et al., 2022b). For both rounds of reproduction, we used the same breeding colony (i.e., F_0), and we used the same two incubators for the control and low treatments, programmed at the same temperatures. For control and low treatments, respectively, hatching success was 25.9% and 35.3% in the first round of reproduction and 25.9% and 23.9% in the second round of reproduction. Although these hatching successes are low, they are still within the range found in other studies that artificially incubate zebra finch eggs (Criscuolo et al., 2011; Von Engelhardt et al., 2006; von Engelhardt et al., 2004; Winter et al., 2013). Additionally, although keeping incubators consistent throughout a study is a standard practice for studies such as this (e.g., DuRant et al., 2010; Hepp et al., 2006; Wada et al., 2015), it is important to acknowledge that incubators are confounded with the temperature treatment. Individuals in this study that were produced in the two rounds of reproduction were evenly distributed among treatments (control: 9 individuals from the first round and 11 from the second round; low: 9 first round and 13 second round).

2.3. F_1 nestling rearing

F_1 nestlings were raised by foster parents (F_0) in individual indoor cages (47.5 x 38 × 51 cm) until they no longer needed to be fed by their foster parents (i.e., nutritional independence; 30 days-old), and we measured body mass on Days 5, 10, and 30. Between 30 days of age and two months before pair formation, we housed birds communally in either indoor (24 birds) or outdoor (18 birds) aviaries. Of nestlings that hatched, 63.3% of control and 48.9% of low birds survived until the time of this study (binomial GLM: $X^2 = 1.98, p = 0.16$). To prevent birds from breeding before the start of the experiment, we removed any eggs that were laid in the aviaries every 2 days. Two months before pair formation, we transferred all birds in this study to the same indoor aviary (3 x 1 × 2 m), to be housed communally (mixed-sex) and reacclimate to the same environmental conditions. The indoor experimental room was kept at a constant 22 °C and the photoperiod was set to a 14:10 day:night cycle.

2.4. Pair formation

After F_1 offspring reached adulthood, we formed same-treatment pairs (N = 10 control and 11 low pairs). We assured that pairs were never biological siblings nor foster siblings (i.e., raised by the same foster parents, even if during a different reproductive bout). Further, we avoided creating pairs with large age differences (i.e., >3 months difference, considering that reproductive maturity takes 3 months). The mean age difference of pairs in this study was 37 ± 35 [SD] days. After accounting for these factors, when a female could be paired equally well with multiple males, we chose her male partner randomly (i.e., random number generator). Eleven control males were excluded randomly from this experiment because there were not enough females to pair them with. At the time of pair formation, birds were at least 143 days-old and, on average, were 241 (control females: 261 ± 59 [SD]; low females: 259 ± 72; control males: 244 ± 67; low males: 227 ± 67) days-old. The number of nestmates that each individual was raised with (i.e., between 0 and 2) was equally distributed between treatment groups. Just before pair formation, we weighed all birds with an electronic scale (±0.01 g) and measured tarsus (±0.01 mm, using a digital caliper), wing (±1 mm, using a wing ruler), and beak length (±0.01 mm, using a digital caliper). All birds were inexperienced breeders (i.e., no experience with incubation or chick rearing). We transferred each pair from their communal housing into an individual cage on April 7, 2021. Birds were allowed to acclimate to their cage and partner for one week without a nest box, and then nest boxes were installed on April 14, 2021. Birds were allowed to reproduce until they had at least one nestling that reached Day 30, or until July 16, 2021 (i.e., 3 months after nest box installation).

2.5. General husbandry during breeding

Pairs were housed indoors in cages (47.5 x 38 × 51 cm) with external nest boxes (12 x 13 × 16 cm), all in the same room. Ambient temperature was kept at a constant 22 °C and the photoperiod was set to a 14:10 day:night cycle. We provided birds with ~7 g of alfalfa hay every day, and then ~0.5 g of coconut fiber once a nest had been built with a cup formation. To stimulate reproduction and nest building, we added 5 pieces of hay and ~1 g of coconut fiber to the interior of each nest box at installation and, further, misted pairs with water once per day until their first egg was laid. We provided birds with *ad libitum* food, water supplemented with vitamins, cuttlefish bone, and grit. We also gave birds ~2g of chopped hard-boiled eggs (including shells) every day from couple formation until nestling Day 30, along with endives and millet sprays once per week. Breeding/rearing conditions were consistent across all generations (i.e., F_0, F_1, F_2).

