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Plasticity in Female Incubation Behaviour Mitigates Effects of Experimentally Increased Nestbox Temperature on House Wren Nestling Growth and Survival

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PLASTICITY IN FEMALE INCUBATION BEHAVIOUR MITIGATES EFFECTS OF

EXPERIMENTALLY INCREASED NESTBOX TEMPERATURE ON

HOUSE WREN NESTLING GROWTH AND SURVIVAL

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43 Pages

In recent years, temperatures have increased globally, and by different amounts locally. As local climates change and temperatures increase, nestlings from a variety of bird species are exposed to the threat of increased oxidative stress, producing adverse effects on growth and survival of offspring. One defence against oxidative stress is to increase the dietary intake of antioxidants. I tested the hypothesis that experimental heating of house wren nests during the incubation period leads to increased oxidative stress in offspring. I predicted that experimental heating of nests would lead to decreased growth and survival of young, but that these negative effects would be ameliorated by dietary supplements of the antioxidant vitamin E, found naturally in the invertebrate prey with which parents provision their young. I employed a split-brood design in which I experimentally manipulated nest temperature of entire broods, while simultaneously providing dietary supplements of vitamin E to half of the nestlings within broods. To control for the possibility that experimental heating of nests might also influence maternal incubation behavior, thereby potentially confounding any treatment effects, I also recorded female incubation effort. There was a significant interaction between nestbox heating treatment and vitamin E treatment in their effect on nestling mass, a trait that is positively correlated with survival and future reproductive success. Vitamin E supplementation promoted increased

nestling mass in heated nests, whereas it had the opposite effect in control nests, but these effects were weak. Heating significantly affected female incubation behaviour, with females in heated nestboxes investing less in incubation than those in unheated boxes. These results suggest that within specific limits, effects of climate change on nestling development in cavity-nesting birds can be mitigated by adjustments in female incubation behaviour.

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CONTENTS

CHAPTER I: INTRODUCTION AND METHODS

Introduction

All birds are oviparous, and there is a narrow range of incubation temperatures that optimize embryo development within the egg (Haftorn 1988). For eggs to be maintained at the appropriate temperature, which, on average, is 33.8 ℃ for all Passeriformes (Huggins 1941) and ≈35.0 ℃ for my study species, the house wren (*Troglodytes aedon*) (Tieleman et al. 2004), females must alter their incubation behaviour based on environmental conditions, especially temperature fluctuations (Coe et al. 2015). Among all North American passerines, those that reside in cooler environments tend, not surprisingly, to spend less time off the nest and, thus, devote more time to incubation than those in warmer environments (Conway & Martin 2000a). Heating of eggs by incubating parents requires an input of valuable resources, such as time and energy, which in different thermal environments can lead to different trade-offs (Stearns 1989) with a variety of life-history traits (Reid et al. 2002).

There is mounting evidence that in recent years temperatures have increased globally (Hansen et al. 2006, Kraaijenbrink et al. 2017), and by varying amounts depending on locality (Gordo & Sanz 2005). Locations across North America and Eurasia have experienced an increase in spring temperatures (Groisman et al. 1994, Menzel & Fabian 1999, Piao et al. 2008, Caignard et al. 2017), and birds have responded to spring warming by laying earlier (Dunn & Winkler 1999, Both et al. 2004, Nussey et al. 2005, Charmantier et al. 2008, Visser et al. 2009). Earlier breeding has led to a decline in numbers in some populations because of a mismatch between timing of migration, onset of breeding, and prey availability (Saino et al. 1999, Thomas et al. 2001, Jones & Cresswell 2010). Across all species and geographic locations surveyed in

1

North America, arrival dates of migratory bird species have shifted, on average, 0.8 days earlier for every 1 °C of warming (Hurlbert & Liang 2012).

Temperature change has influenced not only the onset of breeding dates, but also other aspects of reproduction (Pendlebury et al. 2004, Conradie et al. 2019). Although the rise in spring temperatures is typically small, even small changes in outside ambient temperatures affect temperatures within the nest environment (Maziarz et al. 2017), and this can have important effects on the development of embryos and nestlings. When nestbox temperature was experimentally increased by only 1 °C during the incubation period, prothonotary warblers (*Protonotaria citrea*) had reduced fledging success and nestling Carolina wrens (*Thryothorus ludovicianus*) had lower overall body condition than those in the control group in the temperate climate of western Tennessee (Mueller et al. 2019). Similarly, when nests of free-living northern mockingbirds (*Mimus polyglottos*) were heated during incubation, embryo mass decreased (Londoño et al. 2008), and when American robin (*Turdus migratorius*) eggs were artificially incubated at higher temperatures, nestlings hatching from those eggs were significantly smaller than their non-experimental foster nest mates 7-10 days post-hatch (Ospina et al. 2018).

However, increased nest temperature can produce positive as well as negative outcomes, both on offspring phenotype and the timing of their development. For example, young nestling tree swallows (*Tachycineta bicolor*) hatching from eggs in heated nestboxes were in better condition and weighed more than nestlings from unheated boxes (Pérez et al. 2008; but see Corra et al. 2021), and high incubation temperatures also positively affected morphological traits of blue tit (*Cyanistes caeruleus*) nestlings, with those hatching from eggs incubated at the highest incubation temperature having longer tarsus lengths at two weeks of age (Nord & Nilsson 2011). High incubation temperatures also provide better environments for developing wood ducks (*Aix*

sponsa) compared with cool temperatures, as hatchlings incubated at high temperatures had higher body mass, even though they were structurally smaller than hatchlings incubated at low temperature (Hepp et al. 2006). Wood duck ducklings incubated at intermediate and higher than normal incubation temperatures had an overall 43% higher growth rate post-hatch than those incubated at lower temperatures (Durant et al. 2010).

