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RESEARCH ARTICLE

Interplays between pre- and post-natal environments affect early-life mortality, body mass and telomere dynamics in the wild

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ABSTRACT

Early-life conditions are crucial determinants of phenotype and fitness. The effects of pre- and post-natal conditions on fitness prospects have been widely studied but their interactive effects have received less attention. In birds, asynchronous hatching creates challenging developmental conditions for the last-hatched chicks, but differential allocation in last-laid eggs might help to compensate this initial handicap. The relative importance and potential interaction between pre- and post-hatching developmental conditions for different fitness components remains mostly unknown. We manipulated hatching order in wild pied flycatchers (*Ficedula hypoleuca*), creating three groups: natural asynchrony (last-laid eggs hatching last), reversed asynchrony (last-laid eggs hatching first) and hatching synchrony (all eggs hatching at once). We examined the effects of these manipulations on early-life survival, growth and telomere length, a potential cellular biomarker of fitness prospects. Mortality was mostly affected by hatching order, with last-hatched chicks being more likely to die. Early-life telomere dynamics and growth were influenced by the interplays between laying and hatching order. Last-laid but first-hatched chicks were heavier but had shorter telomeres 5 days after hatching than their siblings, indicating rapid early growth with potential adverse consequences on telomere length. Synchronous chicks did not suffer any apparent cost of hatching synchronously. Impaired phenotypes only occurred when reversing the natural hatching order (i.e. developmental mismatch), suggesting that maternal investment in last-laid eggs might indeed counterbalance the initial handicap of last-hatched chicks. Our experimental study thus highlights that potential interplays between pre- and post-natal environments are likely to shape fitness prospects in the wild.

KEY WORDS: Ageing, Bird, Developmental mismatch, Fitness, Hatching asynchrony, Maternal allocation

INTRODUCTION

Early-life conditions (i.e. conditions experienced during development) can have long-lasting effects on behavior (Weinstock, 2008), physiology (Sheriff et al., 2010) and fitness (Lindström, 1999). In particular, a poor start in early life has been associated with

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negative effects on survival and reproduction later on (Metcalfe and Monaghan, 2001). Developmental conditions both before (Groothuis et al., 2005; Sheriff et al., 2010) and after (Merkling et al., 2014; Trillmich and Wolf, 2008) birth seem to be key determinants of later-life phenotype and performance.

In birds, variation in pre- and post-natal developmental conditions can even arise within a brood, for instance through differential resource allocation to eggs (Groothuis et al., 2005) or unequal parental care allocation (Mainwaring et al., 2011). Female birds deposit in the eggs variable amounts of nutrients (Ramírez et al., 2015), hormones (Gil, 2008), antioxidants (Török et al., 2007) and immunoglobulins (Hargitai et al., 2006), which can create phenotypic variation within a brood (Groothuis et al., 2005; Laaksonen, 2004). Concentrations of these resources often decrease or increase according to the laying order, resulting in different physiological environments between embryos developing in first-laid compared with last-laid eggs (Mentesana et al., 2018). Another common source of variation in developmental conditions within a brood exists if females start incubating before the last egg(s) are laid, resulting in chicks from the last-laid egg(s) to hatch later than others, a widespread phenomenon known as hatching asynchrony (Magrath, 1990). Many hypotheses exist to explain the evolution of hatching asynchrony (Glassey and Forbes, 2002; Laaksonen, 2004; Magrath, 1990). While there may be many factors, such as predation risk or energetic efficiency selecting for synchrony or asynchrony, it is clear that asynchronous hatching leads to a competitive hierarchy within the brood during post-natal development, putting last-hatched chicks in an inferior competitive position compared with their first-hatched siblings (Magrath, 1990). This position can lead to reduced food intake, slower growth rate, lowered body mass at fledging and higher early-life mortality (Hildebrandt and Schaub, 2018; Kilgas et al., 2010; Malacarne et al., 1994).

Pre- and post-natal environments are also known to interact in shaping an individual's phenotype, and potential developmental mismatches between pre- and post-natal conditions are likely to impair subsequent health and fitness (Gluckman et al., 2019). In the case of hatching asynchrony, some evidence suggests that higher allocation of maternal androgen hormones to last-laid eggs could help last-hatched chicks to catch-up with their older siblings, at least if environmental conditions are favorable (Müller and Groothuis, 2013; Stier et al., 2015). Yet, much remains to be done to understand the respective contribution of pre- and post-natal conditions or their potential interactions in shaping fitness prospects in the wild. In the case of hatching asynchrony, it is often impossible to distinguish between the effects of laying order (via egg components) and hatching order because they are intrinsically linked in the natural scenario. Moreover, measuring long-term fitness consequences in the wild is often difficult or even impossible. Fitness consequences could, however, be predicted using indirect proxies, such as body size or body mass at fledging (Starck and Ricklefs, 1998), or as

more recently suggested, using telomere length (Wilbourn et al., 2018).

Telomeres are repetitive non-coding sequences of DNA located at the ends of chromosomes that maintain genomic integrity and stability (De Lange et al., 2006). Telomeres usually shorten with age, and this shortening is accelerated by environmental stressors, such as competition or poor diet (Chatelain et al., 2020). Short telomeres are associated with cellular senescence, and telomeres are considered to be one key hallmark of ageing (López-Otín et al., 2013). Telomere length is known to predict future survival prospects in the wild (Wilbourn et al., 2018), and recent evidence suggests that telomere length could be used as a fitness proxy in wild birds (Angelier et al., 2019; Eastwood et al., 2019). Most telomere shortening happens in early life when growth occurs and cell proliferation is high (e.g. Stier et al., 2020), thus making it a critical period in determining long-term performance and ageing. Alterations of both pre- and post-natal developmental conditions have been shown to shorten telomeres (Monaghan and Ozanne, 2018; Stier et al., 2020).

