

# Experimental manipulation of cavity temperature produces differential effects on parasite abundances in blue tit nests at two different latitudes

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## ABSTRACT

Although different predictive models forecast that climate change will alter the distribution and incidence of parasitic diseases, few studies have investigated how microclimatic changes may affect host-parasite relationships. In this study, we experimentally increased the temperature inside nest boxes of the blue tit *Cyanistes caeruleus* during the nestling period at two different latitudes (central Spain and central Germany) to determine its effect on parasite abundance. The two localities have contrasting climate conditions: the southern one in Spain is warmer and drier than the northern one in Germany. Consistent with this, we observed that the parasitic fauna in nests at the two localities differs. The flea species *Ceratophyllus gallinae* was more abundant in the northern locality, while the blowfly species *Protocalliphora azurea* and biting midge species of the genus *Culicoides* were more abundant in the southern one, as were blood parasites. Moreover, dermanyssid mites and blackflies (Simuliidae) were observed only in the southern locality. The temperature inside nest boxes was increased using heat mats placed underneath the nest material during the nestling period (day 3 to day13 post-hatching). Compared with control nests, the average temperature in heated nests increased by 2.24 °C and 1.35 °C at night in Spain and Germany, respectively. Consequently, the average relative humidity in heated versus control nests decreased 4.93 and 0.82 units in Spain and Germany, respectively. The abundance of blowfly pupae in the heated nests was significantly lower than that of control nests at both localities. The abundance of larval fleas was also lower in the heated nests, but only at the Spanish locality. Infection by the blood parasites *Haemoproteus*/*Plasmodium* was higher in males attending the heated nests in Germany, and the control nests in Spain. Moreover, both male body mass and nestling wing length were negatively related to the abundance of larval fleas. In conclusion, our results indicate that increased temperature at the nestling stage may affect the fitness of blue tits by altering parasite prevalence rates.

## 1. Introduction

The world's climate is changing and is expected to continue changing through the twenty-first century and beyond (IPCC, 2007). In fact, recent predictions forecast more drastic climate change than previously expected (Oldenborgh et al., 2009; Brysse et al., 2012). Many studies have warned about the consequences that climate change may have on ecosystems and populations (e.g., Parmesan and Yohe, 2003; Root et al., 2003; Rosenzweig et al., 2008; Charmantier et al., 2008), particularly as they relate to the abundance and distribution of pathogens, including metazoan and protozoan parasites, and the emergence of infectious

diseases in humans (reviewed in Daszak, 2000; Kovats et al., 2001). Various studies have also suggested that climate change could affect host-parasite interactions (Patz et al., 2000; Dobson et al., 2003; Brooks and Hoberg, 2007). For example, arthropod ectoparasite abundance has been shown to depend mainly on the off-host environment and, to a lesser extent, the abundance of hosts (van der Mescht et al., 2018). The impact of climate change on host-parasite interactions, however, is still difficult to assess as optimal ecological conditions are sometimes not known (Kutz et al., 2009; Merino and Møller, 2010; Penczykowski et al., 2015). In fact, many aspects related to the potential effects of climate change on infectious diseases and host-parasite interactions are not yet

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fully understood (Mouritsen and Poulin, 2002; Rohr et al., 2011). For example, climate change could more severely affect parasites that spend part of their life cycle independent of their vertebrate hosts than those that live on their hosts permanently (Merino, 2019). The potential impacts of climate change on hosts include changes in population size, behavior and physiology, which, in turn, may affect the ability of parasites to complete development (Morley and Lewis, 2014). The complexity of host-parasite interactions makes it challenging to predict how climate change may affect both the ecological and the evolutionary processes influencing such interactions (Kingsolver et al., 2011). By studying the effects of climate change on organisms (Potter et al., 2013; Reyes-García et al., 2016), especially those with host-parasite relationships, at different locations (Penczykowski et al., 2015) and even at broad geographic scales (Parmesan, 2006), we can better understand these processes and make more accurate predictions about future global trends.

Host species (Poulin, 2005), host abundance (Arneberg et al., 1998; Ellis et al., 2017) and host geographic distribution (Ellis et al., 2015; Ricklefs et al., 2016) all play a fundamental role in host-parasite relationships. Environmental change and host-parasite interactions are influenced by multiple factors including host resistance (or parasite infectivity), virulence level, and interactions between parasites, or between parasites and their predators, or between parasites and the host immune system (Lafferty, 2009; Wood and Johnson, 2015). The physiological responses of hosts, which could be influenced by environmental conditions (Morley and Lewis, 2014), also can modify host-parasite interactions. For example, Wegner et al. (2008) showed that an increase in ambient temperature reduced the immune activity of three-spined sticklebacks (*Gasterosteus aculeatus*), resulting in decreased survival in those with a high parasite load.

In the case of birds, it is well known that climate change is severely impacting different aspects of their life cycle (Carey, 2009); however, more studies on how bird populations respond to climate change are needed (Møller and Dunn, 2019). For example, several studies of birds have shown that climate change could lead to microevolutionary changes (Sheldon, 2010; Charmantier and Gienapp, 2013) and selection processes (Teplitsky and Charmantier, 2019). In fact, some bird species show a certain degree of plasticity in their response to climate change (Gienapp et al., 2008), including behavioral changes in response to high temperatures (Smit et al., 2016; Gudka et al., 2019). Likewise, an increase in temperature during incubation can affect nestling mass and body condition (Perez et al., 2008; Nord and Nilson, 2011; Vaugoyeau et al., 2017). Rodríguez et al. (2016), for instance, showed that great tits (*Parus major*) attending nests reaching high temperatures (~40 °C) produced smaller fledglings that had a lower post-fledging survival rate than those from nests with lower temperatures.

Climate change can also affect bird-parasite interactions (Merino, 2019). For example, Garamszegi (2011) showed that the increase in *Plasmodium* prevalence in different bird species is related to the increase in global temperatures over the last 20 years. Likewise, Zamora-Vilchis et al. (2012) showed that increased temperature positively affected vector abundance, and accelerated the development of blood parasites within vectors and, consequently, their transmission to birds. In the same way, Calero-Torralbo (2011) and Amat-Valero et al. (2013) showed the effect of temperature on the emergence and end of diapause of the hematophagous fly *Carnus hemapterus* in avian nest cavities over different breeding seasons. Furthermore, several studies have investigated the effect of temperature and humidity on the abundance of avian nest-dwelling ectoparasites (Heeb et al., 2000; Dawson et al., 2005a; Castaño-Vázquez et al., 2018; Dube et al., 2018). Similarly, Møller et al. (2013) suggested that an increase in temperature could play an important role in host-parasite interactions through changes associated with hosts such as fecundity or body condition. That is, changes in temperature can affect host fitness, specifically clutch size and body condition, which are components associated with parasite abundance. Møller (2010) also showed that environmental factors such as temperature and

rainfall were associated with the emergence of mosquitoes affecting a barn swallow (*Hirundo rustica*) population during the breeding season. As these studies highlight, environmental conditions are an important aspect to consider when assessing the effects of abiotic factors on host-parasite interactions. Therefore, it is important to understand how climate change affects not only bird morphology and physiology but also the relationship between birds and their parasites (Merino, 2019; García del Río et al., 2020).