In addition to these direct effects on nestling phenotype, increased nest temperatures during incubation also can affect other aspects of reproduction. When blue tit nests were artificially heated for two-thirds of the incubation period, not only did hatching success increase with temperature, but the incubation period also decreased (Nord & Nilsson 2011). Likewise, in both prothonotary warblers and Carolina wrens, incubation and nestling periods were reduced when nest temperature was increased (Mueller et al. 2019). Finally, zebra finch nestlings (*Taeniopygia guttata*) hatching from eggs incubated at warmer temperatures (37.9°C) had an overall higher survival rate compared with those incubated at cooler temperatures (35.9°C) (Berntsen & Bech 2016). Such outcomes could very well be caused by an accelerated developmental rate brought about by a reduction in cooling that occurs when the female leaves the nest (Nord & Nilsson 2011).

If the incubation period decreases with increased ambient temperature (Hepp et al. 2006, Nord & Nilsson 2011) and if incubation is energetically taxing for females (Nord & Nilsson 2016; but see Lothery et al. 2014, Sakaluk et al. 2018) female behaviour and condition may also be affected. When the thermal properties of nests of European starlings (*Sturnus vulgaris*) breeding in the harsh climate of the Shetland Islands in the North Atlantic were altered by inserting a small thermal mat under each nest that continually produced heat for the entirety of

the incubation period, females invested less in incubation, which had positive downstream effects on the hatching and fledging success of their subsequent clutches (Reid et al. 2000).

As local climates continue to change and temperatures increase, perhaps beyond a species' optimal thermal range, not only will life-history traits change to cope with the increase (Nussey et al. 2005) but oxidative stress, the imbalance between antioxidants and pro-oxidants (Sies 1997, Costantini & Verhulst 2009, Monaghan et al. 2009), will likely also increase (Vinagre et al. 2012). Pro-oxidants are by-products of metabolic activity (Finkel & Holbrook 2000) and result in the production of reactive oxygen species or ROS (Rahal et al. 2014) that cause damage to nucleic acids, lipids, carbohydrates, and proteins (Marri & Richner 2014). There is clear evidence that ROS production increases in heat-stressed poultry (Altan et al. 2003, Mujahid et al. 2005, Akbarian et al. 2016) and, presumably, in all birds. Although at high levels ROS can cause damage, at intermediate levels they play important roles in circulatory and immune system function (Patel et al. 2018). An important dietary component mitigating oxidative stress by neutralizing ROS molecules is the dietary intake of antioxidants, such as vitamin E, vitamin C, and carotenoids (Sies & Stahl 1995, Ozougwu 2016). However, as with high levels of ROS, higher-than-normal levels of antioxidants can be detrimental, as they reduce ROS levels below that required for normal metabolic function (Dröge 2002). Thus, a shifting balance between antioxidants and pro-oxidants must be maintained during times of environmental change, the success of which is likely to be reflected in adult life-history traits and offspring growth and survival.

The relationship between increased incubation temperature and decreased nestling condition (Murphy 1985, Ospina et al. 2018, Mueller et al. 2019) could result from increased metabolic rates that in turn increase production of ROS in embryos and nestlings. Physiological

4

mechanisms invoked to mitigate oxidative stress could, in turn, result in trade-offs that lead to reduced survival or growth (Smith et al. 2016). If so, any negative effects of increased ambient temperatures during incubation on nestling birds may be counteracted by increasing their antioxidant intake, which may or may not be possible for parents to do as antioxidants, such as vitamin E, are provided to nestling passerine birds by the invertebrates brought to them by their parents (Harrison 2006). One study found that vitamin E-treated great tit (*Parus major*) nestlings had increased survival to fledging as well as higher growth rates than controls (Marri & Richner 2014). Likewise, vitamin E supplemented pied flycatcher nestlings (*Ficedula hypoleuca*) had increased growth (Pérez-Rodríguez et al. 2019). Studies have found that increasing the intake of antioxidants typically reduces oxidative stress in other species of birds (Sahin et al. 2003, Çiftçi et al. 2005, Mujahid et al. 2009). The results of these studies are consistent with the hypothesis that increased vitamin E can improve nestling condition by reducing oxidative stress, and that there is an optimal amount of vitamin E intake that maximizes bird immune system function and overall performance (Sahin et al. 2002, da Silva et al. 2009).

In the present study, I tested the hypothesis that increased nest temperature during the incubation period, within the range of the increase in spring ambient temperatures documented in recent years in my study population (Bowers et al. 2016b), leads to increased oxidative stress in offspring resulting in decreased nestling growth and survival. Specifically, I employed a split-brood design in which I experimentally manipulated nest temperature of entire broods, while simultaneously providing dietary supplements of the antioxidant vitamin E to half of the nestlings within broods. I predicted that experimental heating of nests would result in decreased growth and survival of young, but that these negative effects would be at least partially mitigated by dietary supplements of vitamin E. To control for the possibility that experimentally increased

nest temperature might also influence maternal investment in incubation and nestling provisioning, thereby potentially confounding any treatment effects, I also recorded female incubation effort and nestling provisioning at critical points in offspring development.

Materials and Methods

Study Site and Organism

I conducted the study on part of the Mackinaw study area in McLean County, Illinois (40.665N, 88.89W), where there are 700 nestboxes (5.4 boxes/ha) mounted above aluminium predator guards (see Lambrechts et al. 2010 for information on nestbox design). These nestboxes are used as nest-sites by house wrens, a small (10-12g), secondary cavity-nesting, insectivorous songbird. Egg laying begins in late April-early May shortly after the wrens return from their wintering grounds; a second brood is produced in late June-early July (Bowers et al. 2012, Sakaluk et al. 2018). Clutch sizes range from 4-8 eggs, with one egg laid each day until the clutch is complete (Bowers et al. 2016a). Only the female incubates the eggs, which hatch after ≈12 days of incubation (Baltz & Thompson 1988, Dobbs et al. 2006, Sakaluk et al. 2018). After hatching, both males and females provision their nestlings with food and, on average, nestlings fledge 15-17 days after hatching (Bowers et al. 2016b). Additional information on house wren biology and details about the study population can be found in Johnson (2020).

