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The effect of nest cup heating during incubation on the cold tolerance of Blue Tit (*Cyanistes caeruleus)* chicks

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Abstract

Climate change and stochastic weather events can affect many aspects of avian life cycles, including reproduction. For birds living in hot environments, rising temperatures are often detrimental because they constrain parental provisioning and chick growth. Currently, most of our knowledge regarding temperature effects during avian reproduction comes from correlative data, where cause and effect cannot be determined. More recently, evidence suggests that birds breeding in cooler environments are also affected by rising temperature. For example, increasing spring temperature and a higher frequency of heatwaves may change both parental behaviour and the parent's capacity to maintain optimal egg temperature during incubation, with a range of phenotypic consequences for developing offspring. These effects are not well understood. This study tested whether experimentally increasing nest cup temperature during incubation would influence cold tolerance of Blue Tit (*Cyanistes caeruleus*) chicks in a study area in Western Scotland. This is relevant, because there is a putative trade-off between nestling growth and thermoregulation and manipulating developmental temperature may alter resource allocation between the two. I predicted that a high nest cup temperature would influence the subsequent thermogenic capacity of chicks, the direction of the effect dependent on whether lower incubation temperatures are adaptive or constraining for offspring development. Nests were either experimentally heated during the incubation stage of reproduction or were sham manipulated (controls). After hatching, cooling challenges were performed across the ages of 4 to 10 days, during which chicks were exposed to temperatures of 10-15°C, which is below their thermoneutral zone, for 5 minutes and changes in body surface temperatures were recorded. Chicks from both heated and control nests showed a decrease in cooling rate with age but chicks from heated nests cooled slower than controls. Chicks became more homeothermic with age, but there was no difference in the development of homeothermy between heated and control nests. However, chicks from heated nests had a greater body mass during the first 12 days of life compared to chicks from control nests. My results indicate that nest microclimate can impact thermoregulation in offspring. In light of climate projections for Western Scotland, where average temperatures are expected to increase across seasons, these results may be used to predict some of the future physiological responses of birds to climate change during breeding.

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1. General Introduction

1.1 A warming world

Climate change is one of the most important environmental issues facing animal populations today (Pearce-Higgins and Green, 2014). On a broad scale, rising global and regional temperatures can negatively affect species viability (Kissel et al., 2019), alter distributions and abundance of wild populations (Hamann and Wang, 2006; Johnston et al., 2013) and consequently impact ecosystem services (Mina et al., 2017). Even with stringent measures in place to reduce greenhouse gas concentrations, global temperatures are predicted to rise by up to 1.7°C by 2100 and extreme weather events such as heatwaves are expected to increase in both frequency and intensity (IPCC, 2013).

Climate change has altered multiple aspects of avian life cycles, from breeding phenology (Dunn and Møller, 2014; Bowers et al., 2016) to behaviour (Cunningham et al., 2015) and survival (Bourne et al., 2020). One specific example is the advanced onset of egg laying to maintain timing of peak reproductive effort and an earlier occurrence of peak food abundance in temperate areas of Europe (Visser et al., 1998; Charmantier et al., 2008). In some study populations where no shift in lay date has occurred, reductions in both the growth rate and survival of fledglings have been seen (Sanz et al., 2003). Some spring migrants are now arriving earlier in Europe, due to warmer local temperatures in the wintering areas (Sparks et al., 2005). Species that have altered their arrival dates least in response to climate change also appear to have the largest population declines (Møller et al., 2008).

In addition to altering timing of breeding in response to increasing temperature, birds are also affected by extreme weather events, which are predicted to become more common in the future. Extreme weather events in hot environments can lead to lethal hyperthermia in birds, therefore driving mass mortality events (McKechnie and Wolf, 2010; McKechnie et al., 2012). Ultimately, if birds are unable to cope with severely hot weather that will occur more frequently in the future, collapses in communities are a possibility (Riddell et al., 2019).

1.2 Behavioural and physiological responses of birds to rising temperature

Although mortality and subsequent population declines are extreme examples of climate change effects, rising temperatures can have sub-lethal fitness costs for many animals, including birds. Birds are endothermic homeotherms and will show both behavioural and physiological responses to increasing air temperature. Behaviours that minimise the need for a physiological response can be advantageous as they are less energy demanding and can be sustained for a longer period (Bicego et al., 2007). These may include reduced activity during the hottest part of the day and use of shaded habitats (Wolf, 2000; Cunningham et al., 2021), though it should be noted that frequent use of such responses may lead to missed opportunity costs (Cunningham et al., 2013; Cunningham et al., 2021). If air temperature increases above body temperature, evaporation is the only way to dissipate heat. Birds do not sweat and so instead lose heat by evaporation, by panting, gular fluttering and through the skin (cutaneous heat loss). Passerines are typically limited to panting, which is a moderately effective cooling method that also incurs costs of metabolic heat production (McKechnie et al., 2017). Birds can also use facultative hyperthermia to conserve water and energy that would otherwise be used for evaporative heat loss. By reducing the thermal gradient between the body and the environment in this manner, the bird will lessen its cooling demands, thereby saving both energy (Gerson et al., 2019) and water (Nord and Williams, 2015).

1.3 The costs of responding to rising temperature

Behavioural and physiological responses of birds to avoid overheating in high temperatures can incur direct costs. For example, small birds with a large surface area to volume ratio can experience excessive rates of evaporative water loss, leading to acute dehydration (McKechnie and Wolf, 2010). Furthermore, energy expenditure increases whilst birds thermoregulate in hot environments; this energy is diverted from other processes, resulting in indirect fitness costs. For example, Southern Pied Babblers (*Turdoides bicolor*) living in arid environments showed lower foraging efficiency on the hottest days when they had to thermoregulate more, resulting in body mass loss on these days (du Plessis et al., 2012).

Increasing the need for thermoregulation in hot environments can also affect an individual’s reproduction (Cunningham et al., 2013). Young birds in the nest incur costs for thermoregulation (Andreasson et al., 2018). Additionally, they may have to contend with a lower intake of food, if shifts in energy allocation and behavioural adjustments (e.g. avoiding activity) lead to reduced foraging and subsequent nest provisioning from their parents (Luck, 2001; Cunningham et al., 2013; van de Ven et al., 2020; Cunningham et al., 2021). This is seen in Common Fiscals (*Lanius collaris*), where over the course of the chick period the frequency of days in which the maximum temperature exceeded 33^{o}C was negatively correlated with chick body mass; on these hotter days, the number of provisions per day by adults was lower. Additionally, hot days reduced chick mass, the magnitude of which could not be explained solely by lessened provisioning, suggesting energy was being directed towards thermoregulation (Cunningham et al., 2013).

1.4 Hot versus temperate climates

Whether rising air temperatures have a detrimental or beneficial effect on birds may depend on temperatures commonly experienced in the environment (Salaberria et al., 2014). Increasingly warm temperatures in the nest are likely to have strong negative fitness consequences for birds living in already hot environments (McKechnie and Wolf, 2010). These consequences may extend to birds in cooler climates if historically there has been a weak selection for heat tolerance. Alternatively, birds may be constrained by low temperature in cooler climates and so increasing temperature could remove some of these constraints (Andreasson et al., 2020). Correlational studies of temperature effects on reproduction (Conway and Martin, 2000; Arlettaz et al., 2010; Amininasab et al., 2016; Bambini et al., 2019) make it difficult to conclude on the causal role of warming. Studies manipulating the thermal conditions of the nest allow separation of the effects of nest microclimate from other effects of rising environmental temperature, such as food availability. For example, Bleu et al. (2017) increased nest temperature during egg laying; this cue affected breeding decisions in females resulting in improved chick health. Studies manipulating nest temperature after hatching report mixed responses from offspring depending on the habitat where the manipulation was performed. Rodriguez and Barba (2016a, 2016b) found that both increasing and decreasing nest temperature in a Mediterranean region produced smaller offspring, suggesting current developmental temperatures were already at an optimal. However, at a higher, temperate latitude, Dawson et al. (2005) found that increasing nest temperature led to a greater body mass and enhanced survival in the nest for chicks. At another temperate latitude, Andreasson et al. (2018) reported that heated chicks were smaller than controls before fledgling but had higher long-term survival.

This thesis will focus on the effects of increasing nest cup temperature during incubation, a costly stage of reproduction for parents (Nord and Williams, 2015). Manipulating temperature in this way has the potential to modify female behaviour, which in turn can alter the microclimate for developing embryos (Ardia et al., 2009) and the level of provisioning given to chicks after hatching (Pérez et al., 2008). Furthermore, increased nest temperature can directly affect embryos due to warmer egg temperatures. This can have broad phenotypic effects on offspring hatching success, morphology, physiology, growth, fledging success and long-term survival (DuRant et al., 2019). Ultimately, changes to temperature that occur during this one life history stage have potential implications for birds later in life.

1.5 Aims of the thesis

The main aim of this thesis was to investigate whether changes in nest cup thermal environment during incubation could result in phenotypic changes in the offspring at early life stages. Using the Eurasian Blue Tit (*Cyanistes caeruleus*; henceforth 'Blue Tit') as a model study system, I experimentally manipulated nests in the wild with the aim of increasing mean daytime nest cup temperature, hence increasing the incubation temperature for embryos. After hatching, there is a trade-off between thermoregulation and other energetically costly processes, such as growth. I was interested to see if, by increasing developmental temperature, I could alter resource allocation between the two. Although pulses of hypothermic or hyperthermic developmental temperatures in controlled environments have been shown to increase thermal tolerance in domestic birds after hatching (Shinder et al., 2009; Shinder et al., 2011; Yahav et al., 2004; Piestun et al., 2008), effects of incubation temperature on temperature tolerance are not well understood in free living birds, yet are ecologically important. For precocial species, the more homeothermic a chick is, the more time it can spend foraging independently before body temperature drops and brooding by the parent is necessary (Pedersen and Steen, 1979; Jørgensen and Blix, 1985; Jørgensen and Blix, 1988). For altricial species, achieving homeothermy quickly limits the amount of time spent in the nest where predation risk is high (Wegrzyn, 2013). The purpose of this study was therefore to fill this knowledge gap regarding avian development in the wild. With climate projections predicting a rise in average air temperature, along with increased frequency of heatwaves for temperate environments in the near future (IPCC, 2013), results may improve our understanding of how birds respond to these environmental changes.

My main research question was:

Does a warmer nest cup temperature during incubation affect cold tolerance and the onset of endothermy in chicks after hatching?

I predicted that heating nests during incubation would alter thermoregulatory ability so that heated offspring and control offspring would perform differently when exposed to cooling challenges after hatching.

1.6 Study species and study area

Fieldwork was performed using a wild Blue Tit population breeding in the surrounding woodland of the Scottish Centre for Ecology and the Natural Environment (SCENE). The Blue Tit is a small (10-13g) passerine, commonly found in deciduous woodland in the Western Palearctic. Being hole nesting birds, they readily accept artificial nest boxes for breeding. Their willingness to use these cavities allows easy access in the field, eliminating the need for a lab-based experiment where natural behaviour could be compromised.

Nest building of Blue Tits generally starts in late March and egg laying begins around mid-April. The timing of egg laying and woodland tree phenology is strongly correlated, as the main food source for chicks is caterpillars found on the foliage of deciduous trees (Perrins, 1979). This food source needs to be at its peak during the time when young are in the nest to meet the high energy demands associated with their growth. Clutch size usually ranges from 7-13, although clutches smaller than this have been found in our population. Usually, the female will lay one egg per day, early in the morning and begin incubation after the last egg has been laid. Clutches tend to hatch synchronously 13-14 days later. Due to insufficient thermoregulatory ability in the first week after hatching, chicks receive a high level of brooding from the female during this period (Andreasson et al., 2016). Almost three weeks after hatching, young will leave the nest but will continue to be fed by parents for an additional two weeks after fledging (Perrins, 1979).