Since environmental conditions can differ remarkably between latitudes, parasite prevalence or abundance can vary among populations of the same host species. Szöllosi et al. (2011), for example, found that the prevalence of hemoparasites in blue tit populations across Europe differed consistently according to parasite lineage and host population, indicating that the transmission success of parasites is partly shaped by locality-specific effects. Similarly, Merino et al. (2008) found a lower prevalence of hemoparasites of the genera *Haemoproteus* and *Plasmodium*, but a higher prevalence of *Leucocytozoon*, in several bird species found at higher latitudes. Thus, latitudinal, altitudinal and other ecological gradients, particularly as they relate to temperature or relative humidity, could differentially influence bird-parasite interactions in populations at different localities (Chapa-Vargas et al., 2020; Cuevas et al., 2020; Zamora-Vilchis et al., 2012). In order to assess the effect of temperature on parasites affecting the same bird species but under different climatic conditions, we experimentally increased the temperature (and indirectly decreased the relative humidity) within nest boxes of the blue tit *Cyanistes caeruleus* during the nestling period in two localities, one in Spain and the other in Germany.

Blue tit reproduction attracts many parasites and vectors to their nests. The impact of temperature on these invertebrates could differ depending on the length of time spent in nests. Some ectoparasites spend most of their life cycle inside nests, whereas others only visit nests in search of a blood meal. In any case, many of these ectoparasites could act as vectors for blood parasites. We studied the influence of temperature and relative humidity on the abundance of ectoparasites (mites, larval fleas, blowfly pupae, biting midges and blackflies) and blood parasites (*Haemoproteus*, *Leucocytozoon* and *Plasmodium*) in both localities during the same year. We also investigated the potential effect of temperature on the body condition of adults and nestling birds in both localities. We expected that the experimental increase in temperature and decrease in humidity inside nests would negatively affect the abundance of nest-dwelling ectoparasites (see Castaño-Vázquez et al., 2018) but not necessarily that of biting flying insects, which appear to be attracted to nests by the higher diffusion or production of gases expelled by nestlings under higher temperatures (see Castaño-Vázquez et al., 2020). Also, given the sensitivity of arthropods to humidity levels (Tsiafouli et al., 2005; Barnett and Facey, 2016), we expected to observe a greater effect on ectoparasite abundance in Spain than in Germany, due to the milder temperature and higher humidity characterizing the latter locality (Heeb et al., 2000; Wiedenfeld et al., 2007; Castaño-Vázquez et al., 2018). Finally, we evaluated whether the experimental temperature increase positively affected adult bird condition and nestling growth in these nests as a reduction in ectoparasite load could reduce the parental effort needed to raise nestlings, which grow better without parasites, or an increase in temperature may potentially reduce the time females devote to brooding.

## 2. Materials and methods

### 2.1. Study populations

This study was conducted during the 2017 blue tit breeding season. Experimental manipulation of nest boxes was performed in two populations, one located in Spain and the other in Germany.

#### 2.1.1. Spanish population

The Spanish population of blue tits was located in a Pyrenean Oak

(*Quercus pyrenaica*) deciduous forest in Valsain (Segovia, central Spain, 40° 53' 74" N, 4° 01' W, 1200 m.a.s.l.). During the breeding season, the studied population breeds in wooden nest boxes hanging from tree branches about 5 m above the ground. Nest box dimensions (HxWxD) were 17.5 cm × 11.7 cm × 12.5 cm. Each nest box had an entrance hole that was 4.5 cm in diameter and a small uncovered hollow (11.7 cm × 1.3 cm) just under the roof to air the box.

### 2.1.2. German population

Nest boxes of the German population of blue tits were located in three different habitats (urban, traditional orchards and forest) in central Germany between the towns of Giessen (50° 35' 3" N, 8° 40' 42" E, 159 m a.s.l.) and Pohlheim (50° 30' 30" N, 8° 43' 29" E, 245 m.a.s.l.). The urban sites were in and around Giessen (84,000 inhabitants), and included the university campus, the botanical garden and Old Cemetery Park. These nest boxes were made of breathable wood concrete and hung from trees about 2–3 m above the ground. The sites in the traditional orchards, comprised mainly of apple and cherry trees and extensively managed by sheep grazing, were located in and around the town of Pohlheim. The sites in the forest, which is predominately comprised of the deciduous European beech *Fagus sylvatica*, were located between the two towns. Nest boxes of two different dimensions were used at these sites: model 1 was 26 cm × 17 cm × 18 cm and model 2 was 25 cm × 18 cm × 29 cm.

## 2.2. Study species

The blue tit is a cavity-nesting bird widely distributed across the western Palearctic (Cramp and Perrins, 1993) that readily uses nest boxes for breeding. Most blue tit populations are sedentary, although in Scandinavia, part of the population migrates a short distance during spring and autumn (see Nilsson et al., 2008). Blue tits are insectivorous during the breeding season, and present a slight sexually dichromatic plumage, with males being more intensely colored than females (Cramp, 1998). During the breeding season, females can be easily differentiated from males by the presence of a brood patch on the abdomen. Females lay a single clutch with an average of 8.7 and 7.6 eggs in the Spanish and German populations, respectively.

## 2.3. Experimental design

During the breeding season (April to June), we experimentally manipulated the temperature inside nest boxes occupied by blue tits. Prior to manipulation, nests were paired according to hatching date and number of nestlings, and each nest within a pair was randomly assigned to either the heat treatment or the control group. A total of 40 nests (20 heated and 20 control nests) were matched in Spain. A total of 24 nests (12 heated and 12 control nests) were matched in central Germany. Of these, 11 (5 heated and 6 control) were located in urban sites (10 of model 1 and one of model 2), six (4 heated and 2 control) in orchards (all of model 2) and seven (3 heated and 4 control) in the forest (all model 1). Sample size varied among the sites because some data could not be obtained from some nests.

To manipulate the temperature inside nest boxes, heat mats (70 × 70 mm, 5V/1.5W; thermo Flächenheizungs GmbH, Germany) were installed for 10 days during the nestling period (from day 3 to day 13 post-hatching). For each of the manipulated nests, a metal grid was placed directly underneath the nest to separate the heat mat from the nest material. Heat mats were connected to lithium batteries (130.7 × 77.42 × 29.97 mm, 20 A/74 Wh; Imuto, X4, China) through a cord with a USB output (24-h autonomy). Batteries were replaced daily to ensure that heat mats functioned throughout the entire experimental period. Metal grids were also installed in the control nests boxes, but heat mats and batteries were not. Heated and control nests were visited at the same frequency during the experiment.

