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Source: Ardea, 110(2) : 213-231

Published By: Netherlands Ornithologists' Union

URL: https://doi.org/10.5253/arde.2022.a6

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Incubation before clutch completion predicts incubation time and hatching asynchrony in the Blue Tit *Cyanistes caeruleus*

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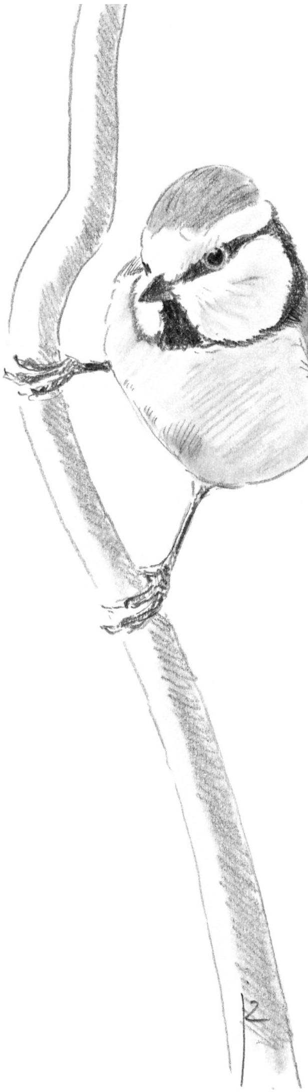

Temizyürek T., Johannknecht M. & Korsten P. 2022. Incubation before clutch completion predicts incubation time and hatching asynchrony in the Blue Tit *Cyanistes caeruleus*. Ardea 110: 213–225. doi:10.5253/arde.2022.a6

Hatching asynchrony is a frequent phenomenon in altricial birds and can lead to brood reduction due to sibling competition. There are a number of adaptive hypotheses to explain its occurrence, relating hatching asynchrony to sibling competition and timing of breeding. Incubation prior to clutch completion (early incubation) is the main cause of hatching asynchrony. We used temperature loggers inside the nests of breeding Blue Tits *Cyanistes caeruleus* to provide a detailed account of female incubation over most of the egg-laying period. We relate this early incubation to the time interval between clutch completion and hatching as well as hatching asynchrony. Our study shows the frequent occurrence of early incubation during the beginning of the laying period, with all females showing more early incubation towards clutch completion. At first, early incubation mostly occurs at night, but as egg laying progresses, it also occurs during the day. However, overall there was more nocturnal than diurnal early incubation. These results were obtained using two different methods for quantifying incubation from temperature profiles, which we compared and cross-validated in this study. Moreover, the amount of early incubation related negatively to the time between clutch completion and first hatching and positively to the extent of hatching asynchrony. While we did not directly investigate the mechanisms driving variation in early incubation, the exceptionally cold March/April period followed by a warm May in our study year may explain the comparatively great amounts of early incubation we observed. We hypothesise that spring temperatures may influence the amount of early incubation, with warmer springs resulting in more early incubation and consequently shorter times from clutch completion until first hatching as well as increased hatching asynchrony. Such a mechanism of adjustment of incubation time and hatching asynchrony may also be important for the adaptation of birds to climate change.

Key words: hatching asynchrony, breeding ecology, early incubation, partial incubation, nocturnal incubation, temperature loggers, Raven software, *Cyanistes caeruleus*

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Hatching asynchrony, where the eggs of the same clutch hatch at different times, is a widespread phenomenon in altricial birds (Stenning 1996). An important consequence of hatching asynchrony is the occurrence of age and size hierarchies within broods, with earlier-hatched chicks having an advantage in the competition over food brought by parents. As a result, later-hatched chicks often experience reduced growth and greater mortality due to starvation (O'Connor 1978, Slagsvold *et al.* 1995, Magrath *et al.* 2009, Hadfield *et al.* 2013), while in some species later-hatched chicks also die from siblicide (Godfray 1986, Johnston 2018). Hatching asynchrony may allow parents to influence the competitive hierarchy or size distribution in their nest in an adaptive way (reviewed in Magrath 1990, Ricklefs 1993, Slagsvold *et al.* 1995, Stoleson & Beissinger 1995, Wang & Beissinger 2009, 2011). For example, in an unpredictable breeding environment later-hatched chicks may die quickly before imposing a severe cost of competition on their surviving siblings in case of poor food availability (Lack 1947, Vedder *et al.* 2019).

The main cause of hatching asynchrony is incubation of the eggs before clutch completion (hereafter we refer to this as 'early incubation'). As many birds lay their eggs at one-day intervals (Vedder 2012), earlier-laid eggs get a developmental head-start when parents start incubation before the laying of the entire clutch is completed. There is a large body of literature showing variation in the occurrence and onset of early incubation both between and within species (e.g. Wiebe *et al.* 1998, for an overview see Wang & Beissinger 2011). Hatching asynchrony and early incubation as its main cause have been relatively well investigated in studies on nestbox-breeding tits (Paridae), in particular Great Tits *Parus major* (Gibb 1950, Kluijver 1950, Neub 1979, Haftorn 1981, Pendlebury & Bryant 2005, Lord *et al.* 2011, Podlas & Richner 2013, Álvarez & Barba 2014a,b, Diez-Méndez *et al.* 2020, 2021) and Blue Tits *Cyanistes caeruleus* (Gibb 1950, Neub 1979, Slagsvold *et al.* 1995, Stenning 2008, Hadfield *et al.* 2013, Vedder *et al.* 2012), where only the females incubate the eggs (Haftorn & Reinertsen 1985).

Previous studies have investigated (early) incubation behaviour using several different methods including daytime nest inspections to establish incubation by finding either an incubating parent on the nest or warm eggs (e.g. Haftorn 1981, Álvarez & Barba 2014a), recording nest attendance using either video cameras (e.g. Bulla *et al.* 2016, Amininasab *et al.* 2017) or automated PIT tag registrations (e.g. Bulla *et al.* 2016, Bambini *et al.* 2019), as well as recording incubation using temperature loggers in between the eggs (e.g. Haftorn 1979, Bulla *et al.* 2016, Diez-Méndez *et al.* 2021). Nest attendance can provide a proxy for incubation, but females sometimes spend extended periods of time at the nest without incubating the eggs (Pendlebury & Bryant 2005). Particularly during the egg laying period, females often roost inside the nestbox during the night without incubating (Pendlebury & Bryant 2005, Vedder *et al.* 2012). Therefore, we used small temperature loggers placed in the nest in between the eggs, which provided continuous recordings of nest temperatures and thus allowed us to infer incubation from the temperature patterns directly. We employed these temperature loggers from the laying of the first egg onward until clutch completion to be able to quantify female early incubation behaviour over most of the laying sequence.

The main aims of our study were (1) to provide a detailed description of the occurrence of early incubation over most of the egg-laying period during both day- and night-time and (2) to investigate to what extent early incubation before clutch completion, during day- and/or night-time, predicts the timespan between clutch completion and first hatching as well as the degree of hatching asynchrony. For extracting patterns of early incubation from the temperature recordings in the nest we applied two different methods which have been used previously: first, we applied a fixed temperature threshold above which we assumed incubation of the eggs, while not taking into account the detailed temporal profiles of the temperature recordings (following e.g. Stoleson & Beissinger 1999, Ardia *et al.* 2006, Vedder 2012, Hadfield *et al.* 2013), and second, we manually analysed the recorded temperature profiles using the Raven/Rhythm software (following e.g. de Jong *et al.* 2016, Bueno-Enciso *et al.* 2017, Schöll *et al.* 2020) to thereby compare and cross-validate the two methods (for further details see Methods section). By comparing the performance of the temperature threshold-based method with detailed manual analysis of the temperature profiles, we aimed to validate the first, more time-efficient, method of quantifying incubation behaviour.

METHODS

Study population

The study was conducted in the spring of 2018 on a Blue Tit population breeding in nestboxes (Nisthöhle 1B, Schwegler, Germany) in the Teutoburger Wald forest (52°01'49"N, 8°29'33"E) and an adjacent garden area, next to Bielefeld University, Germany. The forest area is mostly deciduous, mainly consisting of Beech *Fagus sylvatica*, European Ash *Fraxinus excelsior* and European Oak *Quercus robur*. The forest is managed by the city of Bielefeld and used as recreational area by the general public. The nestboxes in the forest were placed at around 2.5 m height along existing tracks and at least 30 m apart (50 nestboxes in total). The garden area belongs to Bielefeld University and mainly consists of grassland with some forest edges, scattered with a few large European Oaks. In the garden area, the boxes were mounted at a height of 1.5 m, c. 10–20 m apart (14 nestboxes in total). New nestboxes were installed, and existing ones cleaned, in the last week of March. Of the 64 available nestboxes, 41 were occupied by breeding Blue Tits, while 10 were occupied by Great Tits and Marsh Tits *Poecile palustris*. We successfully collected data on Blue Tit incubation behaviour from 26 nests, 20 in the forest and 6 in the garden area.

Recording of nest temperature during egg laying and measuring hatching asynchrony

From the beginning of April, we visited nestboxes regularly to monitor occupation and nest building. Once nest-building was complete, nests were visited daily before 11:30 to determine the start of egg laying, as females typically lay their eggs at one-day intervals early in the morning before leaving the nestbox after roosting (Haftorn & Reinertsen 1985). When we found the first-laid egg, we placed a small, labelled (with a permanent marker pen) temperature logger (Thermochron iButtons, Maxim Integrated, CA, U.S.; Figure 1) in the nest cup next to the egg, which recorded the temperature every 12 min with ±0.5°C accuracy. As the loggers can store 2048 individual measurements, we were able to record the Blue Tits' incubation behaviour over c. 17 days without disturbing the female by accessing the logger. Given that Blue Tits typically lay between 8–14 eggs (Amininasab *et al.* 2016), this ensured we captured the females' incubation during the entire egg-laying period (allowing for laying gaps).