2. The effect of nest cup heating during incubation on the cold tolerance of Blue Tit (*Cyanistes caeruleus*) chicks

2.1 Introduction

Reproduction in birds is characterised by distinct stages of egg laying, incubation and chick rearing. Conditions during one specific life history phase can have carry over effects later in life for both parents and chicks (Reid et al., 2000; Pérez et al., 2008; Bleu et al., 2017). Incubation is an energetically costly stage of reproduction because parents must meet the demands of maintaining an optimum clutch temperature but must also leave the nest intermittently for their own maintenance needs (Nord and Williams, 2015). When air temperature is below that for viable embryonic development, more energy is needed from the parent to keep eggs at a temperature optimal for development (Haftorn and Reinertsen, 1985). Therefore, parental energy expenditure is reduced with higher temperatures during incubation (e.g. Nord et al., 2010) and often this is reflected in an increased investment in keeping eggs warm (Reid et al., 1999) or higher provisioning during the chick stage (Pérez et al., 2008). As a result, chicks incubated in warmer nest microclimates experience fitness benefits, such as greater immunity, body mass and condition (Reid et al., 1999; Perez et al., 2008; Ardia et al., 2009).

Temperature also has direct consequences for developing birds and this is demonstrated well in artificial conditions, where eggs are kept at distinct temperatures throughout incubation. For example, studies on Wood Duck (*Aix sponsa*) have found a range of factors that are positively correlated with incubation temperature, such as hatching success (Hepp et al., 2006), growth rate (DuRant et al., 2010) and body and lipid mass (Hepp and Kennamer, 2012). In wild Blue Tits, clutches incubated at lowest temperature had reduced hatchability, increased developmental time and chicks were smaller close to fledging (Nord and Nilsson, 2011). Furthermore, by cooling Zebra Finch (*Taeniopygia guttata*) eggs periodically, thus imitating parental absence, embryos suffered reduced growth efficiency (Olson et al., 2006) and mass (Olson et al., 2008). Finally, higher metabolic rates have been found in passerines incubated at lower temperature both during the embryonic stage (Olson et al., 2006) and at fledging (Nord and Nilsson, 2011). These examples highlight the importance of parental maintenance of high clutch temperature in the nest throughout incubation.

It is still unclear as to how incubation temperature may affect thermoregulatory capacity and thus cold tolerance in chicks that continue to live in environmental conditions below their thermoneutral zone. The development of thermoregulation is important for hatchlings. Unlike precocial chicks, altricial chicks hatch naked and are completely dependent on parents in the first weeks of life (Winkler, 2016). Individually, they are unable to maintain homeothermy in the early days after hatching and body temperature varies in response to fluctuating ambient temperature (i.e., the chicks are poikilothermic) (Visser, 1998b). Although parents brood their young during cold periods, chicks are sometimes unattended. If, during those periods, they are better at withstanding cooling, they could spend resources on growth rather than keeping warm, thereby minimizing state-dependent predation risk in the nest (Wegrzyn, 2013). As chicks develop, growing feathers provide insulation and as they grow larger their surface area to volume ratio also decreases. These factors reduce heat loss from the chick to the cooler surrounding environment (Visser and Ricklefs, 1993). Most importantly, metabolic heat production capacity also develops with age. Heat is mainly produced by shivering, but thermogenesis is constrained because heat producing muscles are not well developed (Hohtola and Visser, 1998). As chicks age, metabolism, oxygen transport and insulation mature (Debonne et al., 2008; Morton and Carey, 1971). These processes increase heat-producing capacity and allow birds to achieve homeothermy before fledging (Hohtola, 2004).

It has been proposed that a high metabolic rate in response to sub-optimal developmental temperature may prepare birds for future thermoregulatory demands, allowing individuals to be more tolerant to cold climates when they hatch (Tzschentke, 2007). The main body of evidence showing improved thermal tolerance in response to manipulated developmental temperature comes from studies on poultry. Short duration (<24 h) temperature changes from the second trimester of incubation either above (Yahav et al., 2004; Piestun et al., 2008) or below (Shinder et al., 2009; Shinder et al., 2011) the optimal developmental temperature increased chicks' tolerance to hot or cool thermal challenges after hatching. Furthermore, few negative effects of temperature changes on phenotype were found, suggesting physiological changes in response to temperature exposure outside the optimum were adaptive. However, studies on poultry are carried out in controlled environments where changes in developmental temperature are short and perfectly timed. It is therefore unclear as to whether similar results would be seen in the wild, as non-domesticated birds are likely to experience stochastic fluctuations in temperature throughout incubation. Unlike results from short term temperature manipulations, continuously low temperatures throughout incubation, better reflecting wild incubation patterns, produce negative phenotypic consequences for offspring (Hepp et al., 2006; Olson et al., 2006; DuRant et al., 2010; Nord and Nilsson, 2011; Hepp and Kennamer, 2012). More specifically, experiments testing thermoregulation have found that precocial birds exposed to continuously low incubation temperature developed thermoregulatory ability less well with age (DuRant et al., 2013), experienced increased metabolic costs to maintain body temperature (DuRant et al., 2012) or had a greater cooling rate and reduced homeothermy (Nord and Nilsson, 2021) compared to birds from higher incubation treatments.

To date, there are limited studies that have manipulated nest cup temperature in the wild and measured the effect on chick thermoregulation. Andreasson et al. (2016) assessed whether the onset of endothermy in chicks could be influenced by conditions in the nest after hatching, but nests were not heated directly. Instead, changes to thermal conditions of the nest were driven by the manipulation of brood size, where reduced broods were colder early in life. In another study, Andreasson et al. (2018) heated nest cups of Blue Tit chicks and measured effects of heating on chick growth and thermoregulation, but this manipulation of nest microclimate occurred post hatch. I experimentally heated nests of free-living birds during incubation and assessed chick cold tolerance ability at different stages of development after hatching. Chicks were exposed to temperatures below their thermoneutral zone for short periods (<10 min) and changes in surface temperature were measured using thermal imaging. I predicted that nest cup temperature during incubation would influence cold tolerance of chicks. If a lower nest cup temperature during incubation allows for greater cold tolerance, as seen in poultry, chicks from heated nests would show reduced cold tolerance compared to control chicks. This would be seen by a faster cooling rate and a lower level of homeothermy when faced with a cold challenge. If, however development is constrained by a lower incubation temperature, as has been reported in studies where suboptimal conditions continue throughout the course of incubation, chicks from heated nests would show improved cold tolerance.

2.2 Materials and methods

2.2.1 Study area

Fieldwork took place between March and June in 2019 and 2020 at the Scottish Centre for Ecology and the Natural Environment (SCENE), situated in Western Scotland (56.13°N, 4.61°W). I used birds from a wild Blue Tit population, breeding in nest boxes within the surrounding oak (*Quercus robur*) dominated woodland.

Starting at the end of March, nest boxes were checked once a week for signs of nest building and egg laying. To determine the start of incubation (which was assumed to occur on the last day of laying), nests were visited more frequently from day 9 of egg laying (day of first egg = day 1). If nests contained fewer eggs than days of laying (for example, 8 eggs on day 9 of egg laying), it was assumed that laying had finished, and incubation had started the previous day. If nests contained the same number of eggs as days of laying (for example, 9 eggs on day 9), it was possible that the female was still laying, and nests were visited the following day. This process continued until it could be assumed that the female had completed laying and started incubating.

2.2.2 Manipulation of nest cup temperature during incubation

Over both breeding seasons, a total of 57 nest boxes were initially sampled; 29 received a heated treatment and 28 acted as control nest boxes (Table 1). Nests were allocated as either a heated treatment (Figure 1A) or a control treatment (Figure 1B) on day 2 of incubation. The treatment type of the first nest was selected randomly by a coin toss and following this, treatments were allocated alternately to nests as the female began incubating. This ensured heated and control nests were spread evenly throughout the course of the breeding season. Both heated and control nest boxes had a wire mesh platform inserted underneath the nest cup, creating a space between the nest cup and the floor of the nest box (Figure 1). For heated treatments, two small (6×9 cm) heat packs (HotHands®, KOBAYASHI, Osaka, Japan) were inserted between two, 1 cm thick polyethylene sheets, the same size as the nest box floor. The polyethylene sheets were used to reduce heat loss through the nest box floor but also to prevent overheating of eggs (>40^{o}C) due to the heat packs. Heat packs generated heat for up to 7 hours (compared with control nests) and were replaced each day (time of replacement ranged from approximately 08:30 to 14:00 BST, mean: 10:39 am BST). Due to the wire mesh platform, there was no need to move the nest cup or completely remove the nest box door to change heat packs. This ensured that each visit caused as little disturbance as possible to the incubating female. Control nests were visited each day during incubation but heat packs and polyethylene sheets were not added to boxes to avoid altering the insulation properties of the nest.

Throughout the incubation period, air, nest box and nest cup temperature were each recorded. Air temperature (±0.1^{o}C) was recorded every 30 minutes by a MiniMet Automatic Weather Station (Skye Instruments, Powys, UK) in the centre of the study area.

Mean air temperature during incubation was 10.7±0.3°C in 2019 and 11.3±0.1°C in 2020. Nest box and nest cup temperature (±0.0625°C) were each recorded using temperature dataloggers (iButton® DS1922-L, Sunnyvale, CA, USA; accuracy: ±0.5°C) in all nests. The mean deviation of iButtons from actual temperature (recorded by a mercury thermometer) was 0.35±0.06°C. Nest box dataloggers were placed on the inside wall of the nest box, at a height where they were not affected by heat production by the incubating female, and were programmed to record temperature every 15 minutes, allowing recording over the entire incubation period. To insert nest cup dataloggers, eggs were first carefully removed from the nest. Thin nylon material was wrapped around each datalogger and a section of wire was then attached to the material, passed through the bottom of the nest cup and attached to a small weight that sat on the nest box floor (Figure 1). This prevented the female removing the logger, as has been observed with iButtons not attached to the nest. Eggs were then replaced to surround the datalogger to record temperature change in the nest. Nest cup dataloggers were programmed to record temperature at 1 minute intervals in 2019 and were replaced every three days to provide continuous measurements. The purpose of a short sampling frequency in 2019 was to measure female on and off-bouts (see below; Capilla-Lasheras, 2018) to infer whether heating nests altered female incubation patterns. In 2020, these were programmed to record at 5 minute intervals. This sampling frequency allowed measurement throughout the incubation period without replacement to further minimise disturbance to the nest but was unsuitable for analysing changes to female behaviour (see below). Nest cup temperature was analysed between the hours of 04:00-22:00 that approximated photoperiod over the study period (which ranged from 05:33-20:59 to 04:42-21:51). It was assumed during these hours, effects of daytime heating would be more obvious and not masked by overnight incubation when females were continuously on the nest.

Hatch checks commenced 12 days after the incubation start date and continued daily until the first signs of hatching. At this point, treatments ended and all equipment was removed. Nests were not visited again until chicks were four days old, when the first cooling challenge took place.

Table 1. Overview of the 2019 and 2020 breeding season of Blue Tits in the woodland surrounding SCENE in Western Scotland. A total of 57 nest boxes were initially sampled, 29 of these received a heated treatment and 28 were controls. Below is a summary of each breeding season along with an overall summary for both years of experiment combined. Detailed are initial sample sizes (i.e., nest box numbers), mean date of first egg, mean and range of clutch sizes, total number of eggs, mean brood size and average cool box temperature for each treatment.