Field Procedures

Nestboxes were regularly checked to find nests starting in early May. Once the first nest was found, I arbitrarily assigned it to the experimentally heated (n=35 nests) treatment and the second to the unheated control (n=34 nests) treatment (Fig. A-1); thereafter, I alternated the treatment assigned to each new nest. Approximately half-way through the incubation period,

parents at each nestbox were captured, weighed (to the nearest 0.1 g with an American Weight Scales AC-100 digital scale), their tarsus length measured (to the nearest 0.1 mm with dial callipers), and ringed with a uniquely numbered U.S. Geological Survey aluminium ring. In addition to the numbered ring, I added three coloured Darvic rings in unique combinations to males so that they could be identified again without being recaptured.

I conducted the study during the first brood (May-June 2020). On the first day of full incubation (incubation-day 0, the day the last egg is laid; Lothery et al. 2014), I installed a small, battery-powered resistive film heater (Adafruit Industries, product 1481) on the back wall of the inside of the nestbox and secured its battery pack outside the nestbox beneath its bottom panel. The heating pad batteries were replaced each morning between 06:00-12:00 CDT (Central Daylight Time) throughout the incubation period. The heating pads produced heat for about 5-6 h/morning, which is the coolest part of the day (see Fig. A-2). Control nests were equipped with heating pads and battery packs without batteries. On two different days during the incubation period, usually the third and seventh day, I installed two temperature-recording iButtons (Maxim Integrated), one on the inside of each nestbox, about midway up the sidewall, and another outside the nestbox beneath the bottom panel away from direct sunlight. iButtons were retrieved the following morning. iButtons recorded temperature every 5 min, allowing a comparison of inside nestbox temperature with outside nestbox ambient temperature thereby enabling me to validate the temperature difference between experimental and control boxes. Heating pads and iButtons were removed once the first egg hatched (brood-day 0). The length of the incubation period was calculated as the length of time from incubation-day 0 to brood-day 0.

After the eggs hatched, I initiated supplemental feeding of nestlings (n=281) with either vitamin E (DL-alpha-tocopheryl acetate, Sigma-Aldrich T3376) or the vehicle in heated and

7

unheated nestboxes using a split-brood design (Fig. A-1): half of the nestlings in each nest were given a dosage (based on body mass) of vitamin E dissolved in corn (*Zea mays*) oil, whereas the others were given corn oil only, a protocol that significantly increased circulating levels of vitamin E in experimentally dosed nestling barn swallows (*Hirundo rustica*) over that of corn-oil dosed controls (de Ayala et al. 2006). I determined the approximate amount of vitamin E needed to double the intake of vitamin E a nestling would normally obtain from insects provided by their parents, 13.6 mg/kg of nestling mass, following de Ayala et al. (2006). Nestlings were orally dosed with a variable volume of a solution of 9.6 mg vitamin E/mL corn oil in accordance with the increase in their body mass over time: brood-day 2 (body mass ≈2 g) = 3 µL, day 4 (≈4 g) = 6 µL, day 6 = 9 µL, day 8 = 12 µL, day 10 = 15 µL. The vitamin E was dispensed to nestlings between 06:00-12:00 CDT using a pipette inserted into the nestlings' oesophagus to deliver the corn oil. I visited nests to administer the dose every two days to reduce the number of visits and, hence, disturbance to each nest.

On incubation-day 9, I recorded the incubation behaviour of the female for ≈1.5 h using a small digital video recorder (Kodak PlaySport Zx5) in the morning between 06:00-12:00 CDT (Lothery et al. 2014). One day prior to recording, I placed a 1.5-m metal pole with a dummy camera about 1 m from the box and at a 45-degree angle from the nestbox door to allow the parents to acclimate to the device. After recording, I removed the camera and pole.

On brood-day 4, the beginning of the period of most rapid growth of nestlings (Bowers et al. 2014, 2015), I recorded the provisioning behaviour of both parents for ≈1.5 h using the same camera system and procedures used to record incubation behaviour. On brood-day 11, nestlings were weighed, their tarsus measured, and ringed with a numbered U.S. Geological Survey aluminium ring. To determine the length of the nestling period and fledging success, I recorded

8

the date on which the nestlings left the nest and the number of nestlings that survived to leave the nest.

The following breeding season, I recaptured experimental and control nestlings recruited to the breeding population (F1 generation) to assess possible long-term effects of the heating and vitamin E treatments. Each recruit was recaptured, weighed, and their tarsus measured, and their reproductive success (i.e., number and condition of F2 young produced) determined. On brood-day 11, F2 nestlings were weighed, their tarsus measured, and ringed. Finally, I recaptured, weighed, and measured the tarsus of all females returning from the previous year's experiment and recorded their reproductive success.

Laboratory Procedures

The videos of incubation behaviour and nestling provisioning were scored blind with respect to treatment by CFT and PAF, respectively. Once the parents returned to the nestbox to incubate or provision following the disturbance that resulted from the placement and activation of the camera, their behaviour over the following hour was recorded, a time period that provides an adequate sample of incubation and provisioning behaviour (Lothery et al. 2014). When a female was inside the nestbox during the incubation period, she was considered to be incubating (i.e., applying heat to the eggs) as eggs are never cold to the touch when a female is flushed from a nestbox during the incubation period (Lothery et al. 2014; pers. obs.). Four measures of female incubation behaviour were obtained from the digital video recordings: (1) incubation constancy (the proportion of time a female spent in the nestbox during the recording period), (2) mean on-bout length (length of time spent inside the nest-box), (3) mean off-bout length (length of time spent outside the nestbox), and (4) bout frequency (the number of on-bouts per hour) (Lothery et al. 2014).

