Nestling Growth is Impaired by Heat Stress: an Experimental Study in a Mediterranean Great Tit Population

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Samuel Rodríguez and Emilio Barba (2016) During the nestling stage, nestlings of small altricial birds face energetic limitations due to their rapid development and the need to maintain a stable body temperature once homeothermy is achieved. In Mediterranean habitats, high air temperatures reached during the breeding season could negatively affect the health and condition of the nestlings. The aim of this study was to determine the effect of an experimental increase of nest temperatures during the nestling stage on the growth and survival of Great Tit (Parus major) nestlings. Additionally, changes in parental brooding and feeding behavior as a result of the alteration of the nest microclimate were addressed. Increased nest temperatures affected nestling mass, as heated nestlings were lighter than controls on day 15 in the warmer of the two breeding seasons considered. Moreover, females from the heating treatment reduced their brooding time. Fledging success and parental feeding rates were not altered by the experimental treatment. The results of this study suggest that high nest temperatures may impair nestling development and therefore affect post-fledging survival probability. Negative effects are more likely to occur in warm habitats and/or warmer years, where juveniles are liable to suffer from thermal stress.

Key words: Brooding behavior, Feeding behavior, Hyperthermia, Growth, Survival.

BACKGROUND

The nestling stage is one of the most energetically challenging periods of a bird’s lifetime (Lack 1968; Ricklefs 1983). The growth of developing chicks is optimum across a range of nest temperatures that meets their energetic needs, but deviations from this optimum may be harmful. In this sense, the effects of temperature on the metabolic demands of developing nestlings can vary with age, depending on their homeothermic abilities (McCarty and Winkler 1999). Newly hatched altricial young have very limited capacity for conserving heat, because of their high body surface-to-mass ratio (Dawson and Evans 1960) and absence of plumage (Jenni and Winkler 1994; Hinsley et al. 2003). During early development, it is therefore up to the parents to invest energy and resources in the provisioning and brooding of their young, at the expense of their own self-maintenance (Marsh and Wickler 1982; Starck and Ricklefs 1998; Visser 1998; Węgrzyn 2013). Temperature regulation in Great Tit (Parus major) nestlings begins when they are 4-6 days old, and consolidates rapidly once they are 10 days old (Mertens 1977a). Before reaching homeothermy, nestling altricial condition allows the allocation of available resources to tissue growth rather than maintenance (Ricklefs 1968, 1993; Olson 1992; Arendt 1997; Węgrzyn 2013). Once they begin to thermoregulate on their own, however, nestlings face energetic limitations associated with their rapid development and the need to sustain a stable body temperature (Ricklefs 1983; Schew and Ricklefs 1998).

Regardless of their developmental stage, heat...
loading could be a serious threat for nestlings. In Mediterranean habitats, high temperatures reached during the breeding season could exert negative effects on the health and condition of the nestlings (e.g., Belda et al. 1995). Hyperthermia has been shown to lower chick food intake, and consequently reduce their growth rate and muscle development (Murphy 1985; Geraert et al. 1996; Moreno et al. 2002; Cunningham et al. 2013). It also increases their energetic demands, causing them to lose weight (Routman et al. 2003; Catry et al. 2015). High environmental temperatures may impair nestling’s immune response, reducing the levels of total circulating antibodies or altering the phagocytic ability of macrophages (Lara and Rostagno 2013). In addition, chicks exposed to high ambient temperatures enter a stage of oxidative stress, leading to the production of heat shock proteins in several tissues, which have been shown to have negative effects on growth (Moreno et al. 2002). Further direct effects of elevated temperatures on nestling body condition include dehydration (a consequence of the activation of thermolytic mechanisms such as panting to promote evaporative heat loss), cardiovascular mortality and respiratory illnesses (Mertens 1977a; Belda et al. 1995; Patz et al. 2005). Additionally, the adverse effects of high temperatures on nestling fitness could also increase adult foraging costs, as chicks may require more food to compensate the energetic expenses of thermoregulation (Royama 1966; Barba et al. 2009).

Only a handful of experimental studies have shown a correlation between nest temperatures and nestling growth, development and/or survival. Most of these have sought to manipulate temperatures during the egg-laying and incubation stages, testing for subsequent effects on parental behavior and nestling condition (e.g., Nager and Van Noordwijk 1992; Nilsson et al. 2008; Pérez et al. 2008; Álvarez and Barba 2014a). If we focus on the nestling stage, only Dawson et al. (2005) applied heat after hatching. They concluded that, in a temperate environment, increasing nest temperatures had overall positive effects on offspring fitness, benefits that could not be attributed to changes in parental behavior. It remains to be seen whether these results would reproduce under hotter conditions, where high air temperatures could act as potential stressors to which developing nestlings may be vulnerable (see Lobato et al. 2008). For example, Álvarez and Barba (2014a) showed that, in a Mediterranean Great Tit population, females incubating in nests where temperature was experimentally increased allocated less time to incubate. A similar response could be expected for females brooding small nestlings.