	2019		**2020**		**Both Years**	
	Heated	**Control**	**Heated**	**Control**	**Heated**	**Control**
Sample size	13	12	16	16	29	28
Mean date of first egg dd/mm/yy	26/04/19	23/04/19	24/04/20	24/04/20	25/04	24/04
Mean clutch size (±SE)	10 (±0.5)	10 (±0.6)	10 (±0.4)	10 (±0.4)	10 (±0.3)	10 (±0.3)
Range in clutch size	7-12	9-12	6-13	6-13	6-13	6-13
Total number of eggs	125	123	166	161	291	284
Mean date of hatching dd/mm/yy	18/05/19	16/05/19	17/05/20	15/05/20	17/05	16/05
Mean brood size (±SE)	8 (±0.6)	9 (±0.6)	9 (±0.4)	9 (±0.3)	9 (±0.4)	9 (±0.3)
Mean cool box temperature °C (±SE)	11.8 (±0.2)	11.9 (±0.3)	12.3 (±0.1)	12.2 (±0.1)	12.1 (±0.1)	12.0 (±0.1)

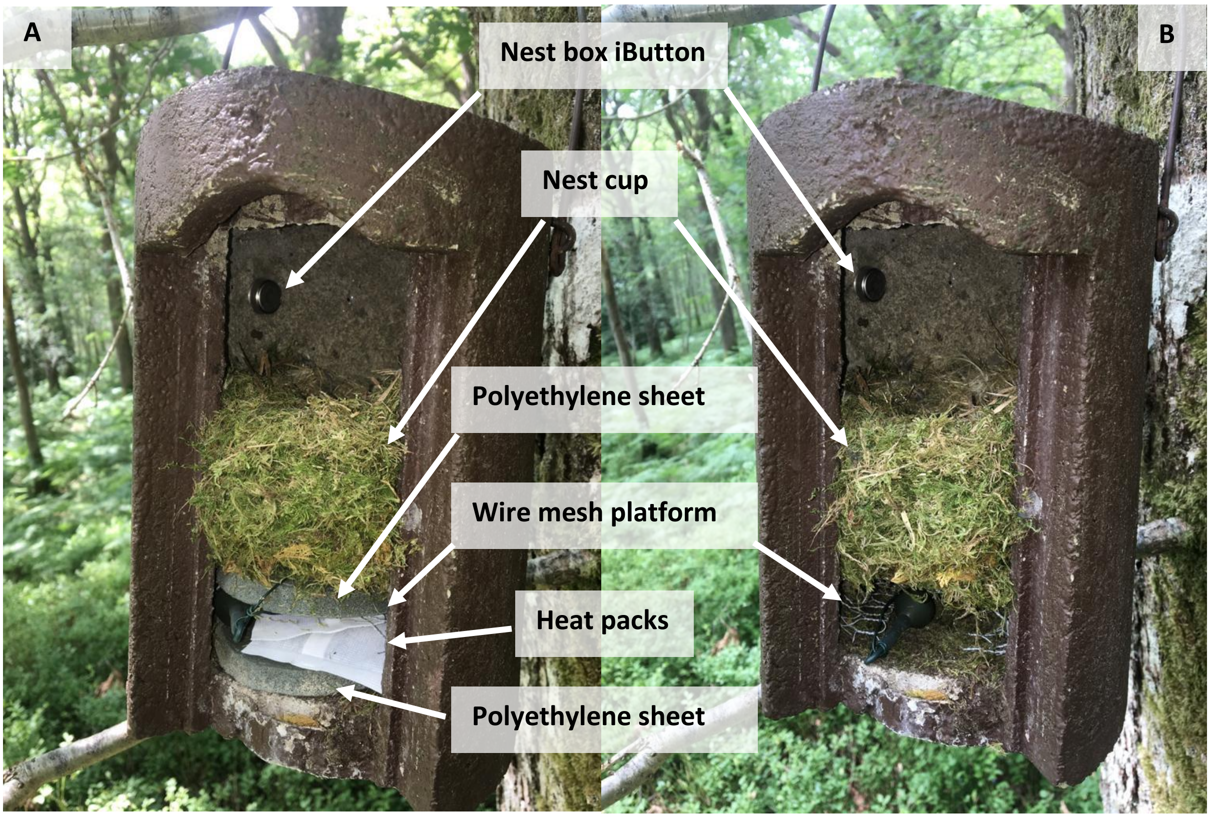

A
B
Nest box iButton
Nest cup
Polyethylene sheet
Wire mesh platform
Heat packs
Polyethylene sheet

Figure 1. Experimental set up for measuring nest cup temperature of Blue Tits breeding during 2019 and 2020 at SCENE in Western Scotland. (A) Experimentally heated nest cups were elevated using a wire mesh platform. This created a space in which two heat packs were placed between a pair of polyethylene sheets. These heat packs were changed daily. (B) Control nests still received a wire mesh platform and were visited daily, but no heat packs or polyethylene sheets were added to nest boxes. (C) Equipment needed to attach a temperature logger to the nest cup floor. (D) A thin piece of nylon material was wrapped around each temperature logger and a section of wire was attached to the material. (E) The wire was passed through the bottom of the nest cup so that the logger sat at the bottom of the nest cup. (F) The wire was attached to a small weight that was placed on the floor of the nest box, beneath the wire mesh platform.

2.2.3 Incubation behaviour analyses

Data from nest cup and nest box temperature loggers were assessed for quality (see Supplementary Material) prior to incubation analyses. Analyses were performed using the ‘incR’ R package version 3.6.2. (Capilla-Lasheras, 2018). Continuous incubation was specified to take place between 22:00 and 3:00 (overnight hours), when the female was assumed to be incubating, to calibrate ‘incR’ functions. The maximum temperature variation allowed between two time points during continuous incubation (‘maxNightVariation’) was set to 1.5°C. ‘Sensitivity’ was set to 0.25 to account for events when nest cup temperature was close to environmental temperature. This was to ensure that shorter off bouts could still be detected. A minimum temperature difference (‘temp.diff.threshold’) of 4°C was allowed between the temperature of the nest and the environmental temperature. Below this value, the sensitivity parameter was triggered. These values were previously found to be the most accurate when determining Blue Tit incubation behaviour (Capilla-Lasheras, 2018). Off-bout frequency (when female was away from the nest) and on-bout frequency (when the female was present on the nest) rates per hour (excluding overnight hours) were calculated, along with bout duration and overall percentage of time the female spent in the nest each day over the course of the incubation period.

2.2.4 Chick cooling challenge

Cooling challenges were undertaken when chicks were 4, 6, 8 and 10 days old (day of hatching = day 0, Figure 2). For each challenge, chicks were selected by reaching into the nest and selecting the first four available. These chicks were placed individually inside a small, open top plastic cup (diameter: 9 cm; rim height: 4.5 cm) which was covered with black matte insulating tape (emissivity: 0.98). Cups were already positioned inside a cool box. In 2019, a Styrofoam cool box (30×22×20 cm) was used in cooling challenges and contained two ice packs (Thermos®, 16×9×3 cm) beneath a wire mesh platform. The platform was elevated 12 cm above the ice packs so that chicks were not being placed directly on top of them. In 2020, an electric cool box (42×41×25 cm) (VonShef, Manchester, UK) was used instead of a Styrofoam cool box. The cool box was connected to a small (12V, 7Ah) battery. A timestamped thermal image was taken of the back of each chick prior to each cooling period using a thermal imaging camera (ThermaCAM E300, FLIR), mounted on a tripod 50 cm from the chicks (Figure 3). After images were taken, the lid was then placed on the cool box for 5 minutes. Air temperature (±0.1°C) inside the cool box was monitored throughout the process using a Tinytag© TK-4023 temperature logger (Gemini Data Loggers, UK Ltd), calibrated to a mercury thermometer. The probe of the temperature logger was secured on the platform adjacent to the plastic cups and took a reading inside the cool box at 2 second intervals. Recorded temperature at the start (0 min), midway (2.5 min) and at the end (5 min) of each challenge were averaged to give a mean cool box temperature. The cool box had a mean temperature of 11.9±0.2°C in 2019 (mean air temperature: 12.8±0.2°C) and 12.3±0.1°C in 2020 (mean air temperature: 13.8±0.2°C) and did not differ between treatment and control chicks in either year (P=0.901). At the end of the 5 minute period, the lid was removed, and a second image was taken of the back of each chick. Chicks were subsequently weighed (±0.1 g) using a digital scale and returned to their nest. The complete procedure lasted on average 9 minutes±2 seconds (range: 6 to 13 minutes).

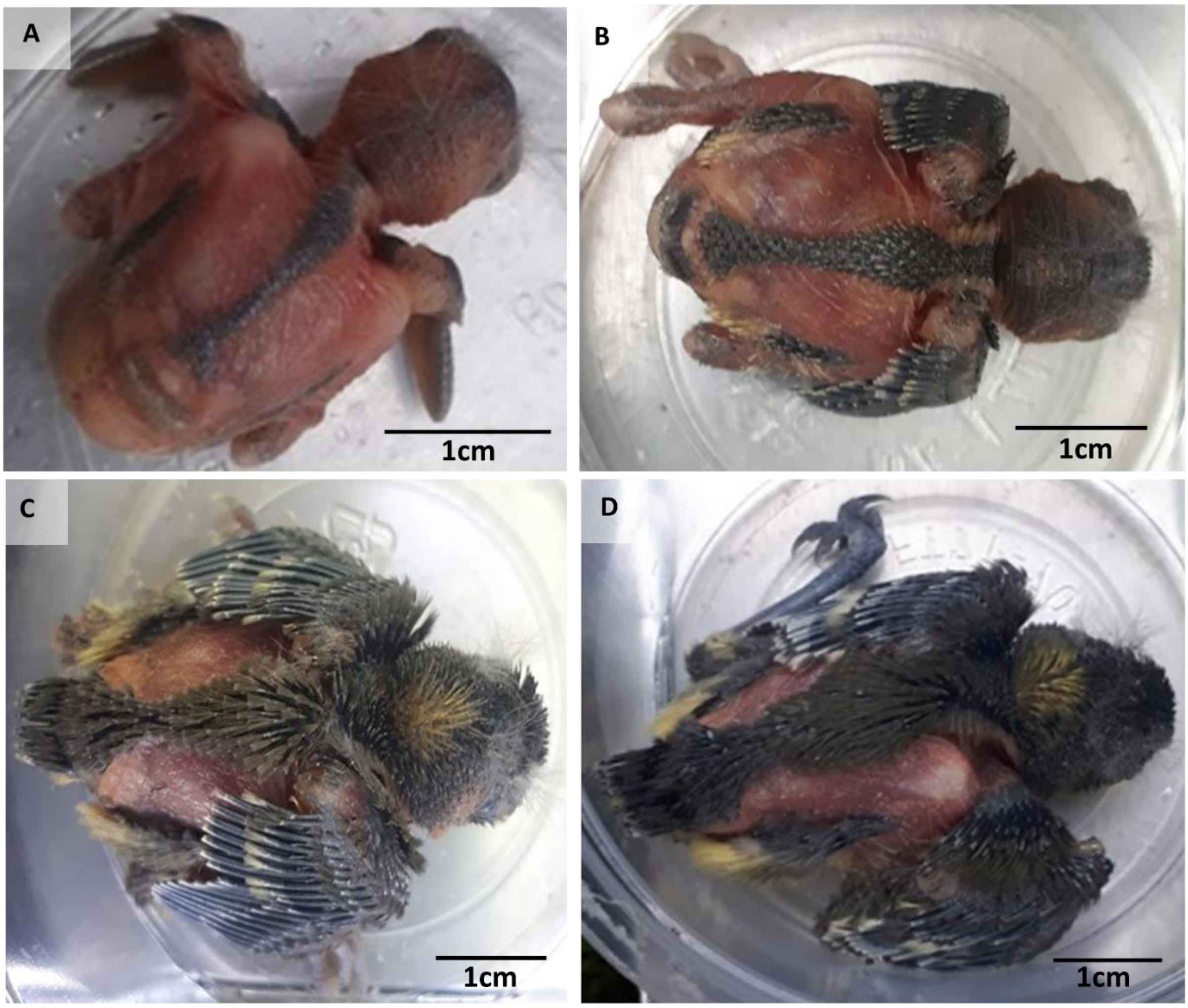


Figure 2. Stages of Blue Tit chick development. For each nest, 4 cooling challenges took place, each at a different stage of Blue Tit chick development. The first cooling challenge took place when chicks were 4 days old (A), followed by cooling challenges on days 6 (B), 8 (C) and 10 (D). The scale bar on each image represents 1 cm.