Consequently, our aim was to investigate the potential interplays between pre- and post-natal developmental conditions in determining chick phenotype and fitness prospects by using the natural opportunity provided by hatching asynchrony in birds. To this end, we conducted an experimental manipulation of hatching order and measured early-life survival, growth rate and telomere dynamics in nestling pied flycatchers [*Ficedula hypoleuca* (Pallas 1764)]. Pied flycatchers are passerines with a laying frequency of one egg per day (Lundberg and Alatalo, 1992), known laying order effect on egg androgen content (Morosinotto et al., 2016) and frequent hatching asynchrony (Slagsvold, 1986). We manipulated hatching order by creating three types of brood (Fig. 1): (1) Natural hatching asynchrony (last-laid eggs hatching last), (2) reversed hatching asynchrony (last-laid eggs hatching first), and (3) hatching synchrony (all eggs hatching in one day). We predicted that: (1) chicks from synchronous nests would exhibit reduced early-life survival, slower growth and a potential acceleration in telomere shortening compared with first-hatched chicks in the asynchrony groups owing to balanced competition among siblings; (2) last-hatched chicks in the natural asynchrony group would partly to fully compensate their initial handicap owing to a potential pre-natal programming by higher testosterone content in last-laid eggs, but could suffer from delayed costs revealed by shorter telomeres (Stier et al., 2015); (3) last-hatched chicks (from the first-laid eggs) in the reversed asynchrony group would suffer enhanced costs in terms of mortality, growth rate and telomere shortening linked to their pre-natal versus post-natal developmental mismatch that could aggravate their competitive disadvantage, possibly resulting in reduced food intake and increased developmental stress; (4) first-hatched (last-laid) chicks in this reversed asynchrony group would benefit from both the competitive advantage of developing in last-laid eggs (i.e. increased exposure to maternal androgens) and of hatching first, thereby enhancing early-life survival and growth rate. Yet, their fast growth could come at a cost in terms of telomere loss (Monaghan and Ozanne, 2018).

MATERIALS AND METHODS
Field experiment
The study was conducted in 2018 on the Island of Ruissalo, Turku, Finland (ca. 60°25′60N, 22°10′0E) in a nest-box population of pied flycatchers that has been monitored since 2004. The pied flycatchers in this population are long-distance migrants that winter in western Africa south of Sahara (Ouwehand et al., 2016) and arrive at the breeding grounds in May (Velmala et al., 2015). After nest construction, females lay one egg per day until the final clutch size of typically six or seven eggs in this population (mean=6.58 eggs in 884 clutches). Around 80% of the females start spending nights in the nest when the fifth egg is laid, but full incubation usually starts around the time the last egg is laid (Lundberg and Alatalo, 1992). The incubation period lasts approximately 14 days and hatching spread between the first and the last chick is typically 0.5–1.5 days (Lundberg and Alatalo, 1992).

There were 290 nest boxes (inner diameter 12.5×12.5×height 25 cm) available for the flycatchers in the area used for this study. The nest-boxes were monitored twice a week from the beginning of May until mid-June to identify new pied flycatcher nests (42 nests identified, of which 2 were deserted before the start of incubation). Flycatcher nests under construction were thereafter checked every other day until the construction was nearly finished. As pied flycatchers lay one egg per day, the nests were subsequently checked every day to determine the exact laying date of the first egg. The nests were visited every day between 10:00 and 12:00 h and every new egg was marked with a consecutive number written using a permanent marker. When there were three eggs in the nest, the eggs were transferred into a closed wooden holding box (13×13×5 cm fitted with a fake nest) attached underneath the original nest box and replaced with dummy eggs, a similar protocol as used by Ouwehand et al. (2017). The nests were visited every day in the following days, to replace the newly laid egg with a dummy egg. The temporary removal of the eggs from the nest was done to experimentally control for the start of the incubation of the actual eggs and to create our different experimental groups as described below and in Fig. 1.

After two consecutive days without a new egg (at which point all the females had started to incubate), the dummy eggs were swapped with the real eggs according to the experimental design described in Fig. 1. In the first group (natural asynchrony), the two last-laid eggs (irrespective of the final clutch size) were left in the holding box while all the other eggs were put back in the nest. The last two laid eggs were put back into the nest the next day. In the second group (reversed asynchrony), third to last-laid eggs were returned to the nest on the first day and the first and second laid ones the next day. In both asynchrony groups, two rather than one egg were returned to the nests the next day, to ensure the hatching of at least one chick. In the third group (hatching synchrony), all the eggs were put back to the nest on the first day. The synchronous nests were visited also the next day, to standardize human disturbance. When putting eggs back to the nest, the same number of dummy eggs were removed.

After 13 days from placing the first (or all) eggs in the nest, the nests were checked daily to determine the hatching date [day when the first chick(s) had hatched=day 0]. When the first nestlings had hatched, they were marked by gently removing the feather tufts on their backs. If there were unhatched eggs in the nest, the nest was visited on the following days. If all the eggs had hatched within one day in the case of the asynchronous groups (three nests), the last-hatched nestlings could easily be identified by body size and state of the feather tufts (i.e. wetness). In the case of synchronous broods, we could not determine each chick's rank in the laying sequence as all the chicks hatched at the same time. All the chicks were ringed on their individual day 5. All the nests were visited 17 days after hatching to determine the fledging success by counting the dead chicks in the nest. The general experimental design and sample size at each stage are illustrated in Fig. 1.

