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Circadian clock period length is not consistently linked to chronotype in a wild songbird

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Abstract

Circadian clock properties vary between individuals and relate to variation in entrained timing in captivity. How this variation translates into behavioural differences in natural settings, however, is poorly understood. Here, we tested in great tits whether variation in the free-running period length (*tau*) under constant dim light (LL) was linked to the phase angle of the entrained rhythm ("chronotype") in captivity and in the wild, as recently indicated in our study species. We also assessed links between *tau* and the timing of first activity onset and offset under LL relative to the last experienced light–dark (LD) cycle. We kept 66 great tits, caught in two winters, in LL for 14 days and subsequently released them with a radio transmitter back to the wild, where their activity and body temperature rhythms were tracked for 1 to 22 days. For a subset of birds, chronotype was also recorded in the lab before release. Neither wild nor lab chronotypes were related to *tau*. We also found no correlation between lab and wild chronotypes. However, the first onset in LL had a positive relationship with *tau*, but only in males. Our results demonstrate that links between *tau* and phase of entrainment, postulated on theoretical grounds, may not consistently hold under natural conditions, possibly due to strong masking. This calls for more holistic research on how the many components of the circadian system interact with the environment to shape timing in the wild.

Wild birds showed chronotypes in the field that were unlinked to their circadian period length tau measured in captivity. In males only, the first onset of activity after exposure to constant dim light did correlate with *tau*. Our study emphasises the need to investigate clocks in the real world, including a need to better understand masking.

Abbreviations: LD, light–dark cycle; LL, constant dim light; Tau, length of the free-running period under constant dim light.

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KEYWORDS
activity rhythm, diel rhythm, great tit, *Parus major*, *tau*, telemetry

1 | INTRODUCTION

Circadian rhythms are present across the whole tree of life and are considered essential for organisms (Jabbur et al., 2024; Krittika & Yadav, 2020; Woelfle & Johnson, 2009). The rationale for the importance of biological clocks, rather than timing in direct response to the environment, assumes two main advantages. Firstly, circadian rhythms allow for temporal coordination of many, sometimes conflicting, behavioural and physiological processes within an organism. Secondly, circadian rhythms provide an internal reference time by which environmental conditions can be correctly interpreted. Thus, organisms respond appropriately to, and anticipate, naturally cyclic environmental conditions, in particular the variation in natural light and darkness. By entraining their circadian clocks to this major synchronizing cue (i.e., *Zeitgeber*), organisms can tell the time of day and thus anticipate cyclically repeating environmental events (Daan & Aschoff, 1982; Jabbur et al., 2024; Krittika & Yadav, 2020; Pittendrigh, 1958; Woelfle & Johnson, 2009).

Biological clocks exhibit properties of physical oscillators, and these similarities in turn enabled predicting and subsequently experimentally testing clock features (Aschoff & Wever, 1962; Johnson et al., 2003; Pittendrigh & Daan, 1976b; Schmal et al., 2020). When a biological oscillator is synchronized by an entraining cycle, it is expected that the oscillator's intrinsic period length (i.e., *tau*) determines its temporal relationship to the entraining cycle. This relationship is quantified as the timing difference between stable cycle-to-cycle reference points in the rhythms of the oscillator and the entraining cycle, called phases (e.g., start of activity and start of the light phase, respectively). Thus, if the oscillator has a shorter period length than the entraining cycle, the phase angle (i.e., phase of the rhythm minus phase of the entraining cycle) becomes negative, indicating a phase lead of the oscillator (e.g., start of the activity occurs before the start of the light phase), and vice versa (Aschoff & Wever, 1962). When this is applied to organisms' activity patterns, typically an individual's activity phase would be predicted by its *tau* relative to the length of the *Zeitgeber* cycle, for example, a 24-h light–dark (LD) cycle. Such predictions could indeed be confirmed empirically in the laboratory, for example by manipulation of LD cycles or by using variation in *tau* (Aschoff & Wever, 1962). Still, even under controlled conditions biological oscillators differed from physical oscillators by changing their properties, for example dependent on season or the social setting (Aschoff, 1979).

Importantly, when applied to the natural environment, the predicted relationships between the natural light cycle and an individual's *tau* should contribute to its particular time-keeping in the wild. Properties of circadian clocks, such as *tau*, and consequently phase angle, are variable between species (Daan & Pittendrigh, 1976), between populations of the same species (Daan & Pittendrigh, 1976; Kyriacou et al., 2008) and even between individuals within the same population (Pivarciova et al., 2016; Salmela & Weinig, 2019). In natural populations, clock properties should be distributed around the population-specific mean, like most other traits (Daan & Beersma, 2002; Helm & Visser, 2010; Jabbur et al., 2024; Michael et al., 2003; Pittendrigh & Daan, 1976a). In several species, consistent individual patterns of temporal behaviour, such early- versus late-phased activity (i.e., behavioural chronotypes), have indeed been described (Roenneberg et al., 2003). In humans, these are traditionally assessed via questionnaires (Roenneberg et al., 2003), while in other animals, consistent early and late behaviours (e.g., Fleury et al., 2000; Helm & Visser, 2010; Nikhil et al., 2016; Strauß et al., 2022) are used as proxy for distinct chronotypes. Studies of chronotypes have provided mixed evidence for a correlation with *tau*. Where correlations existed, they often explained only small parts of inter-individual differences in chronotype (Dominoni et al., 2013).

The discrepancy between predictions and results, especially in the field, is perhaps not so surprising. In contrast to the laboratory, organisms under natural conditions experience a wide range of environmental inputs that contribute to entrain or modify (i.e., mask) their diel time-keeping (Aschoff, 1988; Helm et al., 2017). Main forms of masking are positive masking, which may augment the amplitude of a rhythm, and negative masking, which may suppress it (Mrosovsky, 1999; Schwartz et al., 2017). For example, a nocturnal animal may be kept from displaying nocturnal activity while being exposed to light, and conversely, a diurnal animal may be induced by light to show activity during its circadian rest phase. While masking differs from entrainment by its ephemeral effects during exposure to an external factor, its importance to fitness under natural conditions may equal that of entrainment (Helm et al., 2017;

Mrosovsky, 1999; Rotics et al., 2011). Many modifications of time-keeping by either entrainment or masking are mediated by sensory and physiological pathways that jointly control an individual's phase, for example sensitivity to light and ambient temperature, or metabolic or immune state. If there are advantages to organisms in being earlier or later, natural selection on daily timing should take place and act on any aspects of this integrated circadian system (Helm et al., 2017; Jabbur et al., 2024; Krittika & Yadav, 2020; Roenneberg et al., 2003). Modifications of time-keeping can occur at various levels of the integrated circadian system. For example, when overt behavioural rhythmicity may be absent, rhythmicity could persist in other body function, such as body temperature or gene expression or protein levels in control regions of the brain (Beer & Bloch, 2020). Hence, chronobiologists also assess clock properties through other, putatively more robust measures, such as clock gene expression cycles in cell culture (Brown et al., 2005, 2008), or cycles in body temperature (Duffy et al., 2001; Strauß et al., 2022), and apply more indirect approaches such as (clock) gene-phenotype associations (Allebrandt & Roenneberg, 2008).