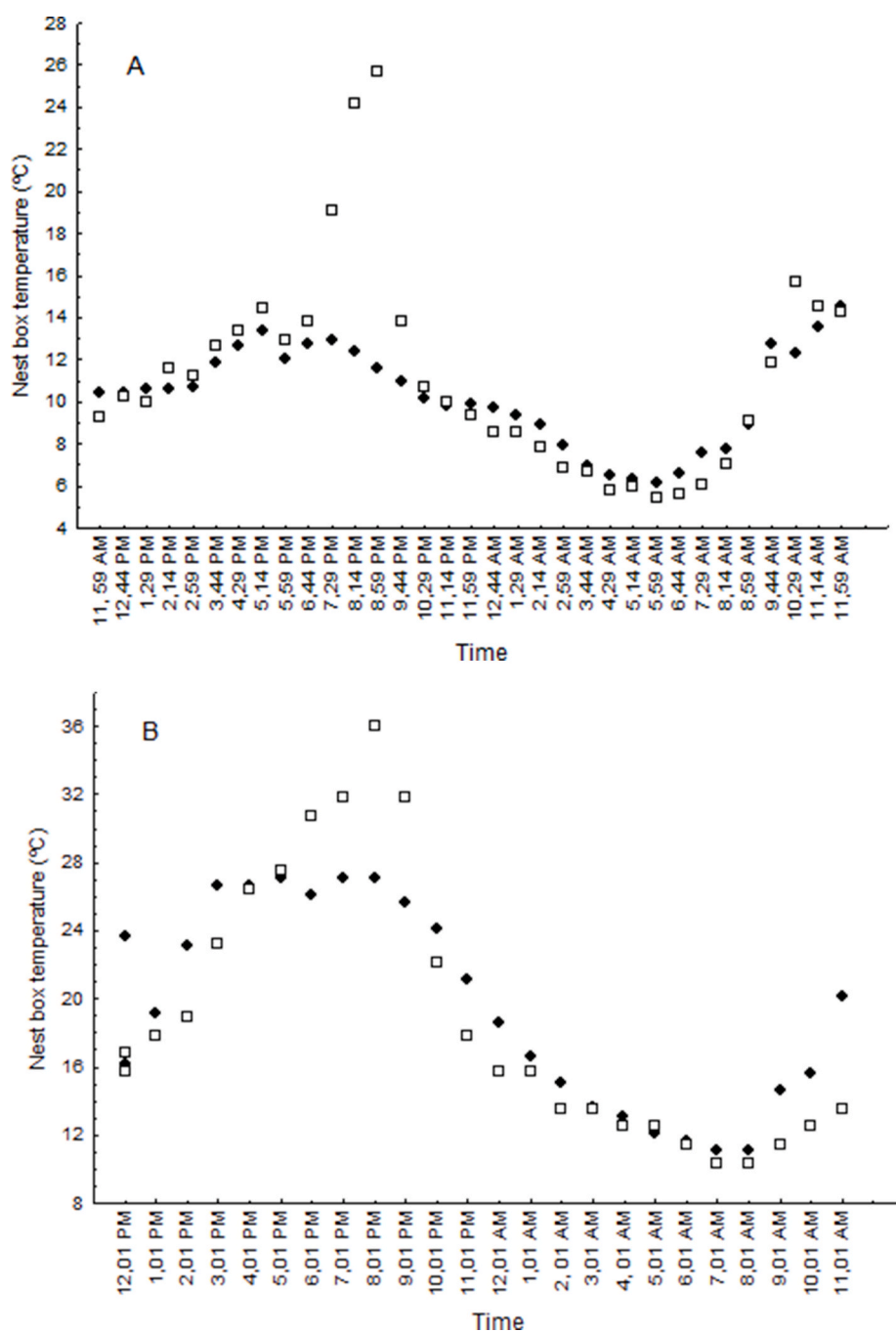
## 2.4. Measuring temperature and relative humidity

The temperature and relative humidity inside and outside of nests were measured during the nestling period (from day 3 to day 13 post-hatching). Nest boxes were fitted with sensors (Thermochron DS1921G-F5 and Hygrochron DS1923; 6 × 17 mm, temperature range: 40–85 °C, resolution 0.0625 °C; humidity range: 0–100%, resolution 0.04%; Maxim IC, USA) attached to the inner wall of the nest box just above the rim of the nest. These sensors registered both environmental variables every 45 min in Spain or every hour in Germany during the experimental period. For each day (24 h) from day 3–13 post-hatching, we calculated the average temperature and relative humidity. We also calculated the averages of both variables during the night (from 00:00 to 8:00 h) over the same period. We selected that hour interval because temperature is decreasing at nests and reaching its lower daily value (see results and Fig. 1). Sensors were removed from the nests once nestlings fledged (day 20 or 21 post-hatching). To measure the ambient temperature and relative humidity, three external sensors (Tinytag Plus 2; TGP-4500; data logger; 7.9 × 5.1 cm, temperature range: –25–85 °C, resolution 0.01 °C; humidity range: 0–100%, resolution 0.3%; Gemini Data Loggers, United Kingdom Ltd) were placed underneath empty nest boxes in both study areas during the nestling period.

## 2.5. Quantifying nest ectoparasites and blood parasites

Several biting flying insects and nest-dwelling ectoparasites, many acting as vectors for blood parasites, are commonly found in these bird populations. Biting midges, *Culicoides* spp. (Diptera: Ceratopogonidae) are among the most abundant of the hematophagous insects, and are distributed worldwide (Mellor et al., 2000). Female midges need a blood meal to lay eggs (although some autogenous species can lay the first batch of eggs without a blood feed). Moreover, biting midges are vectors of haemosporidian parasites of the genus *Haemoproteus* (Order Haemosporida; Valkiunas, 2005; Martínez-de la Puente et al., 2011), which are known to have detrimental effects on bird reproduction and survival (Merino et al., 2000; Martínez-de la Puente et al., 2010). Blackflies (Diptera: Simuliidae) are small insects that need bird blood to complete their life cycle. These insects are vectors of another common blood parasite of birds, *Leucocytozoon* spp. (Order Haemosporida), which produce chronic diseases in infected birds (Merino et al., 2000; Martínez-de la Puente et al., 2010). Mosquitoes (Diptera: Culicidae), although scarcely found in bird nests, act as vectors of *Plasmodium* (Order Haemosporida) for blue tits (Martínez-de la Puente et al., 2011).

