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Hatching asynchrony as a reproductive strategy in birds may explain the hatching failure of the last eggs of the clutch

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Hatching failure, due to infertility or embryo mortality, is an important factor contributing to reduced reproductive success in birds. Although hatching failure and its possible causes have been widely investigated, the stage of development at which embryo death occurs and its association with laying order have rarely been studied. The relative size of eggs laid in different positions within the laying order is a key factor that can lead to different parental reproductive strategies, such as hatching asynchrony. Here we investigate hatching failure in relation to laying order by establishing the developmental stage of dead embryos found in unhatched European Pied Flycatcher *Ficedula hypoleuca* eggs and considering possible causes of failure. We found that variation in egg dimensions showed a quadratic relationship with laying order, with relatively large volumes and sizes in the first and last positions of the clutch. Egg position in the laying sequence was also related to hatching failure, with the first and last positions being more susceptible to failure. The death of embryos late in development was more likely for eggs laid later in the sequence. To our knowledge, this is the first study showing that females may be adaptively allocating more resources to last-laid eggs to avoid competitive disadvantages between siblings, a strategy that seems to fail becasue these eggs suffer greater embryo mortality.

Keywords: development, egg dimensions, *Ficedula hypoleuca*, incubation, laying order, Pied Flycatcher.

Hatching success is a key parameter of fitness in birds (Koenig 1982, Cooke *et al.* 1985). Hatching failure, arising from either infertility or embryo mortality, constitutes a significant factor contributing to diminished avian reproductive outcomes (Jamieson & Ryan 2000, Briskie & Mackintosh 2004, Spottiswoode & Møller 2004). Infertility may be due to either the amount or the quality of the sperm, or both, or to incompatibilities between sperm and ovum (Pizzari & Birkhead 2000). However, multiple factors have been proposed to explain embryo mortality, including rainy or cold weather during incubation and hatching (Järvinen & Väisänen 1984, Webb 1987, Stoleson 1999), poor condition of incubating adults, which could lead to abandonment of the nest, poor egg size or quality (Parsons 1970, Magrath 1992), clutch size (Blackburn 1991), parental age or quality (Reid & Boersma 1990, Bolton 1991, Meathrel *et al.* 1993), or laying order (Robertson & Cooke 1993, Williams *et al.* 1993, Potti & Merino 1996, Cabezas-Díaz & Virgós 2007). Although embryo mortality is the primary cause of hatching failure in birds (Birkhead *et al.* 2008, Hemmings & Birkhead 2016), the specific reasons behind it remain largely unclarified. Embryo mortality can stem from a combination of internal and external factors, including environmental factors and parental behaviour, which may vary across the different stages of development. Consequently, discerning the precise stage at which an embryo dies during development may offer insights

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into the underlying cause of mortality (Hemmings & Birkhead 2016).

Egg size in birds is related to nestling size and growth (Potti & Merino 1996) because chicks hatched from larger eggs possess more energy reserves, grow more quickly and have higher survival rates; egg size is therefore an important factor explaining hatching failure. Embryos from small eggs may die from insufficient energy reserves to complete normal development (Royle *et al.* 1999). The relationship between egg size and hatching failure is often influenced by the quality of the parents, the order in which the eggs are laid or both (Murton *et al.* 1974, Reid & Boersma 1990, Meathrel *et al.* 1993, Williams *et al.* 1993, Potti & Merino 1996, Clifford & Anderson 2002).

The position in the laying order is a factor that has been associated with hatching failure (Robertson & Cooke 1993, Williams *et al.* 1993). Some authors have proposed high copulation frequency before laying the first egg or close to clutch completion as a potential cause explaining infertility, due to sperm depletion (Cordero *et al.* 1999). The specific position of the eggs within the laying order (first-laid and last-laid eggs) has been associated with increased embryo mortality (Williams *et al.* 1993, Potti & Merino 1996). Studies with artificially incubated eggs have found that embryo death occurs more frequently in last-laid eggs, while infertility was the main cause of hatching failure in first-laid eggs (Cabezas-Díaz & Virgós 2007).