Arguably, the most conclusive test of how the variation in *tau* translates into behavioural differences between animals is to combine measures of clock properties in captivity with measures of chronotype in free-living animals. The number of studies that attempted to do this is limited due to the logistical difficulties of measuring the same individual in the wild and under constant conditions. The studies that succeeded in this attempt also presented mixed results: that is, in a comparison of urban and rural blackbirds (*Turdus merula*), a positive relationship between *tau* and activity timing was found only in city birds (Dominoni et al., 2013); in female great tits (*P. major*), a positive relationship was found between incubation activity and *tau* in both city and forest birds (Tomotani et al., 2023), but a previous study with the same species in captivity did not find a relationship (Helm & Visser, 2010; Lehmann et al., 2012).

Therefore, in the present study, we aimed for greater clarity by follow-up investigations of the relationship between clock and chronotype, using locomotor activity rhythms and a larger sample of wild, free-living animals. We used the diurnal songbird great tit as a model because its circadian rhythmicity has been extensively studied in captive and wild contexts (de Jong et al., 2016; Helm & Visser, 2010; Lehmann et al., 2012; Spoelstra et al., 2018; Tomotani et al., 2023). We derived *tau* from free-running activity after the rhythms stabilized (from day 2 or later). We then tested whether variation in *tau* was linked to the phase angle of the entrained activity rhythm ("chronotype") in the wild, and for a subset of birds, also in captivity (i.e., "wild chronotype" and "lab chronotype", respectively). In the wild birds, we also quantified diel timing patterns of peripheral body temperature in parallel to their activity patterns from continuous skin temperature measurements using telemetry. Based on the oscillator theory summarised above, we expected a positive correlation between *tau* and chronotype.

In addition, we investigated the first activity cycles in constant dim light (LL) separately from subsequent cycles because the phase and period length of the first cycles of the rhythm are affected by *tau* and by the previous conditions that the organism was exposed to (after-effects, Pittendrigh, 1960). We calculated the phase angle of the onset and offset relative to the natural LD cycle experienced by the birds on the previous day. This measure, referred to as first onset and offset in LL, is thought to capture an animal's prediction of morning and evening, based on its previous entrainment, but in the absence of overriding environmental cues, as well as based on effects of *tau* (e.g., Tomotani et al., 2012, 2023). It can thereby tentatively be interpreted as a proxy for the phase angle of an individual's rhythm given its circadian period length, without confounding effects of masking. The measure was previously reported to correlate with *tau* in studies with great tits in captivity (Laine et al., 2019; Spoelstra et al., 2018).

2 | MATERIAL AND METHODS

2.1 | Measurements in the lab

All experimental procedures in the lab and the field were carried out underlicenses of the Central Authority for Scientific Procedures on Animals (Project AVD 80100 2019 9005) and the Animal Welfare body (IVD) of the Royal Netherlands Academy of Sciences (KNAW; Protocols NIOO 20.02 and NIOO 21.13).

During the winters of 2021 and 2022, we captured wild great tits (Table S1_1) at night, when the birds were roosting in nest boxes. We captured 66 birds in total (including 2 birds captured twice), at the Zernike campus of Groningen (2021, 2022, 53°14.5′N 6°32.3′E), in the city of Utrecht (2021, 52°6.1′N 5°8.9′E), and in Heikamp forest (2021, 52°1.9′N 5°50.3′E). Birds were immediately taken to the Netherlands Institute of Ecology (NIOO-KNAW, 51°59.2′N 5°40.3′E). Once at the institute, birds were ringed, weighed, and in 2021 kept for 1 day in a cage exactly like the one in the experimental set-up but exposed to the natural LD cycle to acclimatize (Figure 1). Birds were kept in a room with direct access to natural light, supplemented by indoor lighting (experiencing light intensity values ranging from 120 to 600 lx) that