The aim of this study is to experimentally determine the influence of relatively high nest temperatures on Great Tit nestling growth and survival in a Mediterranean habitat. Additionally, we aimed to clarify whether these potential effects may lead to variations in parental care and/or provisioning behavior. We hypothesize that, under high air temperature regimes, a hot nest microclimate will have negative effects on nestling development, as the nestlings will have to allocate resources to thermoregulation rather than growth. Moreover, raising nest temperatures will likely modify parental behavior, as the extra heat provided may reduce female brooding constancy, and the higher energetic demands of the chicks may require an increase in adult provisioning effort.

MATERIALS AND METHODS

Study site and species

Data used for this study were collected from a Great Tit population breeding in nest boxes in Sagunto (Valencia, eastern Spain 39°42’N, 0°15’W, 30 m a.s.l.) during 2011 and 2012. The study area was located within an extensive orange monoculture, and wooden nest boxes [see Lambrechts et al. (2010) for details] were placed each year for the birds to breed (e.g., Rodríguez et al. 2016). Mean air temperatures at this site increase from 15.1°C in April to 24.5°C in July (Agencia Estatal de Meteorología, period 1986-2012). On average, mean maximum air temperatures during these months range from 21.0°C in April to 30.0°C in July, with extreme daily values that may be as high as 43.4°C.

Great Tits are uniparental incubators in which females incubate for about 66% of the day in the studied population (Álvarez and Barba 2014b). The average clutch size is around 8 eggs (Atiénzar et al. 2012). Incubation starts once the clutch is complete, and lasts for about 13 days (Álvarez and Barba 2014c). After hatching, both sexes take part in feeding the young. The nestling period in this species lasts for about 18 days (Atiénzar et al. 2012).
Experimental design

Nest boxes were visited weekly in order to obtain the laying date of the first egg (estimated assuming that one egg was laid per day; Encabo et al. 2001). After the 5th egg was laid, we made daily visits to determine the beginning of incubation as well as the final clutch size (see Álvarez and Barba 2014c). Once incubation started, we paired 20 nests according to clutch size and expected hatching date, with one nest from each pair randomly allocated to the heating treatment, and the other acting as control. Beginning on day 11 after the start of incubation, nests were visited daily until the first egg hatched.

On the date of hatching of the first egg (day 0), we took the nest and its contents (eggs and chicks) out of the nest box and measured the thickness of the nest base (see Álvarez and Barba 2008). We then prepared the nest box for the experiment by introducing a wire mesh 1 cm above the nest box floor (i.e. to standardize treatment conditions), thus creating a chamber where control and heat pads could be inserted (see Álvarez and Barba 2014a, for more details). Finally, we returned the nest and its contents to the nest box, positioning them on the wire mesh.

The temperature manipulation treatment started at 07:00 the next day (day 1), and lasted until 19:00 on day 14, when the heat and control pads, as well as the wire mesh, were permanently removed. Heat pads consisted of commercial warming units (UniHeat Packs, Chrosmack Ventures, Montana, USA, in 2011, and Mycoal warmpacks, Northbrook Industrial Estate, Southampton, UK in 2012), which produced elevated temperatures when exposed to air for 40 and 24 h respectively. We used pads kept at ambient temperature as controls. Heat pads were replaced as necessary to maintain continuously elevated nest temperatures during the experiment (every other day in 2011 and daily in 2012), and control nests were disturbed in a similar way (i.e., control pads were removed and replaced with the same frequency as the heat pads). Regardless of the different longevity of the pads used, their replacement in the field was done before they lost their warming capacity, in an effort to guarantee the effectiveness of the heating treatment in both years. The experiment was conducted between April 27 (i.e., day 1 of the first nest) and May 19 (i.e., day 14 of the last nest) in 2011, and between April 28 and May 23 in 2012.

The experimental nests were collected in sealed plastic bags after fledging, and stored at -20°C. Once the breeding season concluded, the nests were dried in an oven (105°C, for 12 h) and weighed with a digital scale (accuracy 0.01 g) to obtain dry nest masses (details in Álvarez et al. 2013). This parameter has been shown to be a good estimator of overall nest mass, regardless of the possible addition of nesting material during the course of the nesting cycle (Dubiec and Mazgajski 2013). Overall, we have data from 19 nests in 2011 (10 control, 9 heated) and 17 nests in 2012 (9 control, 8 heated). In 2011, 1 nest selected for heating failed during the incubation period (before the temperature manipulation experiment). In 2012, we lost 3 nests during the experiment: 1 control nest failed whereas 2 heated nests were preyed upon.