Figure 3. Set up of the cooling challenge. (A) in 2019, a Styrofoam cool box was used in cooling challenges. A platform was placed 12 cm above two ice packs and individual, open top cups covered in matte insulating tape were placed on this platform. (B) In 2020, individual cups were placed on the same platform but inside an electric cool box that did not contain ice packs. (C) Instead, the cool box was connected to a small battery that could easily be transported in the field. (D) Chicks were placed individually inside these cups during cooling challenges. (E) A thermal imaging camera attached to a tripod was used to take a thermal image of the back of each chick. An image of each Blue Tit chick was taken before a 5 minute cooling period and after.

2.2.5 Thermal image analysis

Overall, 1,584 images (8 images per cooling challenge, per nest) were analysed for this study. All images were analysed using ThermaCAM Researcher Pro (Version 2.10) software (FLIR Systems), using ‘Rain’ colour palette, with temperature scale adjusted to emphasise the outline of the chick and Tinytag in each image. Values for parameters known to affect the amount of radiation that reaches the camera were provided. Emissivity was set to 0.98, according to Kastberger and Stachl (2003) and distance from the camera was 50 cm. Temperature and relative humidity were set according to the Tinytag inside the cool box and the automatic weather station, respectively. The region of interest (ROI) tool was used to fit a polygon around the body and head of the chick, excluding wing and legs as these were not consistently seen (Figure 4). The mean temperature recorded from this region was calibrated using the temperature recorded by the Tinytag probe visible in each thermal image (Figure 4). The mean temperature of the probe taken by the thermal camera was compared to the temperature taken by the probe itself. This difference was used to correct the thermal image temperature within the ROI. The mean difference between thermal image and temperature probe was 0.5±0.01°C. Chick cooling rate was determined by the change in temperature (T_2-T_1).

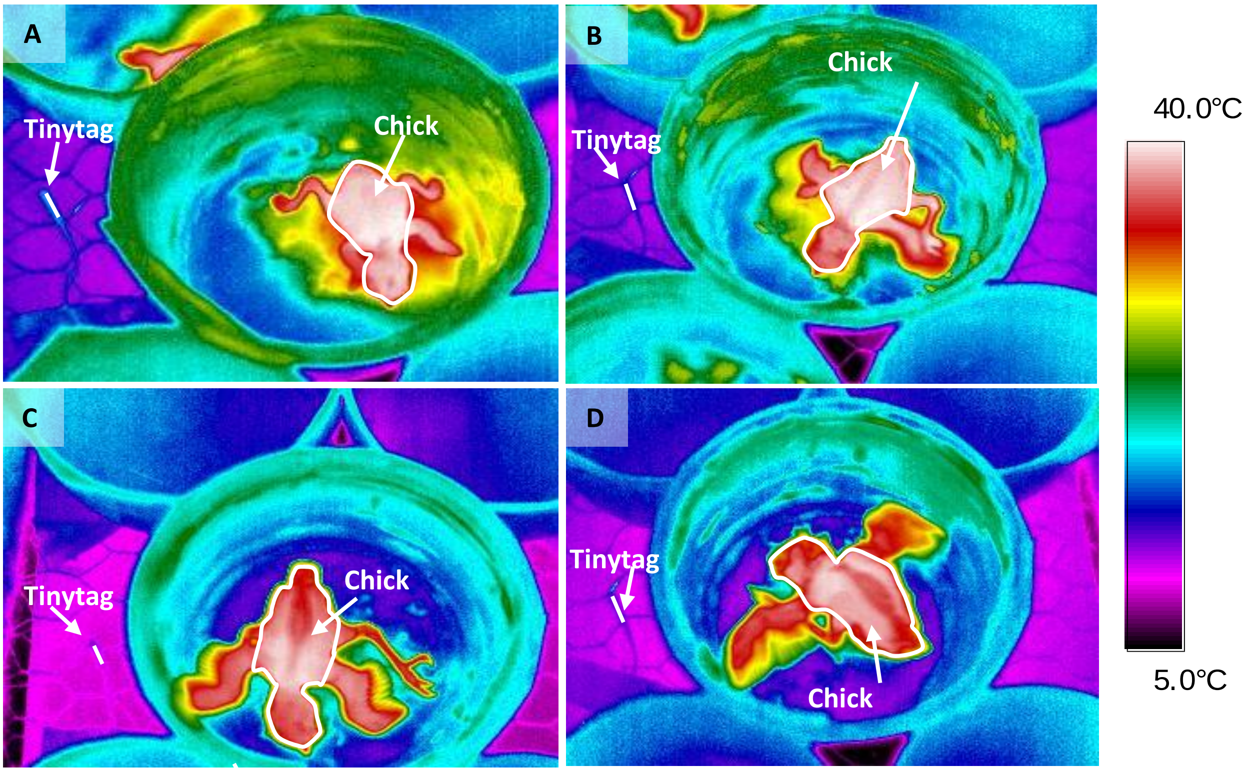


Figure 4. Analysis of Blue Tit chick surface body temperature. (A) shows a thermal image of a 4-day old Blue Tit chick, (B) a 6-day old chick, (C) an 8-day old chick and (D) a 10-day old chick. Using imaging software, a polygon was fitted around the body and head of each chick (wings and leg extremities have been excluded). The data inside the polygon was collected to find the average body temperature of the chick. Body temperature was calibrated by comparing the camera temperature of the Tinytag probe with the temperature taken by the probe itself at the time of the image. Camera deviations from the temperature probe were used to adjust mean body temperatures.

2.2.6 Chick morphometric measurements

On day 12 all chicks were ringed and fitted with a British Trust for Ornithology (BTO) metal ring with a unique ID number on their right leg. During this process, all chicks were weighed for a final time (±0.1 g) and tarsus (±0.1 mm) and wing length (±1 mm) were also measured. At the end of the breeding season, nest boxes were visited, emptied and dead chicks were noted, from this fledging success was determined.

2.2.7 Statistical analyses

Overview

Of the initial 57 nests sampled, three were abandoned by parents during early incubation and thus were excluded from all datasets (heated: *N*=2, control: *N*=1). All statistical analyses were carried out using linear models (LMs) fitted using Base R and linear mixed models (LMMs) implemented in the ‘lme4’ package (Bates et al., 2015) in R version 3.6.2 (R Core Team, 2019). When data from both years of study were analysed, an interaction between treatment and year was included to determine whether the effect of treatment was influenced by external factors that fluctuate between years (e.g., environmental temperature). When data were collected over different stages of chick development, an interaction between treatment and chick age was included to determine whether the effect of treatment differed across ages. Nest box was used as a random intercept in all LMMs to account for repeated measurements. For each response variable, final models were derived by backward elimination of non-significant (P>0.05) terms, starting with interactions and followed by variables with the highest P values as determined using likelihood ratio tests (LRT). Final models contained only significant variables (P≤0.05) and were graphically assessed for parametric assumptions of a Gaussian distribution, using residual plots and normality histograms. If required, response variables were transformed to better meet model assumptions (see below). I calculated model estimates and performed pairwise post hoc tests (Tukey HSD test) for final models using the ‘pairs’ function within the ‘emmeans’ package (Lenth, 2019). Values used in post hoc tests are based on predicted means from final models.

Nest cup temperature and nest box temperature

To assess the effect of nest treatment on mean daytime nest cup temperature (i.e., between 4:00 and 22:00), mean nest cup temperature, nest cup temperature range, maximum nest cup temperature and minimum nest cup temperature were used as response variables in separate LMs, with treatment and year included as factors and air temperature included as a continuous variable. An interaction between treatment and year was also included in each model. Nest box temperature was included as a response variable in a separate LM with treatment, year and treatment×year as factors.

Length of the incubation period

Of the 54 nests included in nest cup temperature analysis, a further two control nests were abandoned during late incubation. These nests were excluded from the incubation length data set. Hence data from 52 nests that hatched were included in the dataset (heated: N=27, control: N=25). Incubation length was used as a response variable in a LM with treatment and year included as factors alongside the interaction treatment×year.

Cooling rate and Homeothermy index

Of the 52 nests included in incubation length analysis, two broods died shortly after hatching. These nests were excluded from the cooling rate and homeothermy index datasets (heated: *N*=1, control: *N*=1). Therefore, data from 50 nests were included in the datasets (heated: *N*=26, control: *N*=24). I calculated the cooling rate of an individual chick according to Andreasson et al. (2016):

$$\left(\frac{\log(T_2-T_C)-\log(T_1-T_C)}{t}\right) / \, m_b^{0.67} \qquad \text{Eq. 1}$$

Where T_1 is chick temperature before cooling, T_2 is chick temperature after cooling, T_c is cool box temperature, t is time of cooling in minutes and m_b is body mass in g. Smaller chicks cool passively at a faster rate than large chicks because of a higher surface area to volume ratio, which is accounted for by dividing the cooling rate by $m_b^{0.67}$. I square root-transformed the absolute value of cooling rate prior to statistical analysis to meet model assumptions.

To consider how the experiment affected how constant chick body temperature remained during cooling challenges I calculated a homeothermy index following Ricklefs (1987):

$$H = \frac{(T_2-T_C)}{(T_1-Tc)} \qquad \text{Eq. 2}$$

The closer *H* is to 1, the more homeothermic the chick is. The averaged cooling rates and homeothermy indices across the 4 chicks of each challenge were used as the response variables in two separate LMMs, with treatment, chick age and year included as fixed factors and brood size included as a continuous variable. Interactions between treatment and year and treatment and chick age were included in the model.

Morphometric analyses

Of the 50 nests that hatched, one control brood died before day 12 measurements could be taken. This nest was excluded from the tarsus and wing length dataset, but I included body mass data until day 10 for this brood. Thus, data from 49 nests were included in the tarsus and wing length models dataset (heated: *N*=26, control: *N*=23) and data from the 50 nests above in the body mass model. Mean brood body mass was measured on days 4, 6, 8, 10 and 12 after hatching and was used as the response variable in a LMM with treatment, chick age and year included as fixed factor variables and brood size as a continuous variable. Interactions of treatment×chick age and treatment×year were included. Mean tarsus length and mean wing length per brood were taken on day 12 and were each used as the response variable in separate LMs, with treatment and year included as fixed factor variables and brood size as a continuous variable. A treatment×year interaction was included in these models.