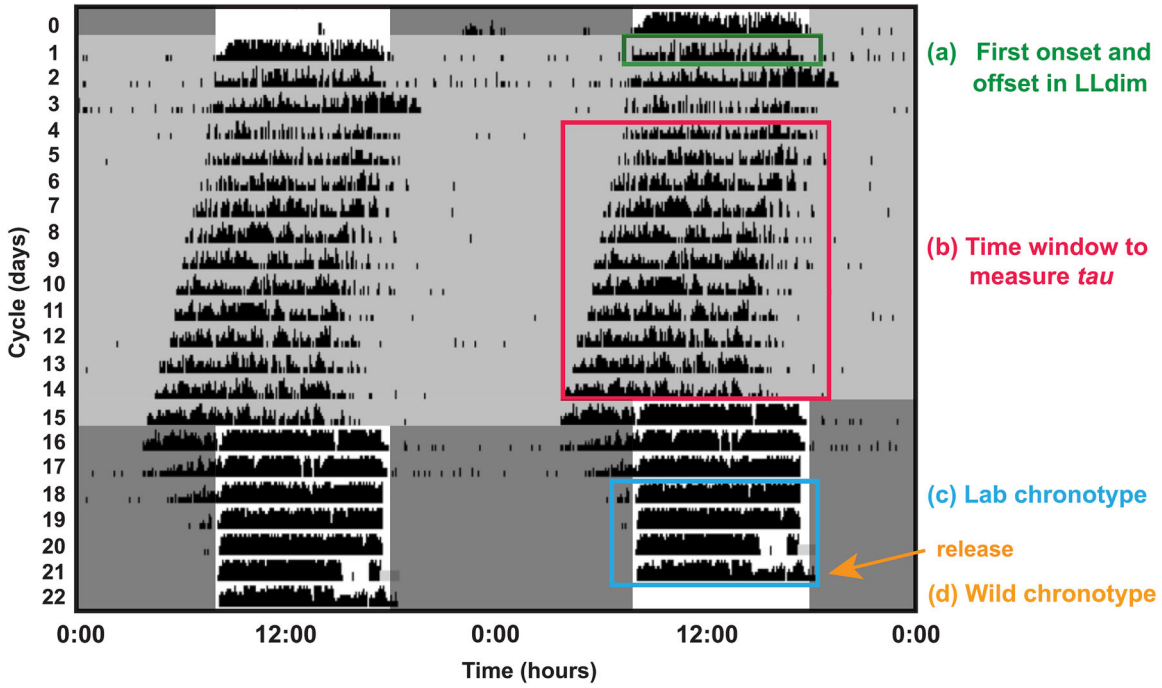

FIGURE 1 Actogram of one of the birds from the 2022 group, showing the measurements collected in this study. Activity (amount of activity per minute measured in increments of 10 s, thus ranging from 0 to 6 per 1-min bin) is plotted in black against time of day, whereby each row represents a day of experiment. Activities on a given day are repeated to the right of each day (i.e., double-plotted) for greater clarity. Measures are as follows: (a) First onset and offset in LL (constant dim light conditions). (b) Tau, the period length of the endogenous clock. (c) Lab chronotype, the onsets and offsets of the rhythm in captivity, once re-synchronized by a light–dark (LD) cycle, based on a subset of birds. (d) Wild chronotype, the onsets and offsets of the activity rhythm and body temperature in the wild (data not shown).

were switched on at sunrise and switched off at sunset. At night, birds were kept without any provided light. Birds were then moved from this acclimatization cage to the experimental set-up (Fig. S1_1) the following night. Thus, at the end of the light phase of the preceding LD-cycle, instead of experiencing darkness, birds were exposed to constant dim light (i.e., LL; .5 lx at perch level; see Supporting Information Part S1 and Fig. S1_2). Birds were kept in the set-up in LL for 14 days (Figure 1). In 2022, the capture procedure was the same but birds were placed directly in the set-up. As in 2021, birds were exposed to one natural LD cycle to acclimatize through windows in the room. The set-up was left uncovered until the start of the LL stage. Then, before the following sunset, windows were covered, cage doors were closed at sunset time, and the constant dim light treatment started.

The experimental set-up (Fig. S1_1) was designed to measure great tit rhythms. It consisted of individual metal cages, placed in stand-alone plywood racks in groups of six cages (three rows and two columns; Fig. S1_1). The racks provided plywood separators to the outside, as well as between each cage, so that only the metal-barred cage fronts remained accessible. We then added a wooden front-door that covered the cage fronts except during feeding, effectively isolating the animals from external cues and from each other. A ventilation grid with a light trap on the side of this wooden door provided ventilation. Each cage was individually equipped with a night lamp that provided dim light continuously for the LL experiment. Cages were also equipped with passive infrared (PIR) sensors that checked for movement every 10 s and binned the data every 60 s. Thus, the intensity of activity varied from 0 to 6 every 1-min bin (software developed by T&M Automation, Leidschendam, The Netherlands). Throughout the study, the whole room with the isolation cages was kept completely dark, with all windows covered by a thick black plastic. White noise, broadly resembling rain, was played continuously in the background to cover any external noise and animals' vocalizations. Birds were offered *ad libitum* water and food (i.e., beef heart mixture, apple, dry bird food, peanuts, sunflower seeds, and live mealworms). Food was refreshed daily but at variable times of the day so that the birds would be unable to use the feeding times as a cue to synchronize their clock. At the end of the experiment, in 2021, birds were returned to a regular cage without recording facilities and were again exposed to the natural LD cycle before being released with a transmitter. In contrast, in 2022, birds

were also re-exposed to LD cycles but remained in the set-up prior to release, allowing their re-entrainment to be measured.

2.2 | Measurements in the field

To measure biological rhythms in the field, we deployed temperature-sensitive radio transmitters (PicoPip Tag PIP31 and PIP51 Ag317 single-celled tag including temperature sensor option, Lotek, Wareham, UK; < 5% of the body weight). Skin temperature measurements have been shown to correlate with core body temperatures in great tits (Nord et al., 2016), to be rhythmic and to deviate from ambient temperature patterns in winter (Strauß et al., 2022). These transmitters emit pulses of a radio wave, each with a tag-specific frequency (150–151 MHz). The detected signal varies in strength with movement and distance of a transmitter so that the variance of signal strength can be used to distinguish active and inactive times of a tagged individual (Dominoni et al., 2014). Additionally, our transmitters were temperature sensitive. The interval between two consecutive pulses depended on the transmitter's temperature so that increasing temperature decreased the pulse interval. This enabled us to simultaneously measure diel skin temperature and activity rhythms (Strauß et al., 2022).

The tags were calibrated before deployment by exposing them to the progressively cooling temperatures of a hot water bath (decreasing from ca. 40 to 20°C). Temperatures of the water bath were recorded simultaneously with a temperature logger (iButton: Thermochron DS1922L-F5, Maxim, USA), and signals were recorded with a telemetry receiver (SRX800 MD2, Lotek, Wareham, UK).

To record the birds' rhythms, we attached the transmitters to the birds' upper backs (Strauß et al., 2022). If the transmitter is firmly attached to the skin, reliable temperature measurements can be taken. Thus, prior to deployment, we sewed the tags to a cotton cloth (1 cm diameter) to increase gluing and attachment surface. A small patch of feathers on the bird's back was trimmed, and the transmitter glued to the patch using eyelash glue and only a small amount of superglue to ensure easy falling-off during moult at the latest. During deployment, the anterior feathers were brushed away and afterwards brushed back in position to cover the patch.

Individuals were automatically recorded using stationary receivers. The receivers were self-constructed using materials from Motus (a collaborative wildlife radio tracking system, Taylor et al., 2017) and the SensorGnome (SG) system (here, Raspberry Pi3 model B, Raspberry Pi Foundation, Cambridge, UK) with software version 2018-10-12 (SensorGnome Project, 2018). One to two SGs were placed per site, each with an omnidirectional antenna (SIRIO CX 148 U – 148–152 MHz, Volta Mantovana, Italy). In Groningen, we additionally used a fixed station on the roof of the university building "Linnaeusborg" (RUG) that had five directional antennas (SIRIO WY 140-6 N, SIRIO Antenne, Volta Mantovana, Italy). The receivers were set to scan through the frequencies of each deployed tag for 10 s before switching to the next frequency, so every tag was recorded in intervals of 1.5–2.8 min (see Supporting Information Part S2 for more details).

2.3 | Data processing

The captivity data were used to obtain (a) the first onset and offset in LL (2021 and 2022), (b) the bird's endogenous free-running period length *tau*, and (c) onsets and offsets of the re-entrained rhythm (lab chronotype, 2022 only) (Figure 1). All 66 individual actograms are shown in Figures S1_3 –S1_10.