Nest and air temperature recording

Temperature was measured with data loggers in a sample of nests: 15 nests in 2011 (8 control, 7 heated), and 14 nests in 2012 (7 control, 7 heated). Temperature sensors were placed between the wire mesh and the nest base (see Dawson et al. 2005). In 2011, we used 4 thermocouple thermometers (Model HOBO U23 Pro v2, Onset Corporation, Bourne, MA, USA), programmed to make internal temperature readings every 2 minutes. The thermometers were moved between nests in order to obtain records of as many nests as possible. In 2012, we used 20 Thermochron iButton data loggers (Model DS1922L-F5, Embedded Data Systems, Lawrenceburg, KY, USA), programmed to make internal temperature readings every 32 seconds during the first 4 days, and every 95 seconds afterwards.

For each of the nests in 2011 we recorded nest temperature for 24 h, once between days 1 and 7, and again between days 8 and 14. These age categories were chosen so as to comprise two different stages of nestling thermoregulatory capabilities. In 2012 we were able to use continuous data recorded during the two periods. Then, we calculated, for each nest and age category, mean diurnal (07:00-20:00) and nocturnal (20:00-07:00) temperatures.

Ambient temperature during the experiment was estimated by calculating, for each nest, average maximum, mean and minimum air temperatures from day 1 to day 15 of age of the nestlings. Air temperature data were collected from a meteorological station located approximately at 4 km from our study site.
Female brooding and parental feeding rates

All the nests under study were visited 4 times a day after the beginning of the experiment (around 07:00, 11:00, 15:00 and 19:00) to record the presence of the female (i.e., we checked if the female was inside the nest box, without causing her to leave; see e.g. Álvarez and Barba 2014a, for a similar methodology during the incubation period). This pattern of visits allowed us to estimate female brooding constancy during the nestling period. We calculated, for each nest box, the proportion of visits with the female present during three age intervals: on days 1-4, 5-9 and 10-14.

Parents were captured at the nest using spring traps when nestlings were 10-12 days old. Adults were ringed with individually numbered metal rings at this time, and we measured their mass with a digital balance, and tarsus length with a digital caliper. In order to quantify their provisioning behavior, we subcutaneously injected them passive integrated transponder tags (PIT tags) specifically designed for small passerines (Álvarez and Barba 2014a) in 2012. The day after both adults were fitted with transponders (on days 11-13), we attached a transponder reading system (Trovan Ltd, Isle of Man, United Kingdom) to the nest box entrance, which recorded the time when a tagged bird entered or left the nest, as well as its identity. The reader was removed the following day. Recording times varied between 4 and 15 h, which allows for a good estimation of feeding frequency (see Pagani-Núñez and Senar 2013). Previous studies in our site have shown that Great Tits feed their nestlings at a constant rate throughout the day (Barba et al. 2009), so we did not limit data collection to a specific time period.

From the collected data, we calculated the absolute number of feedings per hour of males and females. For analyses, transponder reader failure limited our sample size to 7 heated and 5 control nests in the case of male provisioning data, and 7 heated and 7 control nests in the case of female provisioning.

Nestling biometry and survival

To allow for individual recognition of the nestlings during the nestling period, they were marked on day 5 on different parts of the body with a permanent non-toxic pen, remarked on day 7, and ringed on day 9 using individually numbered metal rings. We recorded the number of nestlings alive on days 5, 9 and 15 of age, and weighed them at these ages using a digital balance. On day 15, we also measured their tarsus length with a digital caliper. This age of measuring is a standard procedure in most Great Tit populations (e.g. Barba et al. 1993) and no nestling was force-fledged due to handling at this age. On day 20 we visited each nest box to check whether all the young had fledged (age of fledging in our population is approximately 18 days, per. obs.). Dead individuals were identified.

Statistical analyses

We tested for pre-experimental differences among treatments (control vs. heating) in nest dry mass, base thickness, laying date, clutch size, hatching date, number of hatchlings and proportion of eggs hatched (number of hatchlings/clutch size) using General Linear Models with normal distribution (nest dry mass, nest base thickness, laying date, hatching date) or Generalized Linear Models with Poisson (clutch size, number of hatchlings) and Binomial (proportion of eggs hatched) distributions. We considered including these variables as covariates in further analyses (i.e., nest dry mass in temperature model, see below) in case of significant pre-experimental differences between heated and control nests.