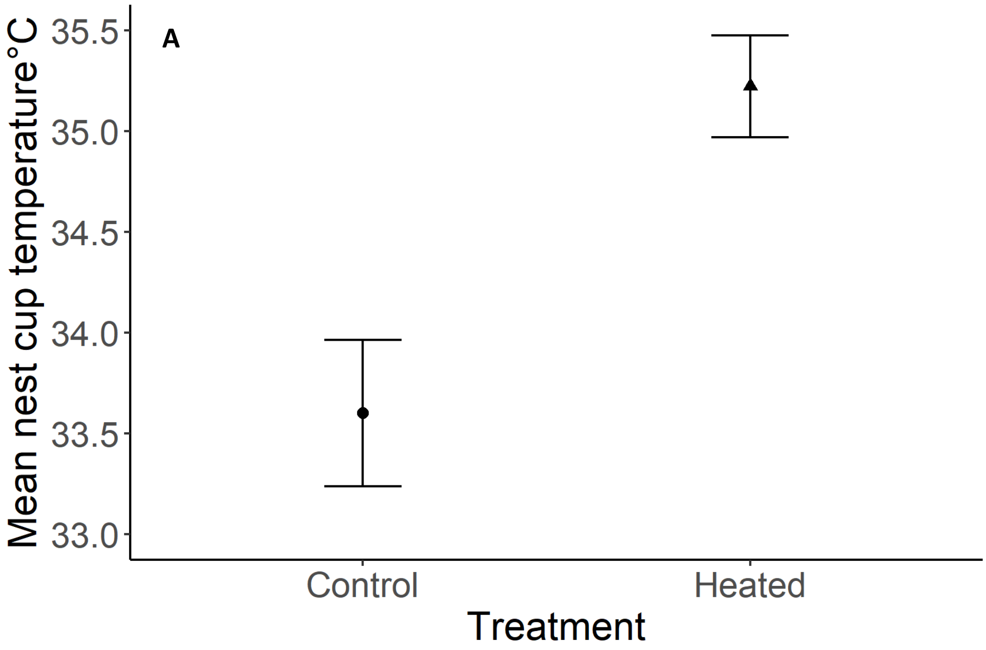
Incubation behaviour

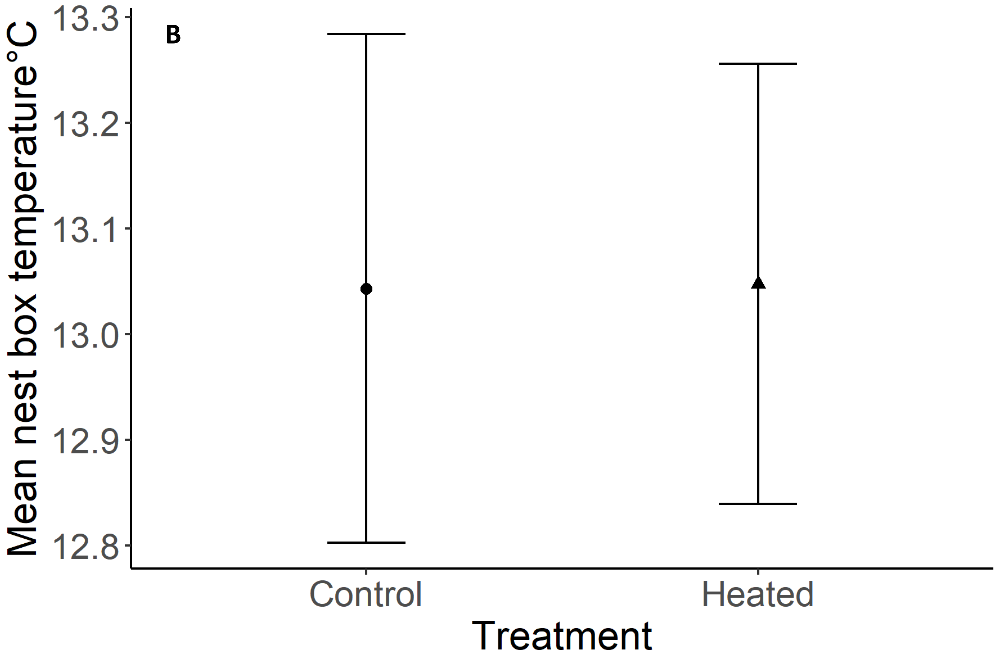
Overall, 123 days of data were included in the dataset (heated: *N*=52, control: *N*=71). Off and on-bout frequency, duration and percentage of time spent incubating were used as response variables in separate LMMs with treatment included as a fixed factor variable.

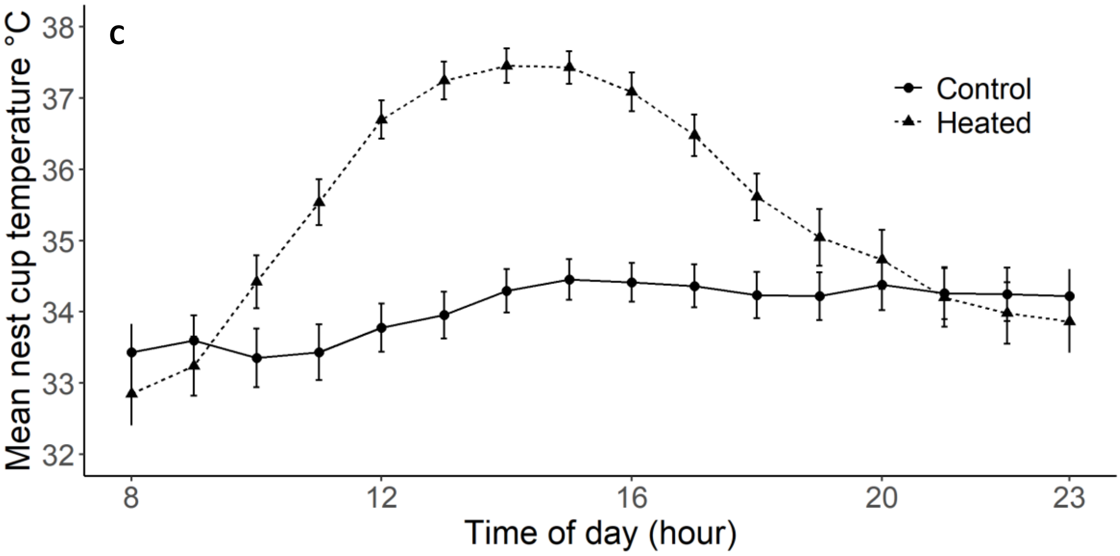
2.3 Results

2.3.1 Effects of heating on nest cup and nest box temperature

Parameter estimates and test statistics are presented in Table 2. The effect of the experiment on nest cup temperature did not differ between years (treatment×year interaction: *P*=0.281). Mean±SE daytime nest cup temperature was 1.6°C higher in heated nests (35.2±0.3^{o}C) compared with control nests (33.6±0.3^{o}C) (*P*<0.001; Figure 5A) during incubation. There was no main effect of year on nest cup temperature (*P*=0.416) and no significant relationship was found between nest cup temperature and air temperature (*P*=0.942). Nest box temperature did not differ between treatments (*P*=0.988; Figure 5B).The effect of the experiment on nest cup temperature range did not differ between years (treatment×year interaction: *P*=0.790). The range (max-min) in daytime nest cup temperature was 2.3°C greater in heated nests (12.3±0.5^{o}C) compared to control nests (10.0±0.5^{o}C). (*P*=0.001). There was no difference between years (*P*=0.209) and no effect of air temperature on range (*P*=0.896). The effect of the experiment on maximum daily temperature did not differ between years (treatment×year interaction: *P*=0.665). Heated nests reached greater maximum temperatures (39.5±0.3°C) than controls (36.5±0.3°C) (*P*<0.001) but there was no difference between years (*P*=0.161) and no effect of air temperature (*P*=0.978). The effect of the experiment on minimum daily temperature did not differ between years (treatment×year interaction: *P*=0.996). Minimum nest cup temperature did not differ between heated and control nests (*P*=0.199), but minimum temperature was lower in 2019 (26.0±0.5°C) than in 2020 (27.4±0.4°C) (*P*=0.023). There was no effect of air temperature on minimum nest cup temperature (*P*=0.867).


A
Mean nest cup temperature°C
35.5
35.0
34.5
34.0
33.5
33.0
Control
Heated
Treatment


B
Mean nest box temperature°C
13.3
13.2
13.1
13.0
12.9
12.8
Control
Heated
Treatment



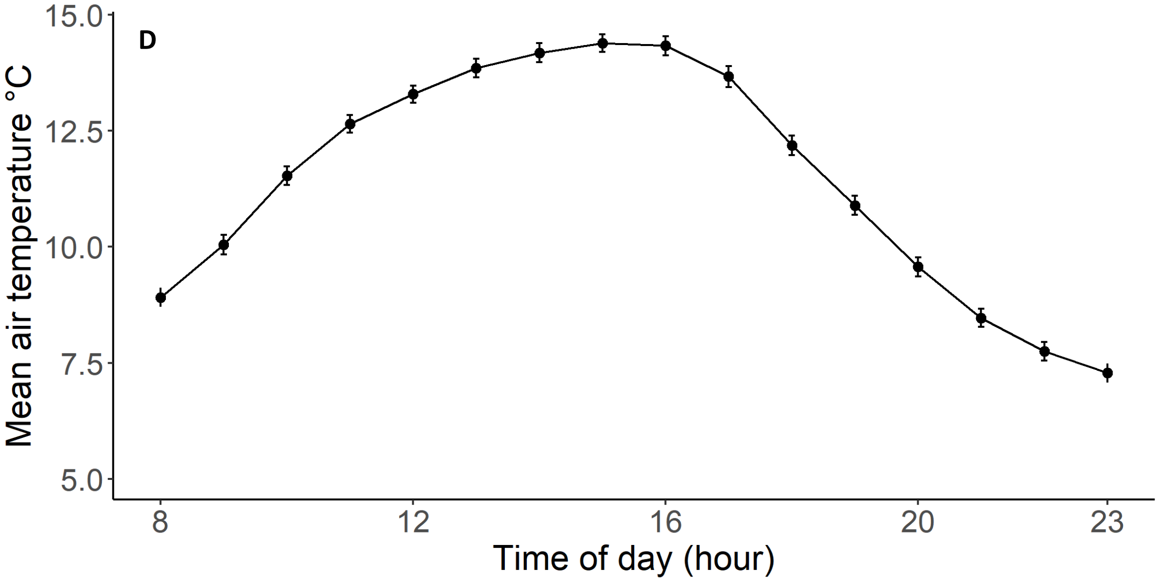


Figure 5. Nest cup and nest box temperature of control and heated nests and air temperature. (A) Mean±SE daytime nest cup temperature for control and heated nests (*P*<0.001) for both years combined, taken from temperature loggers attached to the bottom of the nest cup. Overall, mean daytime nest cup temperature for control nests was 33.6±0.3°C and for heated nests 35.2±0.3°C (B) Mean ±SE daytime nest box temperature for control and heated nests for both years combined, taken from temperature loggers attached to the wall of the nest box. (C) Mean±SE hourly nest cup temperature for heated nests before and after heat pads were changed daily compared to mean hourly nest cup temperature for control nests where no heat pads were inserted. (D) Mean±SE hourly air temperature from 8:00-23:00 (BST). Air temperature data were collected from a Minimet Automatic Weather Station situated in the centre of the study area.

Table 2. Test statistics, degrees of freedom, *P*-values and parameter estimates for final models and *P*-values and test statistics for dropped terms, for models describing daytime mean nest cup temperature, nest cup temperature range, maximum and minimum nest cup temperature and daytime nest box temperature. Final models were derived using backward elimination of non-significant (*P*>0.05) terms.

Mean nest cup temperature °C				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Treatment		12.41	1	<0.001
Heated	35.2 (0.3)			
Control	33.6 (0.3)			
Dropped terms:				
Year		0.66	1	0.416
Air temperature °C		0.01	1	0.942
Treatment×Year		0.16	1	0.281
Nest cup temperature range °C				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Treatment		10.86	1	0.001
Heated	12.3 (0.5)			
Control	10.0 (0.5)			
Dropped terms:				
Year		1.58	1	0.209
Air temperature °C		0.02	1	0.896
Treatment×Year		0.07	1	0.790
Maximum nest cup temperature °C				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Treatment		38.12	1	<0.001
Heated	39.5 (0.3)			
Control	36.5 (0.3)			
Dropped terms:				
Year		1.97	1	0.161
Air temperature °C		0.00	1	0.978
Treatment×Year		0.19	1	0.665
Minimum nest cup temperature °C				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Year		5.16	1	0.023
2019	26.0 (0.5)			
2020	27.4 (0.4)			
Dropped terms:				
Treatment		1.65	1	0.199
Air temperature °C		0.03	1	0.867
Treatment×Year		0.00	1	0.996
Mean nest box temperature °C				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Year		7.60	1	0.006
2019	12.6 (0.2)			
2020	13.4 (0.2)			
Dropped terms:				
Treatment		0.00	1	0.988
Treatment×Year		0.04	1	0.841

2.3.2 Effects of heating on incubation behaviour

All parameter estimates and test statistics for the incubation behaviour models are presented in Supplementary Material, Table S1. Both on-bout and off-bout duration was longer for heated females (47.4±1.8 min and 6.6±0.3 min, respectively) than for unheated females (42.5±1.6 min and 5.7±0.3 min, respectively) (on-bout: P=0.038; off-bout: P=0.033). There was no effect of the experiment on on-bout frequency (P=0.101), off-bout frequency (P=0.101), or on the percentage of time the female spent on the nest (P=0.867).