To account for seasonal changes in day length, we calculated relative timing by subtracting the time of sunrise or sunset from the activity onset and offset time, respectively (i.e., activity onset minus sunrise or activity offset minus sunset). Thus, a bird would have a negative onset phase angle if its activity started before sunrise and a positive onset phase angle if its activity started after sunrise. Likewise, it would have a negative offset phase angle if its activity ended before sunset and a positive offset phase angle if its activity ended after sunset.

The data collected from the set-up were used to produce actograms using the software *Chronoshop* (v. 1.04, 2015, written by Spoelstra, e.g., Spoelstra et al., 2018; Tomotani et al., 2023). *Chronoshop* was also used for obtaining the values of *tau*, and onset and offset of activity in captivity. *Tau* was calculated via the Sokolove and Bushell method (S-B), for all but one bird in which no clear periodicity emerged. In these analyses, we excluded the first cycle that we used to obtain the first onset and offset in LL. We also excluded additional cycles when the rhythm was still displaying after-effects (see Supporting Information). In these excluded cycles, the rhythm was still similar to the previous synchronized state with a period close to 24 h, and the onset of activity was matching the time of sunrise. This lasted from 1 to 7 days depending on the individual, and was easily detected by a change in the actogram where the onset of activity drifted from the synchronized onset (see Figure 1). For example, in Figure 1 (with annotations on the right), cycle 0 is the synchronized onset under a LD cycle; cycles 1 to 3, when the animal had transitioned to LL, show history-

dependent after-effects from the previous synchronization, whereby onset time is similar to the entrained state. The onsets start to drift from cycle 4 onwards as the animal expresses its own internal period length. For extracting the onset and offset of activity, the software calculates the centre of gravity per cycle positioned at the mean vector angle. Then, it estimates the activity onset or offset by going .5 cycles back or forward in time, respectively, to detect the phase when the momentary activity first exceeds the average activity in the current cycle. In order to avoid onsets and offsets being detected at times where small amounts of movements or noise are present, a running mean is fit to the data so only activity bins above those values are classified as the onset or offset of activity (Spoelstra et al., 2018). Because the detection of the onset and offset was sensitive to the activity level of the individual bird, we had to adjust the running mean per individual, per cycle, varying between 10 bins (48 onsets / 32 offsets), 70 bins (14 onsets / 26 offsets), or 180 bins (0 onsets / 4 offsets). In a few instances, the amount of background noise did not allow the detection of an onset or offset of activity regardless of the running mean used, in such instances the onset for that cycle was excluded. The estimation of *tau* is very robust to small amounts of noise in the activity rhythms and was not affected by changing the running mean.

From the wild, we obtained telemetric data that were processed and filtered in R (version 4.3.1, R Core Team, 2023) and R studio (version 2023.06.2) to obtain activity and skin temperature estimates (for details see Supporting Information Part S2 Section 1–4). The raw data were filtered to address several issues associated with the data collection using a SG. In particular, we accounted for carry-over effects from switching from one to the next frequency, due to a time lag between transitioning in the hard- and software. We also accounted for multiple detections per second due to multiple recordings of the same radio frequency along the antenna and for further artefacts visible in the recorded frequency and background noise that were probably caused by the SG software (for details, see Supporting Information Part S2 Section 3). Thereafter, we calculated pulse intervals and applied the tag-specific calibration curves to calculate the skin temperature sensed by the transmitter (Jonasson, 2017). We binned the data into 5-min bins and calculated the deviation of signal strength between two consecutive bins as an indicator of activity.

To extract the onset and offset of activity, we used a behavioural changepoint analysis (BCPA) that finds the most plausible changepoint by fitting two distributions to the data (Dominoni et al., 2014; Strauß et al., 2022, for details see Supporting Information Part S2, Section 5, Fig. S2_5). We selected an 8-h window around 7:20 CET (i.e., the overall mean onset of activity across the whole dataset) for onsets and around 18:20 CET (i.e., the overall mean offset of activity) for offsets. For the BCPA, we set a 70%-threshold to make sure that enough data were available for a reliable analysis. In 158 occasions (67 onsets and 91 offsets), a BCPA was not possible. The birds were recorded well at the night-time but had many data gaps during their active phase. Therefore, we additionally used the first and last intersection of a 4 dB-threshold on a given day (Adelman et al., 2010) to determine activity onset and offset, respectively, when enough data were available at night-time (i.e., >70% between midnight and sunrise or sunset, respectively). Chronotypes from the BCPA and from the 4 dB-threshold were highly correlated in the cases were both methods could be used (onset: Pearson's cor = .94, confidence interval = (.93, .96), $t = 40.21$, df = 197, $p \ll .001$; offset: Pearson's cor = .87, confidence interval = (.82,.90), $t = 19.69$, df = 127, $p \ll 0.001$). To assess the skin temperature minimum at night, we smoothed the temperature data, averaged to 5-min bins, using a three-harmonic sinusoidal curve (Strauß et al., 2022, for details see Supporting Information Part S2 Fig. S2_4.2 in Section 4), and interpolated for data gaps of maximally three bins (i.e., 15 min). We selected a 12-h window around the observed overall mean time of minimum temperature at 4:10 CET, derived from the data from all birds. From the smoothed data, we then extracted for each bird the time of the minimum temperature just before rewarming for its active phase (adjusted from Strauß et al., 2022, for details see Supporting Information Part S2, Section 5). The time of temperature minimum was interpreted as the onset of the anticipatory increase in body temperature prior to wakening.

2.4 | Data analysis

The birds used in this study differed in their origins (caught in distinct sites and years), and we also had males and females. We combined year and site to create four groups (i.e., Groningen 2021: six females, eight males; Utrecht 2021: four females, 10 males; Heikamp 2021: seven females, eight males; Groningen 2022: 10 females, 14 males; Table S1_1) due to the unbalanced study design. In order to test if this would have an impact on our analyses, we first explored the variation of *tau*, using one *tau* measurement per individual ($n = 63$, using only one *tau* estimate per bird), in response to the covariates *group* and *sex*. We also accounted for cage position in the experimental set-up by including rack as random factor to account for the possibility that the six cages in

the same rack could be more similar to one another than to the other cages in the room. As there were significant differences between *groups* and *sexes*, we included *group* and *sex* in all following models (Fig. S1_11 & Table S1_2).