Nests were checked every five days and from a few days before anticipated hatching (assuming a 14-day post clutch-completion incubation period; Álvarez & Barba 2014a) we visited nests daily to record the first day of hatching and the number of hatchlings. On the day of first hatching, the temperature loggers were removed. We then visited the nests for five consecutive days to record hatching of eggs (subsequent nest checks confirmed that remaining eggs did not hatch thereafter). We took the time interval between the hatching of the first and last hatchling in days as a measure of hatching asynchrony.

Quantifying early incubation from the temperature recordings

Before analyses, all temperature recordings were standardized, so that recordings started at the first data point after 11:30 on the morning of temperature logger placement (i.e. on the day of first egg-laying). This was done to account for differences in the timing of temperature logger placement (no logger was placed after 11:30). Further, recordings were truncated at sunrise on the day of clutch completion, which was calculated by reverse counting (assuming one egg was laid per day) taking into account the total clutch size (combined with information from nest checks at five-day intervals during egg laying). Thus, the recordings spanned the complete laying period, except for the morning following the first egg (i.e. until 11:30).

Early incubation was categorized as either diurnal or nocturnal incubation based on sunrise and sunset.

Figure 1. (A) An incubating female Blue Tit inside a nestbox. (B) A temperature logger in between the eggs. Temperature loggers were placed after the first egg of the clutch was laid and recorded the temperature at 12-min intervals until the end of egg laying.

This categorization closely matches the active (during the day) and inactive (during the night) periods of the female (Haftorn 1979, Álvarez & Barba 2014b, Bueno-Enciso *et al.* 2017, Bambini *et al.* 2019). Exact times for sunrise/sunset were obtained from the National Oceanic and Atmospheric Administration (www.esrl.noaa.gov/gmd/grad/solcalc, accessed on 25/3/2021) for 52°03'N, 8°53'E, close to the study area. See Figure 2 for an illustrative example of a recorded temperature profile (see Figure S1 in the online supplement for other recorded profiles).

As mentioned above, we used two methods to quantify incubation from the temperature profiles. First, we used a fixed temperature threshold, counting all recorded temperatures above this threshold as incubation. We chose to apply three temperature thresholds, 27, 30 and 32°C, to ascertain the robustness of our results (following Vedder 2012). To validate the temperature threshold method, we also analysed the temperature recordings with the Raven/Rhythm software (Cooper & Mills 2005; Raven Lite v. 2.0.1, Center for Conservation Bioacoustics 2019), which allows for manual assignment of presence/absence of incubation based on the temperature profiles. When using the Raven/Rhythm software, incubation behaviour was visually identified according to the following main rules: the start of an incubation bout was identified as an increase in temperature steeper than expected by the daily fluctuations in ambient temperature alone. Further, off-bouts were identified if the temperature dropped ≥ 2°C within 12 min (i.e. between two consecutive data-points).

Statistical analysis

All statistical analyses were carried out in R v. 4.1.2 (R Core Team 2021). To test for differences and correlations between the incubation estimates obtained via the different methods, as well as for differences between nocturnal and diurnal incubation we used parametric tests (*t*-tests, Pearson's correlations), which can be robust even in violation of the normality assumption (Knief & Forstmeier 2021). To ensure robustness of inference, we also ran non-parametric tests (Wilcoxon-tests, Spearman's correlations), which resulted in equal inference in all cases (results not shown). To analyse the extent to which early incubation (amount of time of nest temperature above threshold or the sum of time of all on-bouts as identified via Raven/Rhythm) predicts post clutch completion incubation duration until first hatching (days between clutch completion and first hatching) and hatching asynchrony (in days) we fitted univariate linear models. The validity of these linear models was verified by visually inspecting the residuals for heteroscedasticity as well as via Q-Q plots (not shown). Analyses for the 30°C threshold are shown in the text below (see Table S1, S2 for results with 27°C and 32°C). The raw data and R script to reproduce these analyses are presented in the Data Supplement.

RESULTS

Quantifying incubation: Raven/Rhythm versus temperature thresholds

The length of the laying-phase differed between females, resulting in clutch sizes that varied between 7 and 12 eggs (mean ± SD: 9.1 ± 1.3, $n = 26$). As a result of the variation in the length of the laying sequence (and hence clutch size) and due to laying gaps (in case of four nests) the length of temperature recordings during the laying phase varied between 5.8 and 13.8 days (mean ± SD: 8.3 ± 1.7, $n = 26$).

While the temperature recordings showed variation in their lengths and profiles (see Figure S1), the two different methods (temperature threshold versus Raven/Rhythm software-based) for quantifying the amount of incubation over the laying sequence mostly converged (Table 1). While the 27°C threshold overestimates early incubation compared to Raven/Rhythm, 32°C underes-

Table 1. Mean early incubation duration inferred from Raven/Rhythm and different temperature threshold levels, for total, nocturnal and diurnal early incubation. Early incubation duration is shown in the following format: mean ± SD, in hours, (percentage of Raven/Rhythm incubation obtained via threshold) [Pearson's correlation coefficient between the respective Raven/Rhythm and threshold estimates]. All correlation coefficients are significant at $P < 0.001$.

	Total incubation	Nocturnal incubation	Diurnal incubation
Raven/Rhythm	58.7 ± 15	37.4 ± 8.2	21.3 ± 10.3
27°C threshold	71.2 ± 17.2 (122%) [0.68]	41.0 ± 7.4 (108%) [0.92]	30.0 ± 12.3 (143%) [0.80]
30°C threshold	55.7 ± 16.5 (94%) [0.84]	37.0 ± 7.9 (100%) [0.80]	18.7 ± 10.6 (85%) [0.79]
32°C threshold	44.2 ± 16.2 (76%) [0.75]	31.1 ± 8.9 (84%) [0.67]	13.3 ± 8.8 (62%) [0.77]

timates early incubation, and differences between the methods are smallest for the 30°C threshold. For this reason, we present results for the 30°C threshold throughout the manuscript (inference is robust to changes in threshold, Table S1, S2). The overall correlation between the incubation estimates from the two methods (Raven/Rhythm and 30°C threshold) was strong for total incubation (nocturnal + diurnal incubation; $r = 0.84$, $t_{24} = 7.60$, $P < 0.001$), nocturnal incubation ($r = 0.80$, $t_{24} = 6.50$, $P < 0.001$) and diurnal incubation ($r = 0.79$, $t_{24} = 6.40$, $P < 0.001$; Figure 3).

The two methods not only produced incubation estimates that were strongly correlated, but which were also similar in magnitude. On average (±SD), the 30°C threshold resulted in estimates of 55.7 ± 16.5, 37.0 ± 7.9 and 18.7 ± 10.6 h of total, nocturnal, and diurnal incubation, respectively, as compared to estimates of 58.7 ± 15.0, 37.4 ± 8.2 and 21.3 ± 10.3 h based on manual analysis using the Raven/Rhythm software (Table 1). The difference in the estimated incubation times between the two methods was not significant for total (paired t-test: $t_{25} = -1.79$, $P = 0.10$) and nocturnal ($t_{25} = -0.39$, $P = 0.70$) incubation, while it approached significance for diurnal incubation ($t_{25} = -1.99$, $P = 0.06$).

Description of incubation patterns

Incubation patterns over the day and laying period were generally similar across nests in our study population (Figure 2, Figure S1). The raw temperature recordings show that many females started early incubation from the laying of the first egg onward (Figure 4A). During the first part of the egg laying period early incubation peaked during the first hours of the roosting period just after sunset (at c. 21:00; Figure 4A). Nocturnal incubation became more frequent later in the egg-laying period (Figures 4B,C,D), becoming continuous through the entire night in almost all cases during the last two days of egg laying (Figure 4E,F). Diurnal

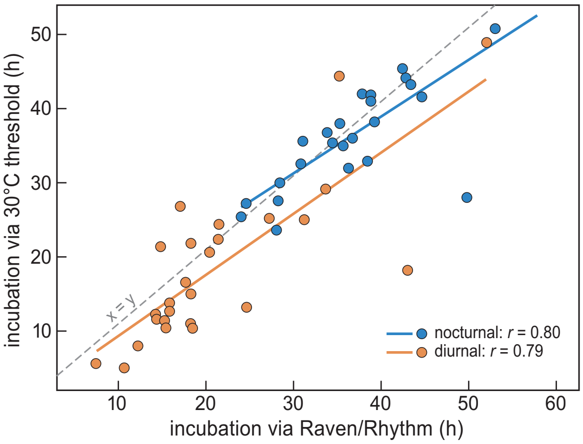

Figure 3. Correlations of the estimates of early incubation extracted either via the 30°C threshold method and Raven/Rhythm, for diurnal (orange) and nocturnal (blue) incubation. As a benchmark the x = y line is included in the plot. Shown are Pearson's correlation coefficients, both significant at $P < 0.001$.