2.6. Nest monitoring of F_1 reproduction

We checked nest boxes every day starting at 10:00. For each nest, we noted the latency for the female to lay her first egg (i.e., number of days between pair formation and first egg laid). Once the first egg was laid in the nest box, we weighed the egg to the nearest 0.01 g, and marked it with its lay-order. We repeated this process every day that a new egg was found, and noted clutch size once the last egg was laid. Incubation start date was defined as the day that the last egg was laid for clutches that were <5 eggs, and as the day that the fourth egg was laid for clutches of ≥5 eggs (Zann and Rossetto, 1991). Nine days after the last egg was laid, we began checking daily at 10:00 for hatching. We noted the date when the first egg hatched (Day 0), and calculated the incubation period as the number of days from the incubation start date until the first hatched nestling. We then noted the number of nestlings that had hatched each day until all nestlings hatched, which we used to calculate hatching asynchrony (i.e., the number of days between the first hatched and last hatched nestling). If no eggs had hatched after 20 days of incubation, or

the parents had clearly abandoned, we removed all eggs, and restarted adding hay or coconut fiber. Thus, some pairs had multiple nesting attempts (e.g., multiple values for clutch size, etc.) but, since we ceased experimentation (i.e., birds released back into communal aviaries) after a given pair had one successful nesting attempt (i.e., at least one nestling living until Day 30) we only have data for one successful reproductive bout per pair.

2.7. Offspring (F_2 generation) measurements

On Day 5, we weighed all nestlings (±0.01 g) and individually marked them by removing distinct patches of down feathers (Adam et al., 2014). To minimize disturbance to nests during the early stages (and thus, the risk of abandonment), we did not mark nestlings before Day 5. Therefore, we could not match individual egg mass to nestling mass. Subsequently, all 'Days' refer to the number of days since the first chick hatched, and not the age of each individual nestling. On Day 10, we weighed all nestlings and banded them with individually-numbered metal bands. Nestling measurements on Days 5 and 10 were conducted starting at 10:00 and were always done before food supplements (i.e., hard-boiled egg, endive, millet sprays) were given.

On Day 14, we began checking twice per day (at 10:00 and 17:00) for fledging, and noted the first day that at least one nestling was out of the nest (hereafter, 'latency to fledge'). From Day 14 until all nestlings had fledged, we did not disturb the nest boxes. Once all nestlings had fledged, we removed the nest box.

On Day 30, starting at 14:00, we weighed each bird (±0.01 g using an electronic scale) and measured tarsus (±0.01 mm, using a digital caliper), wing (±1 mm, using a wing ruler), and beak length (±0.01 mm, using a digital caliper).

2.8. Statistical analyses

We conducted all statistical analyses using R v 3.5.1 (R Core Team, 2022). We built models using the package *lme4* (Bates et al., 2015) for mixed effects models and the base package for simple linear models. We ensured that all models following a Gaussian distribution met the assumptions of normal and homoscedastic residuals using normal quantile and predicted vs. residual plots. We verified that models met the assumption of non-multicollinearity by investigating the variance inflation factors (*vif*); all VIFs were <3. For discrete variables that did not follow a normal distribution, we used either a Poisson (base R) or negative binomial distribution (*MASS* package; Venables and Ripley, 2002), and chose between the two distributions by investigating predicted vs. residual plots. We investigated pairwise comparisons using *emmeans* (Lenth, 2018). For models in which there was a significant effect of incubation temperature, we report effect size as partial eta squared (η_p^2) for linear models using the *eta_squared* function of the *effectsize* package (Ben-Shachar et al., 2020) and Cohen's *d* for pairwise comparisons using the *eff_size* function of the *emmeans* package. We interpret a small effect as $\eta_p^2 = 0.01$ or $d = 0.2$, a medium effect as $\eta_p^2 = 0.06$ or $d = 0.5$, and a large effect as $\eta_p^2 = 0.14$ or $d = 0.8$. For significant terms, we also report post-hoc power using the *pwr* package (Champely, 2020), with an $\alpha = 0.05$, effect sizes that we calculated (above), true sample sizes, and the appropriate function depending on the test statistic of each model. All random effects mentioned refer to random intercepts. We initially included sex as a covariate in all models for which it was possible, but there was never a significant effect (all models $p > 0.14$), so we excluded it for simplicity and to avoid overparameterization. All terms are reported with ± SE, except the variance explained by random effects, which is reported with ± SD.