We then assessed the relationship between *tau*, chronotype measured in the wild and in the lab, and first onset or offset in LL. Models included as response variables chronotypes (i.e., measures of the entrained clock using onset and offset in minutes relative to sunrise or sunset) measured either in the lab (Figure 1(b)) or in the wild (Figure 1(c)), or the first activity onset or offset in LL (Figure 1(a)). *Tau* was used as an explanatory variable in all models, while models with chronotype as response variable also included the first onset or offset in LL as an explanatory variable. Analyses were done in separate linear mixed models with Gaussian error distribution (*lme4* package, Bates et al., 2015). Models included *Individual* as random factor to account for multiple measurements and for studying between-individual differences in chronotype. To assess individual variation, the proportional variance (σ^2) of the *Individual* term was calculated from the model output. All test statistics were obtained via stepwise model reduction using likelihood ratio tests (*drop1* and *anova* function). Estimates were extracted from the model with all non-significant interactions dropped.

For the wild chronotype traits (i.e., activity onset and offset, and time of skin temperature minimum collected in the wild), we excluded the first day after release into the wild to avoid confounding effects from the disrupted night of release. We then chose data from Groningen only (both years), because too few individuals were recorded at the other sites (four in Utrecht, one in Heikamp). As before, we assessed sex- and group-specific relationships with *tau* and onset and offset in LL, and also included Julian day and mean ambient temperature (at night for onset and at daytime for offset) to account for seasonal and temperature-dependent variation (temperature data from the weather station in Elde, (Royal Netherlands Meteorological Institute [KNMI], 2023)). To assess a potential correlation between the times of activity onset and skin temperature minimum, we extracted individual-specific residual variances (best linear unbiased predictor, BLUP) to account for multiple measurements. In order to obtain the BLUPs, we used the same model as from above for both traits, including only days when timing of both, activity and skin temperature minimum, were available ($n = 167$). We then used the BLUPs to check for a correlation between chronotype estimated from activity and skin temperature (Houslay & Wilson, 2017). Because the analysis of BLUP correlation is prone to false positives, multivariate models are preferable, but sample sizes in our study were insufficient for multivariate analyses (Houslay & Wilson, 2017). Thus, using BLUPs, we found no significant relationship and expect therefore that the analysis, here, was not delivering false positives.

For tests involving the first onset or offset in LL or lab chronotypes as responses to *tau*, we included *group*, *sex*, and their two-way interactions with *tau* as covariates. Lab chronotype was only available for Groningen 2022, and its analysis also included the interaction between first onset and offset in LL and *sex*. Then, in separate *post hoc* models, we verified effects of *tau* for males and females.

Finally, as a separate test, we compared if wild chronotype traits were related to lab chronotype using the subset of individuals from 2022 for which both measures were available. In two separate models, we modelled the onset and offset of activity in the wild as response variables. We included as predictors the mean onset or offset in captivity, *sex*, and their interaction, as well as Julian day and mean ambient temperature of night or day, and *Individual* as random effect.

3 | RESULTS

The free-running period lengths *tau* for the 63 individuals in our studies ranged from 23.1 to 24.8 h, and were on average shorter than 24 h (mean $23.72 \pm .04$ h; Fig. S1_11 and Table S1_2). Day-to-day changes in the timing of onset under LL are shown in Figure S1_12. We found that the wild chronotype measurements, in terms of both activity onset and offset, were unrelated to *tau* (slope for onset: −11 min per h, $F_{1,222} = 1.45$, $p = .25$; offset: 0 min per h, $F_{1,177} = .00$, $p = .98$; Fig. S1_2 and Table S1_3). However, individuals differed significantly from each other in wild chronotype (onset: .34 proportional variance (σ^2), $X^2_{1,n\ =\ 223} = 23.30$, $p \ll .001$; offset: $\sigma^2 = .32$, $X^2_{1,n\ =\ 178} = 14.74$, $p < .001$). For the birds of 2022, whose chronotype was also measured in the lab, we found that both activity onset and offset were unrelated to *tau* (slope for onset: −2 min per h, $F_{1,118} = .07$, $p = .80$; offset: 5 min per h, $F_{1,110} = 3.01$, $p = .10$; Figure 2 and Table S1_4), but that these individuals also differed consistently from each other (onset: $\sigma^2 = .33$, $X^2_{1,n\ =\ 119} = 8.52$, $p = .004$; offset: $\sigma^2 = .40$, $X^2_{1,n\ =\ 111} = 19.92$, $p \ll .001$; Table S1_4). In these birds, chronotype measured in the wild could not be explained by chronotype measured in the lab (onset: −1 min per min, $F_{1,142} = .30$, $p = .60$; offset: 0 min per min, $F_{1,124} = .13$, $p = .73$; Table S1_5).

The relationship between the first onset in LL and *tau* depended on *sex* ($F_{1,59} = 4.23$, $p = .04$; Figure 3 and