2.3.3 Effects of heating on incubation duration

Parameter estimates and test statistics are presented in Table 3. The effect of the experiment on incubation length did not differ between years (treatment×year interaction: P=0.798). Treatment had no effect on incubation duration (P=0.191), but incubation duration differed between years, being longer on average in 2019 than in 2020 (P=0.013).

2.3.4 Effects of heating on chick cooling rate

Parameter estimates and test statistics are presented in Table 3. The reduction in cooling rate with age did not differ between treatments (treatment×chick age interaction: P=0.381) and the effect of heating did not differ between years (treatment×year interaction: P=0.370). There was a small but significant difference in the rate of cooling between treatments, with chicks from control nests losing heat 8% faster than chicks from heated nests, over the course of the cooling challenge (P=0.039; Figure 6A). Chick age strongly influenced the rate of temperature change, with less cooling from day 4 to day 10 of age (P<0.001; Figure 6A). Chick cooling rate was 9% faster in 2020 than 2019 (P=0.004). Brood size did not influence the cooling rate in chicks (P=0.496).

2.3.5 Effects of heating on chick homeothermy index

Development of homeothermy with age did not differ between treatments (treatment×chick age interaction: P=0.437) and the effect of heating did not differ between years (treatment×year interaction: P=0.383). Chicks became more homeothermic with age (P<0.001; Fig. 6B), but neither treatment (P=0.068), year (P=0.053) nor brood size (P=0.876) affected homeothermy index.

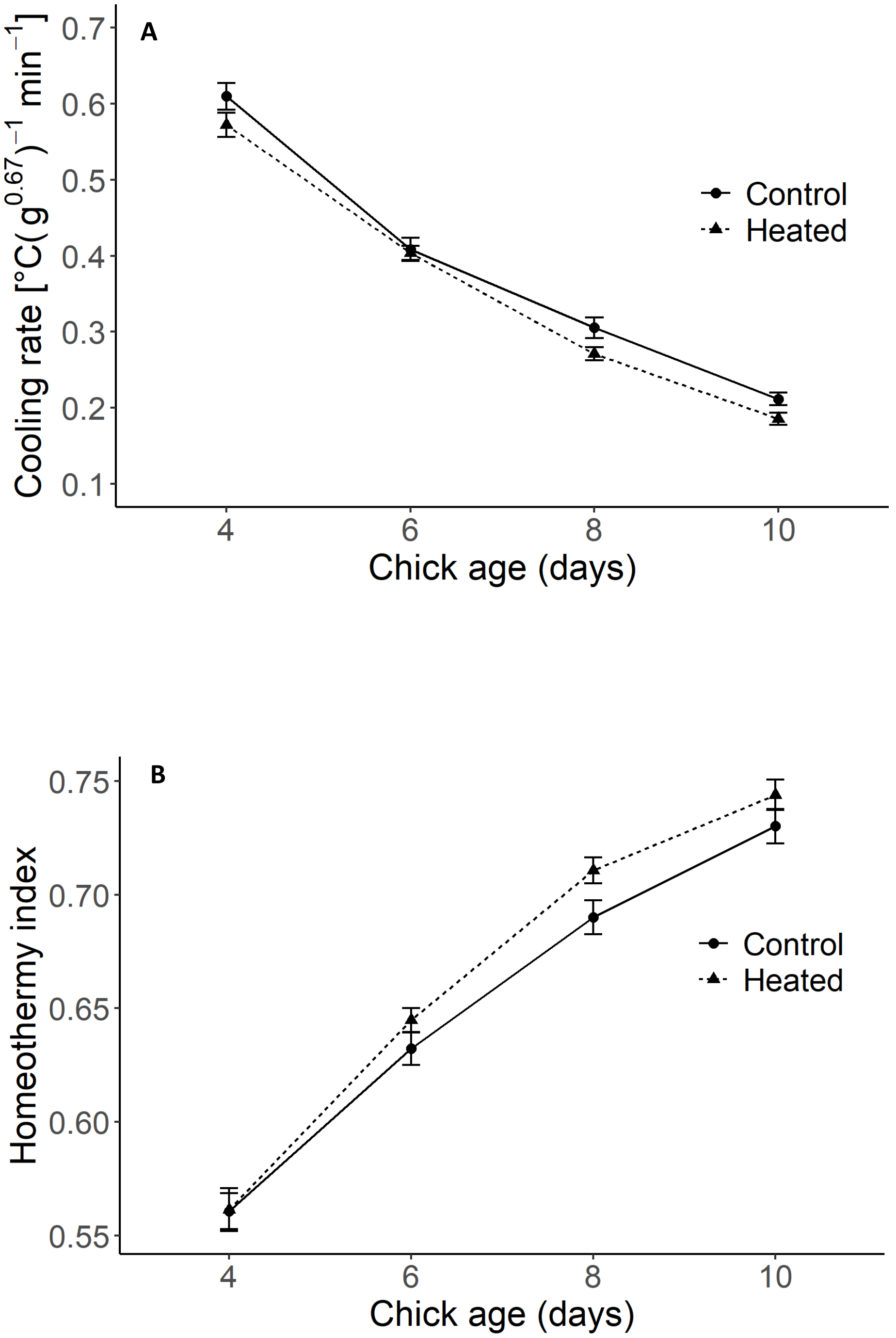


Figure 6. Cooling rate and homeothermy index of Blue Tit chicks from control and heated treatments. (A) Mean±SE surface area specific cooling rate for chicks from control nests and nests that were experimentally heated during incubation (*P*=0.039) on days 4, 6, 8 and 10 of age (*P*<0.001) for both years combined. (B) Mean±SE homeothermy index of chicks from control and heated nests on days 4, 6, 8 and 10 of age (age: *P*<0.001) for both years combined. Cooling rate and homeothermy index were calculated based on the difference in chick surface temperature before and after a 5 minute cooling challenge.

Table 3. Test statistics, degrees of freedom, *P*-values and parameter estimates for final models and *P*-values and test statistics for dropped terms, for models describing incubation length, cooling rate and homeothermy index. For cooling rate estimates, untransformed values are presented. Final models were derived using backward elimination of non-significant (*P*>0.05) terms.

	Incubation length (days)			
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Year		6.23	1	0.013
2019	14.2 (0.1)			
2020	13.8 (0.1)			
Dropped terms:				
Treatment		1.71	1	0.191
Treatment×Year		0.07	1	0.798
	Cooling rate (°C $[g^{0.67}]^{-1}min^{-1}$)			
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Age:		510.83	3	<0.001
Day 4	0.590 (0.012)			
Day 6	0.406 (0.009)			
Day 8	0.288 (0.008)			
Day 10	0.198 (0.006)			
Year:		8.20	1	0.004
2019	0.354 (0.016)			
2020	0.385 (0.016)			
Treatment:		4.25	1	0.039
Heated	0.358 (0.015)			
Control	0.386 (0.017)			
Dropped terms:				
Brood size		0.46	1	0.496
Treatment×Year		0.80	1	0.370
Treatment×Chick age		3.07	3	0.381
	Homeothermy index			
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Age:		308.01	3	<0.001
Day 4	0.561 (0.005)			
Day 6	0.639 (0.005)			
Day 8	0.701 (0.005)			
Day 10	0.737 (0.005)			
Dropped terms:				
Year		3.74	1	0.053
Treatment		3.33	1	0.068
Brood size		0.02	1	0.876
Treatment×Year		0.76	1	0.383
Treatment×Chick age		2.72	3	0.437

2.3.6 Effects of heating on chick morphometrics

Parameter estimates and test statistics are presented in Table 4. The increase in body mass with age did not differ between heated and control nests (treatment×chick age interaction: *P*=0.251) and the effect of heating did not differ between years (treatment×year interaction: *P*=0.532). However, chicks from heated treatments were heavier (7.9±0.1g) than chicks from control treatments (7.5±0.1g), when comparing body mass across all ages (*P*=0.031; Figure 7). Chick mass increased with age (*P*<0.001; Figure 7) and was positively influenced by brood size (*P*=0.001) but was not affected by year (*P*=0.186). The treatment×year interaction did not explain any variation in wing length (*P*=0.687). There was no difference in wing length of chicks from heated and control nests (*P*=0.142) and neither year (*P*=0.750) nor brood size (*P*=0.085) were significant. The treatment×year interaction did not explain any variation in tarsus length (*P*=0.733) and there was no effect of treatment on tarsus length (*P*=0.590). Chicks had longer tarsi in 2020 (16.7±0.1) than in 2019 (16.3±0.1) (*P*=0.002). There was no effect of brood size on tarsus length (*P*=0.693).

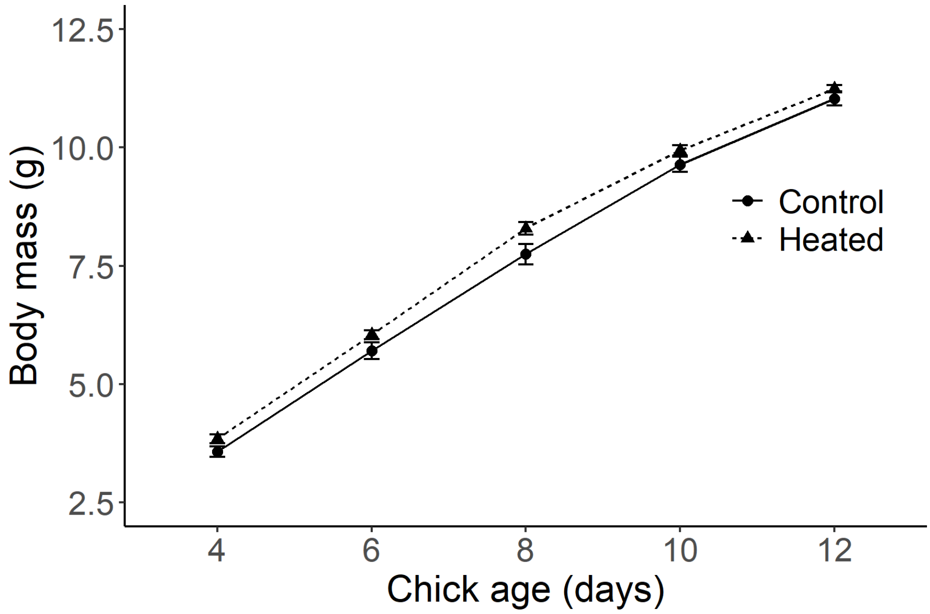


Figure 7. Body mass of Blue Tit chicks from control and heated treatments. Mean±SE body mass for chicks from control treatments and heated treatments (*P*=0.031) on days 4, 6, 8, 10 and 12 days of age (*P*<0.001) for both years combined. Body mass of each chick in the cooling challenge was taken at the end of the 5 minute cooling period on days 4-10. A final body mass measurement was taken on day 12 when chicks were ringed.

Table 4. Test statistics, degrees of freedom, *P*-values and parameter estimates for final models and *P*-values and test statistics for dropped terms, for models describing body mass and day 12 wing and tarsus measurements. Final models were derived using backward elimination of non-significant (*P*>0.05) terms.

Body mass (g)				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Age:		920.62	4	<0.001
Day 4	3.7 (0.1)			
Day 6	5.9 (0.1)			
Day 8	8.0 (0.1)			
Day 10	9.8 (0.1)			
Day 12	11.1 (0.1)			
Brood size:	0.09 (0.03)	12.07	1	0.001
Treatment:		4.65	1	0.031
Heated	7.9 (0.1)			
Control	7.5 (0.1)			
Dropped terms:				
Year		1.75	1	0.186
Treatment×Chick age		5.37	1	0.251
Treatment×Year		0.39	1	0.532
Wing length (mm)				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
-				
Dropped terms:				
Brood size		2.97	1	0.085
Treatment		2.15	1	0.142
Year		0.10	1	0.750
Treatment×Year		0.16	1	0.687
Tarsus length (mm)				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Year:		9.58	1	0.002
2019	16.3 (0.1)			
2020	16.7 (0.1)			
Dropped terms:				
Brood size		0.16	1	0.693
Treatment		0.29	1	0.590
Treatment×Year		0.12	1	0.733

2.4 Discussion

The experimental treatment increased nest cup temperature by 1.6°C, similar to temperature differences in other studies where a range of phenotypic effects have been observed in offspring (Nord and Nilsson, 2011; Durant et al., 2012; Hope et al., 2018). In this study, the change in nest cup temperature did not influence length of the incubation period, but chicks from heated nests grew larger and cooled more slowly when faced with a cold challenge.