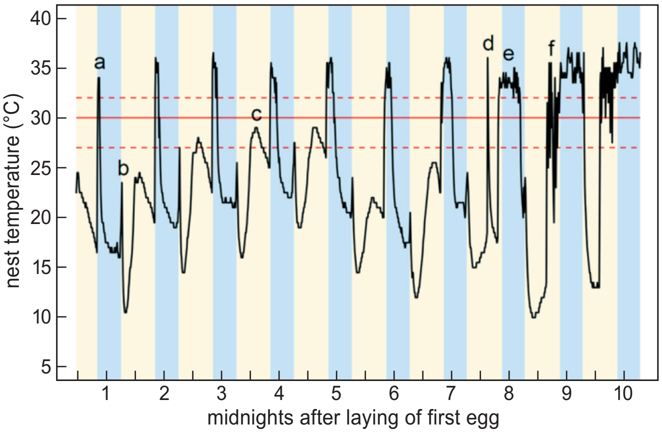

Figure 2. An example of temperature recording of early incubation by a female Blue Tit, from the first day after egg laying (starting at 11:30) until sunrise after clutch completion. Ticks on the x-axis mark midnight and noon. Day and night are shaded in light yellow and light blue, respectively. Clearly visible are incubation bouts shortly after sunset (a), which increase in length over the laying-period, as well as shortly before sunrise (b). Note that in this example temperatures during the day can exceed the 27°C threshold (but not the 30°C threshold) (c), without showing the steep rise characteristic of female incubation (d). These gradual rises in temperature above 27°C during the day (c) may be due to direct sun light warming the nestbox. Towards the end of the laying period the female shows continuous nocturnal incubation (e) and substantial diurnal incubation, in particular during the afternoon (f). Temperature thresholds are indicated at 27°C (dashed), 30°C (solid) and 32°C (dashed).

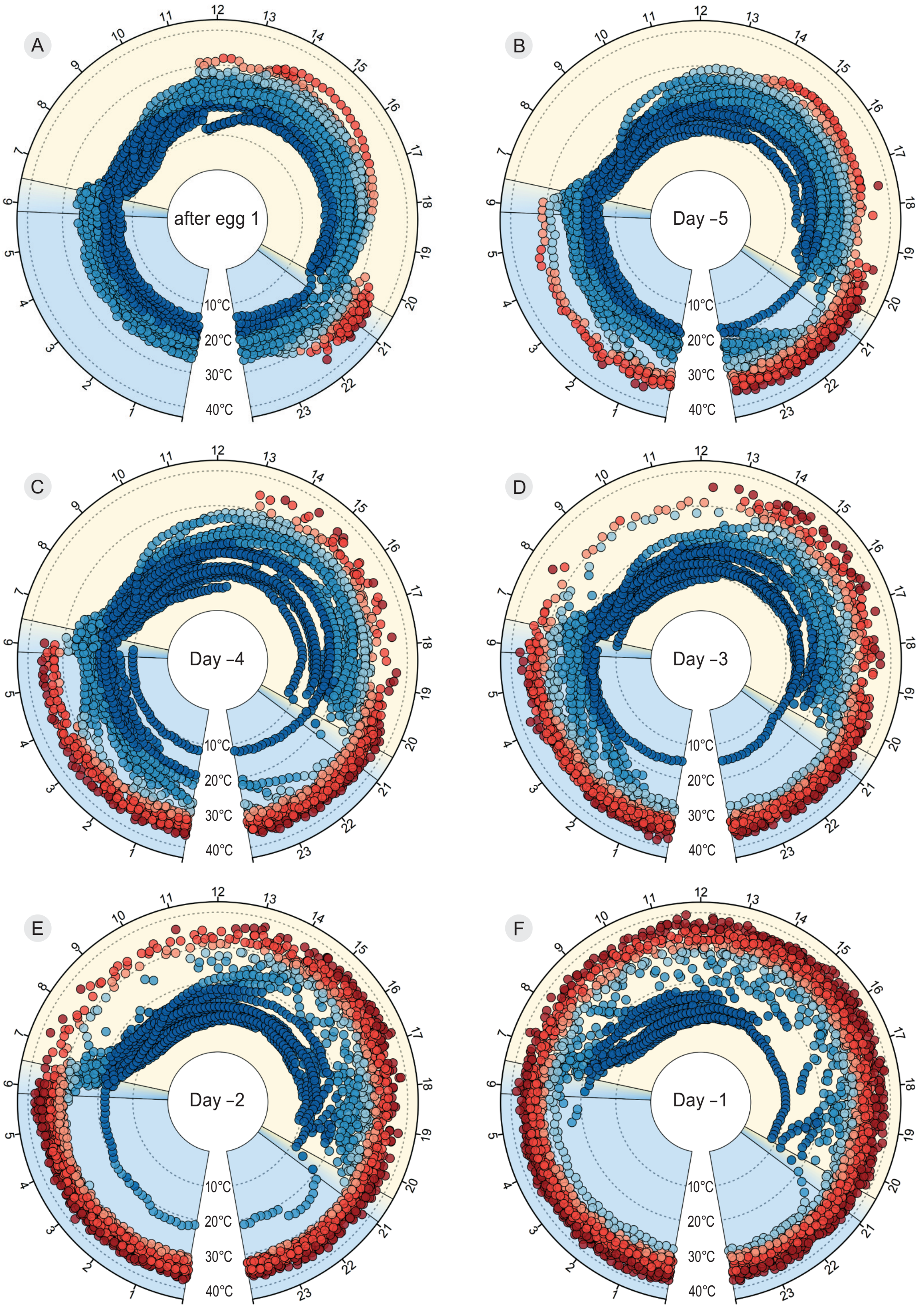

Figure 4. Nest temperatures recorded at 12-min intervals in 26 Blue Tit nests during the egg-laying period for (A) the day after first egg laying and (B–F) the last five days before clutch completion. The circular x-axis displays time of day, starts at 00:00 and runs clockwise until 24:00. Areas of the plots after sunset are shaded in light blue, after sunrise in light yellow. Note that sunset- and sunrise-times differ roughly two minutes between days, so that later in the season there is longer daylight – the timeframe of these differences is indicated by the shaded area. Points are coloured based on temperature: different intensities of red indicate temperatures above 30°C (dark red for >35°C), while different intensities of blue below 30°C (dark blue for <20°C).

incubation occurred only in a few nests during the first part of the egg laying period, mainly in the afternoons (Figure 4A,B); although we suspect that in these cases warming of the nestbox by direct sun light rather than incubation may have caused the temperatures to rise above the 30°C threshold (see Figure S1 and Discussion). The proportion of individuals showing early incubation during daytime increased later in the egg laying period (Figure 4C,D), with the majority of individuals showing diurnal incubation towards the end of laying, particularly in the afternoons (Figure 4E,F).

These daily patterns of incubation over the laying period are also reflected by the proportions of time the females spent incubating during day- and night-time, as a function of the laying sequence (Figure 5A, S2; here expressed relative to the day of the last-laid egg). Nocturnal incubation begins to increase from five days before clutch completion onwards, while diurnal incubation increases from two days before clutch completion onwards (Figure 5A). To investigate if differences in clutch size – which approximately equate to the differences in the length of the egg laying sequence (but which are not exactly equivalent due to the infrequent occurrence of laying gaps) – relate to these incubation patterns, we categorized nests by length of the laying sequence. As displayed in Figure 5B, incubation patterns are remarkably similar regardless of the length of the laying sequence (or clutch size), with a steady increase in incubation in the last five days before clutch completion.

Early incubation predicts time until first hatching and hatching asynchrony

The amount of early incubation estimated via the temperature threshold method significantly predicted the time interval between clutch completion and first hatching (mean ± SD: 12.2 ± 0.95, range: 10–14) when using total, nocturnal, and diurnal incubation (Figure 6A,B, Table 2). Likewise, the Raven/Rhythm-based incubation estimates also significantly predicted the interval between clutch completion and first hatching using total and diurnal incubation, while the association was weaker and marginally nonsignificant for nocturnal incubation (see Figure S3).

Hatching was asynchronous for all clutches, ranging from 1 to 5 days between the first and last hatched chick with a mean (±SD) of 2.8 ± 1.0 days. The amount of early incubation as estimated via the temperature threshold method significantly predicted hatching asynchrony when using total, nocturnal, and diurnal incubation (Figure 6C,D, Table 3). Raven/Rhythm-based incubation estimates predicted hatching asynchrony significantly using total and diurnal incubation, while the association was marginally nonsignificant for nocturnal incubation (see Figure S3). We would like to briefly note that the observed associations between hatching asynchrony and early incubation (obtained both via the threshold and Raven/Rhythm methods) depended on two relatively extreme nests (with much early incubation as well as large hatching asynchrony, see Table S3, S4). When they are removed