Several hypotheses have been proposed to explain hatching asynchrony (reviewed in Węgrzyn *et al.* 2023). The timing of the initiation of incubation is the primary determinant of such a phenomenon (Temizyürek *et al.* 2022). Many birds such as the European Pied Flycatcher *Ficedula hypoleuca* are known to begin incubating before clutch completion (Enemar & Arheimer 1989, Forbes *et al.* 1997, Johnson *et al.* 2009, Lord *et al.* 2011). This strategy could lead to failure in the hatching of the last egg or competitive disadvantages of the last chicks as a consequence of hatching asynchrony (Wiebe 1996, Ostreiher 1997, 2001, Clotfelter *et al.* 2000, Pettifor *et al.* 2001, Jeon 2008, Węgrzyn 2012, Soler *et al.* 2020). Nearly all bird species care for their young soon after hatching, neglecting last-laid eggs due to foraging constraints after hatching of earlier ones, which can lead to the death of embryos before hatching (Terminal Egg Neglect Hypothesis; Cabezas-Díaz & Virgós 2007). Hatching asynchrony creates harsh conditions for last-laid eggs, and often late-hatched chicks tend to exhibit lower survival and growth rates (Węgrzyn *et al.* 2023).

Here we analyse hatching failures in relation to laying order in the European Pied Flycatcher (henceforth, Pied Flycatcher). We also assess whether those hatching failures are the result of infertility or embryo death, paying attention to the development stage of embryo deaths. We hypothesize that hatching failure might be related to egg size and the position within the laying sequence. Based on previous findings, we expect that both first- and last-laid eggs would experience higher hatching failure, the first mainly by infertility and the last mainly by embryo death. To date, studies that have addressed this topic have not taken into account embryo development stage, yet this may be key to understanding the reproductive strategies of some birds. Identifying the stage of embryonic development at death could provide clues about the causes of mortality.

METHODS

Field methods

The study was conducted during spring 2023 in a population of Pied Flycatchers breeding in nestboxes in a montane forest of Pyrenean oak *Quercus pyrenaica* in Valsaín, central Spain (40°54′N, 04°01′W) where long-term studies on cavity-nesting birds have been carried out since 1991. Of 465 nestboxes, 112 were occupied by Pied Flycatchers and the rest by other species, mainly Eurasian Blue Tits *Cyanistes caeruleus*, Rock Sparrows *Petronia petronia*, Eurasian Nuthatches *Sitta europaea* and Great Tits *Parus major*. A total of 106 pairs of Pied Flycatchers fledged at least one chick and were used in this study. In the remaining six nestboxes the female abandoned the nest shortly after clutch completion through natural causes.

From the beginning of April, nestboxes were routinely checked twice a week to monitor flycatcher reproduction and determine signs of nest building. Comprehensive daily checking was done from the moment the females completed the construction of the nest to detect the laying of the first egg until the clutch was completed. Egg-laying within the study population typically commences in mid-May (Cantarero *et al.* 2013), with a usual clutch size of six eggs. The species is considered to be single-brooded, and the female lays only one successful clutch in a given breeding season, laying one egg per day and taking on the responsibilities of incubation and brooding alone (Cantarero *et al.* 2014). Once egg-laying started, each new egg was distinctively marked according to the laying order with an indelible black pencil that did not penetrate the eggshell or damage the embryo. In the last marking visit, clutch size was determined and it was verified that all females had started incubation. No laying gaps were detected.

On the fifth day of incubation (calculated from the penultimate egg) females were captured in the nestbox during daytime without traps as they are not easily frightened from the nest at this stage. They were identified by their metal rings or supplied with individually numbered rings for identification if necessary and weighed to the nearest 0.01 g with a digital balance. We took a digital photograph of the entire clutch by placing the eggs in order into a custom box that contained a reference ruler (Fig. 1); this allowed us to take pictures while reducing the risk of egg breakage. The box was placed on a flat surface and pictures were taken perpendicular to the surface. Digital pictures were analysed with Adobe PhotoShop CS4 version 11.0 (Adobe Systems, San Jose, CA, USA) to estimate the size (area estimated in cm^2), maximum length and breadth (maximum diameter) for each egg to the nearest 0.1 mm. All these data were automatically obtained from the measurement record panel of the software once we had selected the outline of the egg. Repeatability for egg size calculated on a set of random eggs measured twice from the same picture ($n = 50$) was $r = 0.95$ ($P < 0.001$). Mean egg size, length and breadth were calculated for each clutch, and mean egg volume (V) was calculated from egg length (L) and breadth (B) using the formula $V = 0.042 + 0.4976LB^2$ (Hoyt 1979).