Provisioning videos were scored to record the sex of the provisioning parent, the number of visits, and the size of the single prey item delivered by the parent (scored with respect to bill length: shorter, same, or longer; see Barnett et al. 2011).

Statistical Analysis

I used SAS statistical software (version 9.4; SAS Institute, Cary, NC, USA), two-tailed (α=0.05) tests, and the Satterthwaite's degrees-of-freedom approximation, which can result in non-integer denominator degrees of freedom. I included nest (or maternal identity) as a random effect to account for the statistical nonindependence of observations within the same nest or mother. Where appropriate, I also included time-of-season and brood size (number of nestlings on brood-day 4) as covariates. For each response variable, potential distributions were assessed for model fit and adherence to model assumptions.

To determine the effects of experimental heating on nestbox temperature, I compared the inside nestbox temperature of control nestboxes and experimentally heated nestboxes over each of the six consecutive hours pilot data had shown the heater batteries to remain operational. To do so, I used a repeated-measures ANCOVA (PROX MIXED) with inside box temperature as the response variable, temperature regime (heated or control) as the main effect, time as the repeated factor, and incubation day and time-of-season (day of the year) as covariates. I ran this model twice, once with outside box temperature as a covariate and once excluding outside box temperature from the model. This enabled me to visualize how the actual inside box temperature in each treatment varied over time irrespective of outside ambient temperature, but also allowed me to evaluate the effect of the temperature treatment on inside box temperature after controlling for the influence of outside ambient temperature.

To examine the effects of the nestbox temperature manipulation and the vitamin E treatment on offspring body mass at brood-day 11, I used a generalized linear mixed model (GLMM) with a Gaussian response distribution and an identity link function, including time-of-season and brood size at brood-day 4 as covariates. I employed a split-plot design, with vitamin E treatment nested within temperature treatment as the split-plot unit, and temperature treatment included as the whole-plot unit. This same model was used to analyse treatment effects on nestling tarsus length. To evaluate treatment effects on recruitment of offspring to the breeding population, I used a GLMM with a binary response distribution and logit link.

To examine the effect of nestbox temperature treatment on the length of incubation period, I used a GLMM with a Gaussian response distribution and an identity link function (PROC GLIMMIX), including time-of-season as a covariate. The same model was used to analyse the length of the nestling period (i.e., time from hatching to fledging), except that here brood size was also included as a covariate. To compare hatching success of the two nestbox temperature treatments, I used a GLMM with a binomial response distribution and logit link function with the number of nestlings at brood-day 4 as the dependent variable and clutch size as the binomial denominator (i.e., events/trials syntax), including time-of-season as a covariate. To examine the effect of nestbox temperature treatment on number of young present at brood-day 11, I used a GLMM with a Poisson response distribution and a log link function, including time-of-season as a covariate. To determine whether nestbox temperature treatment influenced the likelihood of females producing a second brood, I used a GLMM with a binary response distribution (produced a second brood or did not) and logit link function, including time-of-season as a covariate. I used the same model to determine whether nestbox temperature treatment

influenced the likelihood of females returning to the study site to breed the following year but omitted time-of-season as a covariate.

To assess the effect of the nestbox temperature manipulation on female incubation constancy, I used a generalized linear model with a quasi-binomial distribution and logit link function, which is suitable for bounded proportions, as was the case here (i.e., proportion of time spent on the nest). To assess the effects of the heating treatment on mean on-bout length and mean off-bout length, I used a generalized linear model with a Gaussian response distribution and an identity link function. To examine treatment effects on bout frequency, I used a generalized linear model with a Poisson response distribution and a log link function.

To evaluate the effect of the heating treatment on female provisioning rate, I used a generalized linear model with a negative binomial response distribution and a log link function, including time-of-season and number of young at brood-day 4 as covariates. I reran this model including male provisioning rate as an additional covariate to control for the possibility that female provisioning rate might be contingent, in part, on male provisioning effort. I also examined the effect of heating treatment on the female's share of all feedings using a generalized linear model with a binomial response distribution and logit link function, with the number of female feeds as the dependent variable and total number of feeds as the binomial denominator (i.e., events/trials syntax), including time-of-season and brood size as covariates.

CHAPTER II: RESULTS AND DISCUSSION

Results

Validation of Heating Protocol

Even without accounting statistically for outside ambient temperature, heating pads significantly increased inside nestbox temperatures over that of controls throughout most of the morning (Fig. A-2a; Table A-1), the coolest part of the day. However, the difference in inside nestbox temperature between the two treatments decreased steadily over the 6-h recording period (Fig. A-2a), presumably because the ambient outside temperature steadily increased over the course of the morning or because batteries in heating pads slowly lost their charge leading to diminished heating capacity. This doubtless accounts for the strong significant treatment*time interaction returned by the model (Table A-1). After controlling for outside ambient temperature, the inside temperature of control boxes remained relatively constant over time, suggesting that most of the variation in the inside nestbox temperature of control nestboxes can be attributed to ambient temperature fluctuations (Fig. A-2b). As in the previous analysis, heating pads significantly increased the inside temperature of experimental boxes, but, as before, the difference between treatments declined throughout the 6-h recording period, from a maximum of 3 °C early on, to a minimum of about 0.5 °C by the end of the recording period (Fig. A-2a); here too the treatment*time interaction was highly significant (Table A-1).

Collectively, these results demonstrate that heating pads altered the temperature regime of nestboxes, and thus validate our experimental approach.

Nestling Mass and Size in Relation to Heating and Vitamin E Treatments

There was a significant interaction between heating treatment and vitamin E treatment in their effects on nestling body mass, due apparently to opposing effects of vitamin E in the two temperature regimes, which was positive in heated nestboxes and negative in control nestboxes (Fig. A-3a). Despite the difference in the sign of the effect of vitamin E on nestling body mass, there were no significant differences in any of the pairwise comparisons within or between temperature treatments, although nestlings within heated boxes and dosed with vitamin E had marginally higher body mass than nestlings dosed with the vehicle only ($t_{231.4} = 1.78$, $p = 0.076$). There were no effects of heating treatment or vitamin E treatment on nestling tarsus (Fig. A-3b), nor was the interaction significant (Table A-2).