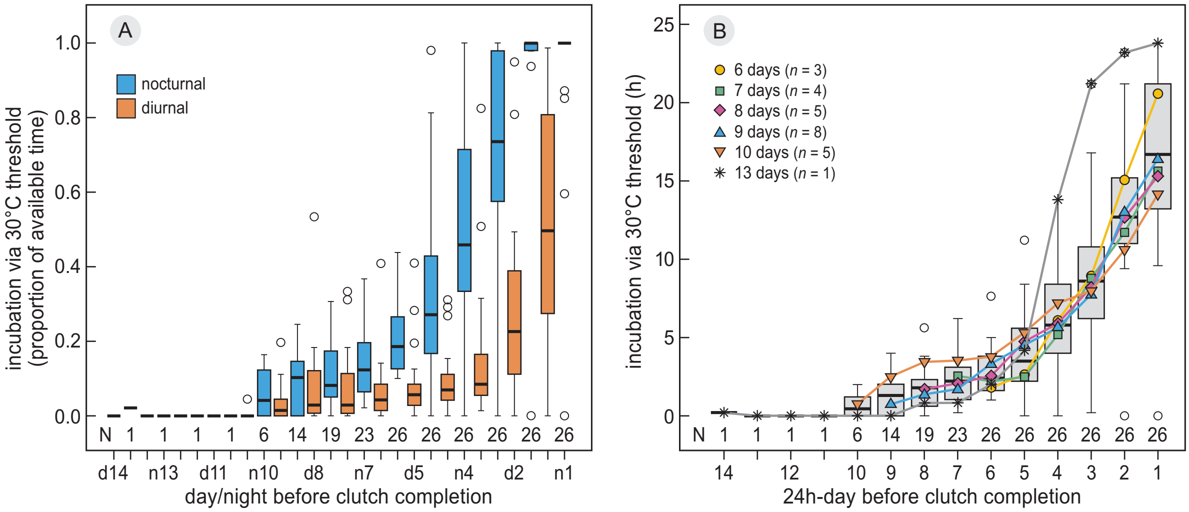

Figure 5. Incubation (threshold method) during egg laying. (A) The proportion of available time that was spend incubating during each night and day before clutch completion. (B) absolute incubation time in hours for each 24-h day before clutch completion. In addition to the boxplot, the lines indicate the patterns of average incubation for females with different laying period lengths (which approximate clutch sizes; note the outlier nest marked by the circles). Sample sizes are indicated below the plots. The complementary figure with early incubation estimated using Raven/Rhythm can be found in Figure S2.

from the analyses, observed associations become nonsignificant (results not shown). The models predicting time between clutch completion and first hatching are robust to the removal of these outliers.

DISCUSSION

By recording temperature at the nest, we found that female Blue Tits generally start incubating their eggs before clutch completion. Females showed early incubation during night-time (early nocturnal incubation) directly after laying the first eggs of the clutch, while they started incubating during the daytime (early diurnal incubation) later in the laying period. Furthermore, we compared two methods to infer incubation from temperature profiles: a fixed temperature threshold-based method versus manual analysis of temperature profiles using the Raven/Rhythm software, showing that both lead to strongly correlated incubation estimates which are similar in magnitude. Using early incubation inferred from both methods, we found that more early incubation resulted in (1) a shorter incubation period between clutch completion and first hatching and (2) a higher degree of hatching asynchrony. Below we will discuss our findings in more detail.

Comparison of methods for quantifying early incubation

The two different methods for quantifying incubation from temperature profiles, using a fixed temperature threshold and manual analysis of temperature profiles, strongly correlate and similarly predict hatching asynchrony. While yielding functionally similar estimates, it can be argued that the two methods measure slightly different aspects of the birds' breeding biology, making them conceptually distinct. The threshold method is somewhat disconnected from the females' actual incubation behaviour. For example, it does not include

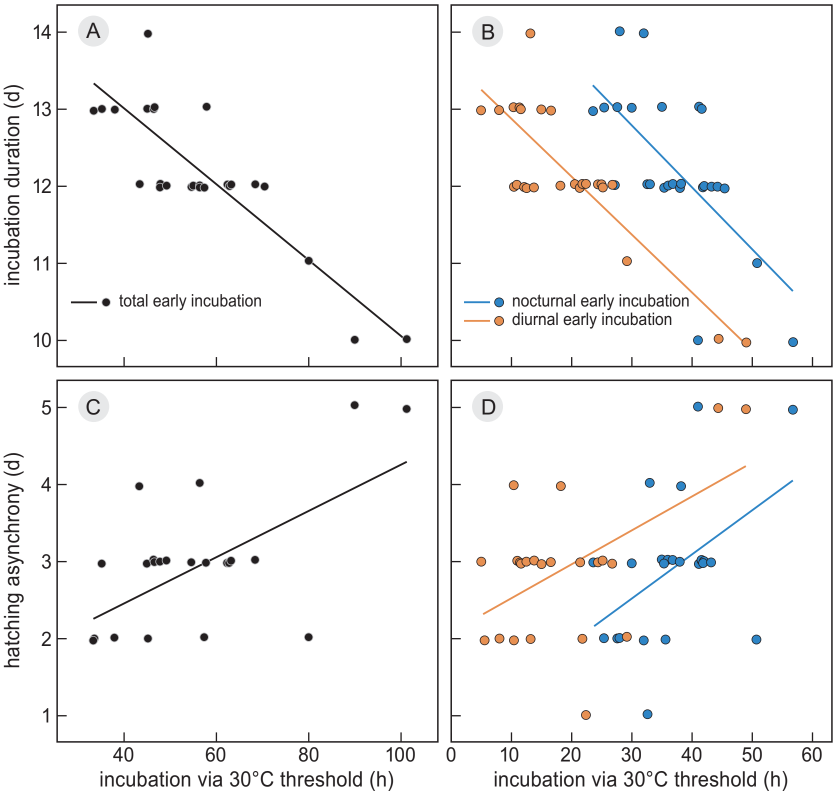

Figure 6. Incubation (threshold method) during the laying period (early incubation) predicts the time interval between clutch completion and first hatching (incubation duration) as well as hatching asynchrony. (A) the relationship between total early incubation (in hours) and incubation duration (in days). (B) Total early incubation split into nocturnal (blue) and diurnal (orange). (C, D) Relationships between early incubation (h) and hatching asynchrony. Note that slight jitter was added to facilitate visibility of all data points. Regression lines were derived from linear models (for details see main text and Tables 2, 3). The complementary figure with early incubation estimated using Raven/Rhythm can be found in Figure S3.

female incubation before the threshold temperature is reached, while on the other hand it may include warming of the eggs above the threshold temperature due to high ambient temperatures or direct sunlight warming the nestbox (Figure 2), regardless of the female's behaviour.

The Raven/Rhythm method allows for the measurement of female on- and off-bouts of incubation on a small timescale and makes it possible to distinguish between warming of the eggs due to high ambient temperatures (or direct sunlight) and female incubation behaviour. These particular features of the two methods match our observation that their incubation estimates are most strongly correlated and most similar in magnitude during night-time when potential discrepancies due to high ambient temperatures or direct sunlight are minimized and incubation is generally more continuous.

All in all, the Raven/Rhythm method would be most suitable for inferring the female's incubation behaviour (i.e. taking the female perspective). However, as the development of the embryos may proceed given a certain minimum temperature (regardless of the female's behaviour; Griffith *et al.* 2016), the temperature threshold method quantifies incubation received by the eggs (i.e. taking the eggs' perspective).

Early incubation

The observed patterns of early incubation are broadly in accordance with the literature, as several authors note that nocturnal early incubation starts after laying of the first egg in Blue Tits and Great Tits (e.g. Haftorn 1981, Stenning 2008, Podlas & Richner 2013, Diez-Méndez *et al.* 2021). The early incubation peak we observed (shortly after nightfall, in our population around 21:00) is also apparent in Great Tits (e.g. Podlas & Richner 2013, Diez-Méndez *et al.* 2021). The function of the short incubation bouts both after sunset and at sunrise, which occur from the start of egg laying onwards, is unclear but might be connected to the maintenance of egg viability (Wang & Beissinger 2011).

Early incubation has been reported to increase the concentration of egg-white antimicrobials (Svobogdová *et al.* 2021) – which are also present in Blue Tit eggs (D'Alba *et al.* 2010) – and influences bacterial communities on the egg-shell (Lee *et al.* 2014, Bollinger *et al.* 2018). It is therefore possible that these short early incubation bouts have an effect on several factors impacting egg viability, like microbial load (Cook *et al.* 2003, Ruiz-De-Castañeda *et al.* 2012), concentrations of antimicrobials, as well as embryo viability (for investigations of egg viability in poultry see e.g. Kosin & Pierre 1956, Gómez-de-Travecedo *et al.* 2014).

Table 2. Model details for incubation duration, predicted via total, nocturnal and diurnal incubation (as measured with Raven/Rhythm and 30°C threshold). The estimate is the effect of one hour of early incubation on the incubation duration in days; significant estimates in bold. See Table S1 for results using different thresholds.

Predictor	Intercept	Estimate (95% CI)	R^2 (adjusted)	*F*(df1,df2)	*P*
Total incubation (Raven/Rhythm)	14.71	**–0.042 (–0.062, –0.022)**	0.424	19.398 (1,24)	<0.001
Nocturnal incubation (Raven/Rhythm)	13.81	–0.042 (–0.088, 0.003)	0.097	3.679 (1,24)	0.067
Diurnal incubation (Raven/Rhythm)	13.59	**–0.064 (–0.092, –0.036)**	0.452	21.661 (1,24)	<0.001
Total incubation (30°C threshold)	14.98	**–0.049 (–0.062, –0.037)**	0.724	66.667 (1,24)	<0.001
Nocturnal incubation (30°C threshold)	15.20	**–0.08 (–0.118, –0.042)**	0.417	18.867 (1,24)	<0.001
Diurnal incubation (30°C threshold)	13.63	**–0.075 (–0.096, –0.055)**	0.692	57.291 (1,24)	<0.001

Table 3. Model details for hatching asynchrony, predicted via total, nocturnal and diurnal incubation (as measured with Raven/Rhythm and 30°C threshold). The estimate is the effect of one hour of early incubation on hatching asynchrony in days, significant estimates in bold. See Table S2 for results using different thresholds.