From the estimated day 12 of incubation (i.e. 1 day before the expected hatching date), we carried out daily visits to record hatching date (day 1). Three days after hatching (day 3), we recorded the number of nestlings and calculated 'hatching success' as the proportion of eggs that hatched. Unhatched eggs were collected in sterile tubes and frozen for subsequent laboratory analysis. All adult birds were captured in their nestboxes by using a passive trap placed inside their nestbox, in which the birds were automatically caught when entering to feed 7-day-old nestlings. The trap was placed for a maximum of 1 h and removed once both adults were captured. All birds were identified by

Figure 1. Customized box used in the field to take pictures of eggs of each nest.

their rings or ringed if necessary. The following traits associated were recorded: age estimated from ring data, wing length (mm) measured with a ruler from the carpal joint to the tip of the longest primary feathers, body mass (g) recorded with an electronic balance and male dorsal blackness as percentage of black plumage on the mantle with values ranging from 0 (0–10%) to 9 (90–100%). This last trait has been associated with male individual quality (Siitari & Huhta 2002). The exact age of adults older than 1 year was established if ringed as nestlings in the study area or classified if they were unringed according to Jenni and Winkler (1994) and Svensson (1984) as yearlings or older from the colour of the outer wing coverts. Once they have bred in our nestboxes, surviving adults typically return each spring to attempt breeding. On day 13, we ringed nestlings and measured them in the same way as adults. Fledging success was calculated as the proportion of hatched chicks that fledged.

Egg examination

Previously collected unhatched eggs were defrosted and dissected in the laboratory. Egg contents were emptied into a Petri dish and examined under a magnifying glass with each being given a score of infertile or inviable. Eggs scored as infertile only contained a yellow yolk, while eggs scored as inviable contained a germinal disc or embryo that had died before hatching. If this was observed, it was cleaned in a saline solution and examined under a microscope to identify the developmental stage at which death occurred, following Hemmings and Birkhead (2016). Developmental stages were grouped as early (germinal disc corresponding to up to 3 days of development), middle (small embryo corresponding to 4–7 days of development) or late (clearly visible embryo which died before hatching).

For an overall analysis of hatching failure, we have grouped together failures due to infertility and those due to death of the embryo. As a result, nests with ‘hatching failure’ were those where at least one egg failed to hatch.

Statistical analyses

We first investigated whether egg morphology varied within and among clutches as a function of laying order or female quality. Here we fitted four separate linear mixed models (LMMs) with ‘egg volume’, ‘egg size’, ‘egg length’ and ‘egg breadth’ as response variables, while fitting ‘laying order’, ‘female weight’, ‘female age’, ‘laying date’ and ‘clutch size’ as explanatory variables. Egg volume, size and breadth were $\log_{10}$ transformed to improve normality of model residuals. For these LMMs, ‘laying order’, ‘female weight’, ‘female age’, ‘laying date’ and ‘clutch size’ were included as explanatory variables. No collinearity was detected among these predictors (variance–inflation factors < 2). We further added the quadratic term for laying order to explore possible non-linear trends. In these models ‘Female ID’ was included as a random factor. The inclusion of ‘clutch size’ in our models determines that potential effects of laying order on egg morphology are present regardless of the final clutch size produced by females. However, to further support our findings, we carried out two further analyses on the relationship of clutch size and egg morphology. First, we ran the above models on two additional sub-datasets containing only the nests with clutch sizes of five or six eggs (the two predominant clutch sizes, representing respectively 22% and 60% of our nests), to test whether the quadratic effect was only due to relatively small or larger clutches. Subsequently, we ran *post hoc* tests to determine significant differences in egg morphology among the laying order categories.