Effects of Heating Treatment on Female Reproductive Success

There were no effects of heating treatment on any aspect of female reproductive success, including length of the incubation period, hatching success, hatchling body mass, number of nestlings alive on brood-days 4 or 11, and length of the nestling period. Heating treatment also had no effect on the likelihood of a female producing a second brood (Table A-3). Least-squares mean trait values and their associated standard errors are reported in Table A-4.

Effects of Heating Treatment on Female Behaviour

Heating treatment had a significant effect on female incubation behaviour. Specifically, incubation effort of control females was significantly greater than that of females in experimentally heated nests, as reflected by differences in incubation constancy and mean on-bout length (Table A-5). Control females spend approximately 10% more time on the nest than experimental females (Fig. A-4), and this difference was highly significant (Table A-5).

14

Additionally, the mean on-bout length of control females was significantly higher than that of females in heated boxes (Table A-5; Fig. A-4). There were no significant effects of heating treatment on either mean off-bout length or frequency of on-bouts (Table A-5).

Heating treatment had no effects on females' subsequent provisioning of nestlings. Specifically, there were no effects of heating treatment on female provisioning rate, and this remained the case even after controlling for number of male visits (Table A-5). Analyses of female provisioning rate by prey size class similarly showed no treatment effects (results not shown). However, female provisioning rate was negatively correlated with male provisioning rate (estimate $\pm$ SE = -0.090 $\pm$ 0.019, $F_{1, 34}$ = 22.24, $p <$ 0. 0001). Heating treatment also did not significantly affect the proportion of all feedings done by the female, but the female's share of provisioning decreased significantly with brood size (estimate $\pm$ SE = -0.405 $\pm$ 0.077, $F_{1, 35}$ = 27.70, $p <$ 0.0001; Table A-5). Least-squares mean trait values and their associated standard errors are reported in Table A-6.

Female Return Rates and Nestling Recruitment

There was no effect of heating treatment on the likelihood of females returning to breed the following year ($F_{1, 67}$ = 0.62, p=0.43), of which only 8 of 69 females did so. There was no effect of heating treatment ($F_{1, 273}$ = 2.13, p = 0.17) or vitamin E treatment ($F_{1, 273}$ = 1.51, p = 0.22) on the number of offspring recruited to the breeding population (Table A-7), but the total number of recruits was small (5 of 281 offspring), hampering any meaningful inferences.

Discussion

Manipulation of nestbox temperature during incubation and subsequent vitamin E dietary supplements to nestlings resulted in a significant statistical interaction in their effects on nestling

body mass; however, there were no treatment effects on nestling tarsus length or recruitment. The significant interaction suggests that the dietary supplement of vitamin E positively affected nestling body mass near the time of nest-leaving in nestboxes heated during incubation, but negatively affected body mass in unheated boxes. The effects within treatments, however, were small, and nestlings within heated nestboxes and dosed with vitamin E had only marginally higher body mass than their vehicle-supplemented nestmates. Body mass in the study population's house wrens is positively associated with recruitment to and reproductive success in future breeding populations (Bowers et al. 2019), as it typically is in other passerines (Tinbergen & Boerlijst 1990, Both et al. 1999, Monrós et al. 2002, Morosinotto et al. 2020); therefore, the significant difference in the effect of vitamin E supplementation on the direction of the difference in nestling body mass is consistent with the hypothesis that increased temperature leads to increased oxidative stress in developing embryos and that negative downstream effects on nestlings are attenuated by antioxidant supplements to their diets.

I have no explanation for the opposite direction of the effect of vitamin E supplementation on nestling mass in the unheated treatment, given the current understanding that vitamin E supplementation positively, not negatively, affects growth in wild birds (Marri & Richner 2014, Pérez-Rodríguez et al. 2019). In blue tits, long-term variation in ambient temperatures can produce different effects on nestling body mass depending on other environmental conditions, for example, manipulation of brood size (Arct et al. 2022). Variation in ambient temperature likely has different effects on a range of phenotypic traits of growing nestlings that find themselves in different environments, including being reared on territories that differ in quality and by parents that differ in condition. Future studies with the goal of

understanding how climate change will affect survival and reproductive success of birds should look for similar interactive effects of temperature on aspects of growth and physiology.

Although the direction of the interaction between heating treatment and vitamin E treatment in their effects on nestling mass align with my predictions, these effects were weak as none of the pairwise interactions between or within heating treatments was significant. The lack of a difference between heated and unheated nests in the mass of nestlings orally dosed with the vehicle undermines the assumption that any increase, either naturally or experimentally, in nestbox temperature inevitably leads to increased oxidative stress and that mounting a defence against the pro-oxidants produced by heating comes at a cost to investing in growth (Smith et al. 2016). Although one explanation for my results is that the magnitude and duration of heating were insufficient to induce levels of oxidative stress that result in reduced growth, I think that this is unlikely as other experiments have found that increasing temperature during incubation in the range achieved here typically produces an observable effect, both positive and negative, on nestling development (Londoño et al. 2008, Pérez et al. 2008, Durant et al. 2010, Nord & Nilsson 2011, Mueller et al. 2019, Skwarska et al. 2021). A second and, I think, more likely explanation is to be found in the major effect that the nestbox-temperature manipulation had on the incubation effort of females, with females in heated boxes investing less in incubation effort. This plasticity in incubation behaviour likely mitigated any negative effects of experimental nestbox heating.