Predictor	Intercept	Estimate (95% CI)	R^2 (adjusted)	*F*(df1,df2)	*P*
Total incubation (Raven/Rhythm)	0.83	**0.035 (0.013, 0.058)**	0.301	10.46 (1,21)	0.004
Nocturnal incubation (Raven/Rhythm)	1.32	0.042 (–0.007, 0.092)	0.092	3.216 (1,21)	0.087
Diurnal incubation (Raven/Rhythm)	1.85	**0.050 (0.016, 0.083)**	0.279	9.514 (1,21)	0.006
Total incubation (30°C threshold)	1.25	**0.030 (0.009, 0.051)**	0.261	8.778 (1,21)	0.007
Nocturnal incubation (30°C threshold)	0.81	**0.057 (0.008, 0.106)**	0.181	5.852 (1,21)	0.025
Diurnal incubation (30°C threshold)	2.10	**0.044 (0.01, 0.077)**	0.228	7.486 (1,21)	0.012

Incubation patterns are very similar among individual females from five days prior to clutch completion onwards (Figure 5). This fits with the idea that increases in incubation behaviour and the cessation of egg-laying are to an extent physiologically integrated, possibly triggered by the same hormonal changes (Sockman *et al.* 2006). Both the end of egg-laying and start of incubation may be partly regulated by prolactin, its levels being influenced by the tactile stimulus provided by the eggs (Sockman *et al.* 2006, Vedder 2012). In line with such a physiological mechanism, experimental addition of model eggs early in the laying period has been found to increase incubation attentiveness in Yellow Warblers *Dendroica petechia* (Hébert & Sealy 1992; for evidence of a similar relationship in Blue Tits see Winkel 1970 and Vedder *et al.* 2010), while removal of eggs during laying suppressed early incubation in Blue Tits (Vedder *et al.* 2012).

We found all of the females to be incubating already before or at clutch completion, which differs from findings by Stenning (2008) who showed, via nest checks, that incubation started at any time between six days before and eight days after clutch completion in a UK population. A similar but smaller spread was recorded for Great Tits via daily nest checks in a Spanish population (Álvarez & Barba 2014a, Diez-Méndez *et al.* 2021). We also found more diurnal early incubation than previously recorded in other Blue Tit (e.g. Vedder 2012, in the UK) and Great Tit populations (e.g. Haftorn 1981, in Norway). These different reports in the literature suggest that incubating birds are flexible and do not have a fixed pattern of early incubation.

One potential factor shaping incubation behaviour could be ambient temperature, as experimentally heating nestboxes to 16°C during the night (mimicking higher ambient temperatures) resulted in more early incubation, sooner hatching, and greater hatching asynchrony (Vedder 2012, for more information on the effect of ambient temperatures see Cresswell & McCleery 2003, Nord & Nilsson 2012, Simmonds *et al.* 2017, Shutt *et al.* 2019, Diez-Méndez *et al.* 2021). Variation in the amount of early incubation depending on ambient temperatures could provide a potential mechanism for previously reported (slight) decreases in incubation times over time in response to climate change in Belgian Blue Tit and Great Tit populations (Matthysen *et al.* 2011).

Early incubation predicts time until first hatching and hatching asynchrony

The average time from clutch completion until first hatching of 12.2 days which we found is very close to the average of 12.9 days reported by Vedder (2012), while other studies report slightly longer intervals: 14.2 days (Gibb 1950) and 14.6 days (Winkel 1970). Regarding hatching asynchrony, we measured on average 2.8 days of hatching spread, while others reported mean hatching spreads ranging from 1.8 to over 3 days for Blue Tits (Neub 1979, Slagsvold *et al.* 1995, Magrath *et al.* 2009). While hatching asynchrony is a general finding, the association between early incubation and hatching asynchrony is found in some (Stenning 2008, Lord *et al.* 2011, Hadfield *et al.* 2013), but not in other studies (Podlas & Richner 2013). These mixed results, in addition to the findings presented here, may suggest additional mediating factors, possibly intrinsic to the eggs or related to storage time in the nest (Hadfield *et al.* 2013, Thomson & Hadfield 2017), that are not included in analyses.

Another potential factor influencing early incubation and hatching asynchrony could be the ambient temperature of the study year. Blue Tits raise comparatively large broods (Gibb 1950, Amininasab *et al.* 2016), resulting in a high peak in food demand. In order to feed the offspring, Blue Tits rely on a caterpillar food supply which peaks during a narrow time window (Perrins 1991, Naef-Daenzer & Keller 1999, Cole *et al.* 2015). The birds need to start egg laying about 30 days before the caterpillar food peak for it to coincide with the peak food demand by the nestlings (van Noordwijk *et al.* 1995). If temperatures after the start of egg laying are relatively low or high (i.e. slowing down or speeding up caterpillar development, respectively), the timing of peak food demand and food availability may not match (Visser *et al.* 2006). During a relatively warm spring, it may be an adaptive response for females to start early incubation sooner, which then leads to earlier first hatching as well as increased hatching asynchrony (see Slagsvold *et al.* 1995, Cresswell & McCleery 2003, Matthysen *et al.* 2011, Vedder 2012, Shutt *et al.* 2019).

Data from the Deutsche Wetterdienst (www.dwd.de, accessed 25/3/2021) show that in the spring of our study year (2018) there was a colder than average March, followed by the second warmest April (and warmest May) recorded between 1990 and 2019. Thus, we speculate that the Blue Tits in our study area started their breeding relatively late (due to the cold March, mean lay date of the first egg = 18.19 ± 1.94 SD in April days; see Shutt *et al.* 2019) and subsequent widespread early incubation resulted from females attempting catch up with the earlier than anticipated caterpillar food peak due to the subsequent warm weather in April.

Conclusion

In conclusion, our study presents an account of Blue Tit early incubation and the relationship of early incubation to incubation time and hatching asynchrony. We found that Blue Tits start incubating early in the laying phase, in particular during the night. Furthermore, both the time from clutch completion until first hatching and hatching asynchrony were related to the amount of early incubation. We also found that two previously used methods for inferring incubation from temperature profiles, detailed manual analysis of actual temperature profiles and using a fixed temperature threshold, lead to comparable estimates. We therefore suggest that the use of a fixed temperature threshold can be a reliable and efficient method for inferring incubation from temperature data recorded in the nest. Although we have not directly investigated what causal factors drive the observed variation in incubation patterns, we suggest that differences in the amount of early incubation observed among study years and populations may be partly explained by the prevailing spring weather conditions, with higher temperatures generally leading to more early incubation, and consequently, shorter incubation times until hatching and a higher degree of hatching asynchrony.

ACKNOWLEDGEMENTS

We are grateful to the Umweltamt of the city of Bielefeld for the permission to set up nestboxes and carry out our research in the Teutoburger Wald. We thank Henrik Jazwinksi of the Umweltbetrieb of the city of Bielefeld for introducing us to the forest, for providing us with a tall ladder, and for showing us a tree-friendly way of attaching nestboxes during the setup of the study. The Institute of Avian Research 'Vogelwarte Helgoland' in Wilhelmshaven granted permission to ring the birds in our study. We thank Harold Mills who was quick to help with troubleshooting when using the Rhythm plugin for Raven. We thank the two anonymous reviewers, who provided constructive feedback that allowed us to improve the manuscript. The study was funded by Bielefeld University ('Förderung von Kleinforschungsprojekten' from the Faculty of Biology).

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Supplementary Material is available online
www.ardeajournal.nl/supplement/s110-213-225.pdf
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SAMENVATTING

Vaak verschilt bij vogels het tijdstip van uitkomen van de eieren binnen hetzelfde nest. Leeftijdsverschillen tussen de kuikens als gevolg hiervan kunnen veel invloed hebben op hun ontwikkeling en zelfs leiden tot extra sterfte in het nest. Er zijn verschillende adaptieve hypothesen die het voorkomen van dergelijke spreiding in het uitkomen van de jongen proberen te verklaren, vaak in relatie tot de concurrentie in het nest en de timing van het broeden. Incubatie van de eieren nog voordat een legsel compleet is – hier ‘vroege incubatie’ genoemd – is waarschijnlijk de belangrijkste oorzaak van uitkomstverschillen tussen eieren in hetzelfde nest. In dit onderzoek dat plaatsvond in het Teutoburger Wald in 2018 beschrijven we in detail het voorkomen van vroege incubatie over bijna de gehele legperiode bij in nestkasten broedende Pimpelmezen *Cyanistes caeruleus*. Hiervoor hebben we gebruikgemaakt van kleine temperatuurloggers die we tussen de eieren van in totaal 26 Pimpelmeesnesten geplaatst hebben. Elke 12 min sloegen deze loggers een temperatuurmeting op, waarmee we na afloop van de metingen de incubatiepatronen precies konden reconstrueren. We vonden dat vroege incubatie voorkwam bij alle broedende vrouwtjes. Dit gedrag nam toe tijdens de eilegperiode (welke in lengte varieerde van 6 tot 13 dagen, afhankelijk van de legselgrootte; de vogels leggen ongeveer 1 ei per dag). In het begin van de eileg trad vroege incubatie vooral ’s nachts op, tegen het einde van de eileg ook steeds meer overdag. We hebben deze incubatiepatronen op twee verschillende manieren vastgesteld: door middel van handmatige analyse van de gemeten temperatuurprofielen en op basis van een drempelwaarde, bijvoorbeeld 27, 30 of 32°C. Als de temperatuur boven deze drempelwaarde kwam, namen we aan dat de eieren bebroed werden. Deze laatste methode is veel minder tijdrovend en gaf vergelijkbare uitkomsten. De hoeveelheid vroege incubatie – die varieerde van ongeveer 35 tot meer dan 100 uur op basis van de 30°C drempelwaarde – bleek een goede voorspeller voor de incubatieduur vanaf het laatst gelegde ei tot het eerste uitkomen (dit tijdsinterval varieerde van 10 tot 14 dagen). Meer vroege incubatie leidde tot sneller uitkomen. Bovendien hing zoals te verwachten de mate van spreiding in het uitkomen af van de hoeveelheid vroege incubatie (de spreiding in uitkomst varieerde van één tot vijf dagen verschil tussen het eerst en laatst uitgekomen ei). We hebben niet onderzocht door welke factoren de hoeveelheid vroege incubatie wordt bepaald. Het zou kunnen dat de omgevingstemperatuur tijdens het voorjaar hierop van invloed is en dat bij warm weer de vogels meer vroege incubatie vertonen. Zo’n mechanisme waarmee vogels de broedduur en uitkomstspreiding aanpassen aan de heersende temperaturen zou ook van belang kunnen zijn bij de aanpassing aan klimaatverandering.