In order to evaluate the natural relevance of our experimental design in our study population, we measured incubation behavior in
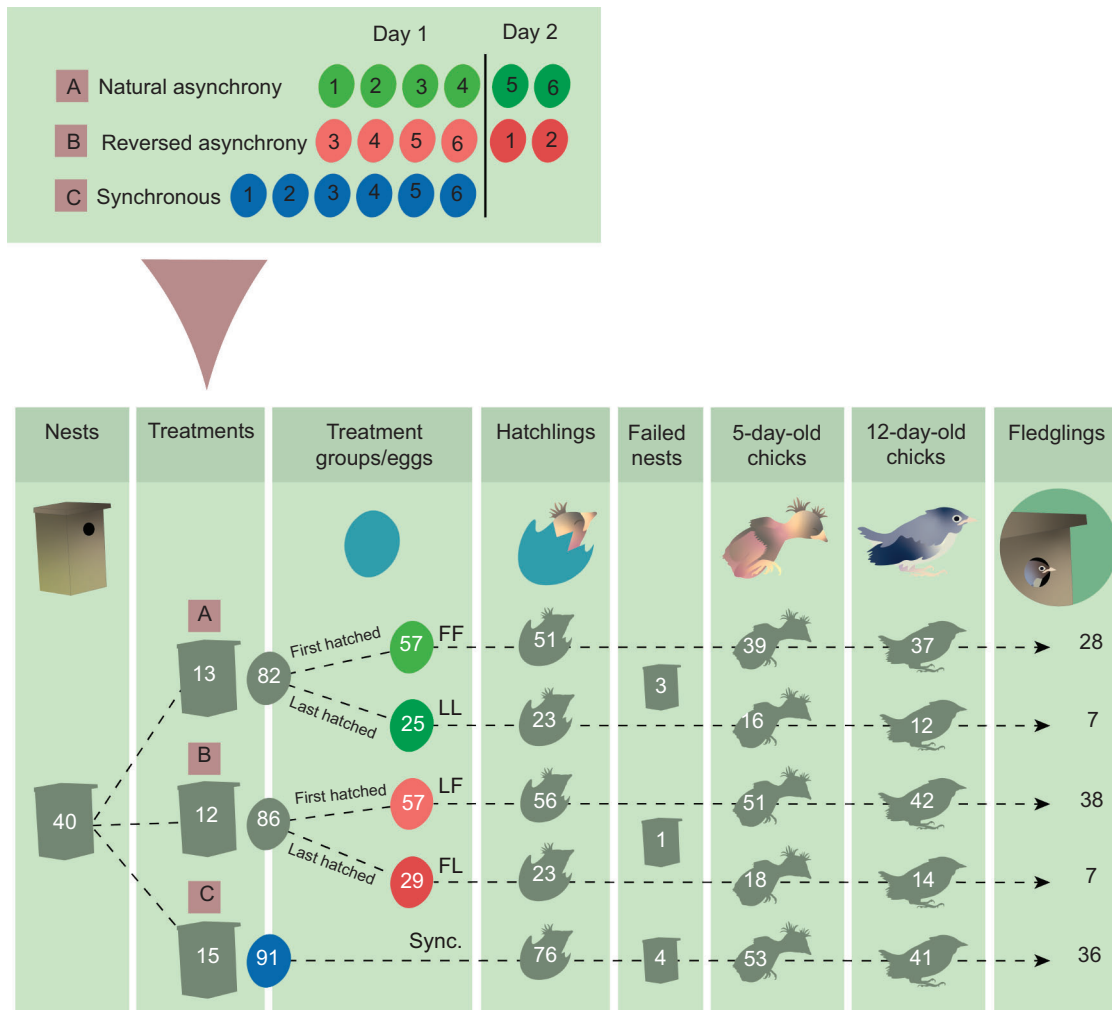
Fig. 1. Schematic representation of the experimental set-up and the sample sizes for nests, eggs and nestlings at different stages with the three experimental nest groups and the five experimental eggs/chick groups and their respective schedule for returning the eggs back to their nest. Day 1 refers to the second consecutive day during egg-laying without any new eggs, and Day 2 refers to the following day. The number inside the egg represents the position in the laying order. FF, 'first to first', chicks hatching first from first-laid eggs; LL, 'last to last', chicks hatching last from last-laid eggs; FL, 'first to last', chicks hatching last from first-laid eggs; LF, 'last to first', chicks hatching first from last-laid eggs; Sync., all chicks hatching at the same time.

a subset of the nests (N=23) with temperature loggers (iButton Thermochron, iButtonLink, Whitewater, WI, USA). Temperature loggers were placed in the nest after the third egg was laid and removed after hatching (measuring at 5 min intervals with 0.0625°C accuracy) to estimate the occurrence of hatching synchrony and asynchrony. We confirmed that both hatching synchrony and asynchrony are likely to occur in our population since 57% of the females started continuous incubation at the time of laying the penultimate egg (i.e. second last egg, expected to result in asynchronous hatching), while 43% started continuous incubation after laying the penultimate egg (i.e. expected to result in synchronous hatching).

Blood sampling

When the first-hatched nestlings were 5 and 12 days old, they were weighed to the nearest 0.01 g and blood sampled. At these times, the last-hatched nestlings were 4 and 11 days old and were not handled but were kept together with the other chicks the whole time. The next day (days 5 and 12 of the last-hatched chicks) the nests were visited again to measure and sample the last-hatched chicks at the same age as the first-hatched ones. In the synchronous group, all the

measurements and blood samples were taken at days 5 and 12 (unless nestlings were too small to be ringed, in which case they were ringed and measured on day 6; N=6 nestlings). Blood samples (~35 µl) were taken from the wing vein with non-heparinized capillary tubes and diluted in 75 µl of phosphate buffered saline (PBS, Medicago AB, Uppsala, Sweden). All the blood samples were stored in a cold bag while in the field and transferred to −80°C at the end of the day.

Laboratory work

DNA was extracted from whole blood samples using salt extraction alcohol precipitation method (Aljanabi and Martinez, 1997) within 3 months of sample collection. DNA concentration and quality were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was then diluted to a concentration of 2.5 ng µl^{-1} for subsequent qPCR analysis. DNA integrity was checked using gel electrophoresis [50 ng DNA, 0.8% agarose gel at 100 mV for 60 min, Midori^{Green} staining (NIPPON Genetics Europe, Düren, Germany)] on randomly selected samples and was deemed satisfactory (Kärkkäinen et al., 2020).