We also tested for post-treatment differences in number of fledglings and proportion of young fledged (brood size at fledging/initial brood size) using Generalized Linear Models with Poisson (number of fledglings) and binomial (proportion of young fledged) distributions. All models incorporated year, treatment group (control or heat), and treatment-by-year interaction term as fixed factors.

Differences between treatments in nestling survival to day 15 were tested with a Generalized Linear Mixed Model with binomial distribution. We considered year, treatment, and treatment-by-year interaction term as fixed factors and nest box as a random factor. We used General Linear Mixed Models to examine whether nestling biometry differed among treatments and years. The variables examined individually in these analyses were nestling mass at ages 5, 9 and 15 days, tarsus length at age 15 days, and mass differences between days 15 and 9, and between days 9 and 5. The models included year, treatment group and treatment-by-year interaction term as fixed factors, and nest box as a random factor. Given that brood size may affect the growth and survival of each
individual nestling (Pettifor et al. 2001), we initially included the number of nestlings present at each age as a covariate in the models, and eventually simplified them in case its inclusion did not lead to an improvement in model fit.

To test for differences among experimental treatments in parental feeding rates late in the nestling period, we used a Generalized Linear Model with Poisson distribution. We included sex, treatment group and treatment-by-sex interaction term as fixed factors. In addition, given that provisioning rates may vary with brood size (Rauter et al. 2000; Barba et al. 2009) and temperature (Rauter et al. 2000; García-Navas and Sanz 2012), we initially included the number of nestlings present on day 9 (as proxy of brood size during the recording period) and mean air temperature as covariates, and eventually simplified the model because their inclusion did not lead to an improvement in model fit.

We compared female body condition between heated and control nests using a General Linear Model. We included the body mass-to-tarsus ratio as dependent variable, the treatment group and treatment-by-year interaction term as fixed factors, and the number of nestlings on day 9 (as proxy of brood size during the analyzed period) as a covariate. Given that the inclusion of the covariate had no significant effect on the results of the model, it was eventually simplified.

Differences among treatments in the presence of brooding females in the nest boxes were analyzed with Generalized Linear Models with binomial distribution, including the proportion of visits with the female present during days 1-4, days 5-9 or days 10-14 of age of the nestlings as response variable, and year, treatment group, and treatment-by-year interaction term as fixed factors. As female attendance may be influenced by temperatures and brood size (Rauter et al. 2000; Leckie et al. 2008), we initially included mean air temperature during each period and the number of nestlings present on day 5, 9 or 15 (i.e., as proxy of the number of nestlings present during each of the analyzed periods) as covariates, and eventually simplified the models because their inclusion provided no significant improvement in model fit.

Variables affecting internal nest box temperatures were tested using a General Linear Mixed Model. We included nest temperature averages as dependent variable, treatment group, nestling age category (1-7 days or 8-14 days), time of day (day-time or night-time) and year (2011 or 2012) as fixed factors, and nest box as a random factor. Nest dry mass and mean air temperature were initially considered as covariates, although nest dry mass was eventually simplified from the final model, as it provided no improvement in model fit. Given that nest base thickness did not vary between treatments (see Results), it was not included into this analysis. For the sake of simplicity, we only considered in the model first-order interactions involving the treatment factor.

Nestling survival analysis was performed using package lme4 v. 1.1.9 (Bates et al. 2014) in software R v. 3.2.0 (R Development Core Team 2015). The remaining analyses were performed using IBM SPSS Statistics 22 software. We considered results significant at the 0.05 level.

RESULTS

There were no differences between heated and control nests in either the nest base thickness, laying date, clutch size, hatching date or number of hatchlings. We did find differences in nest dry mass ($F_{1,31} = 4.549, p = 0.041$; Table 1), as heated nests were on average lighter than control nests.

Internal nest box temperatures varied significantly with year, treatment and time of day (Table 2). Average temperatures in 2012 were approximately 5°C higher than in 2011. In both years, temperatures inside the heated nests were higher than in control nests. Additionally, temperatures in experimental nests were higher during day-time than during night-time (Fig. 1). We also detected a significant treatment-by-time of day interaction (Table 2), implying that the effect of the heating treatment was greater during day-time than during night-time (Fig. 1). Mean air temperatures in our study area during days 1-15 of age of the nestlings were 18.44°C in 2011 and 19.10°C in 2012. Maximum temperatures during this period were, on average, 2.05°C higher in 2012 than in 2011.