Females in heated boxes reduced their investment in incubation compared with those in unheated boxes, spending significantly less time incubating their eggs and reducing the length of their on-bouts when they were incubating. My results align with other studies that have increased temperatures and found a reduced investment in incubation (White & Kinney 1974, Siegfried et

17

al. 1976, Davis et al. 1984). Great tits reduced nest attentiveness and increased off-bout duration when nest temperatures were experimentally increased during incubation (Álvarez & Barba 2014). Likewise, when nest temperature was experimentally increased in the cool morning, female northern mockingbirds spent less time incubating (Londoño et al. 2008). These responses to experimental increases in nest temperature reflect what occurs naturally during diurnal temperature fluctuations (Conway & Martin 2000b). For example, on-bouts of female goldcrests (*Regulus regulus*) were shorter during the warmest parts of the day (Haftorn 1978), and as ambient temperatures increased during the day female blue tits spent less time incubating their eggs (Amininasab et al. 2016).

Birds may decrease their investment in incubation when temperatures increase because they need to increase food intake or simply because they can afford to invest in various forms of self-maintenance without cost to their reproductive investment (Conway & Martin 2000a). Thus, changes in the thermal environment of the nest can lead to females maintaining a better overall body condition (Cresswell et al. 2004, Pérez et al. 2008) because of a decrease in energetic costs during this period (Bryan & Bryant 1999; but see Sakaluk et al. 2018). An increase in clutch size in common terns (*Sterna hirundo*) decreased the ability of females to rear young later in the nestling period because of the increased energy needed to keep more eggs warm during incubation (Heaney & Monaghan 1996). This shows that energy required from the female during the incubation period can affect the energy allocated at later stages of the breeding cycle, although this is not always the case (Dobbs et al. 2006, Sakaluk et al. 2018).

When conditions make it possible for parents to invest less in the incubation of their current clutch, selection should favour them doing so, thereby enabling greater investment at later stages of development (Mueller et al. 2019), enhanced body condition (Cresswell et al.

18

2004, Pérez et al. 2008), or increased investment in future reproduction (Erikstad et al. 1998). However, natural and experimental increases in temperature do not always result in decreased investment in incubation because ambient conditions may be suboptimal (Ardia et al. 2009, Morton & Pereyra 1985) or other aspects of the environment, such as food supply and predation risk, influence incubation behaviour (Conway & Martin 2000a, Ghalambor & Martin 2002, Vafidis et al. 2018). Whatever the reason for changing investment in incubation, these studies show that avian incubation behaviour is influenced not only by ambient temperatures, but also by other aspects of the environment, and that it is surprisingly flexible (Reid et al. 2000, Ghalambor & Martin 2002).

Although birds can clearly adjust their incubation behaviour in the face of a wide range of environmental changes, we need to consider the limitation of this plasticity and whether additional adaptive responses in life history might be required to cope with more extreme conditions. The plasticity of some behaviours, such as incubation and provisioning, appear to play a crucial role in allowing birds to adjust to environmental changes (Ghalambor & Martin 2002). However, as temperatures continue to increase and become more variable, at what point will these plastic behaviours cease being sufficient if evolved responses have not occurred? The ability of birds to adjust their incubation and provisioning behaviours to gradual changes in climate may protect them from decreased individual fitness in the near future (Reid et al. 2000, Ghalambor & Martin 2002, Charmantier et al. 2008, Lescroël et al. 2014). The greater danger for many bird species at the moment appears to be the well-documented mismatch between the timing of breeding and availability of the food supply (Saino et al. 1999, Thomas et al. 2001, Jones & Cresswell 2010).

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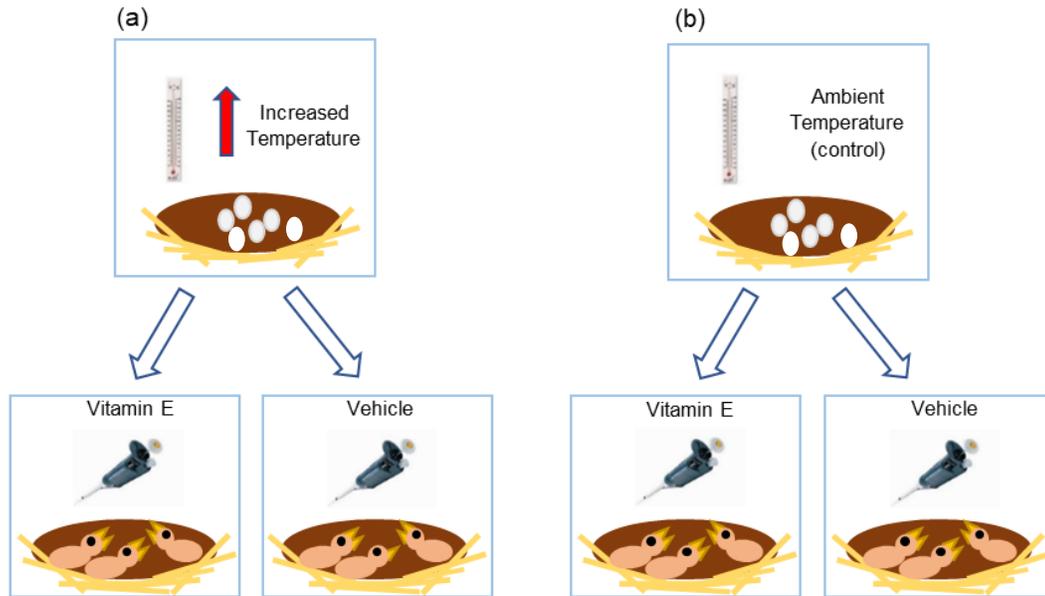
Figure A-1: Schematic of split-brood experimental design. Nests (whole plot unit) were assigned to either an (a) increased nestbox temperature treatment or (b) control treatment (ambient temperature) during incubation. Within each temperature treatment, nestlings (split-brood unit) were either supplemented with vitamin E or supplemented with the vehicle only.

Figure A-2: Inside nestbox temperatures for both heated and unheated nestboxes over 6 h of heating a) not controlling for ambient temperature and b) controlling for ambient temperature.