2.8.1. Effects of F_1 incubation temperature on development and phenotype of F_1 birds

First, we investigated whether the development and phenotypes of the F_1 generation (i.e., the parents in this study) differed as a function of their incubation temperature using six linear mixed effects models, with the dependent variables of incubation period, body mass during nestling development (Days 5, 10, and 30), and body mass, tarsus length, wing length, and beak length at the start of reproduction. Incubation temperature treatment was the independent variable in all models. For the model investigating body mass throughout development, day (5, 10, or 30) and its interaction with incubation temperature were also included as independent variables. For this model, we are missing data from two low individuals because they were not a part of the previous study in which body mass was monitored throughout development; however, these individuals are included in all other analyses. For the models investigating tarsus length, wing length, and beak length at reproduction, body mass at the start of reproduction and its interaction with incubation temperature were also included to test for a potential difference in body size/mass ratio between birds incubated at different temperatures. All models included the identity of the biological parents (i.e., F_0 generation) as a random effect. The identity of the foster parents was also originally included in all models except the model for incubation period (i.e., before foster parents were introduced); however, this random effect explained 0% of the variance in all models besides the model investigating tarsus length, and was thus only retained in this one model.

2.8.2. Effects of F_1 incubation temperature on F_1 egg-laying

To investigate whether incubation temperature affected aspects of egg-laying during the reproduction of the F_1 generation, we built three models. All three models included incubation temperature as the independent variable. The first model was a general linear model with a negative binomial distribution with the latency to lay the first egg (i.e., number of days from pair formation until first egg laid) as the dependent variable. The next model was a general linear model with a Poisson distribution, with clutch size as the dependent variable. The third model was a linear mixed effects model with egg mass as the dependent variable; this model included 'pair' as a random effect because there were multiple eggs per nest. For all three of these models, only the first attempt at reproduction for each pair, even if ultimately unsuccessful, was included, to avoid overrepresentation of pairs that had multiple nesting attempts.

2.8.3. Effects of F_1 incubation temperature on F_2 hatching

Next, to investigate whether the incubation temperature treatment of the F_1 generation affected aspects of hatching of F_2 embryos, we built three models. The first two models were general linear models with Poisson distributions, with the dependent variables of incubation period (i.e., number of days from the incubation start date until the first hatched nestling) and hatching asynchrony (i.e., number of days between the first hatched and last hatched nestlings). The third model was a general linear model with a binomial error distribution (binary regression for proportion data) with hatching success as the dependent variable (success = number of eggs in a clutch that hatched; fail = number of eggs unhatched). In these three models, only successful nests (i.e., at least one egg hatched) were included.

2.8.4. Effects of F_1 incubation temperature on F_2 post-hatch phenotype and survival

To investigate whether the incubation temperature treatment of the F_1 parents affected the body mass of their F_2 offspring, we built one linear mixed effects model. The independent variables were incubation temperature, Day (5, 10, 30), and their interaction. The dependent variable was the mean body mass of the offspring in each nest, which was log-transformed to meet model assumptions. We chose to investigate mean body mass because we did not know the exact age of each nestling (i.e., 'Day' is calculated from the day the first nestling in each nest hatched, and does not necessarily reflect the age of later-hatched siblings). Importantly, mean body mass is a measure that is relevant to the parents, because it indicates how heavy their nestlings are a set

number of days (5, 10, and 30) after their first offspring hatched. To further correct for differences of true chick age within nests, we also included hatching asynchrony (i.e., number of days between the first hatched and last hatched nestlings) as a covariate in this model. Only successful nests (i.e., at least one offspring survived until Day 30) were included in the analysis. Each nestling was measured three times (i.e., on Days 5, 10, and 30), meaning that each nest had 3 measures of mean body mass, and thus we included the random effect of 'pair' to account for repeated measures. No offspring died between Days 5 and 30, and thus all individuals were included in the calculations at all timepoints.

Then, to investigate whether the incubation temperature treatment of the parents affected F_2 offspring body size at Day 30, we built three linear mixed effects models with tarsus length, wing length, and beak length as the dependent variables. The independent variables were incubation temperature, offspring body mass, and their interaction. We included body mass to test for a potential difference in body size/mass ratio between offspring raised by parents that had been incubated at different temperatures. Body mass was scaled and centered to reduce