Corresponding editor: Roeland Bom
Received 28 January 2022; accepted 17 May 2022

SUPPLEMENTARY MATERIAL

Table S1. Model details incubation duration with 27 and 32°C threshold (significant estimates in bold).

Predictor	Intercept	Estimate (95% CI)	R^2 (adjusted)	*F*(df1,df2)	*P*
Total incubation (32°C threshold)	14.34	**–0.048 (–0.062, –0.033)**	0.642	45.854 (1,24)	<0.001
Nocturnal incubation (32°C threshold)	14.44	**–0.071 (–0.105, –0.038)**	0.419	19.016 (1,24)	<0.001
Diurnal incubation (32°C threshold)	13.40	**–0.088 (–0.115, –0.062)**	0.655	48.483 (1,24)	<0.001
Total incubation (27°C threshold)	15.27	**–0.043 (–0.058, –0.028)**	0.581	35.733 (1,24)	<0.001
Nocturnal incubation (27°C threshold)	15.05	**–0.069 (–0.114, –0.023)**	0.259	9.734 (1,24)	0.005
Diurnal incubation (27°C threshold)	13.99	**–0.058 (–0.08, –0.037)**	0.554	32.115 (1,24)	<0.001

Table S2. Model details hatching asynchrony with 27 and 32°C threshold (significant estimates in bold).

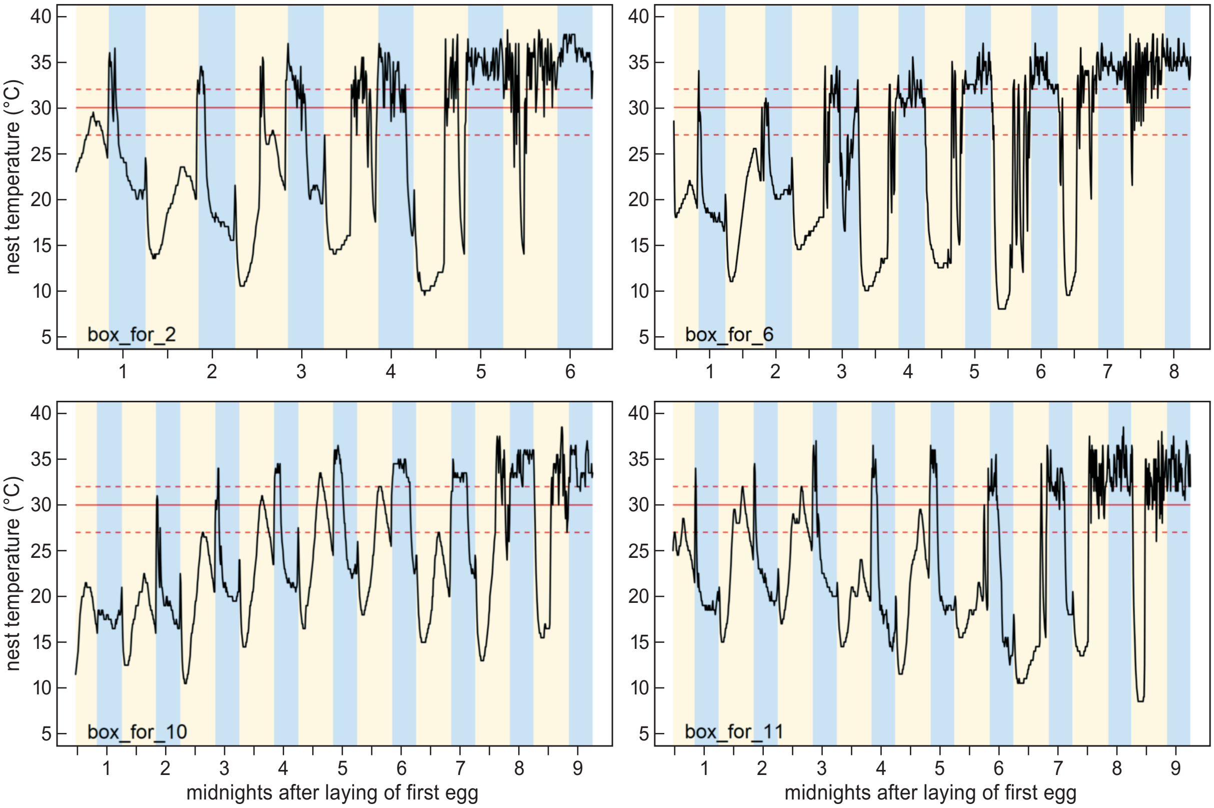
Predictor	Intercept	Estimate (95% CI)	R^2 (adjusted)	*F*(df1,df2)	*P*
Total incubation (32°C threshold)	1.73	**0.027 (0.004, 0.049)**	0.190	6.154 (1,21)	0.022
Nocturnal incubation (32°C threshold)	1.57	**0.043 (0.001, 0.086)**	0.136	4.458 (1,21)	0.047
Diurnal incubation (32°C threshold)	2.28	**0.047 (0.006, 0.089)**	0.172	5.57 (1,21)	0.028
Total incubation (27°C threshold)	0.96	**0.028 (0.007, 0.048)**	0.233	7.701 (1,21)	0.011
Nocturnal incubation (27°C threshold)	0.58	**0.057 (0.003, 0.111)**	0.150	4.885 (1,21)	0.038
Diurnal incubation (27°C threshold)	1.87	**0.035 (0.006, 0.065)**	0.188	6.082 (1,21)	0.022

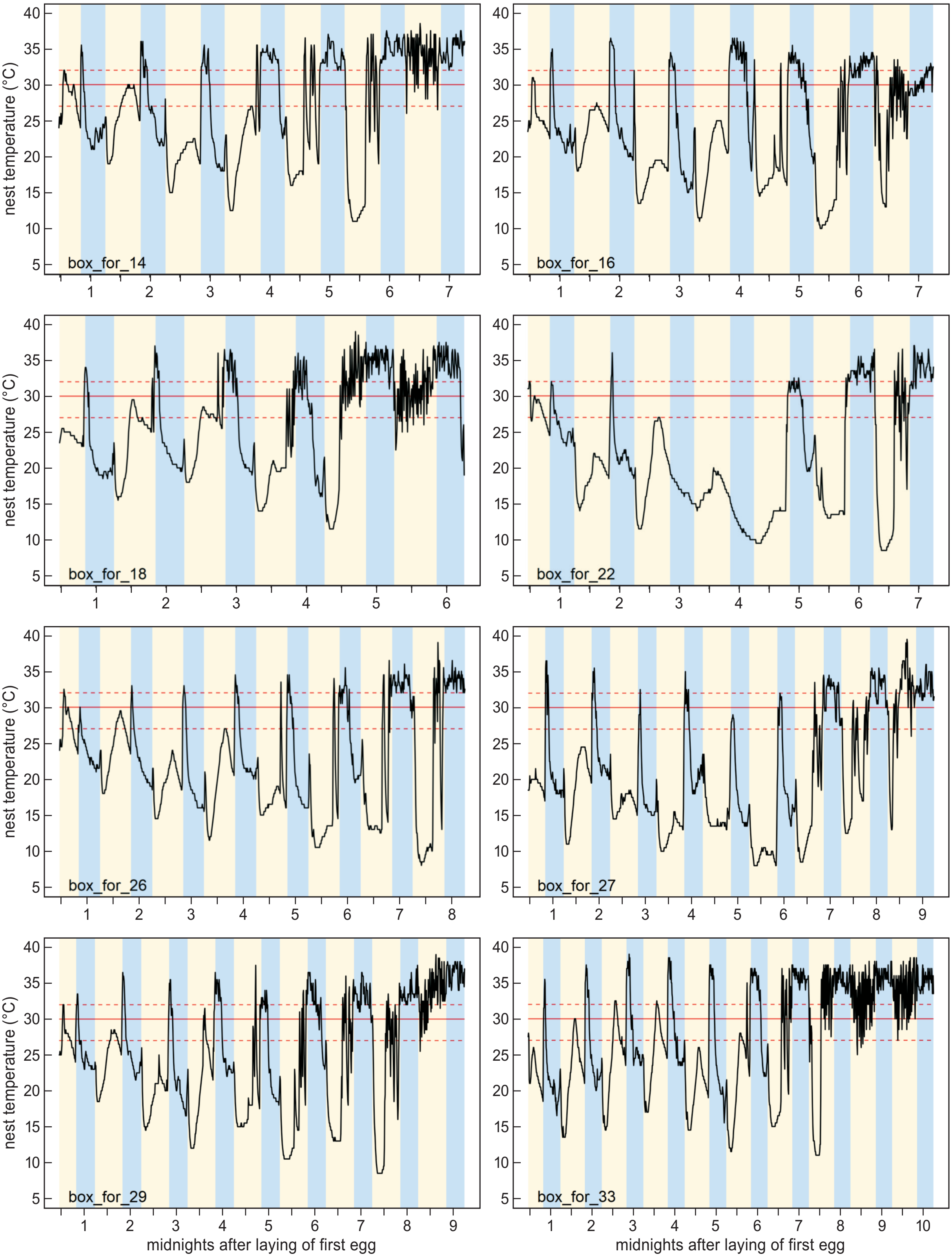
Table S3. Model details incubation duration (outliers omitted; significant estimates in bold).