Several nest-dwelling parasites are commonly found in blue tit nests. One is the blowfly *Protocalliphora azurea* (Diptera: Calliphoridae), a dipteran flying insect that lays eggs in bird nests. Blowfly larvae, after emerging from eggs, feed on the blood of nestlings before pupating in the nest material from which adult flies emerge. Other ectoparasites that feed on bird blood include adult fleas (*Ceratophyllus gallinae*, Siphonaptera: Ceratophyllidae) and mites (*Dermanyssus* spp., Mesostigmata: Dermanyssidae); however, it is unclear whether adult fleas are vectors of any diseases in blue tits. Infestation by fleas is mainly evidenced by the presence of larvae in nests because the adults remain attached to the birds or easily escape when nests are collected to quantify parasites (see below). Flea larvae develop in the nest material and feed on bird debris. *Dermanyssid* mite adults and nymphs feed on bird blood, and may play an important role as vectors of trypanosomes (Macfie and Thomson, 1929). Fleas and mites colonize bird nests by attaching to adult birds while they inspect potential nest cavities. Although not their main mean of transport, mites can also reach bird nests by phoresy on midges and blackflies (Marshall, 1981). Once nestlings fledged (day 20 or 21 post-hatching), the nest material was collected from each nest, sealed in a plastic bag and then transported to the laboratory where ectoparasite abundance was assessed. Nests were stored at 4 °C from 2 to 4 days prior to assessment. To defaunate the nests, they were placed in Berlese funnels for 48 h, under conditions of constant temperature and light



**Fig. 1.** Daily variation of temperature in nests of blue tits. Temperature is decreasing at 00:00 and lower values for the day are attained close to 8:00 h. Data from two different nests with nestling of 7 days old are represented from A) Spain and B) Germany.

provided by lamps (40W) placed 6 cm above the nest material (Merino and Potti, 1996; Tomás et al., 2007). Ectoparasite abundance (mites and larval fleas) was estimated by counting the material obtained from the funnels under a magnifying glass microscope [an OLYMPUS-SZX7 (ACH, Tokyo, Japan) and a MOTIC SMZ-143 stereo microscope (Germany) was used to count ectoparasites collected, respectively, in Spain and in Germany; see Merino and Potti, 1995]. Once defaunated in the funnels, the nests were dismantled and the number of blowfly pupae was counted.

We used a simple trapping method to estimate the abundance of biting midges and blackflies that visited the nests. We placed a plastic Petri dish (8.5 cm diameter; 55.67 cm<sup>2</sup>) containing a commercially available body oil gel (Johnson's baby oil gel with chamomile; Johnson

& Johnson, Düsseldorf, Germany) on the ceiling of each nest box (for more details, see Tomás et al., 2008). The petri dishes were placed inside nest boxes on day 10 post-hatching and retrieved on day 13. Subsequently, petri dishes were observed under a magnifying glass microscope and the number of biting midges and blackflies adhered to the gel was counted.

To detect for the presence of blood parasites, adult birds were captured from the nests when the nestlings were 13 days old and a blood sample was obtained from their brachial vein. One drop of blood was smeared on a slide and immediately air-dried. Smears were then fixed with methanol (96%) and stained with Giemsa for 45 min. Slides were visualized under a microscope (an OLYMPUS-BX43 in Spain and a Zeiss Primo Star in Germany). Half of the smear was scanned at 20X to search



for large parasites, such as *Leucocytozoon* and *Trypanosoma*; small intra-erythrocytic parasites, such as *Haemoproteus*, *Plasmodium* and *Lankesterella* spp., were detected using an 100× oil immersion objective (see Merino et al., 2000). Infections by *Plasmodium* were not detected or were indistinguishable from those by *Haemoproteus*. The abundance of *Haemoproteus/Plasmodium* was quantified as the number of parasites per 10,000 erythrocytes.

After capture and when necessary, adult birds were ringed for individual identification with numbered aluminum rings. Nestlings were also ringed at this time (day 13 post-hatching). We measured the mass of the adults and the mass and wing length of the nestlings at 13 days post-hatching to estimate body condition. Body mass and wing length were measured using an electronic balance ( $\pm 0.1$  g) and a ruler ( $\pm 0.5$  mm).

## 2.6. Statistical analyses

We first checked that both climatic variables (temperature and relative humidity) complied with normality assumptions. When these assumptions were not met, the variables were transformed logarithmically and non-parametric statistics were used. For the German population, differences in nest box size and habitat could influence several variables (see e.g. Vaugoyeau et al., 2016; Möller et al., 2014a, b). To check for any potential effect of box size and habitat, we used ANOVAs that included temperature and relative humidity as dependent variables, and nest box size or habitat and treatment, and their interactions (habitat location  $\times$  treatment or nest box size  $\times$  treatment), as factors. When an effect was detected in the data from the German population, we presented alternative analyses to control for the significant variable.

In order to compare the abundances of ectoparasites and hemoparasites between heated and control nest boxes, we used Generalized Linear Models (GzLM), specifically negative binomial regression models with a log function. The number of ectoparasites (mites, blowfly pupae, flea larvae, biting midges and blackflies) and hemoparasites (*Haemoproteus/Plasmodium* and *Leucocytozoon*) were dependent variables, and treatment (heated or control) and locality (Spain or Germany) were factors. Then, we used a likelihood-ratio chi-square test to compare the current model versus the null (intercept) model. This test assesses the overall significance of the model, indicating whether explained variance in our data is significantly higher than unexplained variance. A significant result of this test indicates that the model fit the data. In these cases, we explored the significance of the independent variables and their interactions.

ANCOVA was used to explore the relationship between different abundances of ectoparasites, heat treatment, locality and their interaction on body condition (mass of adult birds and mass and wing length of nestlings). Graphical and statistical analyses were performed in STATISTICA 7 ([www.statsoft.com](http://www.statsoft.com)) and IBM SPSS Statistics for Windows (Version 25.0. Released, 2017. Armonk, NY: IBM Corp).

## 2.7. Data deposition

Data are available at the Digital CSIC repository: <<http://hdl.handle.net/10261/148411>>

## 3. Results

### 3.1. Effect of heat treatment on temperature and relative humidity in blue tit nests

The temperature inside nest boxes was highly variable during the day. Given this, we did not observe any significant differences in temperature between heated and control nest boxes. In the nest boxes in Spain, the average nest temperature of the heated nest boxes were similar to that of the control boxes ( $20.38 \pm 1.91$  °C and  $19.24 \pm 2.00$  °C, respectively; paired *t*-test:  $t = -2.08$ ,  $df = 17$ ,  $p = 0.052$ ), however, relative humidity differed significantly between heated and control nests ( $60.77 \pm 5.88\%$  and  $64.56 \pm 6.29\%$ , respectively; paired *t*-

test:  $t = 2.57$ ,  $df = 17$ ,  $p = 0.019$ ). In the nest boxes in Germany, both the average nest temperature and relative humidity of heated boxes were similar to those of the controls (temperature:  $16.02 \pm 1.87$  °C and  $15.82 \pm 1.99$  °C, respectively; paired *t*-test:  $t = -0.39$ ,  $df = 9$ ,  $p = 0.703$ ; relative humidity:  $83.50 \pm 6.85\%$  and  $84.75 \pm 6.85\%$ , respectively; paired *t*-test:  $t = 0.34$ ,  $df = 8$ ,  $p = 0.742$ ). However, we observed significant and clear differences in these variables between heated and control nests at night (from 00:00 to 8:00 h), in the absence of variation due to direct solar radiation on nest boxes. Temperature during that interval are decreasing at nests and reaching its lower daily value (Fig. 1). Differences during that period are mainly related to heated nests having a higher temperature or lower relative humidity than controls during the night. All data reported hereafter refer to temperature and relative humidity conditions at night, unless otherwise stated.