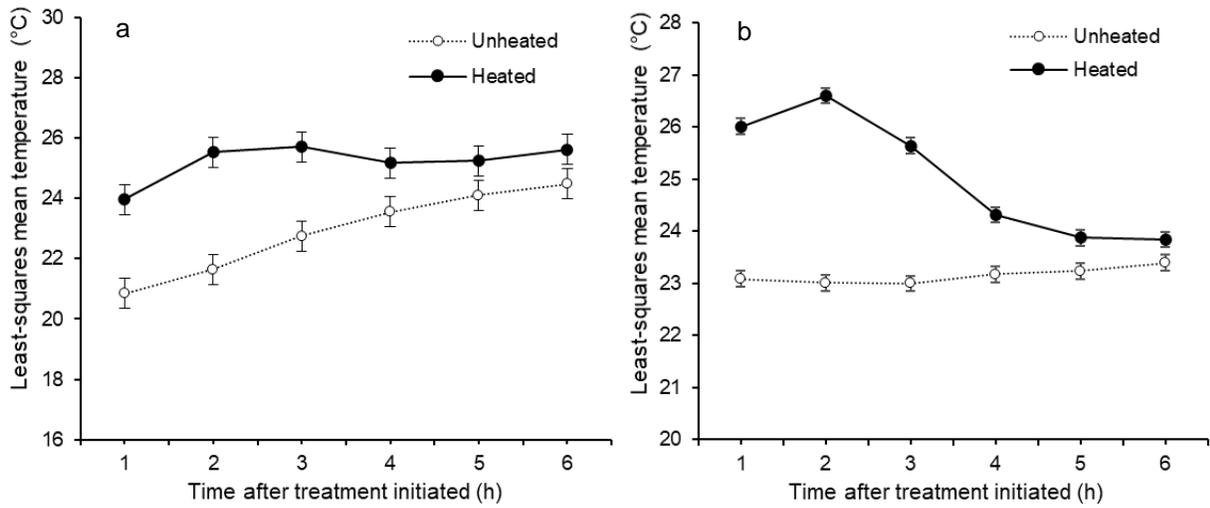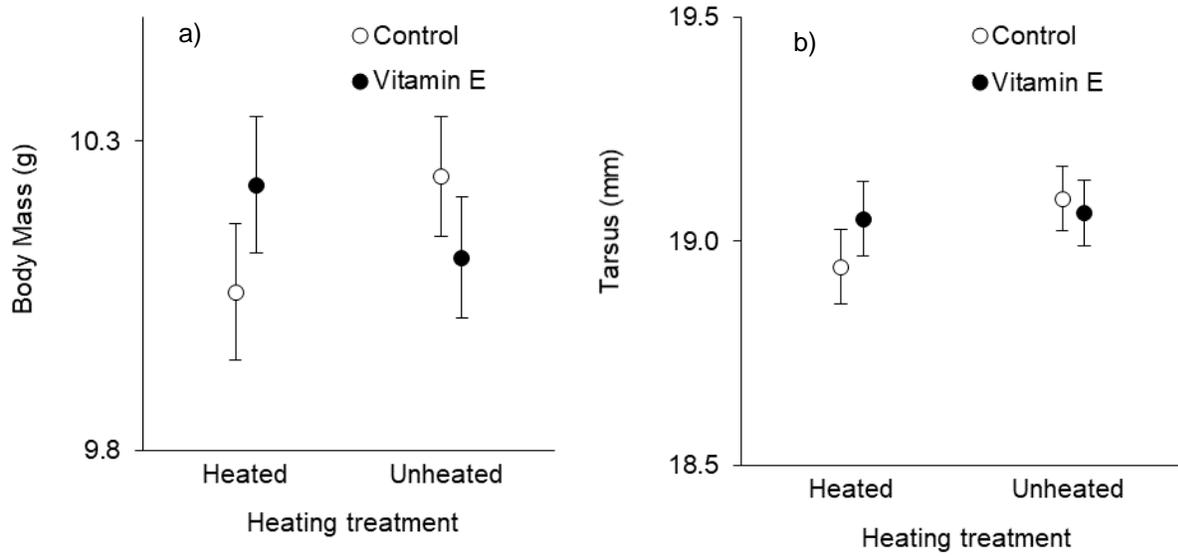Figure A-3: Nestling a) mass and b) tarsus in relation to heating and vitamin-E treatments (least-squares mean ± SE).
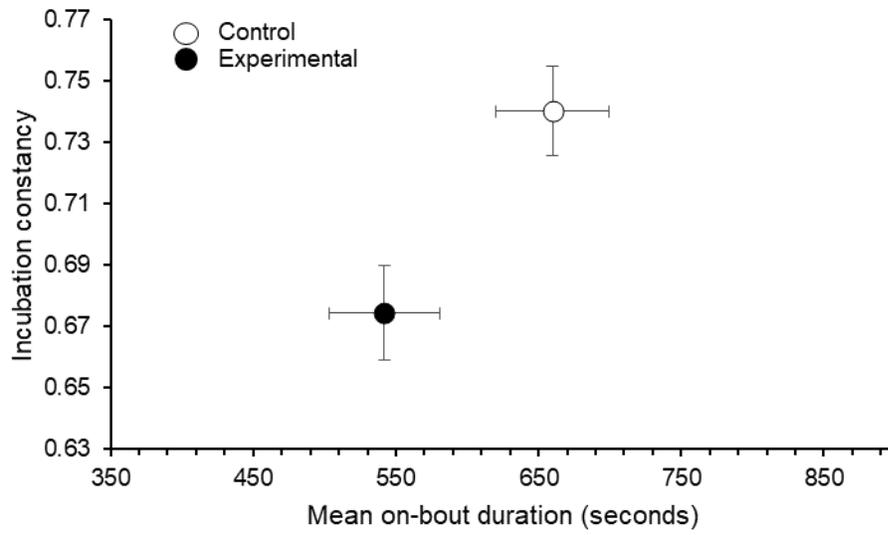
Figure A-4: Incubation consistency and mean on-bout duration for females in heated and

unheated boxes (mean ± SE).