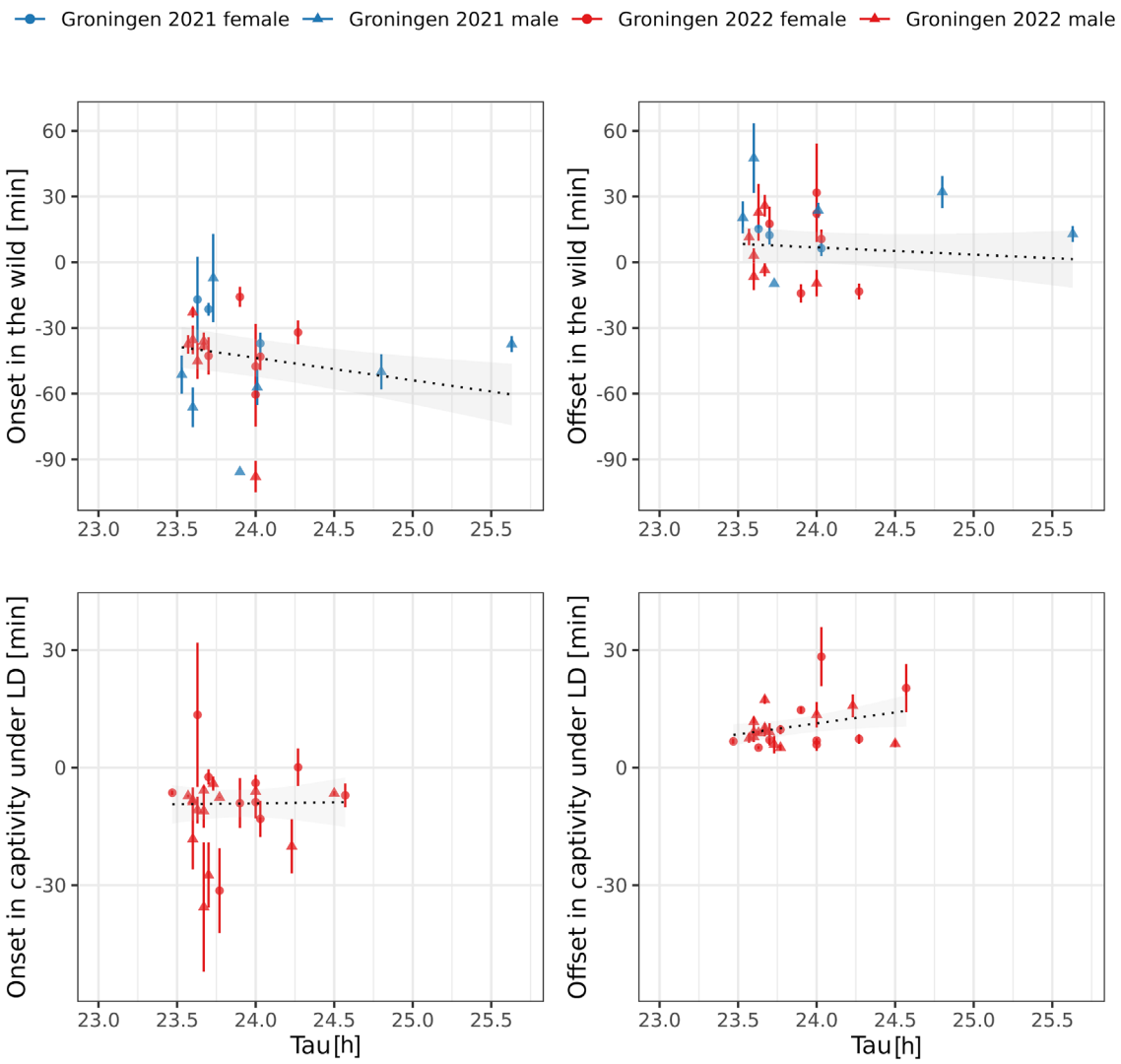

FIGURE 2 Relationships between free-running period length *tau* and activity onset (left) and offset (right) in the wild and in captivity. Top: wild chronotype, i.e., onset or offset in the wild relative to sunrise and sunset after release (only Groningen 2021 and 2022). Bottom: lab chronotype, i.e., onset or offset in captivity under light–dark cycles (LD) relative to lights-on and lights-off, respectively (based on data only collected in Groningen 2022). Raw data are shown as means with standard errors for every individual and model estimates are presented as lines with 95% confidence interval. Colours represent different groups, shapes represent sex (circles in females, triangles in males), and line types show significance level: solid for $p < .05$, dotted for not significant.

Table S1_6). Specifically, in males, the first onset in LL was significantly positively related to *tau*, so that males delayed onset by 45 min per hour of longer *tau* (post hoc: $F_{1,34} = 5.58$, $p = .02$). No significant relationship was detected in females (post hoc: −10 min per h, $F_{1,24} = .27$, $p = .61$; Table S1_7). First offset in LL was also positively, but not significantly, related to *tau* such that offset was delayed with increasing *tau* (slope of 78 min per h, $F_{1,60} = 2.79$, $p = .10$). For the offset, we found no effects of *sex* ($F_{1,60} = 2.96$, $p = .09$) and of its interaction with *tau* ($F_{1,60} = .03$, $p = .85$; Table S1_6). First onset or offset in LL were not significantly related to entrained onset and offset of activity, neither in the wild (onset: 0 min per min, $F_{1,222} = 3.08$, $p = .10$; offset: 0 min per min, $F_{1,177} = .20$, $p = .66$) nor in the lab (onset: 2 min per min, $F_{1,118} = .15$, $p = .71$; offset: −1 min per min, $F_{1,110} = 1.50$, $p = .24$).

For the time of the skin temperature minimum, we also failed to detect significant relationships with predictors. There was no relation with *tau* (slope: −23 min per h, $F_{1,158} = 2.13$, $p = .20$; Fig. S1_13 and Table S1_8) and with first onset in LL (slope: 1 min per min, $F_{1,158} = 4.83$, $p = .10$), nor with lab chronotype in the subset of the 2022 birds (slope: −3 min per min, $F_{1,119} = 0.97$, $p = .37$). Timing of the skin temperature minimum also did not differ between individuals ($\sigma^2 = .02$, $X^2_{1,n=159} = 0.00$, $p = 1.00$; Table S1_8). Further, we could not find a correlation between the timings of