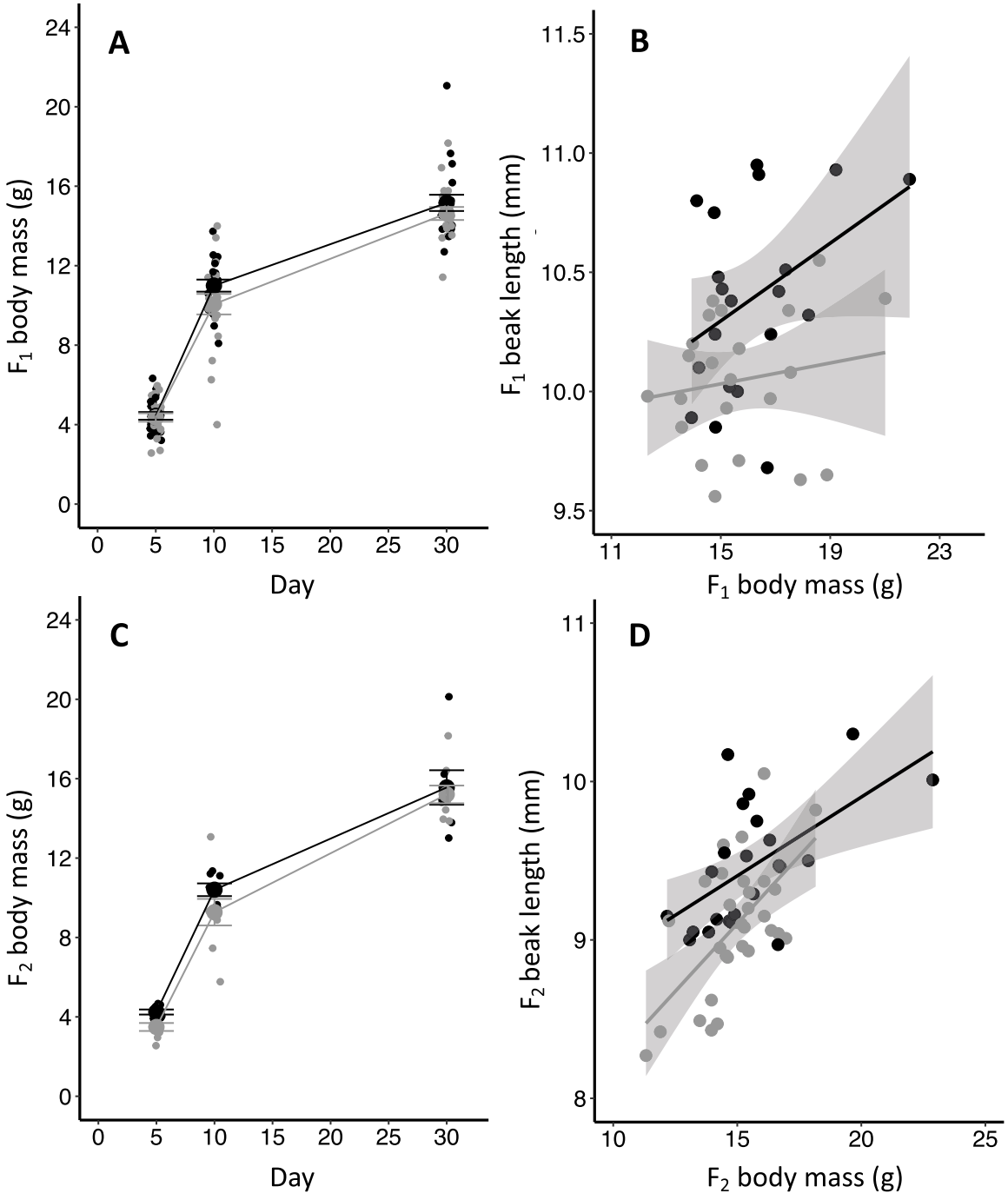

Fig. 1. The effects of incubation temperature (grey = low; black = control) on F_1 (A,B) and F_2 (C,D) body mass (g; A,C) and beak length (mm; B,D). Body mass (A,C) was measured when individuals were 5, 10, and 30 days-old; large points and error bars represent mean ± SE. Beak length (B,D) is plotted as a function of body mass; the grey area around regression lines indicates a 95% CI. Small points in Panel C represent mean body mass of F_2 offspring in each nest (see *Statistical analyses*). In all other panels, small data points represent individuals.

multicollinearity. 'Pair' was included as a random effect to account for similarities among siblings. We conducted this analysis at the individual level, and not the mean nest level, because at ~ Day 30 zebra finches are fully grown and a 1–2 day difference in age would not affect our results. Only successful nests (i.e., at least one offspring survived until Day 30) were included in the analysis.

Lastly, to investigate whether the incubation temperature treatment of the F_1 generation affected F_2 fledging and survival, we built two general linear models with Poisson distributions with incubation temperature as the independent variable. The dependent variable of the first model was the latency to fledge (i.e., the number of days between the day that the first egg hatched and the first nestling fledged), and the dependent variable of the second model was the number of offspring that survived until Day 30. Only successful nests (i.e., at least one offspring survived until Day 30) were included in the analyses.