Quantitative PCR method (i.e. qPCR) was used to assess the relative telomere lengths, as previously described and validated in this species (Kärkkäinen et al., 2020, 2019). The qPCR analyses were performed on a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using 384-well qPCR plates. The final reaction volume was 10 µl, consisting of 5 ng genomic DNA, 200 nmol l^{-1} forward and reverse primers and SensiFAST SYBR Lo-ROX mix (Bioline, London, UK) as MasterMix. Tel 1b was used as a forward telomere primer (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel 2b as a reverse telomere primer (5′-GGCTTGCCTTACCC-TTACCCTTACCCTTACCCTTACCCT-3′) and RAG1 (verified as single copy using a BLAST analysis on the collared flycatcher *Ficedula albicollis* genome) was used as a single copy gene (forward primer 5′-GCAGATGAACTGGAGGCTATAA-3′ and reverse primer 5′-CAGCTGAGAAACGTGTTGATTC-3′). Telomere and RAG1 reactions were performed in triplicates on the same plates. Multiple samples from the same individual and samples from the same nest were always analysed on the same plate while nests in each treatment were distributed evenly across all plates (N=6). All plates contained a negative control and three internal standards. The qPCR conditions were: an initial denaturation (1 cycle of 3 min at 95°C), 40 cycles with first step of 10 s at 95°C, second step of 15 s at 58°C and third step of 10 s at 72°C with melting curve analysis at the end.

LinRegPCR v.2017.1 (obtained from https://www.medischebiologie.nl/files) (Ruijter et al., 2009) was used to determine the baseline fluorescence, the qPCR efficiencies of each reaction (mean±s.d. efficiencies were 2.009±0.029 for telomere and 1.958±0.021 for RAG1) and the quantification cycle (Cq) values. Telomere lengths were calculated based on plate-specific efficiencies using the mathematical model presented in Pfaffl et al. (2001). Technical repeatability of triplicate telomere lengths was 0.848 (95% Cl [0.82, 0.871], P<0.001) and the inter-plate technical repeatability based on control samples and one repeated plate was 0.882 (95% Cl [0.806, 0.928], P<0.001).

Statistical analyses

Generalized linear mixed models were used to examine the effects of laying and hatching order on hatching success (i.e. probability of hatching), mortality (i.e. probability of dying), body mass, growth rate (i.e. change in body mass from day 5 to day 12 measurement) and telomere dynamics of the nestlings. To do this, both the natural and the reversed asynchrony groups were first divided into two subgroups: the first-hatched and last-hatched chicks (Fig. 1). Therefore, there were five levels in the treatment variables: (1) 'first to first' group (FF) contains the chicks that were both laid and hatched first; (2) 'last to last' (LL), the chicks that were both laid and hatched last; (3) 'last to first' (LF), chicks that were laid last but hatched first; (4) 'first to last' (FL), the chicks that were laid first but hatched last; and (5) 'synchronous' (sync.), the chicks that hatched at the same time (Fig. 1). We had measurements from 32 out of 40 nests as 8 nests failed before any measurements from the chicks were taken (Fig. 1).

Possible differences in mortality during the nestling phase between the treatment groups were examined by fitting two mixed logistic regression models with binary distribution (alive or dead) and logit link function (N=228 nestlings, Fig. 1). We tested separately whether the treatment affected the probability of dying before nestling day 5 or before fledging (i.e. including all the chicks found dead during nestling phase and post-fledging check). The potential effect of individual hatching date on mortality was tested but excluded from the final models since it was non-significant. Random intercept for nest identity (brood) was included in all the

models. Mortality analyses did not include unhatched eggs but did include failed nests. Hatching success was tested separately with a similar model as for mortality (N=259 eggs, Fig. 1) to see if the experimental design affected the hatching probability. As in mortality models, we tested the potential effect of individual hatching date (predicted hatching date in the case of unhatched eggs) on hatching success. Treatment group did not affect the hatching probability significantly ($F_{4,116.7}$=1.91, P=0.11), while there was a trend for lower hatching success of the eggs that were to be hatched later in the season (estimate±s.e.=−0.06±0.03, $F_{1,37.49}$=3.11, P=0.09). Hatching probabilities of the groups ranged from 79 to 99% (FL: mean±s.e.=79±0.09%; sync.: 86±0.04%; FF: 90±0.5%; LL: 93±0.05%; and LF: 99±0.02%).

The effects of hatching order manipulation on chick body mass and telomere length at days 5 (N=154 nestlings) and 12 (N=134 nestlings), as well as in the changes in body mass (i.e. growth rate) and telomere length (i.e. telomere dynamics) between days 5 and 12 (N=129 nestlings) were analysed separately by fitting linear mixed models with normal distribution and identity link function. The change variables were calculated by subtracting the first measurement value from the second. Telomere change values were corrected using the equations from Verhulst et al. (2013) to avoid statistical artefacts due to the regression to the mean phenomenon. Treatment group and individual hatching date were first included as an explanatory factor in all analyses. However, hatching date was excluded from the telomere models and mass change-model to reduce parameters, as the effects were not significant and sample sizes relatively limited. All the models included nest ID as a random term. Although qPCR plate identity was initially included as a random term in initial telomere models, it was removed from the final models since it explained virtually no variance and did not affect the results.

The models were estimated using restricted maximum-likelihood (REML) and Kenward–Roger method was used to calculate degrees of freedom of fixed factors and assess parameter estimates and their standard errors. Least square means and Tukey–Kramer adjustment for multiple comparisons were used to evaluate the specific differences between treatment groups. Tukey–Kramer test is designed for unbalanced data and it compares the means of two treatment groups in each pairwise comparison and detects any difference that is greater than the expected standard error and is fairly conservative against type I errors (Ramsey and Ramsey, 2008). Normality and heteroscedasticity assumptions were checked visually from the model residuals and deemed satisfactory. Statistical analyses were conducted with SAS statistical software v.9.4 (SAS Institute, Cary, NC, USA).