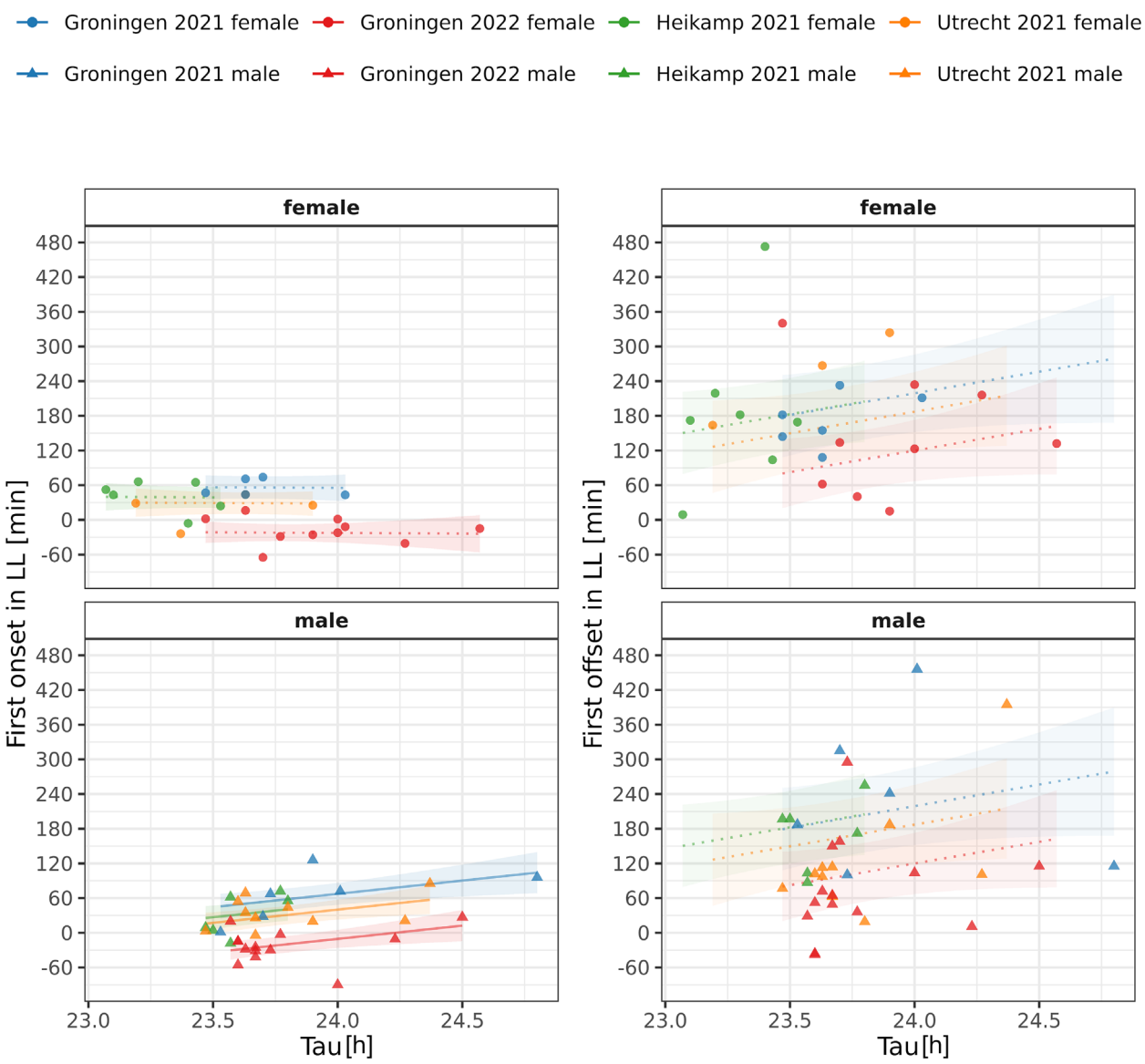

FIGURE 3 Relationships between free-running period length *tau* and the first activity onset (left) and offset (right) in constant dim light (LL) relative to lights-on and lights-off, respectively, on the preceding day in females (top) and males (bottom). Raw data are shown for every individual and model estimates are presented as lines with 95% confidence interval. Colours represent different groups, shapes represent sex (circles in females, triangles in males), and line types show significance level: solid for $p < .05$, dotted for not significant. A significant relationship was only found for first onset in LL in males.

activity in the wild and of skin temperature minima (correlation of BLUPs: Pearson's cor = −0.06, confidence interval = [−.50,.41], $t = -.24$, df = 17, p-value = .82; Fig. S1_13).

4 | DISCUSSION

In our study, we confirmed that while free-living great tits displayed individual chronotypes under entrained conditions, these chronotypes were unrelated to *tau*. The lack of a relationship contrasts with what has been postulated in earlier theoretical and laboratory studies and thus adds to the evidence that predictions made using lab animals may not consistently hold in the wild (Calisi & Bentley, 2009; Daan, 2011; Daan et al., 2011; Tomotani et al., 2012). Earlier studies of the same species also yielded inconsistent results. A large-scale captivity study of hand-raised great tits and follow-up research involving temperature manipulations also reported that *tau* was unrelated to chronotype in the lab in the birds' first autumn of life (Helm & Visser, 2010; Lehmann et al., 2012). Conversely, a recent, smaller-scale study of incubation rhythms revealed that activity onset of wild

female great tits did correlate with *tau* (Tomotani et al., 2023). Such inconsistent findings are perhaps not surprising given the complex interactions between the circadian system and the environment (Helm et al., 2017).

Classical laboratory studies showed systematic relationships between *tau* and phase of entrainment, leading to the formulation of "rules" on theoretical grounds (Floessner & Hut, 2017). Such conclusions were particularly based on testing ranges of entrainment and manipulating the period length of the *Zeitgeber* (Aschoff, 1978). Chronobiologists studying humans also attempted to link chronotype with *tau* (e.g., Allebrandt & Roenneberg, 2008; Brown et al., 2008; Duffy et al., 2001), and in some cases, showed the expected positive correlations between longer *tau* and later chronotype. Intriguingly, Steve Brown and co-authors showed that *tau* also correlated with a molecular measurement for chronotype, the entrained phase of a reporter on a clock gene in cultured dermal fibroblasts (Brown et al., 2008). Positive correlations between longer *tau* and later chronotype have also sometimes been found in wild and wild-derived animals (Fleury et al., 2000; Nikhil et al., 2016; Wicht et al., 2014). However, the evidence has been mixed for birds, including as mentioned above for great tits. While Tomotani et al. (2023), and also Dominoni et al. (2013), showed a relationship between *tau* and chronotype or activity phase in at least some populations, other studies failed to do so (Helm & Visser, 2010; Lehmann et al., 2012). One possible explanation for such discrepancy relates to *tau* as estimated during LL. Despite the fact that the variation in *tau* has a genetic basis (Konopka & Benzer, 1971) and a high heritability (Helm & Visser, 2010), period length is still a labile trait (Pittendrigh & Daan, 1976a). *Tau* has been reported to change seasonally (Aschoff, 1979; Pohl, 1972; Gwinner, 1975; but see Dixit & Singh, 2016) and is affected by changes in light intensity (e.g., Pohl, 1974) and previous entrainment (i.e., after-effects Pittendrigh, 1960), as well as by other aspects such as housing conditions and hormones (Aschoff, 1979).

Despite lacking correlations between *tau* and chronotype, our study provides some support for links between *tau* and phase of entrainment. We found that in males, but not in females, the first onset of activity in LL correlated positively with *tau*. The first onset in LL can be interpreted as approximating the phase angle of entrainment of an individual with a given free-running period length in the absence of masking. This is because on the one hand, the first day(s) after moving an animal from entrained to constant conditions often show after-effects of the previous entrainment on period, phase, and amplitude (Fig. S1_12) that are missing in later stages of a stabilized free-running rhythm (Pittendrigh, 1960). On the other hand, these after-effects take place during exposure to constant conditions, when all external influences on timing are removed and activity can occur at the entrained phase, it would assume without masking. That correlations with *tau* are nonetheless weak is perhaps expected since history-dependent after-effects are a combined reflection of *tau*, of previous entrainment and of other influences on the response of organisms to altered light conditions (Pittendrigh & Daan, 1976a). We thus found a discrepancy between lacking correlations of *tau* with chronotype and some correlation of *tau* with first activity timing in LL. This discrepancy might indicate strong effects of masking in the wild. Chronotype under natural, masking conditions would therefore arise from influences on timing other than of circadian period length.