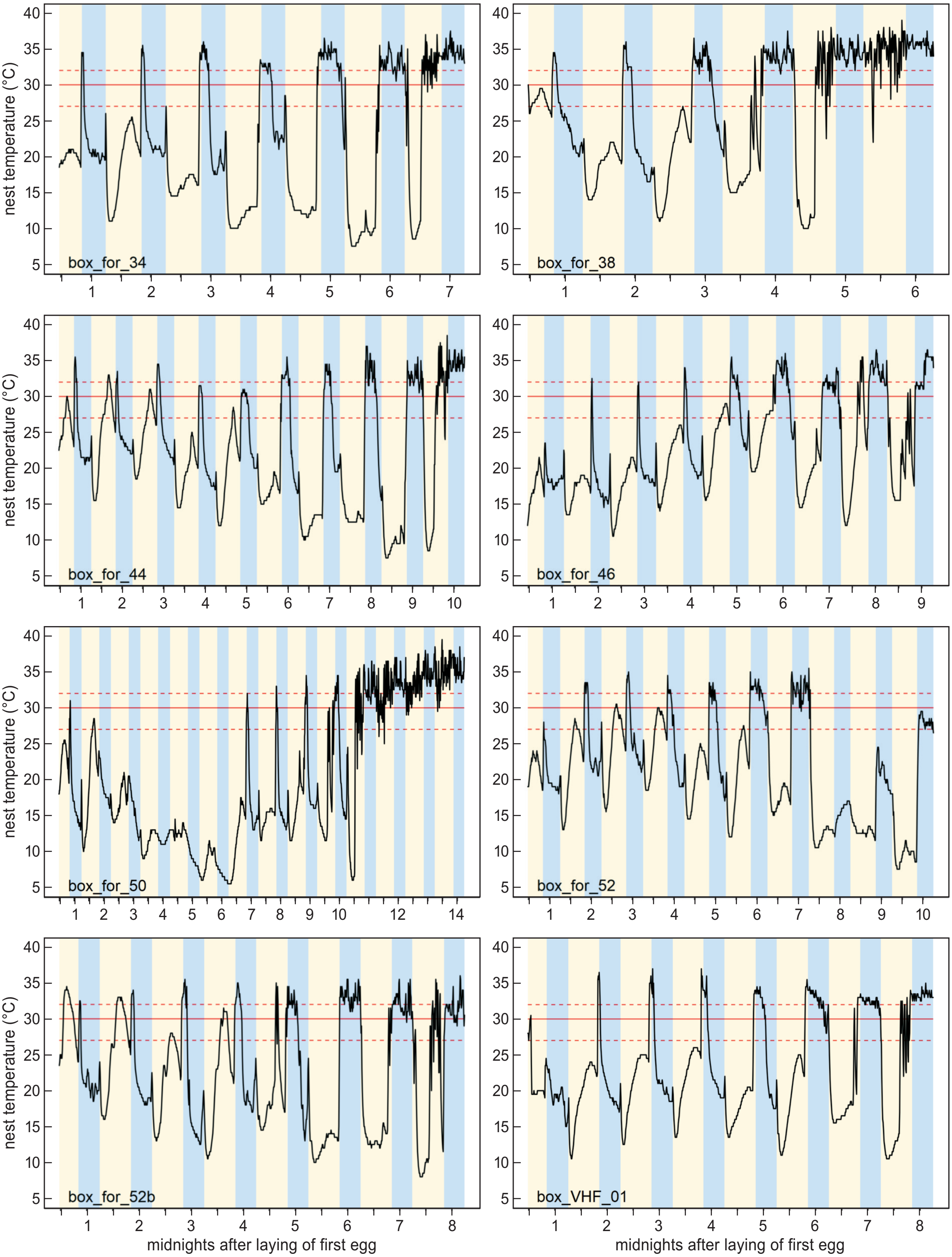
Predictor	Intercept	Estimate (95% CI)	R^2 (adjusted)	*F*(df1,df2)	*P*
Total incubation (Raven/Rhythm)	13.76	–0.024 (–0.048, 0)	0.122	4.194 (1,22)	0.053
Nocturnal incubation (Raven/Rhythm)	12.95	–0.015 (–0.057, 0.028)	–0.021	0.519 (1,22)	0.480
Diurnal incubation (Raven/Rhythm)	13.24	**–0.043 (–0.078, –0.007)**	0.181	6.099 (1,22)	0.022
Total incubation (32°C threshold)	13.98	**–0.038 (–0.057, –0.019)**	0.410	17.008 (1,22)	<0.001
Nocturnal incubation (32°C threshold)	13.98	**–0.052 (–0.086, –0.019)**	0.293	10.512 (1,22)	0.004
Diurnal incubation (32°C threshold)	13.27	**–0.075 (–0.115, –0.034)**	0.370	14.481 (1,22)	0.001
Total incubation (30°C threshold)	14.69	**–0.044 (–0.062, –0.025)**	0.507	24.675 (1,22)	<0.001
Nocturnal incubation (30°C threshold)	14.56	**–0.060 (–0.097, –0.023)**	0.306	11.149 (1,22)	0.003
Diurnal incubation (30°C threshold)	13.54	**–0.069 (–0.103, –0.035)**	0.422	17.806 (1,22)	<0.001
Total incubation (27°C threshold)	14.63	**–0.033 (–0.053, –0.012)**	0.307	11.196 (1,22)	0.003
Nocturnal incubation (27°C threshold)	14.13	–0.043 (–0.088, 0.002)	0.113	3.928 (1,22)	0.060
Diurnal incubation (27°C threshold)	13.56	**–0.041 (–0.071, –0.012)**	0.247	8.537 (1,22)	0.008

Table S4. Model details hatching asynchrony (outliers omitted; significant estimates in bold).

Predictor	**Intercept**	**Estimate (95% CI)**	**R^2 (adjusted)**	***F*(df1,df2)**	***P***
Total incubation (Raven/Rhythm)	2.04	0.012 (–0.016, 0.040)	–0.010	0.799 (1,19)	0.38
Nocturnal incubation (Raven/Rhythm)	2.26	0.012 (–0.035, 0.060)	–0.037	0.296 (1,19)	0.59
Diurnal incubation (Raven/Rhythm)	2.38	0.017 (–0.026, 0.060)	–0.014	0.716 (1,19)	0.41
Total incubation (32°C threshold)	2.69	0.000 (–0.027, 0.027)	–0.053	0.001 (1,19)	0.98
Nocturnal incubation (32°C threshold)	2.34	0.012 (–0.031, 0.055)	–0.033	0.362 (1,19)	0.56
Diurnal incubation (32°C threshold)	2.92	–0.019 (–0.074, 0.036)	–0.026	0.501 (1,19)	0.49
Total incubation (30°C threshold)	2.51	0.004 (–0.025, 0.032)	–0.048	0.081 (1,19)	0.78
Nocturnal incubation (30°C threshold)	1.89	0.023 (–0.028, 0.074)	–0.005	0.902 (1,19)	0.35
Diurnal incubation (30°C threshold)	2.85	–0.009 (–0.057, 0.040)	–0.044	0.150 (1,19)	0.70
Total incubation (27°C threshold)	2.62	0.001 (–0.026, 0.029)	–0.052	0.011 (1,19)	0.92
Nocturnal incubation (27°C threshold)	1.93	0.020 (–0.036, 0.076)	–0.023	0.547 (1,19)	0.47
Diurnal incubation (27°C threshold)	2.88	–0.006 (–0.044, 0.032)	–0.046	0.118 (1,19)	0.74




nest temperature (°C)
5
10
15
20
25
30
35
40
box_for_14
box_for_16
box_for_18
box_for_22
box_for_26
box_for_27
box_for_29
box_for_33
1
2
3
4
5
6
7
8
9
10
midnights after laying of first egg


nest temperature (°C)
40
35
30
25
20
15
10
5
box_for_34
1 2 3 4 5 6 7
box_for_38
1 2 3 4 5 6
box_for_44
1 2 3 4 5 6 7 8 9 10
box_for_46
1 2 3 4 5 6 7 8 9
box_for_50
1 2 3 4 5 6 7 8 9 10 12 14
box_for_52
1 2 3 4 5 6 7 8 9 10
box_for_52b
1 2 3 4 5 6 7 8
box_VHF_01
1 2 3 4 5 6 7 8
midnights after laying of first egg
midnights after laying of first egg