Nestling mass on day 15 differed significantly between experimental treatments and between years (Table 3). The treatment-by-year interaction effect was also close to significance (Table 3). We therefore analyzed both years separately, showing that heated nestlings were 1.56 g lighter than control ones in 2012 (Table 4, Fig. 2). Tarsus length on day 15 also varied significantly with year (Table 3), but neither the treatment nor the treatment-by-year interaction factors were significant. Mass differences between days 15 and 9 and between
days 9 and 5 did not differ significantly between treatments. Neither did nestling survival to day 15, the number of fledglings per nest, or the proportion of young fledged.

Presence/absence data evidenced that heated females spent 15% less time in the nest than control females during the first 4 days of age of the nestlings (Table 5). There were no differences in female attentiveness between treatments either during days 5-9 of age of the

Table 1. Breeding parameters and characteristics (± SE) of heated and control nests during the 2011 and 2012 field seasons. Sample sizes in parenthesis refer to number of nests. Statistics associated to the year, treatment and treatment-by-year interaction are provided, as computed by the Linear Models. Significant p values (≤ 0.05) are indicated in bold font.

<table>
<thead>
<tr>
<th>Year</th>
<th>Heated</th>
<th>Control</th>
<th>Factor</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nest base thickness (mm)</td>
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<tr>
<td>2011</td>
<td>7.07 ± 3.17 (9)</td>
<td>9.54 ± 5.04 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 14.579$</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>2012</td>
<td>4.06 ± 3.10 (8)</td>
<td>3.00 ± 2.96 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.318$</td>
<td>0.577</td>
</tr>
<tr>
<td></td>
<td>Nest dry mass (g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2011</td>
<td>14.80 ± 2.30 (8)</td>
<td>19.95 ± 5.11 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 3.392$</td>
<td>0.075</td>
</tr>
<tr>
<td>2012</td>
<td>14.34 ± 3.70 (8)</td>
<td>15.21 ± 4.58 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 4.549$</td>
<td><strong>0.041</strong></td>
</tr>
<tr>
<td></td>
<td>Laying date</td>
<td></td>
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<tr>
<td>2011</td>
<td>11.33 ± 3.00 (9)</td>
<td>11.50 ± 2.88 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 0.735$</td>
<td>0.398</td>
</tr>
<tr>
<td>2012</td>
<td>12.14 ± 2.80 (7)</td>
<td>12.56 ± 3.91 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.071$</td>
<td>0.792</td>
</tr>
<tr>
<td></td>
<td>Clutch size</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2011</td>
<td>8.44 ± 1.01 (9)</td>
<td>8.80 ± 1.48 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 0.166$</td>
<td>0.686</td>
</tr>
<tr>
<td>2012</td>
<td>8.25 ± 1.39 (8)</td>
<td>8.67 ± 0.71 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.918$</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>Hatching date</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>30.22 ± 2.77 (9)</td>
<td>30.80 ± 3.08 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 4.421$</td>
<td><strong>0.043</strong></td>
</tr>
<tr>
<td>2012</td>
<td>32.63 ± 2.50 (8)</td>
<td>32.67 ± 3.61 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.093$</td>
<td>0.762</td>
</tr>
<tr>
<td></td>
<td>Number hatched</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>7.33 ± 1.41 (9)</td>
<td>7.50 ± 1.96 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 2.509$</td>
<td>0.123</td>
</tr>
<tr>
<td>2012</td>
<td>8.13 ± 1.36 (8)</td>
<td>8.33 ± 0.71 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.133$</td>
<td>0.718</td>
</tr>
<tr>
<td></td>
<td>Proportion hatched</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2011</td>
<td>0.87 ± 0.12 (9)</td>
<td>0.85 ± 0.19 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 9.739$</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>2012</td>
<td>0.99 ± 0.04 (8)</td>
<td>0.96 ± 0.08 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.589$</td>
<td>0.449</td>
</tr>
<tr>
<td></td>
<td>Number fledged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>6.56 ± 2.24 (9)</td>
<td>7.10 ± 1.97 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 0.005$</td>
<td>0.944</td>
</tr>
<tr>
<td>2012</td>
<td>7.38 ± 1.41 (8)</td>
<td>6.22 ± 1.86 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.199$</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td>Proportion fledged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>0.88 ± 0.22 (9)</td>
<td>0.95 ± 0.10 (10)</td>
<td>Year</td>
<td>$F_{1,32} = 2.168$</td>
<td>0.151</td>
</tr>
<tr>
<td>2012</td>
<td>0.91 ± 0.11 (8)</td>
<td>0.75 ± 0.22 (9)</td>
<td>Treatment</td>
<td>$F_{1,32} = 0.163$</td>
<td>0.689</td>
</tr>
</tbody>
</table>

Table 2. Factors affecting temperatures inside experimental nest boxes. Treatment group, nestling age category, time of day, and year are considered as factors, air temperature as a covariate, and nest internal temperature averages as dependent variable. Significant p values (≤ 0.05) are indicated in bold font.