RESULTS
Nestling mortality

Overall nestling mortality (of the hatched chicks) during the 2018 breeding season was 49% (46% died before day 5 and the remaining 54% from day 5 to fledging). Early life mortality (before nestling day 5) did not statistically differ between treatment groups ($F_{4,100.5}$=1.50, P=0.20). However, overall mortality (before fledging) was influenced by our hatching order manipulation ($F_{4,82.81}$=4.53, P=0.002, Fig. 2). The general pattern of mortality did not differ significantly between Natural and Reversed asynchrony groups (Fig. 2). Indeed, last-hatched chicks from first-laid eggs (FL) were more likely to die than their first-hatched chicks from last-laid eggs (LF) siblings (Fig. 2, Table 1), and similarly last-hatched-chicks from last-laid eggs (LL) were more likely to die than their first-hatched chicks from first-laid eggs (FF) siblings, although
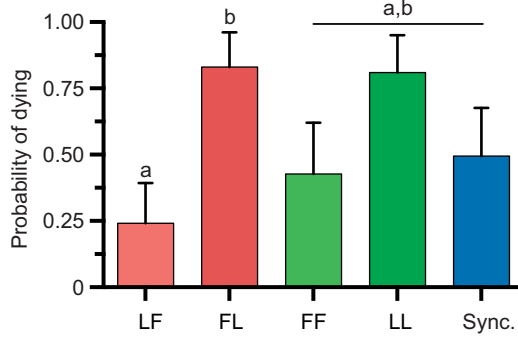
Fig. 2. Probability of dying before fledging in relation to hatching order manipulation in nestling pied flycatchers. Statistically significant differences after Tukey-Kramer adjustment for multiple comparisons are indicated with different letters (see also Table 1). Values are estimated marginal means±s.e.m. See Fig. 1 for explanation of groups and numbers in each group.

the latter result did not remain significant after *P*-value adjustment for multiple comparisons (Table 1). Chicks from synchronous nests had an intermediate probability of dying, as they were not significantly different from any other group (Fig. 2, Table 1).

Body mass and growth
Our experimental manipulation of hatching order had an overall effect on chick body mass at day 5 (Table 2A, Fig. 3A). Specifically, chicks that hatched first from the last-laid eggs (LF) were the heaviest, although the difference was statistically significant only to their siblings (FL, i.e. chicks hatching last from first-laid eggs; Fig. 3A). At day 12, the effect of hatching order manipulation was even more pronounced (Table 2A, Fig. 3A). Last-hatched chicks from first-laid eggs (FL) were the smallest at day 12, and the difference was significant to their first-hatched siblings (LF), synchronous chicks, and first-hatched chicks in Natural asynchrony group (FF, although the latter only before *P*-value adjustment, Table 3A, Fig. 3A). Synchronous chicks were the heaviest at day 12, the difference being significant with the LL group (although only before *P*-value adjustment) in addition to aforementioned FL group (Table 3A, Fig. 3A). Chicks in both natural asynchrony groups (FF and LL) did not statistically differ in their body mass either at day 5 or 12 (Table 3A, Fig. 3A).

Table 1. Results of pairwise comparisons estimating the differences in marginal means (±s.e.) between treatment groups and obtained from mixed logistic regression model investigating the effects of treatment on nestling mortality

Pairwise comparison	Nestling mortality			
	Estimate±s.e.	t_{df}	P	P_{adj}
LF×FL	−2.73±0.77	$−3.58_{223}$	**<0.001**	**0.005***
LF×FF	−0.85±1.15	$−0.74_{34.29}$	0.462	0.946
LF×LL	−2.59±1.24	$−2.10_{45.4}$	**0.042**	0.232
LF×sync.	−1.13±1.11	$−1.02_{33.77}$	0.317	0.847
FL×FF	1.88±1.22	$1.54_{43.07}$	0.130	0.538
FL×LL	0.14±1.30	$0.11_{55.27}$	0.914	1.000
FL×sync.	1.61±1.18	$1.36_{43.12}$	0.179	0.652
FF×LL	−1.74±0.75	$−2.31_{223}$	**0.022**	0.151
FF×sync.	−0.27±1.08	$−0.25_{33.09}$	0.803	0.999
LL×sync.	1.47±1.17	$1.25_{45.43}$	0.216	0.720

Statistical significances both before and after Tukey–Kramer adjustment for multiple comparisons are reported. Statistically significant differences (*P*<0.05) are indicated with bold letters.

Table 2. Summary of linear mixed models investigating the effect of hatching order manipulation (i.e. treatment) on body mass, growth, telomere length and early-life telomere dynamics

A

Independent variable	Body mass day 5				Body mass day 12				Growth (Δ mass day 12–day 5)			
	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P
Fixed effects												
Intercept	8.63±0.48	27.84			14.48±0.60	22.69			5.36±0.40	24.29		
Treatment	−0.09±0.03	4,64.58	3.36	0.015	−0.10±0.03	4,52.99	9.58	<0.0001		4,55.26	6.99	0.0001
Hatching date		1,28.43	11.71	0.002		1,23.15	11.94	0.0021				
Random effect												
Nest box	1.07±0.30	1	106.81	<0.0001	1.21±0.43	1	45.69	<0.0001	1.24±0.42	1	60.94	<0.0001
Residual	0.33±0.04			0.98±0.14				0.73±0.10				

B

Independent variable	Telomere length day 5				Telomere length day 12				Early-life telomere dynamics (Δ telomere length day 12–day 15)			
	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P	Estimate±s.e.	d.f.$_{num,dem}$	F/χ^{2*}	P
Fixed effects												
Intercept	3.66±0.17	35.56			3.38±0.22	24.73			−0.40±0.16	34.44		
Treatment		4,67.43	2.81	0.03		4,54.59	1.16	0.34		4,58.25	3.80	0.008
Random effect												
Nest box	0.11±0.07	1	4.24	0.04	0.32±0.13	1	24.91	<0.0001	0.05±0.05	1	1.55	0.22
Residual	0.81±0.10				0.49±0.07				0.66±0.09			