The conclusions from males are weakened by the lack of an association of *tau* and first onset in LL in females, as well as by non-significant associations of *tau* with first offset in LL. However, sex differences in activity patterns and circadian rhythms have been previously reported (Helm & Visser, 2010; Stuber et al., 2015; Walton et al., 2022). Thus, sex-specific differences in the clock-chronotype link could stem from selection pressures that differentially affect phase and masking responses, as shown for example in fruit flies (Ghosh et al., 2021). Furthermore, although not significant, in both sexes *tau* was positively associated with first offset of activity in LL. The weakness of this association could be due to the large variation in offset derived from our birds. As evident from individual actograms (Figs. S1_3–S1_10), there was a tendency for the evening component of activity to dissociate from the morning component, leading to highly divergent timings of activity offset. Nonetheless, we maintain that after-effects could be interesting for studies with wild animals as they can reveal aspects of entrainment in the wild. Animals under constant conditions in the absence of masking may retain — at least for a few cycles — the same period length and phase of their entrained state (Fig. S1_12). Therefore, after-effects could arguably serve as a closer measure of the clock-predictive ability of the animal than its entrained activity rhythm (Oda & Valentinuzzi, 2023; Tomotani et al., 2012, 2023).

In addition to masking, environmental factors modify the entrained rhythm also in other ways, for example via modulations of clock amplitude and robustness (Daan & Pittendrigh, 1976; Oda & Valentinuzzi, 2023; Pittendrigh & Daan, 1976c, 1976b; Schmal et al., 2015), via modulations of sensory input and output pathways (Chellappa, 2021; Gwinner et al., 1997; Schmal et al., 2020; Shimmura et al., 2017) or via effects of other oscillators (Bartell & Gwinner, 2005;

Gänshirt et al., 1984; Mistlberger, 1994; van der Vinne et al., 2014). The strength of photic entrainment may change due to either environmental changes in exposure to light or to organismic changes in light sensitivity (Marimuthu, 1984; Schmal et al., 2015, 2020). Although chronotype is broadly consistent within individuals as also shown in our study (Schwartz et al., 2017), various environmental variables may modify timing. For example, at higher latitudes, winter has a much shorter light phase (i.e., day length) and lower light intensity than summer. Thus, some models have predicted the strength of the entrainment to be weaker in winter, possibly allowing the variation in chronotypes to be larger (Schmal et al., 2020). Modifying effects can arise from other environmental factors such as light pollution (Sanders et al., 2021), ambient temperature (Lehmann et al., 2012), reproductive stage, season (Daan & Aschoff, 1975; Strauß et al., 2024), social cues (Davidson & Menaker, 2003), and sound (Dominoni et al., 2020). It is noteworthy that the two cited avian studies that showed links between *tau* and chronotype did so for birds experiencing reduced perceived strength of the Zeitgeber. Dominoni et al. (2013) found a relationship only in an urban habitat (Dominoni et al., 2013), where light pollution could result in a reduced contrast between the light and dark phases. This weaker *Zeitgeber* in cities could thus lead to greater variation in chronotypes. Furthermore, Tomotani et al. (2023) had derived chronotype of females during the incubation phase when nest box-breeding females experience greatly reduced exposure to daylight. This reduced Zeitgeber amplitude may contribute to greater expression of inter-individual differences. Some of these environmental factors may have contributed to the inconsistent findings in the case of the great tit.

By which traits chronotype and the circadian clock are measured may also impact the results (e.g., Roenneberg et al., 2003). It is possible that results differ when using other physiological processes (e.g., melatonin levels, e.g., Zawilska et al., 2006, body temperature e.g., Strauß et al., 2022, gene expression or protein levels, e.g., Beer & Bloch, 2020) or other behaviours than the locomotor rhythm (incubation behaviour, e.g., Tomotani et al., 2023). While a relationship should be expected between the different rhythms, there is ample room for variation. Physiological rhythms such as melatonin and (core) body temperature are often considered a more precise way of assessing the phase of entrainment (Roenneberg, 2012; Strauß et al., 2022). In our present study, the timing of the increase in peripheral body temperature during early morning was also unrelated to activity-derived *tau*. The low accuracy of determining phase markers of the measured body temperature rhythm (i.e., timing of the temperature minimum) made it less precise than the activity rhythm, thus further blurring the relationship between clock and chronotype. We cannot exclude that in our birds, other measures of both chronotype or *tau* might have revealed different findings.

How does variation in clock relate to variation in behaviour then? As discussed above, it is perhaps not surprising that theoretical predictions are not consistently met in the natural environment. The clock *versus* behaviour relationship in the wild is more tenuous due to direct influences of the environment on the behaviour itself and to differences between organisms in all of the implicated pathways. *Tau* is only one feature of the circadian rhythms and, although it affects other properties such as the shape of phase response curves (Daan & Pittendrigh, 1976), its influence is subject to many factors that jointly exert phase control. Next to the phase set by the clock relative to the environment, multiple levels of organization ultimately lead to variation of rhythms in nature (Helm et al., 2017).

5 | CONCLUSION

Our study shows that variation in *tau* is not consistently related to chronotype in the great tit. Because the variation in both *tau* and chronotype depends on environmental and internal state conditions, the relationship between clock and chronotype may only appear in certain circumstances, times of the year, or in specific traits. If this is true, literature support of a seemingly straightforward relationship could also be a result of reporting bias. However, evidence for individual differences in chronotype and in diel behaviour, including those reported here, indicates that the suite of components of the circadian system interact with the environment to form broadly consistent temporal behaviour. For wild animals, such consistency matters, especially because of a possible link between chronotype and fitness (Martorell-Barceló et al., 2018; Womack et al., 2023). From an ecological perspective, the important question now is which are the other factors — beside the clock's free-running period length — that explain variation in chronotype and diel timing of behaviour. To solve this challenge, we reaffirm that studying clock features particularly through the combination of measurements in captivity and in the natural settings will be crucial for going forward. A holistic approach, as always embraced by Steven Brown, will benefit chronobiological, ecological, and behavioural research alike.

AUTHOR CONTRIBUTIONS

Barbara M. Tomotani: Conceptualization; data curation; funding acquisition; investigation; project

administration; supervision; visualisation; writing—original draft; writing—review and editing. **Aurelia F. T. Strauß:** Conceptualization; data curation; formal analysis; investigation; methodology; visualisation; writing—original draft; writing—review and editing. **Dmitry Kishkinev:** Methodology; software; writing—review and editing. **Huib van de Haar:** Investigation; writing—review and editing. **Barbara Helm:** Conceptualization; funding acquisition; methodology; investigation; supervision; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16535.

DATA AVAILABILITY STATEMENT

Dataset and code used in this manuscript are available as Figshare repository (https://doi.org/10.6084/m9.figshare.26090455).

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