Secondly, we explored the predictors of hatching success. Here we fitted generalized linear mixed models (GLMMs) by using hatching success as a binomial response variable (family = binomial). ‘Laying order’ and its quadratic term (Laying order^2), ‘male plumage blackness’ and ‘clutch size’ were included as potential predictors. ‘Nest ID’ and an observation-level random effect were included as random factors to account for non-independence of data and overdispersion. Also for this analysis, we carried out two additional models with sub-datasets of clutch sizes of five and six eggs.

We performed all LMMs and GLMMs with the ‘lmer’ function in the ‘lme4’ package (Bates *et al.* 2015) in the R environment (R Core Team 2017). To test the significance of the main effects and interaction terms in the LMMs, we estimated degrees of freedom and *P*-values of the F-tests with the Kenward–Roger approximation implemented in the ‘pbkrtest’ package (Halekoh & Højsgaard 2014). Significance of the fixed effects in the GLMMs was estimated with the function ‘Anova’, implemented by the ‘car’ package. Significance was taken at $\alpha = 0.05$. Collinearity between model predictors was calculated as the variance–inflation factor using the 'vif' function in the 'car' package. Model assumptions were assessed and met (all *P* values from Kolmogorov–Smirnov tests, dispersion and outlier tests > 0.05) via the 'DHARMa' package (Hartig 2022). Finally, χ^2-tests were performed to assess potential relationships between hatching failure and laying order. A significant effect in these tests would indicate that variation in observed proportion of failed eggs across the egg-laying order is statistically different from the random expectation that hatching success is equal along the laying sequence.

RESULTS

Laying order, egg morphology and hatching failure

The mean clutch size was 5.6 eggs (standard deviation = ±0.9, range 3–7). A total of 56 of 594 Pied Flycatcher eggs (9.42%) failed to hatch. From those 56 unhatched eggs, 23.2% ($n = 13$) and 76.8% ($n = 43$) were classified as infertile and as inviable, respectively. Of the inviable eggs, 37.3% ($n = 16$) were classified as early development stage, 20.93% ($n = 9$) as middle stage and 41.86% ($n = 18$) as late stage. We found a significant effect of the quadratic term of laying order on egg volume ($F_{1,418} = 103.58$, $P < 0.001$), size ($F_{1,419} = 53.2$, $P < 0.001$) and breadth ($F_{1,420} = 69.8$, $P < 0.001$), but not for egg length ($F_{1,420} = 0.48$, $P = 0.486$, Table 1). More precisely, *post hoc* tests indicated that earliest and latest laid eggs were bigger in volume, and larger in size and breadth compared with the ones laid in the middle of the laying period (Fig. 2). The significant quadratic effect of laying order observed in our models while correcting for 'clutch size' indicates that within-clutch variation in morphology across the laying sequence occurs regardless of final clutch size. This pattern is confirmed by the additional analyses for different clutch sizes: egg volume ($F_{1,83} = 28.79$, $P < 0.001$ and $F_{1,285} = 34.39$, $P < 0.001$ for the quadratic term for clutch sizes of five and six eggs, respectively), egg size (respectively $F_{1,83} = 10.05$, $P = 0.002$ and $F_{1,286} = 26.42$, $P < 0.001$) and egg breadth (respectively $F_{1,83} = 8.50$, $P = 0.004$ and $F_{1,286} = 35.54$, $P < 0.001$). We detected that laying order significantly predicted the viability of the eggs. Hence, first and last positions in the laying sequence were more prone to fail ($\chi^2_6 = 13.99$, $P = 0.029$; Fig. 3a). The incidence of neither infertile eggs nor early and mid-stage embryos was associated with laying order (infertile: $\chi^2_6 = 4.61$, $P = 0.595$; early development stage; $\chi^2_6 = 5.30$, $P = 0.506$; middle development stage; $\chi^2_6 = 2.73$, $P = 0.842$). However, death of late development embryos depended on laying order ($\chi^2_6 = 14.32$, $P = 0.027$, Fig. 3b), especially in the last positions of the laying sequence.