*F-tests were were used for significance tests of fixed effects, likelihood ratio tests (χ²) with mixture distributions and one-sided P-values were used for random effects.
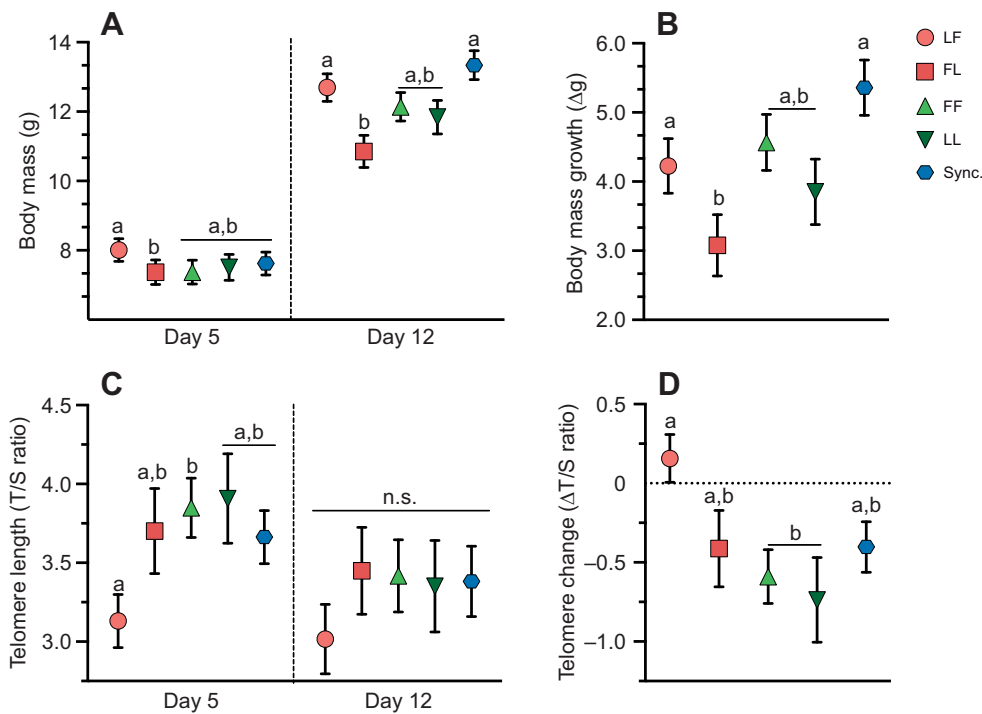
Fig. 3. Effects of hatching order manipulation on body mass, growth, relative telomere length and early-life telomere dynamics in nestling pied flycatchers. (A) Body mass at days 5 and 12. (B) Growth (∆ mass between days 5 and 12). (C) Relative telomere length at days 5 and 12. (D) Early-life telomere dynamics (∆ telomere length between days 5 and 12). To obtain more intuitive estimates for the corrected telomere change values, the difference of the averages of first and second measurement was added to each telomere change value, as recommended by Verhulst et al. (2013). Statistically significant differences after Tukey–Kramer adjustment for multiple comparisons are indicated with different letters (see also Table 3). Values are estimated marginal means±s.e.m. See Fig. 1 for explanation of groups and numbers in each group.

Hatching order manipulation also significantly influenced body mass change between days 5 and 12 (i.e. growth rate; Table 2A, Fig. 3B). Specifically, growth rate differed significantly within the reversed asynchrony group, with first-hatched chicks from last-laid eggs (LF) gaining more mass than their last-hatched siblings from first-laid eggs (FL, Fig. 3B), while such difference did not remain significant after *P*-value adjustment in the case of the natural asynchrony group (Table 3A, Fig. 3B). Finally, synchronous chicks had the highest growth rate, although only significantly higher than LL chicks (before *P*-value adjustment) and FL chicks (Table 3A, Fig. 3B).

Telomere length and dynamics
Telomere length at day 5 was also significantly influenced by our hatching order manipulation (Table 2B, Fig. 3C). Last-laid but first-hatched chicks (LF) had shorter telomeres at day 5 than all the other groups (Fig. 3C), although this difference remained statistically significant after *P*-value adjustment only to the first-laid/first-hatched (FF) chicks (Table 3B, Fig. 3C). Hatching order manipulation did not, however, significantly influence telomere length at day 12 (Table 2B, Fig. 3C). This was because the early-life telomere dynamics was significantly influenced by hatching order manipulation (Table 2B), with chicks from the last-laid but first-hatched chicks (LF) showing less telomere shortening between days 5 and 12 than chicks from all the other groups (Table 3B, Fig. 3D).

DISCUSSION
Our experimental manipulation of hatching order in a wild bird model revealed that different fitness components or proxies are influenced by the interplays between pre- and post-natal environmental conditions. Specifically, we showed that hatching order influences nestling mortality more than laying order, but that both growth and telomere dynamics are impacted by the combined effects of laying and hatching order. Chicks from natural asynchrony nests did not differ in telomere dynamics or final body mass. However, chicks that hatched first from the last-laid eggs were heavier and had shorter

telomeres at day 5 than their first-laid but last-hatched siblings. They also exhibited faster post-natal growth, but no telomere shortening subsequently (day 5 to day 12). Chicks from synchronous nests did not seem to have paid any cost of synchronous hatching. Indeed, they exhibited intermediate early-life survival probability, high body mass at day 12, fast growth, and unaltered telomere length/dynamics compared with naturally asynchronous chicks.