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>$F_{1,25} = 36.652$</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Age</td>
<td>$F_{1,80} = 1.583$</td>
<td>0.212</td>
</tr>
<tr>
<td>Time of day</td>
<td>$F_{1,76} = 44.967$</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,26} = 20.023$</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Treatment*Age</td>
<td>$F_{1,79} = 0.156$</td>
<td>0.694</td>
</tr>
<tr>
<td>Treatment*Time of day</td>
<td>$F_{1,76} = 4.228$</td>
<td><strong>0.043</strong></td>
</tr>
<tr>
<td>Treatment*Year</td>
<td>$F_{1,25} = 1.178$</td>
<td>0.288</td>
</tr>
<tr>
<td>$T_{air}$</td>
<td>$F_{1,88} = 11.609$</td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>
Table 3. Factors affecting nestling biometry. Year, treatment group, and the treatment-by-year interaction term are considered as factors, and nestling mass on day 15, tarsus length on day 15 or mass differences as dependent variable. Models include the number of nestlings present on day 5, 9 or 15 as a covariate in case of significance. Significant $p$ values ($\leq 0.05$) are indicated in bold font.

<table>
<thead>
<tr>
<th></th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass day 5 (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,29} = 0.351$</td>
<td>0.558</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,29} = 1.423$</td>
<td>0.242</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,29} = 0.440$</td>
<td>0.512</td>
</tr>
<tr>
<td>Mass day 9 (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,29} = 0.890$</td>
<td>0.353</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,29} = 0.216$</td>
<td>0.646</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,29} = 0.208$</td>
<td>0.652</td>
</tr>
<tr>
<td>Mass day 15 (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,33} = 14.360$</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,33} = 4.172$</td>
<td>0.049</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,33} = 4.034$</td>
<td>0.053</td>
</tr>
<tr>
<td>Mass day 9 - Mass day 5 (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,30} = 2.846$</td>
<td>0.102</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,30} = 1.786$</td>
<td>0.191</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,31} = 0.794$</td>
<td>0.380</td>
</tr>
<tr>
<td>Mass day 15 - Mass day 9 (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,29} = 2.126$</td>
<td>0.155</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,30} = 1.567$</td>
<td>0.220</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,30} = 1.701$</td>
<td>0.202</td>
</tr>
<tr>
<td>Number of nestlings on day 9</td>
<td>$F_{1,31} = 5.328$</td>
<td>0.028</td>
</tr>
<tr>
<td>Tarsus length day 15 (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>$F_{1,31} = 12.002$</td>
<td>0.002</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,31} = 0.015$</td>
<td>0.903</td>
</tr>
<tr>
<td>Year*Treatment</td>
<td>$F_{1,31} = 0.289$</td>
<td>0.595</td>
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</table>

Fig. 1. Temperatures of experimental nests in relation to time of day. Temperature means (± SE) for experimentally heated (black) and control nests (white) of Great Tits in relation to time of day (day-time: 07:00-20:00; night-time: 20:00-07:00). We show data for two consecutive years: 2011 (dots) and 2012 (triangles). Sample sizes above error bars refer to number of nests.

Fig. 2. Nestling mass on day 15 in experimental nests. Average nestling mass on day 15 for experimentally heated (black) and control nests (white). We show data for two consecutive years: 2011, and 2012. Sample sizes above error bars refer to number of nests.
nestlings or during days 10-14 (Table 5). Female body condition was not altered by the experimental treatment ($F_{1,31} = 0.048, p = 0.828$). We found no significant year ($F_{1,31} = 0.276, p = 0.603$) or treatment-by-year ($F_{1,31} = 0.016, p = 0.900$) effect. In addition, the manipulation of nest microclimate did not significantly influence parental provisioning behavior ($F_{1,22} = 0.092, p = 0.765$). There was no significant effect of sex ($F_{1,22} = 0.323, p = 0.576$), although we did find a significant treatment-by-sex interaction ($F_{1,22} = 5.109, p = 0.034$), meaning that the heating treatment had a different, but non-significant effect on males than on females (i.e., heated males tended to increase their feeding rate in comparison with control males, whereas heated females tended to decrease it).