TABLE A-1

Treatment effects on inside nestbox temperature. Significant effects bolded.

Trait	F	df	p
Not controlling for ambient temp.			
Treatment	18.92	1, 61.4	**<.0001**
Time	13.16	5, 709	**<.0001**
Treatment*Time	5.04	5, 709	**<.0002**
Incubation Day	0.42	1, 668	0.52
Day of Year	20.99	1, 61.6	**<.0001**
Controlling for ambient temp.			
Treatment	103.95	1, 65.1	**<.0001**
Time	52.60	5, 668	**<.0001**
Treatment*Time	134.97	5, 635	**<.0001**
Ambient Temperature	3296.92	1, 640	**<.0001**
Incubation Day	0.34	1, 82.4	0.56
Day of Year	17.28	1, 67.3	**<.0001**

TABLE A-2

Treatment effects on nestling body mass and tarsus. Significant effects bolded.

Trait	F	df	p
Nestling Body mass			
Heating Treatment	0.07	1, 44.66	0.80
Vitamin E treatment	0.11	1, 230.9	0.74
Temperature x Vitamin E	5.51	1, 231	**0.0198**
Time-of-season (Brood-day 0)	1.88	1, 43.52	0.18
Brood size (N nestlings brood day 4)	2.33	1, 49.42	0.13
Nestling Tarsus			
Heating Treatment	0.70	1, 43.89	0.41
Vitamin E treatment	0.57	1, 230.5	0.45
Temperature x Vitamin E	1.90	1, 230.6	0.17
Time-of-season (Brood-day 0)	1.40	1, 42.7	0.24
Brood size (N nestlings brood-day 4)	2.89	1, 48.87	0.10

TABLE A-3

Treatment effects on female reproduction. Significant effects bolded.

Trait	F	df	p
Incubation Period			
Heating Treatment	0.25	1, 60	0.62
Time-of-season (Egg one day)	12.67	1, 60	**0.0007**
Hatching success			
Heating Treatment	0.92	1, 53	0.34
Time-of-season (Brood-day 0)	0.99	1, 53	0.32
Hatchling Body Mass			
Heating Treatment	0.19	1, 44.45	0.67
Time-of-season (Egg one day)	0.03	1, 43.09	0.86
Number of young on brood-day 4			
Heating Treatment	0.92	1, 53	0.34
Time-of-season (Brood-day 0)	0.99	1, 53	0.32
Number of young on brood-day 11			
Heating Treatment	0.00	1, 46	0.99
Time-of-season (Brood-day 0)	0.03	1, 46	0.85
Nestling Period			
Heating Treatment	0.24	1, 45	0.63
Time-of-season (Brood-day 0)	0.21	1, 45	0.65
Brood Size (N nestlings brood-day 4)	0.88	1, 45	0.35
Likelihood of second brood			
Heating Treatment	1.66	1, 46	0.20
Time-of-season (Brood-day 0)	1.38	1, 46	0.25

TABLE A-4

Female reproductive success in relation to heating treatment.

	Control			Experimental		
	n	Mean	SE	*n*	Mean	SE
Incubation period (days)	30	13.09	0.20	33	12.95	0.19
Hatching success	28	0.87	0.03	28	0.83	0.03
Hatchling body mass (g)	116	1.15	0.03	86	1.17	0.03
No. young on brood-day 4	28	5.93	0.22	28	5.67	0.20
No. young on brood-day 11	27	5.73	0.46	22	5.74	0.52
Nestling period (days)	27	14.85	0.20	22	15.0	0.22
Likelihood of 2nd brood	27	0.46	0.10	22	0.66	0.10

TABLE A-5

Treatment effects on female incubation behaviour and nestling provisioning. Significant effects bolded.

Trait	*F*	*df*	*p*
INCUBATION BEHAVIOUR			
Incubation consistency			
Heating treatment	9.62	1, 61	**0.0029**
Mean length of on-bouts			
Heating treatment	4.55	1, 61	**0.037**
Mean length of off-bouts			
Heating treatment	0.61	1, 61	0.44
Frequency of on-bouts per hour			
Heating treatment	1.93	1, 61	0.17
PROVISIONING BEHAVIOUR			
Total female trips with food			
Heating Treatment	0.89	1, 35	0.35
Time-of-season (Brood-day 0)	0.01	1, 35	0.94
Brood Size (N nestlings brood-day 4)	2.16	1, 35	0.15
Controlling for male provisioning rate			
Heating Treatment	2.85	1, 34	0.10
Brood Size (N nestlings brood-day 4)	0.48	1, 34	0.50
Time-of-season (Brood-day 0)	5.24	1, 34	**0.0284**
Male trips with food	22.24	1, 34	**<.0001**
Proportion of Female Trips			
Heating Treatment	0.43	1, 35	0.52
Time-of-season (Brood-day 0)	9.61	1, 35	**0.0038**
Brood Size (N nestlings brood-day 4)	27.70	1, 35	**<.0001**

TABLE A-6

Female nestling provisioning rate in relation to heating treatment.

	Control			Experimental		
	n	Mean	SE	*n*	Mean	SE
Female total trips	19	9.18	1.15	20	7.77	0.93
Female trips when controlling for male visits	19	9.00	0.89	20	7.10	0.69
Proportion of female trips	19	0.65	0.03	20	0.62	0.03

TABLE A-7

Treatment effects on return rates of females and recruitment of offspring. Significant effects
bolded.

Trait	*F*	*df*	*p*
Female return rate			
Heating Treatment	0.62	1, 67	0.43
Offspring recruit rate			
Heating Treatment	2.13	1, 273	0.15
Vitamin Treatment	1.51	1, 273	0.22