In both localities, the average temperature of heated nests was significantly higher than that of the controls (Table 1). Conversely, the average relative humidity of heated nests was significantly lower than that of control nests only in Spain (Table 1). For the population in Germany, no significant differences in average nest temperature were observed among the nest boxes in different habitats (urban, forest and orchards; ANOVA:  $F_{2,16} = 0.47$ ,  $p = 0.635$ ) or of different sizes (ANOVA:  $F_{1,18} = 0.36$ ,  $p = 0.553$ ). The interaction between habitat or box size and treatment were also not significant (ANOVA, habitat  $\times$  treatment:  $F_{2,16} = 0.20$ ,  $p = 0.821$ ; size box  $\times$  treatment:  $F_{1,18} = 0.35$ ,  $p = 0.560$ ). Furthermore, the overall average relative humidity of these heated nests was similar to that of control nests (Table 1). However, the average relative humidity of nests in the orchard habitat ( $82.76 \pm 1.69\%$ ) was significantly lower than those of nests in the forest ( $92.32 \pm 1.57\%$ ) and the urban habitats ( $87.88 \pm 1.57\%$ ) (ANOVA:  $F_{2,14} = 6.55$ ,  $p = 0.009$ ). Moreover, the average nest relative humidity in the wider and deeper boxes (model 2:  $82.76 \pm 1.83\%$ ) was significantly lower than that in the shallow boxes (model 1:  $90.10 \pm 1.20\%$ ) (ANOVA:  $F_{1,16} = 7.89$ ,  $p = 0.012$ ). However, the interaction between habitat or box size and treatment were not significant (ANOVA: habitat  $\times$  treatment:  $F_{2,14} = 1.55$ ,  $p = 0.246$ ; size box  $\times$  treatment:  $F_{1,16} = 2.06$ ,  $p = 0.169$ ).

Comparison of nests between the two localities revealed that the average nest temperature inside control nests in Spain was significantly higher than that of control nests in Germany (Table 1). Similarly, heated nests in Spain had a higher average temperature than those in Germany. By contrast, the control and heated nests in Germany both had a significantly higher average relative humidity than those nests in Spain (Table 1). The average ambient temperature during the nestling period at night was significantly higher in Spain than in Germany ( $10.70 \pm 1.42$  °C and  $9.59 \pm 1.28$  °C, respectively; Mann-Whitney *U* test:  $Z = 2.96$ ,  $p = 0.003$ ). Conversely, the average relative humidity was significantly higher in Germany than in Spain ( $87.76 \pm 1.82$  °C and  $72.50 \pm 6.66$  °C, respectively; Mann-Whitney *U* test:  $Z = -6.44$ ,  $p < 0.001$ ).

### 3.2. Effect of heat treatment on ectoparasite and hemoparasite abundance

The abundance of larval fleas in heated versus control nests did not differ significantly at either locality ( $B = -0.17$ ,  $F_{1,60} = 3.86$ ,  $p = 0.054$ ); however, it was significantly lower in nests (heated and control) in Spain than in those in Germany ( $B = -4.76$ ,  $F_{1,60} = 25.60$ ,  $p < 0.001$ ; Table 2). Moreover, a significant interaction between treatment and locality was observed for larval flea abundance, indicating an effect of heat treatment occurred in nests in Spain but not in those in Germany ( $B = 2.89$ ,  $F_{1,60} = 4.86$ ,  $p = 0.031$ ). Specifically, in Spain, the heated nests had fewer larval fleas than the control nests, however, no significant differences in larval flea abundance were observed between these nests in Germany. Heat treatment had no effect on larval flea abundance in nests in Germany ( $B = 1.32$ ,  $F_{1,18} = 0.01$ ,  $p = 0.931$ ), even after controlling for the significant effect of habitat ( $B = 7.06$ ,  $F_{2,18} = 25.76$ ,  $p < 0.0001$ ).

Blowfly pupae abundance was significantly higher in control nests compared with heated nests ( $B = 1.10$ ,  $F_{1,60} = 7.99$ ,  $p = 0.006$ , Fig. 2)

**Table 1**

Average temperature and relative humidity differences between heated and control nest boxes of blue tits (*Cyanistes caeruleus*) in Valsain, Spain (20 control and 20 heated nests) and in Giessen and Pohlheim, Germany (12 control and 12 heated nests) at night (from 00:00 to 08:00 h). The results of paired *t*-tests comparing treatments are shown. Unpaired *t*-tests were used to compare localities within groups (A vs B), except in the case of relative humidity for the heated groups, which was compared by using the Mann-Whitney *U* test. \* values corresponding to an ANOVA that controlled for the significant effect of habitat (urban, forest or orchard).

	Temperature				Relative Humidity			
	Control	Heated	<i>t</i>	<i>p</i>	Control	Heated	<i>t</i>	<i>p</i>
	Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD		
A: Spain	14.33 ± 2.20	16.57 ± 2.89	−3.16	0.005	68.87 ± 6.49	63.94 ± 5.19	3.13	0.006
B: Germany	11.67 ± 1.82	13.02 ± 1.87	−2.55	0.031	89.16 ± 4.72	88.34 ± 5.86	1.55*	0.246*
A vs B	<i>t</i> : 3.29 <i>p</i> = 0.003 <i>t</i> : 3.88 <i>p</i> < 0.001				<i>t</i> : 4.06 <i>p</i> < 0.001 <i>U</i> : 10.78 <i>p</i> < 0.001			

**Table 2**

Ectoparasite abundance (mean ± SD) in heated and control nest boxes of blue tits (*Cyanistes caeruleus*) in Spain (20 control and 20 heated nests) and in Germany (12 control and 12 heated nests).

Ectoparasites	Spain		Germany	
	Control	Heated	Control	Heated
Flea larvae	34.2 ± 72.3	2.3 ± 8.9	223.2 ± 318.7	263.9 ± 338.8
Blowflies	24.5 ± 15.4	10.5 ± 8.0	1.7 ± 3.5	0.6 ± 1.2
Mites	117.2 ± 147.2	221.9 ± 223.9	0.0 ± 0.0	0.0 ± 0.0
Biting midges	9.3 ± 20.4	6.6 ± 8.0	0.2 ± 0.6	0.2 ± 0.6
Blackflies	1.6 ± 1.8	0.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0