3. Results

3.1. F_1 incubation period, mass, and body size

The incubation treatment of the F_1 generation (i.e., the parents in this study; $N_{control} = 20$, $N_{low} = 22$) affected their rate of embryonic development (i.e., incubation period), but not body mass. As expected, eggs incubated at the low temperature took longer to hatch than those incubated at the control temperature (η^2_p [95% CI] = 0.77 [0.66–1.00]; power = 0.99; $X^2 = 130.7$, $p < 0.001$; mean ± SE: control = 13.7 ± 0.1 days; low = 15.2 ± 0.1 days; random effects – biological parents: variance ± SD = 0.024 ± 0.15; residual: variance = 0.17 ± 0.42). However, body mass of F_1 individuals did not differ between incubation temperature treatments either throughout development (incubation temperature: $X^2 < 0.01$, $p = 0.99$; day: $X^2 = 817.0$, $p < 0.001$; interaction: $X^2 = 2.54$, $p = 0.28$; random effects – biological parents: variance = 1.30 ± 1.14; residual: variance = 1.43 ± 1.20; Fig. 1A) or at the start of reproduction (incubation temperature: $X^2 = 0.29$, $p = 0.59$; random effects – biological parents: variance = 4.81 ± 2.19; residual: variance = 1.25 ± 1.12). F_1 parents weighed, on average 15.9 ± 0.3 [SE] g at the start of reproduction.

Incubation temperature treatment had an effect on aspects of F_1 body size at the start of reproduction. There was evidence for an interactive effect of incubation temperature and body mass on tarsus length (interaction: $\eta^2_p = 0.13$ [0.00–1.00]; power = 0.13; $X^2 = 4.50$, $p = 0.034$; main effects - incubation temperature: $\eta^2_p = 0.03$ [0.00–1.00]; $X^2 = 0.75$, $p = 0.39$; mass: $\eta^2_p = 0.49$ [0.26–1.00]; $X^2 = 25.31$, $p < 0.001$; random effects – biological parents: variance = 0.10 ± 0.31; foster parents: variance = 0.01 ± 0.10; residual: variance = 0.16 ± 0.40), where body mass and tarsus length were positively correlated, but the correlation was greater in control individuals compared to low individuals. Further, birds incubated at the control temperature had longer beaks than those from the low temperature (incubation temperature: $\eta^2_p = 0.40$ [0.17–1.00]; power = 0.74; $X^2 = 18.53$; $p < 0.001$; random effects – biological parents: variance = 0.09 ± 0.30; residual: variance = 0.05 ± 0.22; Fig. 1B), with a trend for an interaction with body mass (interaction: $\eta^2_p = 0.08$ [0.00–1.00]; $X^2 = 3.26$, $p = 0.071$; mass: $\eta^2_p = 0.01$ [0.00–1.00]; $X^2 = 1.98$, $p = 0.16$; Fig. 1B). However, there was no effect of incubation temperature on wing length (main effects-incubation temperature: $X^2 = 0.11$, $p = 0.74$; mass: $X^2 = 2.47$, $p = 0.12$; interaction: $X^2 = 1.73$, $p = 0.19$; random effects – biological parents: variance = 0.68 ± 0.82; residual: variance = 2.19 ± 1.48). F_1 birds had, on average, a wing length of 58.5 ± 0.30 mm.

3.2. General nest success of F_1 adults

The probability of raising at least one nestling until Day 30 did not differ between F_1 pairs that were incubated at different temperatures as eggs. Out of 21 pairs (10 control and 11 low), there were 16 pairs (7 control and 9 low) that successfully raised at least one nestling until Day 30 (test of proportions: $X^2 = 0.40$, $p = 0.53$). All pairs at least started nest-building; however, there were two nests (1 control and 1 low) that never laid an egg in the nest box (effect of treatment on the probability to lay at least one egg was not significant; $X^2 = 0.01$, $p = 0.94$). Further, there were 5 pairs (1 control and 4 low) that failed at their first nesting attempt at the egg-laying/incubation stage ($X^2 = 2.0$, $p = 0.16$) and, thus, the second time that they laid eggs was counted as their second nesting attempt. Of these, 4 pairs (1 control and 3 low) succeeded during their second attempt ($X^2 = 0.31$, $p = 0.58$). There was one nest (control) where the female died during early incubation of her first nest, for reasons unrelated to the experiment, and the male abandoned the eggs and, thus, a second nesting attempt was impossible. There were 2 nestlings (out of 24 control and 32 low) from 2 nests (1 control and 1 low) that died between hatch and Day 5. The probability of a nestling dying between hatch and Day 5 did not differ between treatments ($X^2 = 0.04$, $p = 0.84$). No offspring died between Day 5 and Day 30.