**DISCUSSION**

The experimental heating of the nests affected nesting condition in the warmer year, as heated nestlings were lighter on day 15 than control chicks in 2012. Nest temperatures reached in 2011 did not seem to have been high enough to cause significant impacts on the measured nesting parameters, although we cannot reject further effects in non-measured indicators of chick health, such as immune response or plasma osmolality (see Saito and Grossmann 1998; Mashaly et al. 2004; Dawson et al. 2005).

Increasing nest temperatures during chick development resulted in heated females spending less time in the nest during the first 4 days post-hatching. We are aware of the limitations of the data used to estimate female brooding constancy, which do not allow for a precise record of changing behavior patterns, but this effect was apparent despite the low resolution of the data. This decrease in parental attentiveness in response to experimental heating has already been shown by Álvarez and Barba (2014a) during the incubation stage, and it seems also to occur during the brooding period. The extra-heat provided during

<table>
<thead>
<tr>
<th>Table 4. Biometrics (±SE) of heated and control nestlings during 2012. Least-squared means and standard errors are provided, as computed by the General Linear Mixed Models. Values in parenthesis refer to the degrees of freedom. Significant $p$ values ($\leq 0.05$) are indicated in bold font</th>
</tr>
</thead>
</table>
| Rotating the table correctly...

<table>
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<tr>
<th>Table 5. Factors affecting female attentiveness. Year, treatment group, and the treatment-by-year interaction term are considered as factors, and the proportion of visits with the female present during days 1-4, days 5-9 or days 10-14 as dependent variable. Significant $p$ values ($\leq 0.05$) are indicated in bold font</th>
</tr>
</thead>
</table>
the first days of the experiment may have allowed adult females to increase their investment in self-maintenance activities outside the nest, although we did not detect a positive effect on female condition. This decrease in brooding time had no measurable effects on nestling mass on day 5, so it seems that the extra heat provided compensated the lower brooding attentiveness.

We predicted that increased nest temperatures would alter parental provisioning behavior at the time of greater food demand by the nestlings, as result of the adults having to intensify their feeding effort in order to compensate the higher thermoregulation costs incurred by the chicks. Contrary to expectation, the heating treatment had no significant impact on parental feeding rates on days 10-12 of age of the young. This agrees with the results of Dawson et al. (2005) with heated nestling Tree Swallows (Tachycineta bycicolor), and suggests that the adults may have sacrificed investment in their young in favor of their own fitness. Food provisioning imposes high energetic demands on the parents (see Bryant and Tatner 1991), and they may trade-off current reproductive effort to guarantee their own survival and the success of future breeding attempts.

High nest temperatures have been shown to impose negative effects on nestling condition due to their greater vulnerability and restricted ability to regulate body temperature (Dunn 1979; Belda et al. 1995; Geraert et al. 1996). Nestlings must rely on evaporative cooling and the elevation of body temperature above normal levels as main defenses to cope with heat stress (O’Connor 1984), mechanisms that can be energetically-demanding and may lead to severe dehydration risks and lowered fitness (Du Plessis et al. 2012; Cunningham et al. 2013). Moreover, exposure of nestlings to elevated temperatures may eventually result in higher mortality, when the heat-loss mechanisms are inadequate to dissipate the total heat produced by the chicks and body temperature reaches lethal levels (Mertens 1977a; Warriss et al. 2005). In our case, increasing nest temperatures during nestling development did not alter nestling survival to day 15. Neither there were differences in the number of fledglings and proportion of young fledged. As far as we could tell, temperatures inside heated nests, although high enough to impose a net cost to developing nestlings in 2012 (see Dawson 1958; Quinteiro-Filho et al. 2010), did not reach lethal thresholds.

Our results are in agreement with previous experiments that have tried to determine the optimal thermal range in Great Tit nestlings (see Mertens 1977b). The upper and lower limits of this temperature range are dependent on factors such as brood size, nestling age and the dimensions and thermal properties of the nest (e.g., water content of the nest and heat conductance). For example, for broods consisting of 6 nestlings of 9 days of age, the upper and lower temperature limits in nests with a water content of 8% would be around 31 and 12°C respectively. Temperatures inside this optimal range meet nestling metabolic requirements for growth, but values above and below it may have negative effects on nestling physiology, condition and survival (Salaberria et al. 2014). In his study, Mertens (1977a) distinguished between long-term and immediate risk of hyperthermia based on the maximum percentage of the basal heat production that nestlings could safely dissipate by water evaporation. Nestlings incurred in long-term risks of hyperthermia when they were forced to dissipate more water, leading to dehydration and eventual mortality if conditions persisted. Immediate risk of hyperthermia was evoked when the highest possible rate of water evaporation could not match the evaporation rate required to keep body temperature below lethal levels. Based on our experience, temperatures above 34°C (i.e., temperatures reached in heated nest boxes in 2012) would be needed to start causing negative effects on nestling physiology. Other studies have reported similar upper-temperature thresholds for nestlings of altricial species (see Ardia 2013 and Cunningham et al. 2013 for studies with Tree Swallows and Common Fiscals Lanius collaris respectively).