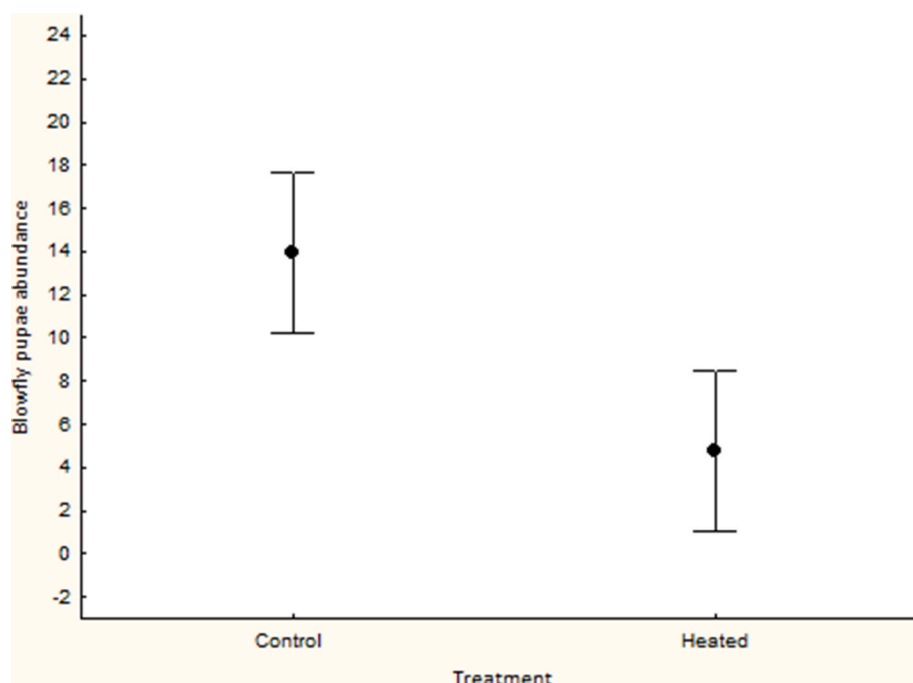
and they were also more abundant in nests in Spain than in nests in Germany ( $B = 2.89$ ,  $F_{1,60} = 58.02$ ,  $p < 0.001$ ). However, the interaction between locality and heat treatment on blowfly abundance was not significant ( $B = -0.25$ ,  $F_{1,60} = 0.13$ ,  $p = 0.722$ ).

Biting midge abundance did not differ significantly between heated and control nests ( $B = 0.09$ ,  $F_{1,59} = 0.06$ ,  $p = 0.809$ , Table 2) at either locality; however, nests in Spain had a significantly higher abundance compared with those in Germany ( $B = 3.68$ ,  $F_{1,59} = 28.56$ ,  $p < 0.001$ ). In this case, the interaction between locality and heat treatment was not significant ( $B = 0.26$ ,  $F_{1,59} = 0.02$ ,  $p = 0.885$ ). Blackflies and mites were

only observed in nests in Spain, and the abundances of these ectoparasites were not significantly different between heated and control nests ( $B = 0.63$ ,  $F_{1,38} = 2.26$ ,  $p = 0.141$  and  $B = -0.64$ ,  $F_{1,38} = 3.08$ ,  $p = 0.087$ , respectively).

No significant differences were observed in *Haemoproteus/Plasmodium* abundance in female blue tits attending heated nests versus those in control nests ( $B = 0.83$ ,  $F_{1,50} = 1.42$ ,  $p = 0.238$ ), nor between the females from the two localities ( $B = 0.63$ ,  $F_{1,50} = 0.63$ ,  $p = 0.430$ , Table 3). Furthermore, the interaction between locality and treatment was not significant ( $B = -0.47$ ,  $F_{1,50} = 0.23$ ,  $p = 0.634$ ). Differences in *Haemoproteus/Plasmodium* abundance in males attending heated versus control nests were also not significant ( $B = -2.08$ ,  $F_{1,50} = 3.01$ ,  $p = 0.089$ ). However, male blue tits from nests in Spain had a significantly higher abundance of *Haemoproteus/Plasmodium* than the males in Germany (average ± SD:  $38.47 \pm 51.60$  in Spain and  $8.16 \pm 16.11$  in Germany;  $B = 0.64$ ,  $F_{1,50} = 16.84$ ,  $p < 0.001$ , Table 3). In addition, the interaction between locality and heat treatment on *Haemoproteus/Plasmodium* abundance in blue tit males was significant ( $B = 2.65$ ,  $F_{1,50} = 8.81$ ,  $p = 0.005$ , Fig. 3), indicating that males attending control nests in Spain had higher abundances of *Haemoproteus/Plasmodium* than those attending control nests in Germany (Tukey HSD test:  $p = 0.035$ ), though there were no differences in abundance between males attending the heated nests in the two localities, nor between them and males attending the control nests (Tukey HSD test:  $p > 0.05$  in all cases).

Evidence of infections by *Trypanosoma*, *Lankesterella* spp. or



**Fig. 2.** Blowfly pupae abundance observed in control and heated nests of blue tits (*Cyanistes caeruleus*) in both localities (Spain and Germany). The data presented was controlled for the locality and the interaction between locality and treatment. Means ± intervals of confidence at 95% are shown.

**Table 3**

*Haemoproteus/Plasmodium* abundance (mean  $\pm$  SD) in male and female blue tits (*Cyanistes caeruleus*) attending heated and control nest from Spain and in Germany. Sample size is shown between brackets.

	<i>Haemoproteus/Plasmodium</i>			
	Spain		Germany	
	Control	Heated	Control	Heated
Males	48.27 $\pm$ 61.39 (18)	27.43 $\pm$ 36.62 (16)	1.80 $\pm$ 4.04 (10)	14.50 $\pm$ 21.02 (10)
Females	60.33 $\pm$ 72.52 (18)	42.23 $\pm$ 55.58 (17)	51.30 $\pm$ 83.67 (10)	22.44 $\pm$ 66.95 (9)

*Leucocytozoon* was not detected by microscopy in any of the birds sampled in Germany, and only *Leucocytozoon* was detected at low abundances in males in Spain (average  $\pm$  SD: 0.02  $\pm$  0.15). Given this result, these blood parasite infections were not further analyzed.

### 3.3. Effect of heat treatment and ectoparasites on body mass and wing length

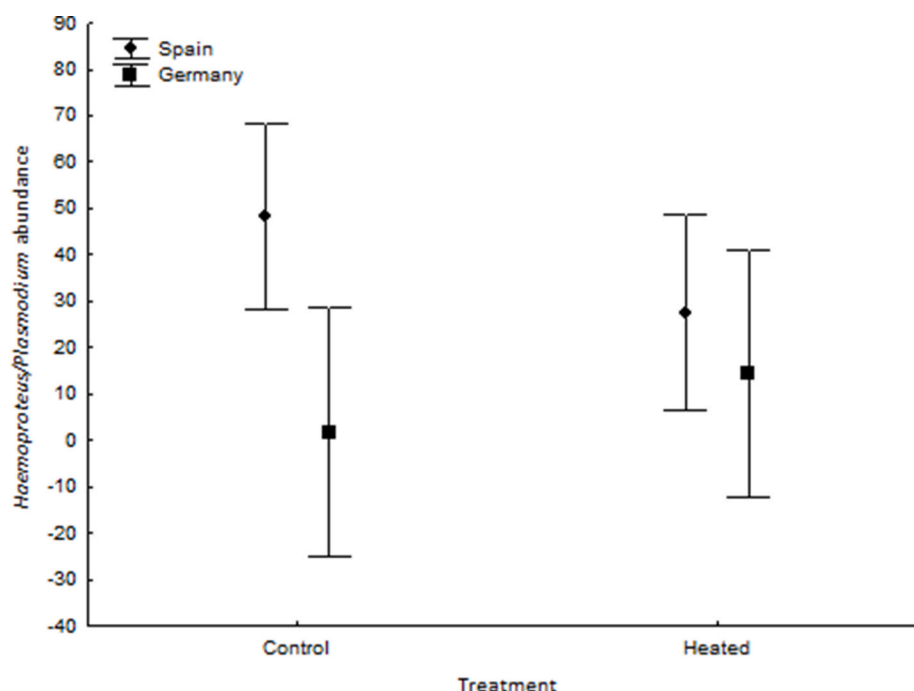
Heat treatment was positively and significantly related to the body mass of males (ANCOVA,  $F_{1,47}$ : 4.12,  $p = 0.048$ ), although this effect was only due to differences observed in the males from Germany (ANCOVA, locality  $\times$  heating treatment interaction;  $F_{1,47}$ : 4.24,  $p = 0.045$ ; Fig. 4). Locality also had a significant effect on male body mass (males in Germany weighed more than those in Spain; ANCOVA,  $F_{1,47}$ : 7.34,  $p = 0.009$ ), whereas larval flea abundance (ANCOVA,  $F_{1,47}$ : 4.36,  $p = 0.042$ ), but not those of other ectoparasites, (ANCOVA,  $p > 0.05$  for all cases) had a negative effect. The body mass of females and nestlings did not vary with heat treatment, locality or ectoparasite abundance (ANCOVA,  $p > 0.05$  for all cases).