3.3. Egg-laying of F_1 adults

We found no evidence that incubation temperature affected egg-laying of the first clutch of F_1 adults ($N_{control} = 9$, $N_{low} = 10$). The latency to lay the first egg was, on average, 39.7 ± 3.8 days and was not related to incubation temperature ($X^2 = 0.68$, $p = 0.41$). Further, females laid, on average, 4.1 ± 0.27 eggs, but clutch size was not related to incubation temperature ($X^2 = 0.45$, $p = 0.50$). Lastly, females laid eggs that weighed, on average, 1.20 ± 0.01 g, but egg mass was not related to incubation temperature ($X^2 = 0.98$, $N_{control} = 45$ eggs, $N_{low} = 33$, $p = 0.32$; random effects – nest ID: variance = 0.005 ± 0.07; residual: variance = 0.003 ± 0.05).

3.4. Hatching of F_2 offspring

We found no evidence that the incubation temperature experienced by the F_1 parents affected the hatching of their F_2 offspring (nests in which at least one nestling hatched: $N_{control} = 7$, $N_{low} = 9$). Offspring took, on average, 11.8 ± 0.45 days to hatch, but incubation period was not related to incubation temperature ($X^2 = 0.85$, $p = 0.36$). Further, there was, on average, 1.4 ± 0.2 days of hatching asynchrony within clutches, but hatching asynchrony was not related to incubation temperature ($X^2 = 0.07$, $p = 0.79$). Lastly, hatching success was 83.1 ± 6.3 %, on average, in nests in which at least one nestling hatched, and hatching success was not related to incubation temperature ($X^2 = 0.38$, $p = 0.54$).

3.5. Mass and body size of F_2 offspring

We found evidence that the average mass of the F_2 generation ($N_{control} = 7$, $N_{low} = 9$ nests) was affected by the incubation treatment of their parents, and that this effect was dependent on offspring age. There was a significant interaction between incubation temperature and day on mean nestling body mass (interaction – 'incubation temperature x day': $X^2 = 8.77$, $p = 0.012$; main effects - incubation temperature: $X^2 = 7.34$, $p = 0.007$; day: $X^2 = 753.4$, $p < 0.001$; hatching asynchrony: $X^2 = 1.40$, $p = 0.24$; random effects – nest ID: variance = 0.013 ± 0.11; residual: variance = 0.008 ± 0.09). Pairwise comparisons revealed that control nestlings weighed significantly more than low nestlings on Day 5 ($p = 0.013$; Cohen's d ± SE = 2.2 ± 0.9; power = 0.98; Fig. 1C), but that average body mass only tended to be different between temperature treatments on Day 10 ($p = 0.099$; $d = 1.4 \pm 0.8$; Fig. 1D), and the difference was no longer significant on Day 30 ($p = 0.89$; $d = 0.1 \pm 0.8$; Fig. 1D).

We also found a body mass-dependent effect of incubation temperature treatment on beak length of individual nestlings at Day 30 ($N_{control} = 23$, $N_{low} = 31$ offspring), but no evidence of an effect of treatment on tarsus or wing length. There was a significant interactive effect of body mass and incubation temperature treatment on beak length (interaction

– 'incubation temperature x body mass': η_p^2 [95% CI] = 0.08 [0.00–1.00]; power = 0.09; $X^2 = 4.27$, $p = 0.039$; main effects - incubation temperature: $\eta_p^2 = 0.20$ [0.00–1.00], $X^2 = 3.47$, $p = 0.063$; mass: $\eta_p^2 = 0.36$ [0.19–1.00], $X^2 = 5.36$, $p = 0.021$; random effects – nest ID: variance = 0.056 ± 0.24; residual: variance = 0.06 ± 0.25) where, in nestlings with small body masses, control nestlings had longer beaks than low nestlings; however, in nestlings with large body masses, beak length converged between temperature treatments (Fig. 1D). Although body mass was positively related to both tarsus length and wing length, there was no relationship with incubation temperature (tarsus length: interaction - incubation temperature X body mass: $X^2 = 1.33$, $p = 0.25$; main effects - incubation temperature: $X^2 = 0.012$, $p = 0.91$; mass: $X^2 = 39.2$, $p < 0.001$; random effects – nest ID: variance = 0.059 ± 0.24; residual: variance = 0.15 ± 0.38; wing length: interaction - incubation temperature X body mass: $X^2 = 0.54$, $p = 0.46$; main effects - incubation temperature: $X^2 = 2.38$, $p = 0.12$; mass: $X^2 = 19.25$, $p < 0.001$; random effects – nest ID: variance = 0.065 ± 0.25; residual: variance = 0.81 ± 0.90). On average, F_2 individuals had a tarsus length of 15.2 ± 0.09 mm and a wing length of 58.8 ± 0.15 mm.