The alteration of nest microclimate above reported optimal temperature levels led to reduced body mass in Great Tit nestlings. Although mass differences in the measured periods were not significant, it seems that the delay in mass gain occurred at older ages (i.e., between 9 and 15 days of age; see Table 4), when energy demands are higher. Nestling size, measured by tarsus length, was apparently unaffected. In this sense, previous studies have suggested the capacity of developing nestlings to selectively allocate resources towards growth of specific sets of tissues when exposed to limiting conditions, thus prioritizing those structures that maximize survival (Schew and Ricklefs 1998; Gil et al. 2008). This way, when exposed to suboptimal temperatures, the energetically-limited nestlings could have sacrificed fat accumulation in favor of skeletal growth or development of other non-
measured morphological traits with direct effects on survival, such as muscle or wing length. Given the advantage of extra fat reserves at fledging to withstand periods of food shortage (Odum and Connell 1956; Perrins 1965; Blem 1990; Perrins and McCleery 2001), adverse temperatures encountered during nesting development could ultimately handicap post-fledging survival. Therefore, although we have been unable to detect an effect of experimental nest heating on immediate juvenile survival at fledging, we cannot discard possible implications on future recruitment (see Monrós et al. 2002; Greño et al. 2008). Further consequences of elevated nest temperatures on nestling physiology that may affect future survival include immunosuppression (e.g., reduced antibody response; see Mashaly et al. 2004), oxidative stress and alterations of thermoregulatory behavior (e.g., release of heat-shock proteins; see Lara and Rostagno 2013).

Recent studies have reached similar conclusions to those presented here regarding the negative effect of high temperatures on nestling fitness. Ardia (2013) evidenced a decrease in reproductive success of nestling Tree Swallows as a result of increased nest temperatures, and Salaberria et al. (2014) reported a reduction in nestling body mass and wing length when analyzing the effects of heat exposure late in the season on development of Spotless Starlings (*Sturnus unicolor*). Similarly, Cunningham et al. (2013) detected reductions in body mass gain of Common Fiscal nestlings in response to high nest temperatures, and Catry et al. (2015) evidenced a relationship between high maximum daily temperatures and mass loss of Lesser Kestrel (*Falco naumanni*) and European Roller (*Coracias garrulus*) nestlings. Our work thus complements previous findings, being, to our knowledge, the first study providing experimental evidence on the negative impact of elevated nest temperatures on nestling fitness in warm environments. Deviations from the results of Dawson et al. (2005), where heating nest boxes caused positive effects on nestling growth and survival, could be attributed to differences in air temperatures (and, consequently, nest box temperatures) between study sites, considerably higher in our case (i.e., temperatures in our control nests where, on average, more than 6.8°C higher than in Dawson’s), which may have led to the heated nestlings being more often exposed to temperatures capable of inducing heat stress (i.e. temperatures above the upper limit of the optimal thermal range of the species). The negative effects of increased nest temperatures on offspring fitness are therefore more likely to manifest under warm air temperature regimes such as the Mediterranean, where birds are more frequently faced with energetically-challenging conditions.

In the current climate change scenario, bird species living in warm climates such as the Mediterranean are predicted to face hot-weather events of greater frequency (Pipoly et al. 2013), duration and intensity (IPCC 2013), which are likely to have a negative impact on breeding success (Sanz 2002). In this sense, individuals with small thermal ranges, such as developing young, are more susceptible to suffer the increased severity of climatic events (Jiquet et al. 2006). In this article we have shown experimentally how nestlings of a Mediterranean bird population are sensitive to nest temperatures surpassing their optimal thermal range, which may constrain their growth and eventually reduce their future recruitment probabilities. It remains to be seen whether these results would hold across the distribution range of the species, considering the possibility of local acclimation. Further manipulative approaches, as the one presented here, are therefore needed in order to effectively assess the effects of temperatures on breeding performance and, ultimately, predict the global effects of climate change on avian biodiversity.

**CONCLUSIONS**

In warm habitats, high temperatures experienced during the nestling period can affect nestling development. As a lower mass at fledging may limit post-fledging survival, the effects of adverse nest microclimates can eventually compromise offspring recruitment. This is the first study providing experimental evidence on the negative effects of elevated nest temperatures on fledgling mass.

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