Heat treatment did not affect the wing length of nestlings (ANCOVA,  $p > 0.05$ ). However, nestling wing length was significantly and negatively related to the abundance of larval fleas (ANCOVA,  $F_{1,53}$ : 4.04,  $p = 0.049$ ), but not of other ectoparasites (ANCOVA,  $p > 0.05$  for all cases). The wing of nestlings in Germany was significantly longer than the wing of nestlings in Spain (ANCOVA,  $F_{1,53}$ : 17.52,  $p < 0.001$ ).

## 4. Discussion

Under the current scenario of climate change, a potential effect of temperature and humidity on different organisms is expected (Hoegh-Guldberg et al., 2018). Birds and their parasites are no exception. In the case of birds, their flight ability allows them to look for habitats with more favorable climatic conditions via short or even long migrations. In this context, their parasites may also travel with them to new habitats, potentially infecting new bird species, or the birds could come into contact with new parasites in the new localities (Phillips et al., 2010). However, some birds are non-migratory and will likely have to adapt somehow to changing climatic conditions. Climate effects on birds are complex (Møller and Dunn, 2019), and may also influence their interaction with parasites (Merino, 2019). Although several models predict diseases will spread due to climate change, higher temperatures and lower humidity levels could also negatively affect parasites, especially those that have a free-living stage (Castaño-Vázquez et al., 2018). In order to better understand how changes in some climatic variables potentially affect host-parasite interactions, we experimentally manipulated nest temperature, and indirectly relative humidity, during blue tit nestling development in two localities with differing climates (one in Spain and one in Germany). To our knowledge, this study is the first to report such a manipulation to test whether an increase in temperature differentially changes parasitic abundances in nests of the same species found at different latitudes. Although nest temperature during nestling development was highly variable in both heated and control nests during the day, our experimental design was able to significantly increase the temperature in heated versus control nests at night (from 00:00 to 8:00 h) in both localities. We observed that nest temperature in control and heated nests in Spain was significantly higher than that of those nests in Germany, probably due to the fact that the ambient temperature during the bird breeding season was significantly higher in Spain than in Germany.

Heat treatment caused a reduction in relative humidity in heated nests compared with control nests, but only in Spain. The ambient relative humidity was higher in Germany than in Spain; therefore, the heat treatment may not have had a significant impact on the relative humidity inside the nests in that country. In fact, the average relative



**Fig. 3.** Differences in the abundance of *Haemoproteus/Plasmodium* in blue tit males by treatment and locality. Means  $\pm$  intervals of confidence at 95% are shown.

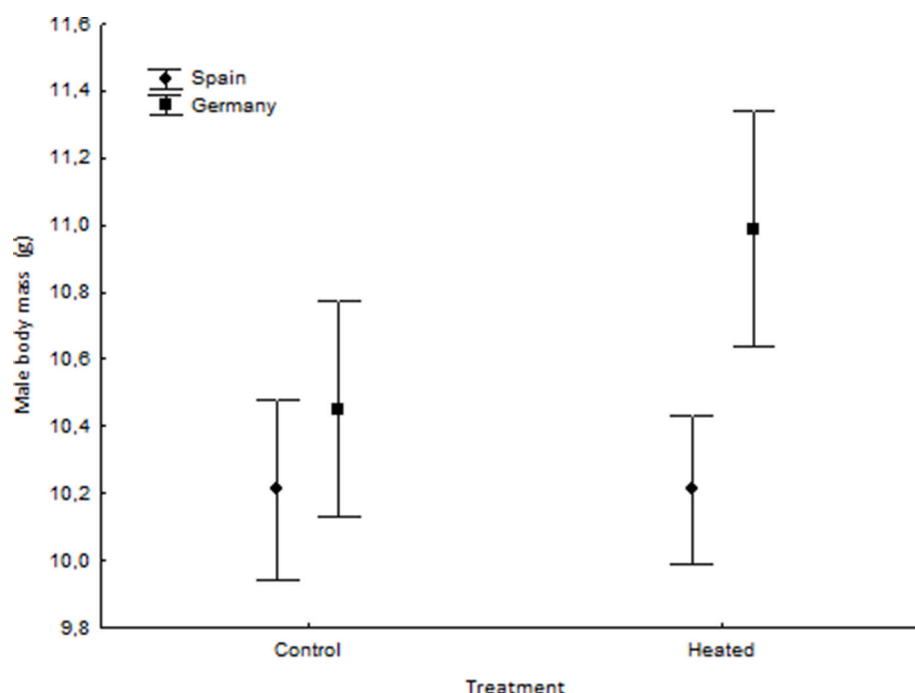


Fig. 4. Differences in the body mass of adult blue tit males by heat treatment of nest boxes and locality. Means  $\pm$  intervals of confidence at 95% are shown.

humidity of both nest groups (control and heated) in Germany was higher than that of those nests in Spain. In Europe, ambient temperatures are lower at higher latitudes (Schönwiese and Rapp, 2013); thus, local conditions likely account for the humidity differences observed between the two localities. Likewise, differences in nest box material and dimensions between the localities may have also contributed to their differential response to heat treatment.

We found a higher abundance of larval fleas and a lower abundance of biting midges and blowfly pupae in the nests in Germany compared with those in Spain. These results suggest that a higher level of humidity inside nests, together with a lower temperature, may favor flea development inside nests. Indeed, Hebb et al. (2000) showed that high humidity levels are necessary for the development of ectoparasites such as fleas inside nests. Furthermore, differences in the number of larval fleas between heated and control nests were nearly significant, and the interaction between locality and treatment in relation to larval flea abundance clearly indicates that heat treatment had an effect on nests in Spain but not on those in Germany. Overall, these findings support the importance of humidity level for fleas, which were affected by treatment at the less humid locality. Consistent with our results, nest humidity is not especially important for biting midges as these insects only visit nests for a short period of time to obtain a blood meal. The lower abundance of parasitic flying insects in the nests in Germany may be due to the more urban condition of this locality. For instance, there may be more reproductive sites for dipterans (e.g., small ponds and streams), and less pesticide use and pollution, in the mountainous study area in Spain compared with the urban one in Germany. Furthermore, blackflies need running waters to reproduce and therefore, as expected, were more abundant in the Spanish locality, which has been previously characterized as having abundant sources of water (Merino et al., 1997, 2008). Regarding the mites, we hypothesize that their presence in only the nests in Spain may be associated with the lower humidity levels found in this warmer locality.