Hatching success

Hatching success was significantly affected by laying order and its quadratic term (Table 2, Fig. 4). Eggs in the middle of the laying sequence had a higher predicted hatching success than earlier and

Table 1. Model estimates for egg morphology; LMM, linear mixed model; se, standard error.

	Estimate	se	df	*t*-Value	*P*-value
(a) LMM for egg volume					
Intercept	2.65	0.46	84.1	5.78	< 0.001
Laying order	−0.23	0.02	418.9	−12.55	< 0.001
Laying order^2	0.02	0.01	418.9	10.18	< 0.001
Female weight	−0.01	0.03	83.2	−0.60	0.547
Female age	0.01	0.01	83.3	0.13	0.890
Laying date	−0.01	0.01	83.6	−0.76	0.447
Clutch size	−0.04	0.03	86.2	−1.27	0.207
(b) LMM for egg size					
Intercept	2.18	0.35	83.8	6.16	< 0.001
Laying order	−0.12	0.01	419.5	−9.11	< 0.001
Laying order^2	0.01	0.00	419.5	7.29	< 0.001
Female weight	0.01	0.02	82.9	0.38	0.704
Female age	0.01	0.01	83.0	0.56	0.574
Laying date	−0.01	0.01	83.3	−0.59	0.558
Clutch size	−0.03	0.02	85.6	−1.27	0.209
(c) LMM for egg length					
Intercept	184.78	22.34	83.9	8.27	< 0.001
Laying order	−1.45	1.06	419.5	−1.37	0.172
Laying order^2	0.10	0.14	419.5	0.70	0.486
Female weight	0.42	1.36	82.5	0.31	0.757
Female age	0.11	0.64	82.7	0.17	0.864
Laying date	−0.06	0.13	83.2	−0.45	0.657
Clutch size	−1.71	1.56	86.7	−1.09	0.278
(d) LMM for egg breadth					
Intercept	179.71	15.86	84.5	11.32	< 0.001
Laying order	−8.27	0.84	419.9	−9.81	< 0.001
Laying order^2	0.98	0.11	420.0	8.35	< 0.001
Female weight	−0.93	0.96	82.7	−0.96	0.338
Female age	0.35	0.46	82.9	0.77	0.442
Laying date	−0.14	0.09	83.5	−1.44	0.154
Clutch size	−1.91	1.11	87.9	−1.71	0.089