The rate of cooling decreased with age in both treatments, but heated chicks had a slower cooling rate than control chicks, suggesting that higher incubation temperature can lead to an improved cold tolerance in offspring. This supports results from other cooling challenges, where birds incubated at higher temperatures were better equipped to deal with cold exposure (Durant et al., 2012; Durant et al., 2013; Nord and Nilsson, 2021). Differences in physiological development, such as neuroendocrine pathways, between treatments provide one explanation for cooling rate results. Heat production is largely dictated by the maturation of the hypothalamic-pituitary-thyroid (HPT) axis. Once activated by thermoreceptors, the hypothalamus produces Thyrotropin releasing hormone (TRH), which stimulates the pituitary to secrete thyroid stimulating hormone (TSH). This leads to increased production of thyroid hormones (T3 and T4) and these hormones are largely involved in temperature regulation (Debonne et al., 2008; Ruuskanen et al., 2021). If warmer nest conditions during incubation allow for accelerated maturation of the HPT axis, the resultant elevated heat production in response to cool temperatures could lead to slower body cooling for heated chicks. Alternatively, because of their larger size, heated chicks may have had a greater capacity to produce heat due to a larger amount of thermogenic tissue (Morton and Carey, 1971). That is, if heated chicks possessed a higher proportion of skeletal muscle, this would have allowed for more efficient shivering thermogenesis.

In this study, chick metabolism and shivering were not measured and therefore it is not known if cooling rate differences are a result of increased heat production or reduced heat loss. Other than a change in heat-producing capacity, differences in cooling rate could also be directly determined by size which was larger in heated chicks and has bearing for thermal mass. When altricial chicks first hatch, they are prone to high levels of heat loss, as their small size means they have a high surface area to volume ratio (Visser and Ricklefs, 1993a). Undeveloped insulation adds to this effect. As chicks age, their increased body mass leads to a decrease in surface area to volume ratio (Morton and Carey, 1971). This, and consequent growth of feathers, reduces heat loss rate. Therefore, body mass is one factor that aids in heat conservation of chicks (Visser and Ricklefs, 1993b). However, body mass differences between treatment groups were small. Additionally, body mass and surface area of chicks were accounted for in cooling rate calculations. Therefore, I believe that cooling rate results were not due solely to a difference in size between treatments and that earlier maturation of thermal physiology is a more probable factor.

Although similar patterns in cooling rate and homeothermy index were observed, there was no significant difference in homeothermy index between heated and control nests. Whilst cooling rate measures heat flux from the body surface, homeothermy index measures heat loss from the whole chick. It is arguable that for chicks facing cold challenges, overall heat loss from the animal is the most important consideration and therefore whilst heat flux was lower in heated chicks, this difference was not large enough to influence the degree of homeothermy at the level of the chick.

The homeothermy index results in this study differ to those of Nord and Nilsson (2021) who found that Japanese Quail (*Coturnix japonica*) incubated at low temperature were less homeothermic than birds from higher incubation treatments. Quail eggs were incubated in artificial conditions, therefore exact egg temperature was known and kept constant throughout development. In comparison, it is not certain that temperatures recorded in this study were a true reflection of exact egg temperature because dataloggers were not positioned inside the eggs. Despite this, results demonstrate that mean nest cup temperature was higher in heated nests by 1.6°C, similar temperature increments were used for Quail incubation. However, a key difference between these two studies is that one focuses on the response of altricial chicks, the other on the response of precocial chicks. Blue Tit chicks were cooled individually so that changes in body temperature were unaffected by the overall brood temperature. However, altricial chicks huddle together in the nest and so rarely experience situations where they are separate from the brood for any length of time. Indeed, a Blue Tit brood as a unit is homeothermic already from about day six after hatching (Andreasson et al., 2016). Additionally, they are provided with food from parents and are not required to forage independently. In contrast, precocial chicks feed independently from hatching. For these individuals, higher levels of heat production and thus an accelerated onset of homeothermy could be largely beneficial, as this would allow for increased foraging efficiency, even in cool environments (Jørgensen and Blix, 1988). If the benefit for altricial birds is not as great, this may explain why large differences in homeothermy were not observed in this study.

Neither cooling rate nor homeothermy index results support the hypothesis that cooler temperatures during incubation lead to an adaptive response in chicks, contrary to the responses of precocial birds in controlled experiments (Tzschentke, 2007, 2008; Shinder et al., 2009). Although studies on passerines have found higher metabolic rates with lower temperatures (Olson et al., 2006; Nord and Nilsson, 2011) it is still unclear as to whether this response is adaptive. If this were the case, a lower cooling rate and higher homeothermy index in control chicks would have been expected, as these individuals experienced a lower incubation temperature. However, in cooling rate at least, the opposite effect was seen suggesting cooler incubation temperatures do not lead to positive fitness consequences.

A positive effect of incubation temperature on body mass is commonly observed in lab studies (Göth and Booth, 2005; Hepp et al., 2006; Olson et al., 2006; Bertin et al., 2018). If chicks from control nests with lower incubation temperatures had higher metabolic rates (Nord and Nilsson, 2011), a greater proportion of food consumed post hatch may have been allocated to this increased energy demand, thus reducing the amount available for mass gain. Alternatively, corticosterone is found in larger quantities when incubation conditions are demanding (Tona et al., 2005; DuRant et al., 2010) and levels of corticosterone in eggs have been found to be lower in faster growing young birds (Saino et al., 2005). Therefore, if heated embryos have lower levels of this hormone due to the different nest cup temperature, this could lessen constraints on their growth. Although body mass was the only morphometric difference, mass is often found to be positively correlated with survival (Perrins, 1965; Smith et al., 1989; Råberg et al., 2005) indicating that the effect of nest heating may have long lasting benefits for chicks.

In contrast to body mass, there was no difference in wing or tarsus length between treatments, suggesting mass gain was compromised in favour of structural growth. Other studies involving nest cup temperature manipulation have found similar results in terms of temperature effects on tarsus and wing length (Rodríguez and Barba, 2016; Andreasson et al., 2018; but see Nord and Nilsson, 2011 where chicks from low incubation temperatures had shorter tarsi). These findings make sense as in the face of predation, quick development of wings and tarsus over body mass is likely to help with escape and reduce the amount of time chicks are constrained to the nest (Cheng and Martin, 2012).

Past studies have shown that temperature manipulation during incubation leads to modified behaviour of the incubating female. In a cross-fostering experiment, Perez et al. (2008) found that early chick condition was driven by egg temperature, as heated chicks had a higher body mass regardless of whether their rearing female had also received this treatment. However, the best indicator of mass and condition during the later chick stages was whether the female rearing the brood had received a heated treatment during incubation, as these heated females had a higher rate of nest provisioning. It is possible that heated females used less energy during incubation and therefore had greater energy reserves for post hatch activities like chick rearing. Supporting studies show that earlier in the chick stage, differences in phenotype are determined by developmental conditions whereas later in the chick stage, the environment the rearing female experienced whilst incubating plays more of a role in influencing offspring phenotype (Nilsson et al., 2008; Ardia et al., 2010).

It cannot be determined whether differences in chick cooling rate and body mass were the result of incubation environment or carry over effects from modified female behaviour. However, the experiment did not affect temperature in the nest box, suggesting the females incubated in similar thermal environments. Additionally, in the overnight hours (with no heating) where the female was continually in the nest, nest cup temperature was similar in both treatments. Finally, though results from incubation behaviour analysis show slight differences in on and off-bout durations between treatments, there was no effect of heating on the percentage of time the female spent in the nest. Therefore, I believe it is likely that female energy expenditure during incubation was similar in both treatments. If so, chick phenotypic changes were a result of warmer nest cup temperature rather than improved female performance.

2.5 Conclusion

This study investigated the effects of nest heating on chick phenotype and gave an insight as to how offspring respond to elevated developmental temperature. Although incubation temperature did not affect incubation duration, heated chicks were better able to withstand a cooling challenge than controls and were heavier between days 4-12 of life. Proximate explanations for slower cooling rates in heated chicks are unknown but may reflect quicker maturation of heat producing systems or a larger body mass and reduced surface area to volume ratio. It is arguable that heat loss at the whole animal level rather than heat flux has more biological significance for how a chick responds to cooling challenges. Studies testing the direction and magnitude of avian responses to changing environmental conditions are important, as extreme weather events are predicted to increase in frequency with climate change (IPCC, 2013). Slight increases in air temperature may remove some constraints for birds in cooler environments, but the likelihood of birds breeding or developing during heatwaves will increase and this may be problematic if individuals have a low tolerance to these conditions. Although no negative effects of heating were observed in this study, increases in nest cup temperature of a similar magnitude in already hot environments could have severe negative consequences for birds (Carroll et al., 2018). We should aim to further our knowledge of how developmental conditions can shape avian phenotypes across a range of environments, by carrying out similar studies across different latitudes to ascertain at what point increasing temperature during incubation ceases to be beneficial and instead becomes detrimental.

2.6 Ethics statement

All work involving nest disturbance was covered by licences 117614 (2019) and 156597 (2020) issued by Scottish Natural Heritage (SNH), held by Dr Davide Dominoni. I was permitted to ring chicks under supervision in 2019 (licence no. T0000) and alone in 2020 (licence no. C6823) by the British Trust for Ornithology.

3. Conclusions and future perspectives

The main aim of this thesis was to study variation in chick thermoregulation depending on nest cup temperature. I used a wild population of Blue Tits and experimentally increased nest cup temperature throughout the course of incubation. Most studies concerning developmental temperature effects on offspring thermoregulation do so in domestic birds, therefore ecological relevance is debatable. This study aimed to bridge a knowledge gap regarding effects of temperature change in free living birds.

My conclusions are as follows:

1. Nest microclimate during development can affect chick body mass and cooling rate when exposed to temperatures below their thermoneutral zone.
2. Although heated chicks may have had a small advantage due to their size, it seems differences in heat flux between treatments were not large enough to significantly influence heat loss at the whole animal level, i.e., the degree of homeothermy.
3. The heating treatment did not influence the percentage of time the female spent on the nest. Therefore, it seems nest cup temperature influenced chick phenotype more than carry over effects due to changes in female behaviour.

This study considered the effects of heating on nestling thermoregulation during the first 10 days of life and body mass during the first 12 days of life, before nestlings fledged. Thus, it is unknown whether effects of heating on chick cold tolerance would become more or less noticeable at later stages in life. There are contrasting findings in past studies regarding the long-term effects of heating during reproduction. In a study by Nord and Nilsson (2016), embryonic survival was lower in birds from the low incubation temperature but birds from the higher incubation temperature experienced longer term costs to survival. However, in a study where nests were heated post hatch, heated chicks were constrained early on in life, yet long term survival was greater in these individuals (Andreasson et al., 2018). Therefore, it would be beneficial to take similar studies further, to gauge whether greater temperatures in the nest translate into cold tolerance ability in adult life, particularly during the winter when birds face greatest cold challenges.

Although it can be speculated that cooling rate difference between treatments is due to a smaller surface area to volume ratio in heated chicks, proximate explanations are not known. It would be interesting in future studies to determine whether heated chicks have a greater capacity to produce heat at an early age by measuring metabolic rate (e.g., by the rate of oxygen consumption) during cooling challenges.