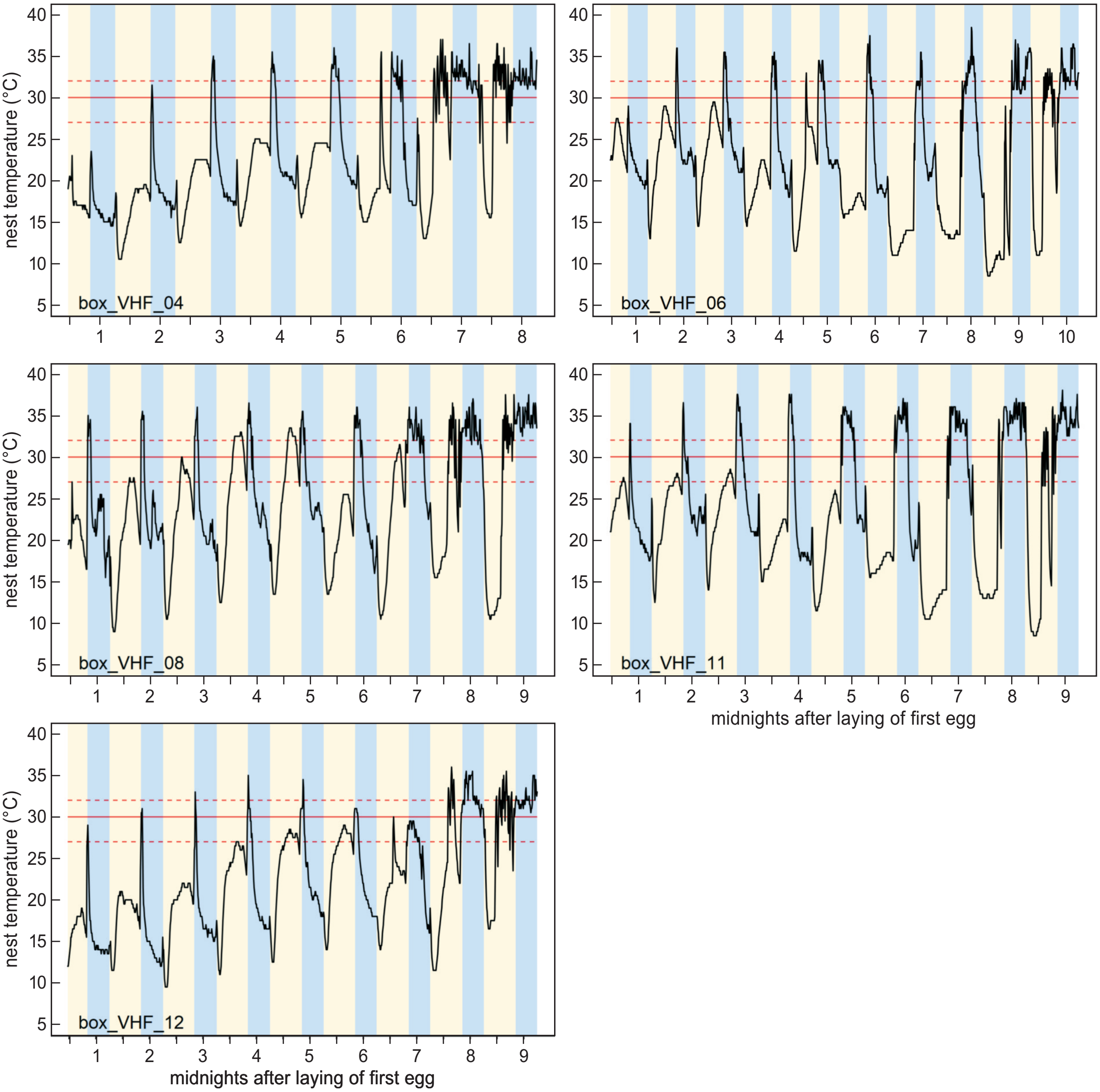


Figure S1. Temperature profiles of all nestboxes (ID bottom left which corresponds to the name of the respective raw data file). For nestbox 24 see main text (Figure 2).

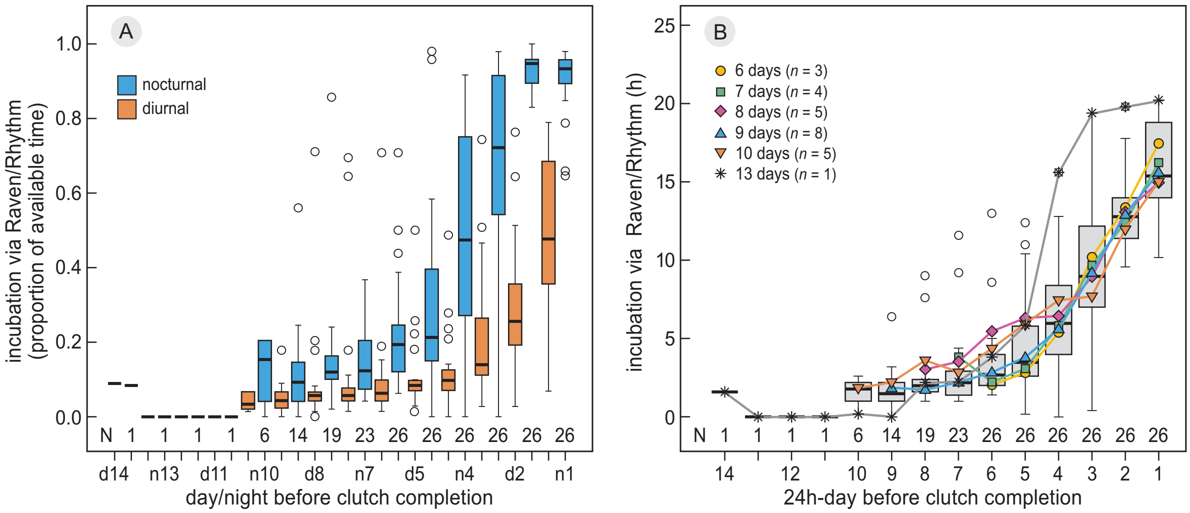


Figure S2. Incubation (Raven/Rhythm method) during egg laying. (A) The proportion of available time that was spend incubating during each night and day before clutch completion. (B) Absolute incubation in hours for each 24-hour day before clutch completion. In addition to the boxplot, the lines indicate the patterns of average incubation for females with different laying period lengths (which approximate clutch sizes; note the outlier nest marked by the circles). Sample sizes are indicated below the plots.

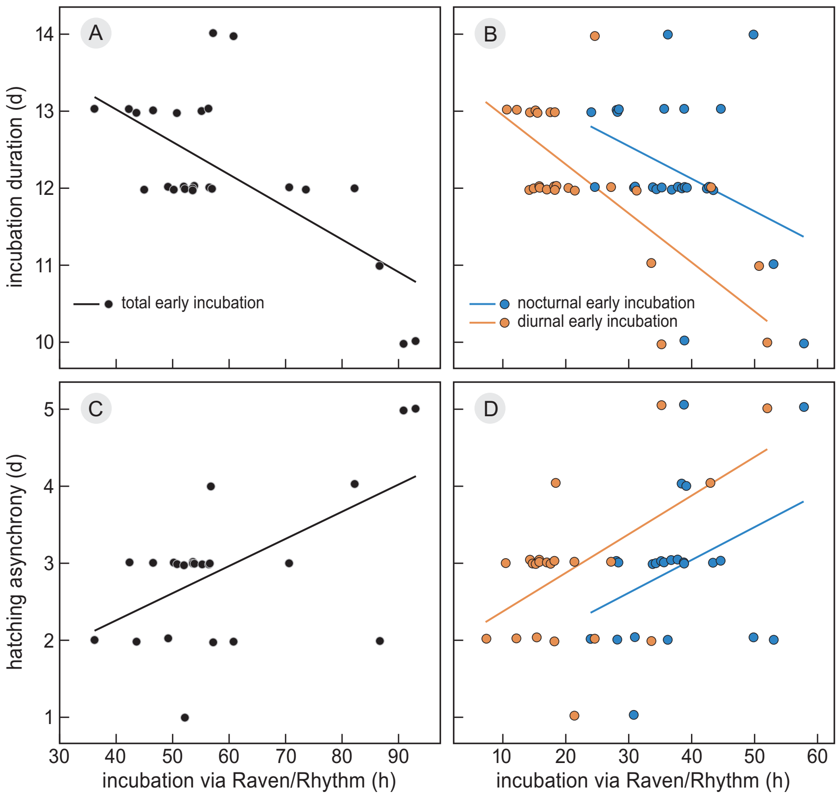


Figure S3. Incubation (Raven/Rhythm method) during the laying period (early incubation) predicts the time interval between clutch completion and first hatching (incubation duration) as well as hatching asynchrony. (A) The relationship between total early incubation (in hours) and incubation duration (in days). (B) Total early incubation split up into nocturnal (blue) and diurnal (orange). (C, D) The relationships between early incubation (hours) and hatching asynchrony. Note that slight jitter was added to facilitate visibility of all data points. Regression lines were derived from linear models (for details see main text and Tables 2–3).