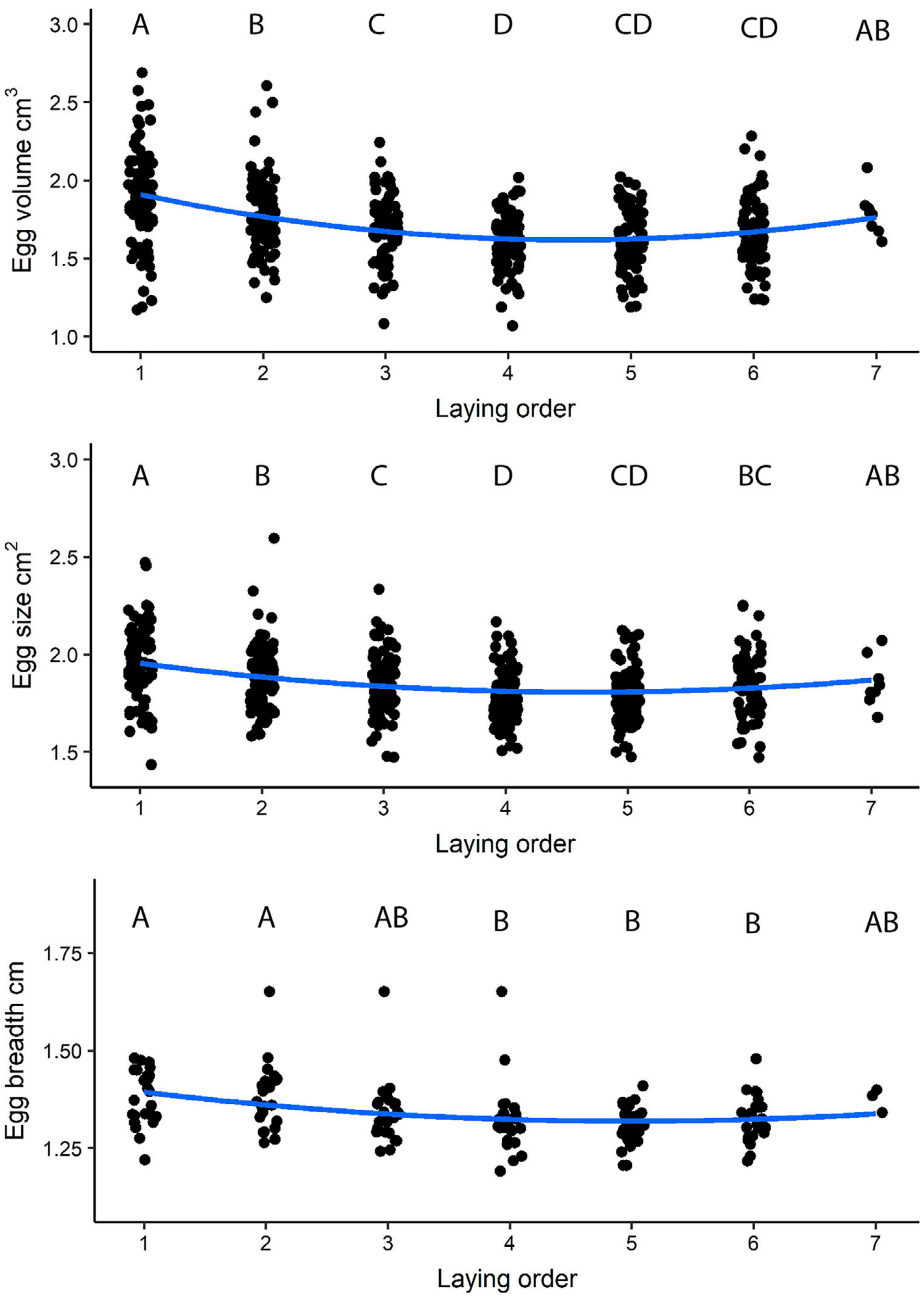

Figure 2. Differences in egg volume, size and breadth in relation to laying order. Different letters on top of the data points indicate significant differences among laying order categories in *post hoc* tests.

later eggs Hatching success also increased with darker dorsal male coloration (Table 2, Fig. 5). Some of these effects were still significant or marginally non-significant when sub-setting the dataset into different clutch sizes ($\chi^2_1 = 3.11$, $P = 0.077$, $\chi^2_1 = 2.76$, $P = 0.096$ and $\chi^2_1 = 3.63$, $P = 0.056$ for laying order, its quadratic term and male blackness for clutch sizes of five; $\chi^2_1 = 5.6$, $P = 0.018$,