Hatching order as the main determinant of nestling survival
We did not observe differences in early-life survival (from hatching to day 5) between treatment groups, but nestling mortality before fledging was the highest in last-hatched chicks in both asynchrony groups, indicating that the position in the laying order or a developmental mismatch between pre- and post-natal conditions had no significant impact on the survival to fledging. This is in accordance with another recent hatching order manipulation (Braasch and Becker, 2019) and supports the adaptive brood reduction hypothesis, suggesting that last-hatched chicks would quickly starve to death owing to their competitive disadvantage under constraining environmental conditions (Lack, 1954; Magrath, 1990). Competitive advantage of the first-hatched nestlings have been demonstrated in previous studies (e.g. Malacarne et al., 1994), and also to some extent in the pied flycatcher (Gottlander, 1987). Additionally, the adaptive brood reduction can be induced by parental food distribution. Other passerine bird studies showed that the parents feed the largest, first-hatched nestlings more than the smallest, last-hatched ones even when there were no differences in the begging behavior between the chicks (Cotton et al., 1999), or when the smallest nestlings begged more intensely (Smiseth et al., 2003). Indeed, the last-hatched chicks in this study did gain body mass slower than other chicks between days 5 and 12. While the breeding season of 2018 (from 15 May, first-laid egg, until 8 July, the last fledged chick) did not stand out in terms of temperature from the previous or the following breeding seasons, it was notably drier than breeding seasons in 2017 or 2019 (average daily temperature/rainfall: 13.6°C/1.37 mm in 2017, 16.6°C/0.75 mm in 2018, and

Table 3. Results of pairwise comparisons estimating the differences in marginal means (±s.e.) between treatment groups and obtained from linear mixed models investigating the effects of treatment on body mass, growth, telomere length and early-life telomere dynamics.

A

Pairwise comparison	Body mass at day 5				Body mass at day 12				Growth (Δ mass day 12–day 5)			
	Estimate±s.e.	t_{df}	P	P_{adj}	Estimate±s.e.	t_{df}	P	P_{adj}	Estimate±s.e.	t_{df}	P	P_{adj}
LF×FL	0.64±0.18	$3.47_{122.9}$	**0.001**	**0.008***	1.84±0.32	$5.70_{106.2}$	**<0.001**	**<0.001***	1.15±0.28	$4.12_{100.6}$	**<0.001**	**0.001***
LF×FF	0.64±0.47	$1.35_{27.73}$	0.187	0.660	0.55±0.57	$0.97_{23.18}$	0.341	0.867	−0.34±0.57	$-0.60_{24.3}$	0.554	0.974
LF×LL	0.50±0.49	$1.01_{33.14}$	0.319	0.849	0.85±0.63	$1.37_{32.5}$	0.182	0.652	0.37±0.62	$0.61_{33.12}$	0.549	0.974
LF×sync.	0.39±0.46	$0.84_{27.25}$	0.407	0.917	−0.64±0.57	$-1.12_{22.45}$	0.275	0.796	−1.13±0.56	$-2.01_{23.73}$	*0.056*	0.275
FL×FF	0.00±0.49	$0.00_{32.77}$	0.999	1.000	−1.29±0.62	$-2.08_{31.35}$	**0.045**	0.242	−1.49±0.60	$-2.47_{30.72}$	**0.019**	0.112
FL×LL	−0.14±0.51	$-0.27_{38.55}$	0.790	0.999	−0.99±0.67	$-1.48_{41.45}$	0.147	0.580	−0.77±0.65	-1.19_{40}	0.240	0.756
FL×sync.	−0.25±0.48	$-0.52_{32.57}$	0.607	0.985	−2.49±0.62	$-4.02_{30.66}$	**<0.001**	**0.002***	−2.28±0.60	$-3.81_{30.11}$	**<0.001**	**0.003***
FF×LL	−0.14±0.20	$-0.70_{123.2}$	0.488	0.957	0.30±0.35	$0.85_{107.8}$	0.397	0.914	0.71±0.32	$2.24_{103.5}$	**0.028**	0.182
FF×sync.	−0.25±0.47	$-0.53_{27.79}$	0.603	0.984	−1.20±0.58	$-2.05_{23.76}$	*0.051*	0.255	−0.79±0.57	$-1.39_{24.86}$	0.177	0.637
LL×sync.	−0.11±0.50	$-0.23_{33.39}$	0.823	0.999	−1.50±0.64	$-2.35_{33.74}$	**0.025**	0.146	−1.51±0.62	$-2.43_{33.72}$	**0.021**	0.123

B

Independent variable	Telomere length at day 5				Telomere length at day 12				Early-life telomere dynamics (Δ telomere length day 12–day 15)			
	Estimate±s.e.	t_{df}	P	P_{adj}	Estimate±s.e.	t_{df}	P	P_{adj}	Estimate±s.e.	t_{df}	P	P_{adj}
LF×FL	−0.57±0.28	$-2.03_{135.2}$	**0.045**	0.264	−0.43±0.23	$0.23_{106.7}$	*0.061*	0.334	0.57±0.26	$2.18_{110.3}$	**0.032**	0.204
LF×FF	−0.72±0.25	$-2.84_{39.31}$	**0.007**	**0.046***	−0.40±0.32	$0.32_{25.3}$	0.218	0.714	0.75±0.23	$3.29_{36.06}$	**0.002**	**0.014***
LF×LL	−0.78±0.33	$-2.35_{75.69}$	**0.021**	0.141	−0.34±0.36	$0.36_{40.61}$	0.363	0.888	0.89±0.31	$2.91_{67.05}$	**0.005**	**0.039***
LF×sync.	−0.53±0.24	$-2.23_{35.44}$	**0.032**	0.180	−0.37±0.31	$0.31_{24.12}$	0.254	0.769	0.56±0.22	$2.55_{32.26}$	**0.016**	0.094
FL×FF	−0.15±0.33	$-0.45_{80.25}$	0.655	0.991	0.03±0.36	$0.36_{39.75}$	0.930	1.000	0.18±0.29	$0.60_{74.73}$	0.551	0.975
FL×LL	−0.21±0.39	$-0.53_{104.4}$	0.599	0.984	0.10±0.40	$0.40_{55.7}$	0.808	0.999	0.32±0.36	$0.90_{91.85}$	0.371	0.896
FL×sync.	0.04±0.32	$0.12_{79.05}$	0.906	1.000	0.07±0.35	$0.35_{38.45}$	0.851	1.000	−0.01±0.29	$-0.04_{71.65}$	0.972	1.000
FF×LL	−0.06±0.30	$-0.20_{138.9}$	0.846	1.000	0.07±0.25	$0.25_{110.8}$	0.790	0.999	0.15±0.29	$0.50_{116.9}$	0.615	0.987
FF×sync.	0.19±0.25	$0.73_{39.44}$	0.468	0.948	0.04±0.32	$0.32_{25.91}$	0.912	1.000	−0.19±0.23	$-0.80_{38.2}$	0.427	0.930
LL×sync.	0.24±0.33	$0.74_{75.9}$	0.461	0.946	−0.03±0.37	$0.37_{41.27}$	0.934	1.000	−0.33±0.31	$-1.07_{68.25}$	0.287	0.820

Statistical significances both before and after Tukey–Kramer adjustment for multiple comparisons are reported. Statistically significant differences ($P \leq 0.05$) are indicated with bold letters and trends ($P \leq 0.1$) are indicated with italics.