3.6. Fledging and survival of F_2 offspring

We found no evidence that incubation temperature was related to either the latency for the first nestling to fledge or nestling survival ($N_{control} = 7$, $N_{low} = 9$ nests). F_2 nestlings fledged after, on average, 19.1 ± 0.34 days, and there was no significant difference between treatments ($X^2 = 0.26$, $p = 0.61$). Further, F_1 pairs produced an average of 3.4 ± 0.3 nestlings that lived until Day 30, however, the number of offspring at Day 30 was not related to incubation temperature treatment ($X^2 = 0.03$, $p = 0.86$).

4. Discussion

In this study, we aimed to determine whether the temperature that birds experience as embryos affects their reproductive performance in adulthood and/or has transgenerational effects on the phenotypes of their offspring. Overall, we found evidence that, compared to those incubated at the control temperature, F_1 embryos that were incubated at the low temperature experienced a slower embryonic development, had shorter beaks in adulthood, and produced F_2 offspring with lighter body masses on Day 5 and smaller beaks on Day 30 (although beak length differences were greater in individuals with low body masses; Fig. 1D). However, we found no evidence that incubation temperature affected F_1 reproduction or F_2 survival, suggesting that long-term fitness consequences are likely minimal. To our knowledge, this is the first study to investigate whether avian incubation temperature has long-lasting effects on the F_2 generation post-hatch. However, because a study like this requires complex and long-term experimental designs in which obtaining large sample sizes is difficult, our study had small sample sizes, which led to reduced statistical power in some models; thus, more work is needed to corroborate our results.

4.1. Does low incubation temperature lead to suboptimal or adaptive effects?

Our results may illustrate yet another negative consequence of suboptimal incubation in birds. Other studies on zebra finches and other bird species show that low incubation temperatures are related to suboptimal phenotypes and lower survival (Berntsen and Bech, 2016; DuRant et al., 2010, 2012a, 2012b; Hepp and Kennamer, 2012; Hopkins et al., 2011; Nord and Nilsson, 2011; Olson et al., 2006; Ospina et al., 2018; Wada et al., 2015). In line with this, we found that F_2 offspring from low pairs had lighter body masses on Day 5 than those from control pairs. A smaller body mass, especially during development, is likely suboptimal because it is typically related to lower survival (Naef-Daenzer and Grüebler, 2016; Ronget et al., 2018). Thus, it is likely that a small body mass, especially on Day 5 when nestlings are vulnerable to starvation and hypothermia, would be disadvantageous.

We also found that, compared to the controls, F_1 individuals incubated at the low temperature had shorter beaks and produced offspring with shorter beaks on Day 30. These results also agree with other studies that found low incubation temperatures lead to smaller beaks (Babacanoğlu and Güler, 2018; Hope et al., 2020b; Prince et al., 1969). In zebra finches, variation in beak size is important for foraging, singing, and sexual signaling (al-Mosleh et al., 2021; Collins and Ten Cate, 1996; Goller et al., 2004), although how a longer beak might affect these different aspects is unclear. However, in the context of temperature, it is possible that the relationship that we found between beak length and temperature could be adaptive. One function of avian beaks is thermoregulation, particularly heat dissipation, and thus a smaller beak would lead to more heat retention than a larger beak (Tattersall et al., 2017). Thus, if being incubated at a low temperature indicates that ambient temperatures are low, it may be advantageous for a bird developing in a cold environment to have a smaller beak, and even be an example of "environmental matching" (Monaghan, 2008). Interestingly, our results agree with Allen's rule (Allen, 1877), which is the observation that endotherms in colder climates tend to have shorter appendages than those in warmer environments, possibly to reduce heat dissipation. However, in our study, it is interesting that individuals of the F_2 generation also had smaller beaks if their parents had been incubated at the low temperature, considering that the ambient temperature during F_2 development was consistent across treatments. This may explain why the effect was weaker in the F_2 compared to the F_1 generation (Fig. 1B, D).

Although we found effects of incubation temperature on F_1 and F_2 phenotype, in our study, all F_2 fledglings survived until Day 30 and body mass between treatments tended to converge on Day 30, suggesting that there may be no long-term effects on F_2 survival. Nevertheless, in this study, all birds were kept in controlled captive conditions with food and water *ad libitum* and this may have hidden a potential effect of our experimental treatment on survival. To further investigate the consequences of low incubation temperature for the survival of the next generation, future studies that would be complementary to ours might investigate whether transgenerational effects persist either in the wild or in challenging captive conditions.