Finally, with predictions of warmer and wetter weather increasing in frequency, future breeding seasons are likely to see birds developing in more extreme or variable conditions in the West of Scotland (Adaptation Scotland, 2014). Temperature effects in this study are close to the extremes of long-term temperature increases if greenhouse gas emissions continue to rise (2050 high emissions scenario; summer mean temperature: 1.5°C; range: -0.1°C-3.1°C, Adaptation Scotland, 2014). Therefore, the work carried out for this thesis may be used to inform how changes in weather, in particular temperature, may affect breeding birds in temperate climates. These effects are likely to be even larger when birds face a heatwave, the likelihood of which occurring over the time of breeding is increasing (Adaptation Scotland, 2014).

I recommend that future experiments aim to manipulate nest temperature both within and beyond current climate model predictions to determine at which point, strong temperature effects on offspring may be seen. Furthermore, birds differ in their response to rising temperature, depending on the thermal conditions of the environment they inhabit. Carrying out similar comparative studies at different latitudes could highlight parts of the range of animals where increasing temperature becomes detrimental for reproduction. The climate of these geographical areas can then be highlighted as hazardous for this species and targeted for species conservation.

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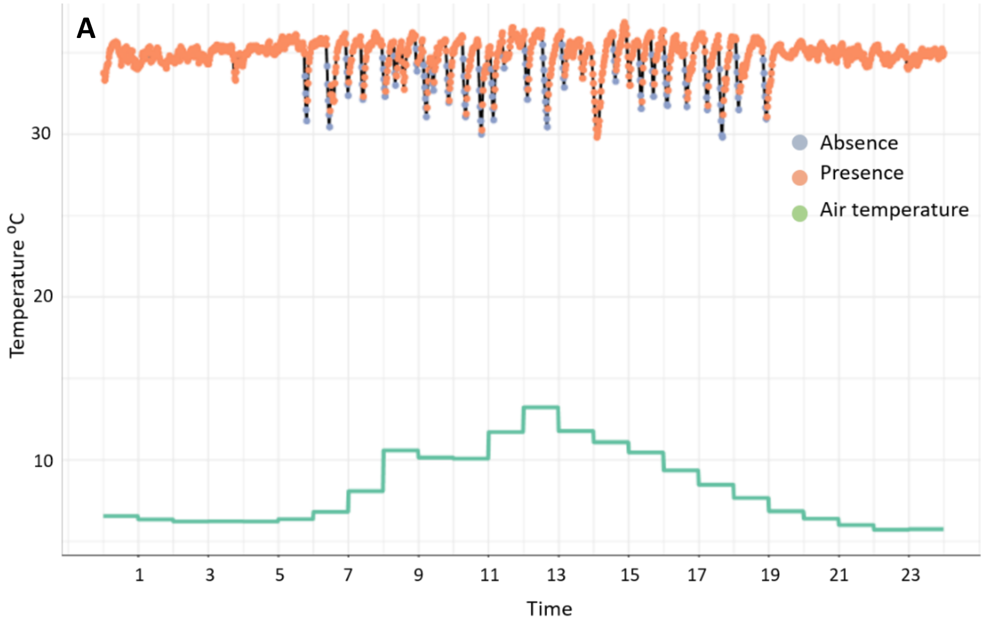
5. Supplementary Material

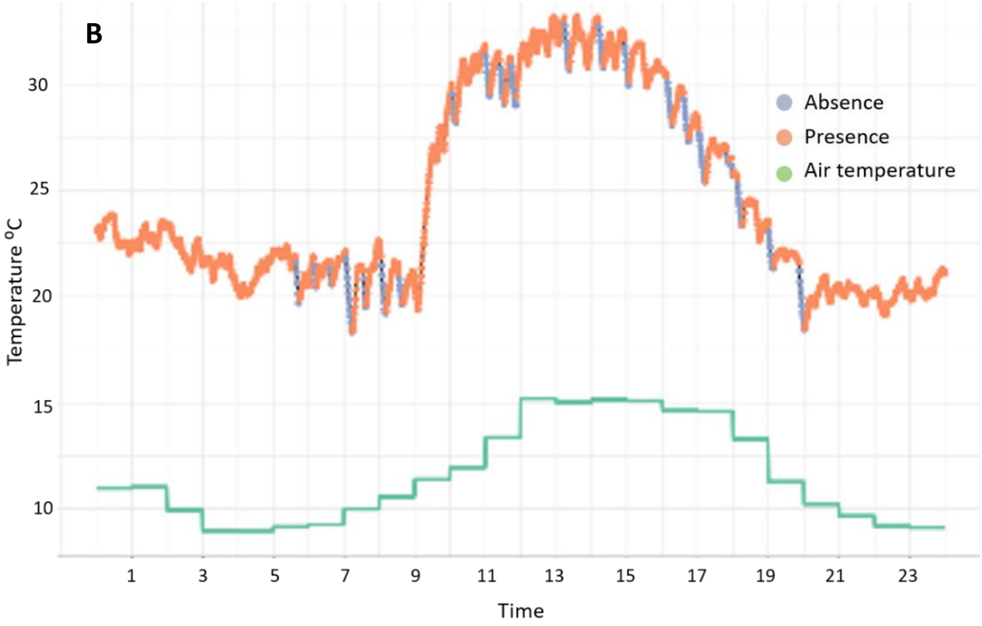
This supplementary material includes: Assessment of data quality for incubation behaviour analyses, Figure S1. and Table S1.

Assessment of data quality for incubation behaviour analyses

For a visual analysis of data, ‘incRplot’ within the ‘incR package’ was used to assess the quality of each day of data per nest (Figure S1). Nest cup temperature data of a high quality showed elevated, constant temperatures during the night followed by sharp peaks and drops in temperature during the day, due to the female leaving and returning to the nest. On occasion, it was still possible for the female to shift the position of the nest cup iButton despite fixing it to a weight. When this occurred, nest cup temperature tended to follow a similar trend to the air temperature inside the nest box. As a result, lower quality data were obtained, where differences in incubation between night and day were less distinguishable. In these instances, lower quality days were removed from the dataset.

In cases where iButtons were likely to be influenced by environmental temperatures during the night (again due to a shift in their positioning), a large maximum drop in nest cup temperature between two consecutive points would set high thresholds for incubation off-bouts. As a result, true off-bouts during the day were missed. Therefore, recordings with many off-bouts missing were removed from the dataset. Temperature loggers needed to be changed every 3 days and so gaps in data resulted in an incomplete incubation time series for that day. Data from these days were therefore removed from the dataset.


A
Temperature °C
30
20
10
Absence
Presence
Air temperature
1
3
5
7
9
11
13
15
17
19
21
23
Time


B
Temperature °C
30
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Absence
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Air temperature
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Time

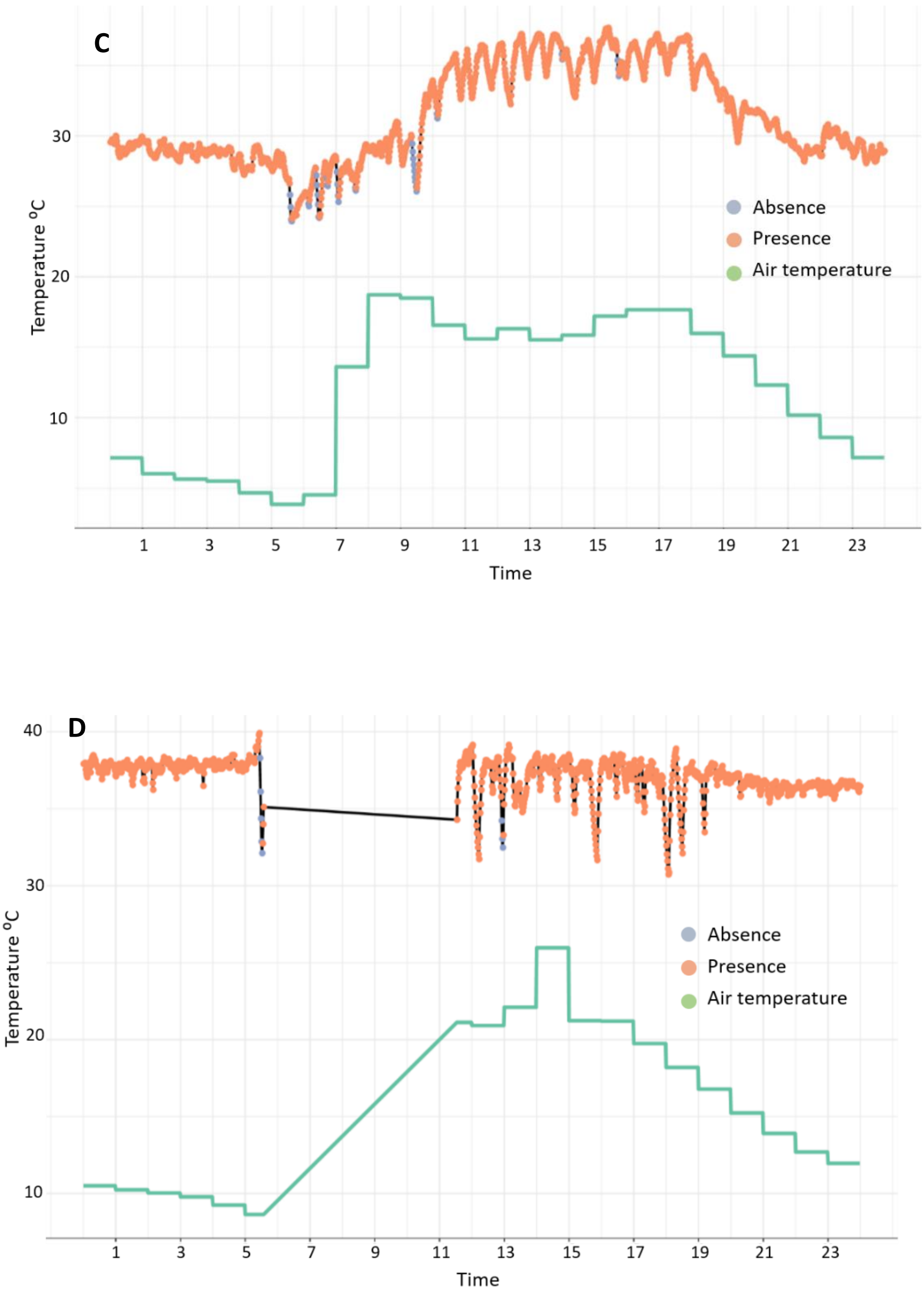

Figure S1. Plots highlighting examples of the range in data quality. (A) Good quality temperature logger data produced plots that showed high, constant nest cup temperatures during the night, easily distinguishable from daytime nest cup temperatures, where peaks and troughs indicate on-bouts and off-bouts. The temperature of the nest cup does not follow air temperature trend in the nest box. When the iButton was shifted in the nest, this could produce plots where nest cup temperature was lower than expected and followed a similar trend to nest box air temperature (B) or true off-bouts were missed (C). As a result of replacing temperature loggers every three days in 2019, there was often an incomplete time series for that day (D). Days with poor quality data were removed from the final data set.

Table S1. Output table for Linear mixed models (LMMs) used in incubation analyses. The table shows test statistics, degrees of freedom, *P*-values and estimates for final models and *P*-values and test statistics for dropped terms, for models describing on and off-bout frequency (per hour), bout duration and percentage of time spent incubating during the incubation period. Final models were derived using backward elimination of non-significant (*P*>0.05) terms based on likelihood ratio tests (LRT).

On-bout frequency				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
-				
Dropped terms:				
Treatment		2.69	1	0.101
Off-bout frequency				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
-				
Dropped terms:				
Treatment		2.69	1	0.101
On-bout duration (minutes)				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Treatment:		4.29	1	0.038
Heated	47.4 (1.8)			
Control	42.5 (1.6)			
Off-bout duration (minutes)				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
Treatment:		4.53	1	0.033
Heated	6.6 (0.3)			
Control	5.7 (0.3)			
Percentage of time spent on the nest				
Parameter	**Estimate (SE)**	**LRT**	**DF**	***P***
Final model:				
-				
Dropped terms:				
Treatment		0.03	1	0.867