16.2°C/1.29 mm in 2019). Prolonged dry periods likely reduce the insect availability making early life conditions harsher for the pied flycatcher chicks, which in turn likely contributes to the low fledging success (51%) in our study compared to the average fledging success of the species (82%; Lundberg and Alatalo, 1992), or this specific population [92.2% in 2017 (Sarraude et al., 2020) and 88.9% in 2019 (A.S., unpublished results)]. Weather data is obtained from a meteorological station in Artukainen in Turku (60°27′N, 22°10′E), 2 km from the study area and provided by the Finnish Meteorological Institution (https://www.ilmatieteenlaitos. fi). Yet, our sample size being relatively limited, we cannot completely exclude that hatching synchrony might increase early-life mortality, or that first-hatched chicks from last-laid eggs (LF) could have a higher survival than first-hatched chicks from first-laid eggs (FF) (non-significant trend in Fig. 2).

No apparent cost of synchronous hatching on chick phenotype

Lack's theory (1954) suggests that, in the case of synchronous hatching, poor environmental conditions could lead to poor growth of the whole brood as all the chicks would be equally competitive. Our results in a particularly harsh year (as reflected by the low fledging success, see above) do not provide evidence supporting this hypothesis, and do not support our own prediction of shared costs among siblings, as chicks from synchronous broods grew fast and ended up with fledging body mass and telomere length relatively similar to the first-hatched chicks from natural asynchrony broods. This observation might be explained by a parental compensatory strategy. Accordingly, Slagsvold and Wiebe (2007) showed that pied flycatcher parents of synchronous broods feed their chicks more often and with bigger prey items than parents of asynchronous broods. Thus, the costs of synchronous hatching, if any, might be mostly paid by the parents rather than by the offspring. Yet, this hypothesis remains to be rigorously investigated. Additionally, we might expect some laying order effects within the synchronous broods (e.g. between the chicks from the first and the last laid egg) arising from potential differences in egg composition. Yet, we were unable to test for such effects since it was not possible to track each chick's position in the laying sequence for synchronous broods in the present study.

Developmental match and mismatch determine post-natal phenotype and fitness proxies

Contrary to previously published results (Stier et al., 2015), last-hatched chicks from naturally asynchronous broods (LL) did not exhibit increased early-life telomere shortening despite a somatic investment enabling them to reach a body mass similar to their older siblings. This could indicate that the developmental match between laying order and hatching order (potentially through elevated maternal androgen levels in last-laid eggs) was efficient in optimizing chick phenotype and prevented potential costs of sibling competition on body mass and telomere length (Nettle et al., 2015). However, this result could be biased by the high-mortality of last-hatched chicks compared with the first-hatched chicks (70% vs. 38%), giving rise to a possible selective disappearance of weak last-hatched chicks exhibiting low body mass and short telomeres. Therefore, the possible delayed fitness costs of asynchronous hatching might be more easily seen under more favourable environmental conditions where direct fitness costs (i.e. mortality) are reduced (e.g. 92.3% survival to fledging in Stier et al., 2015).

By contrast, the developmental mismatch we induced by making last-laid eggs to hatch first and vice versa had an influence on both body mass and telomere dynamics, suggesting that there is likely an adaptive match between laying and hatching order (Müller and Groothuis, 2013). Chicks hatching last from first-laid eggs (FL) were not able to maintain their body mass at similar levels as their older siblings (hatching first from last-hatched eggs, LF), thereby indicating a more unbalanced sibling competition than in the natural scenario (see above). Quite unexpectedly, chicks in the more favourable position regarding sibling competition (hatching first from last-laid eggs) had shorter telomeres than all other groups 5 days after hatching. This could potentially be explained by their fast growth in early stages of the development (i.e. being the heaviest at day 5), possibly induced by higher testosterone levels in the last-laid eggs compared with the first-laid eggs (Morosinotto et al., 2016), as we know that both pre- and post-natal growth acceleration can accentuate telomere shortening (Monaghan and Ozanne, 2018; Stier et al., 2020). Similarly, a mismatch between prenatal cues and realized post-natal competitive conditions resulted in faster growth and increased telomere shortening in yellow-legged gull (*Larus michahellis*) (Noguera and Velando, 2020). However, although all experimental groups experienced some telomere shortening during early life, those first-hatched chicks from last-laid eggs (LF) having shorter telomeres at day 5 did not exhibit any shortening between days 5 and 12 post-hatching while still growing faster than their last-hatched siblings. This could for instance be explained by their higher competitive ability, enabling them to obtain more food from their parents and to invest both in fast growth and in telomere-maintenance processes (Pinto et al., 2011).

Conclusions

Our results show that despite a direct fitness cost (i.e. nestling mortality) being mainly determined by post-natal conditions (i.e. hatching rank), proxies of future fitness prospects (i.e. body mass at fledging and telomere length) were determined by the combined effects of pre- (i.e. laying order) and post-natal (i.e. hatching order) conditions. Importantly, inducing a developmental mismatch by reversing hatching order impaired the phenotype of the young, which may have consequences for later life performance. In the future, the geometric fitness building up through the future of both the parents and the offspring should be investigated to better understand the evolutionary origin of hatching asynchrony, laying-order effects on egg composition and their interplay.

Data availability
Data used in this study are available in Figshare at: https://doi.org/10.6084/m9.figshare.13055996.v1.

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