4.2. What is the mechanism by which incubation temperature affects F_1 and F_2 phenotypes?

Although many studies have now found direct effects of incubation temperature on avian phenotype (reviewed in DuRant et al., 2013), the mechanism by which incubation temperature may cause effects during the development of the F_1 generation is still unclear. For example, incubation temperature may affect the functioning of endocrine axes (e.g., the Hypothalamus-Pituitary-Adrenal axis (reviewed in DuRant et al., 2013; Henriksen et al., 2011; Rubin et al., 2020), metabolic mechanisms (Hope et al., 2022b; Page et al., 2022; Stier et al., 2022), or modify the ontogeny of neurologic functions (Amiel et al., 2017; Bertin et al., 2018), which may then lead to phenotypic differences post-hatch. In our study, we also cannot rule out the possibility that there was selective mortality in each temperature treatment. Because overall hatch success was low in the F_1 generation and not all hatchlings survived until reproduction, it is possible that, for example, embryos and/or nestlings that inherited genes for large body sizes died before reproduction at low temperatures and those that inherited genes for small body sizes died before reproduction at the control temperature. We investigated this in a *post-hoc* analysis by building four general linear models with binomial distributions with either F_1 hatching success (yes or no) or survival until reproduction (yes or no) as the dependent variable and the interactive effect of incubation temperature with parent (mean of F_0 male and female) body mass or beak size as independent variables. However, we did not find any main or interactive effects of incubation temperature (all

incubation main effects and interactions: $p > 0.15$), suggesting that the differences in beak length that we found in the F_1 generation at reproduction were not due to differential survival between incubation treatments. This provides some evidence that our results may not be due to a difference in selection pressure between incubation treatments, but instead represent a plastic response to developmental temperature.

There are multiple mechanisms by which the incubation of F_1 birds could have led to differences in mass and beak length in F_2 offspring. First, F_2 offspring may have simply inherited phenotypes from their parents. For example, incubation temperature could have led to epigenetic changes in F_1 birds (Dunislawska et al., 2022), and their offspring could have inherited these changes. Although these could explain differences in F_2 beak length, which were similar in F_1 and F_2 generations, it would not necessarily explain differences in F_2 body mass on Day 5, considering that F_1 body mass did not differ as a function of incubation temperature. Instead, it is possible that body mass differences in the F_2 generation could be generated by either 1) differences in parental care provided by the F_1 generation (e.g., food provisioning) or 2) differential allocation of nutrients/hormones to the egg. Again, it is unclear what might underlie any potential differences in parental behavior, such as food provisioning, between F_1 incubation treatments, but differences in glucocorticoid hormones (DuRant et al., 2010; Rubin et al., 2020; Wada et al., 2015) or metabolic mechanisms (Hope et al., 2022b; Page et al., 2022; Stier et al., 2022) are potential mechanisms. Future work should examine these potential mechanisms, as well as investigate potential behavioral mechanisms, such as differences in incubation behavior (e.g., consistency) and food provisioning throughout the nestling stage.

4.3. Conclusions

Here, we investigated whether avian incubation temperature affects reproduction in adulthood and, for the first time, whether it can have effects that last until the F_2 generation post-hatch. We did not find any evidence for an effect of incubation temperature on F_1 egg-laying, F_2 hatching, or F_2 survival. There were no ultimate differences in reproductive output (i.e., number of offspring at independence) between treatments, and we thus found little evidence that incubation temperature has a long-term effect on zebra finch fitness. However, we did find some effects of incubation temperature on beak length in both F_1 and F_2 generations, and body mass in the F_2 generation on Day 5, suggesting that small changes to the thermal developmental environment can have effects that span generations. However, our study had a limited sample size due to the logistical difficulties of multi-generational studies. Thus, more experimental work and long-term studies are needed to determine how incubation temperature may affect lifetime reproductive success and reproductive output under different environmental contexts. Further, more work is needed to investigate the underlying behavioral (e.g., parental incubation and provisioning behaviors) and physiological (e.g., endocrine and metabolic axes, brain functions) mechanisms that underlie the transgenerational effects of incubation temperature.

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CRediT authorship contribution statement

Sydney F. Hope: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Frédéric Angelier:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

No competing interests declared.

Data availability

The data that support the findings of this study can be found on Figshare: doi: 10.6084/m9.figshare.22335301.

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