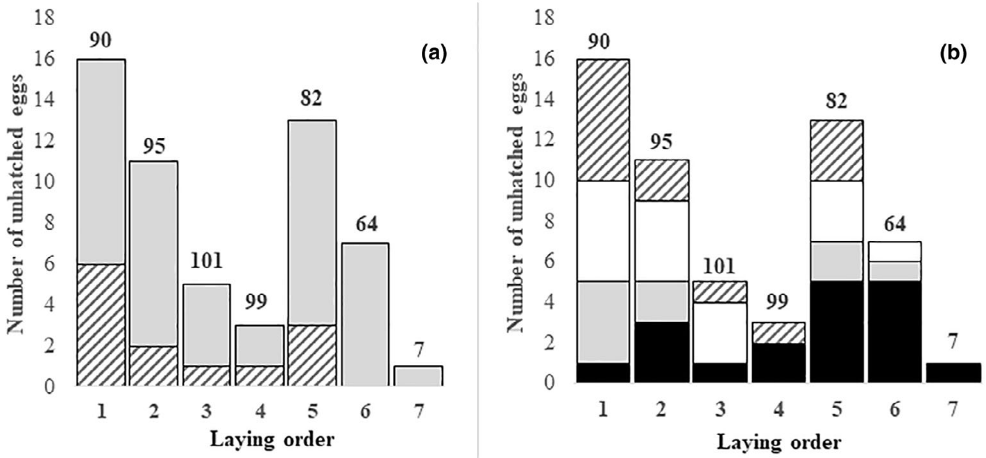

Figure 3. (a) Frequency of failed eggs due to infertility (striped) and embryo mortality (grey) in relation to laying order. (b) Frequency of failed eggs due to infertility (striped) and embryo mortality (early (white), mid-stage (grey) and late (black) development embryos) in relation to laying order. In both cases the numbers above each column correspond to the number of viable eggs.

Table 2. Model estimates for hatching success; GLMM, generalized linear mixed model; se, standard error.

GLMM for hatching success	Estimate	se	χ^2	df	*P*-value
Laying order	1.46	0.45	10.60	1	0.002
Laying order^2	−0.18	0.06	10.54	1	0.002
Male blackness	0.04	0.02	4.17	1	0.041
Clutch size	0.34	0.30	1.21	1	0.223

$\chi^2_1 = 5.39$, $P = 0.020$ and $\chi^2_1 = 0.48$, $P = 0.485$ for laying order, its quadratic term and male blackness for clutch sizes of six, respectively).

DISCUSSION

Our results showed that laying order had a significant effect on egg viability, revealing that the first and last positions in the laying sequence were more prone to fail. In addition, the death of late-stage embryos also depended on the laying order, particularly later in the laying sequence. We found that 8.97% of eggs failed to hatch, a proportion consistent with rates documented in previous studies of Pied Flycatchers and other cavity-nesting birds (Morrow *et al.* 2002, Spottiswoode & Møller 2004, Stewart & Westneat 2013, Di Giovanni *et al.* 2023).

Hatching failure varied with laying order following a quadratic function and predicted late development embryo mortality. Higher failure rates were observed in both the initial and final positions within the clutches, most of which contained five or six eggs. This pattern clearly suggests optimal hatchability at intermediate positions within the clutch despite smaller egg size. These findings are in line with those reported by Potti and Merino (1996), and Ylimaunu and Järvinen (1987) in their studies of Pied Flycatchers, who also observed a reduction in hatching or fledging success associated with both the first and the last egg in the laying sequence.

On the one hand, lower hatchability of eggs laid early in the sequence may be attributed to greater physiological effectiveness in egg production as the laying sequence progresses (Leblanc 1987). Alternatively, the greater exposure duration of early-laid eggs to environmental factors could also contribute to reduce their viability (Arnold *et al.* 1987, Veiga 1992, Veiga & Viñuela 1993). On the other hand, the lower hatchability of the last egg may be due to the asynchronous hatching of passerines such as the Pied Flycatcher (Slagsvold 1985, Enemar & Arheimer 1989, Forbes *et al.* 1997, Johnson *et al.* 2009, Lord *et al.* 2011). Female Pied Flycatchers in our population are known to begin incubation on the day of laying the fourth egg (Ruiz-De-Castañeda *et al.* 2012). Several hypotheses have been proposed to explain this strategy, which could cause the last eggs to not hatch. One plausible hypothesis to explain high failure rates observed in last eggs is 'Terminal Egg Neglect' (Cabezas-Díaz & Virgós 2007).