Heat treatment negatively affected the abundance of blowfly pupae in both localities, consistent with the findings of previous studies (Dawson et al., 2005a; Castaño-Vázquez et al., 2018). However, this treatment did not affect the abundance of other ectoparasites found in nests including blackflies and mites (found only in Spain) and biting

midges (in both localities). Differences in the abundance of biting midges between localities may be associated with the prevalence of blood parasites in the birds. Biting midges are the main vector of *Haemoproteus* (Atkinson and Riper, 1991), and both the vector and the blood parasite were more abundant in the nests/birds in Spain than in those in Germany. However, other factors such as rainfall may be directly related to the abundance of midges and *Haemoproteus* in blue tits (Martínez-de la Puente et al., 2009; Illera et al., 2017). Males attending the control nests in Spain had more *Haemoproteus/Plasmodium* infections than those attending the control nests in Germany; however, no significant differences in infection were observed between males attending heated nests in both localities, or between these males and those attending the control nests. The lower incidence of these blood parasites in Germany, along with the slight positive effect of treatment on males attending heated nests in Spain, could be indicative of, for example, a reduction in parental effort due to low ectoparasite abundances, which may explain the lower impact of blood parasites in males in the different localities and treatments compared with those attending control nests in Spain. In addition, males attending heated nests could also indirectly benefit from energy savings due to treatment on females while brooding nestlings although that potential effect on females was not detected.

The experimental increase in nest temperature did not affect the body mass of adult blue tit females or the body mass and wing length of nestlings. However, males from the heated nests had a higher average body mass compared with those from control nests, but only in Germany. The link between heat treatment and male body mass may be due to a reduction in male reproductive effort. For instance, if the heat treatment led to a reduction in nestling investment in terms of thermal regulation, and also a reduction in the abundance of ectoparasites attacking nestlings, males but apparently not females may have reduced their parental effort, allowing them to increase their mass. In this sense, Dawson et al. (2005b) found that nestlings of the tree swallow *Tachycineta bicolor* between 4 and 16 days of age presented higher survival, weight gain and growth rate of the ninth primary feather when the nest temperature was increased by approximately 5 °C. However, we did not observe any effect of heat treatment on indicators of nestling condition. Other studies relating temperature and nestling growth have found contrasting results. For example, Rodríguez and Barba (2016) observed



a reduction in nestling mass in the great tit *Parus major* at day 15 post-hatching after they were subjected to an average temperature increase of around 5.2 °C during the nestling period. Andreasson et al. (2018) found a lower mass gain in blue tit nestlings when nest temperatures approached 50 °C. Similarly, Rodríguez et al. (2016) showed that great tit nests with temperatures around 40 °C produced smaller fledglings that had a lower post-fledging survival rate. Our experimental manipulation (an increase of 2.24 °C in Spain and 1.35 °C in Germany; see Table 1) was clearly soft compared with the aforementioned studies. Perhaps the temperature increase was not sufficient to cause changes in nestling body condition; however, very high temperatures inside nests are known to be detrimental for nestlings (Greño et al., 2008). Another difference between our study and the previous ones is that we assessed the effect of nest temperature (and relative humidity) changes produced mainly at night rather than during the entire day (24 h). We could increase the temperature even more to affect nestlings and parasites during the entire day, however, in view of our results, we would expect a clear detrimental effect on parasites and perhaps also on nestlings. In any case, these possibilities need further investigation.

The body mass of adults and body mass and wing length of nestlings did not vary with the abundance of ectoparasites and blood-sucking insects inside nests, except in those cases that appeared to be negatively related to larval flea abundance. These relationships could be due to the known detrimental effects of parasites on hosts (Price, 1980), such as nestling susceptibility to ectoparasites during development (Merino, 2010). In fact, Heeb et al. (2000) found that flea infestation in great tit nests reduced nestling body mass and wing length. Likewise, a lower body mass in males could be due to a greater effort expended to feed nestlings parasitized by fleas. In this sense, Hillström (1995) showed a body mass reduction in males of the pied flycatcher *Ficedula hypoleuca* that was associated with reproductive effort.

In conclusion, we observed that heat treatment altered the microclimatic conditions of nests in two different localities with contrasting temperature and humidity. The local conditions determined the composition and abundance of the ectoparasitic and, indirectly, endoparasitic fauna affecting the studied blue tit populations. The heat treatment negatively affected the abundance of some ectoparasites, especially of those in the southern locality where ambient temperatures were higher. It is likely that some of these parasites were near their limit of thermal/humidity tolerance. Although precise tolerance thresholds are not known for these organisms, Shapiro et al. (2017) showed that the optimum temperature for parasites and vectors involved in malaria transmission is around 26 °C. Along these lines, Gehman et al. (2018) suggested that a temperature increase of only 2 °C could negatively affect the prevalence of parasites. Interestingly, a positive effect of heat treatment was observed in male birds in the northern locality, implying that a slight increase in temperature in a colder environment might be generally favorable for birds. In contrast, the detrimental effect of a similar temperature increase on some parasites in a warmer environment may be offset by a detrimental effect on nestlings. In fact, several studies have shown that warmer temperatures have negative effects on nest-dwelling ectoparasites but varied effects on nestlings (Andreasson et al., 2018; Mennerat et al., 2019). Conversely, a decrease in temperature and an increase in humidity could be beneficial for parasites, especially those in the southern locality. These changes, however, would likely prove detrimental for birds, as they feed on invertebrates that may be sensitive to climatic changes. As alluded to earlier, the effects of climate change on parasites and birds can be complex (Merino and Potti, 1996; Merino, 2019; Møller and Dunn, 2019). Overall, our results suggest that the effects of climate change on host-parasite-environment interactions depend on multiple factors that differ at each locality, as well as on the ecological and physiological characteristics of the parasites and hosts (Martínez and Merino, 2011). Therefore, broad knowledge of the factors modulating bird-parasite interactions, including the tolerance limits of species to thermal and humidity conditions, is needed to accurately assess the effect of climate change on such interactions, in

order to make predictions about future trends.

## Ethics statement

The animal study was reviewed and approved by the Dirección General de Agricultura, Ganadería y Alimentación, Comunidad de Madrid (Spain). In Germany, bird capture and sampling were authorized under licenses from Regierungspräsidium Giessen: GI 15/8-Nr.109/2012 and GI 15/8-Nr.77/2016.

## Declaration of competing interest

There are no conflict of interest to declare for the manuscript entitled “Experimental manipulation of temperature produces differential effects on parasite abundances in blue tits at two different latitudes”, authored by F. Castaño-Vázquez, Y. R. Schumm, A. Bentele, P. Quillfeldt and Santiago Merino.

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