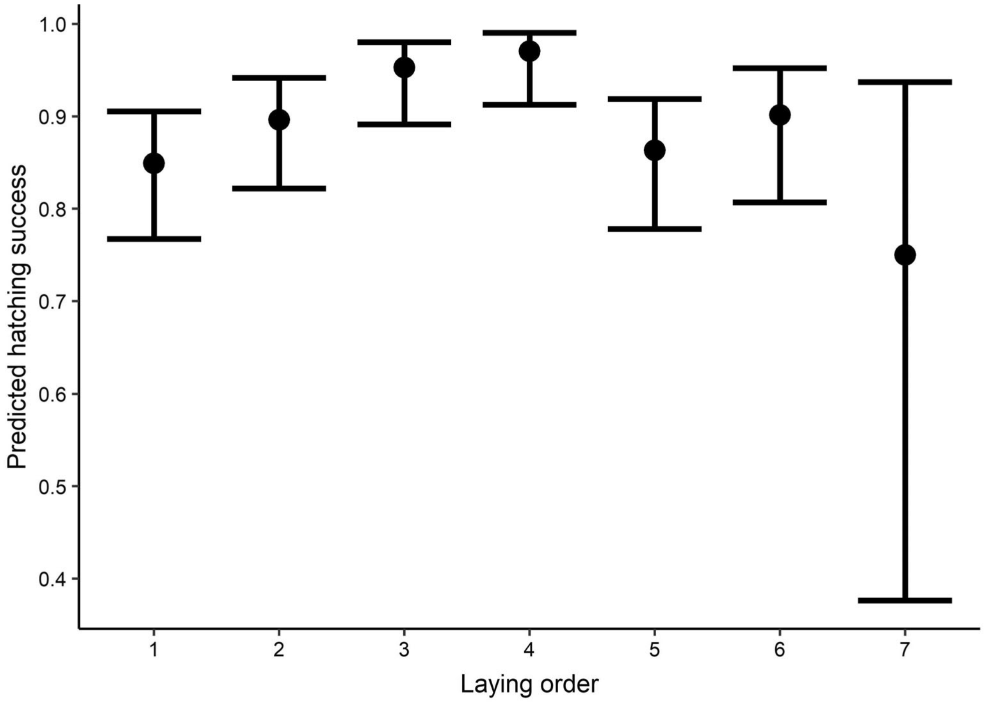

Figure 4. Predicted hatching success in relation to laying order. Hatching success probabilities (mean ± standard error) are estimated via the generalized linear mixed models and plotted using the R function 'effect_plot' (R package 'jtools').

According to this hypothesis, females may exhibit a tendency to neglect the incubation of the final eggs, potentially influenced by the dietary demands of the newly hatched nestlings. Alternatively, final embryonic development and hatching of last eggs could be hampered by the presence of already hatched siblings that prevent correct egg placement and/or female ability to maintain optimal incubation. This scenario would also explain why late development embryos disproportionately died close to hatching. An alternative hypothesis could be the 'Brood Reduction Hypothesis' (Pijanowski 1992). This hypothesis postulates that asynchronous hatching is advantageous in unpredictable environments (bad years), leading to a decrease in brood size at the cost of mortality of late eggs or late-hatching individuals.

Although the increase in infertile eggs in the first and last eggs of the clutch could be explained by an increase of physiological efficiency of the ovaries and oviduct with laying order (Leblanc 1987) or a decrease of sperm efficiency, sperm depletion or ovum viability (Graves 1992), no relationship was found between infertile eggs and laying order. It has also been suggested that females may invest in some infertile eggs within the clutch to favour the survival of fertile ones by spreading predation risk (Cabezas-Díaz & Virgós 2007). Furthermore, there was no association between embryonic death in the early and middle stages of development, and laying order. This may be due to different causes of infertility or embryo death that are not associated with the laying sequence. Some of these causes could include lethal genetic characters, malposition of the embryo and/or gross structural abnormalities and teratism (Romanoff 1949). Hatching failure was not related to any morphological characteristics of the egg. Egg volume may have implications for offspring fitness at different stages of the life cycle (Williams *et al.* 1993, Anderson *et al.* 1997), but there is conflicting evidence on the impact of egg characteristics on hatchability. While some studies have found that probability of hatching is associated with volume (Potti & Merino 1996, Pinowska *et al.* 2002, Serrano *et al.* 2005), others have failed to find such an effect (Smith *et al.* 1995, Clifford & Anderson 2002). Our study was conducted in a single year, so sample size may not have been sufficient to detect significant effects like those found by Potti and Merino (1996) in the same study

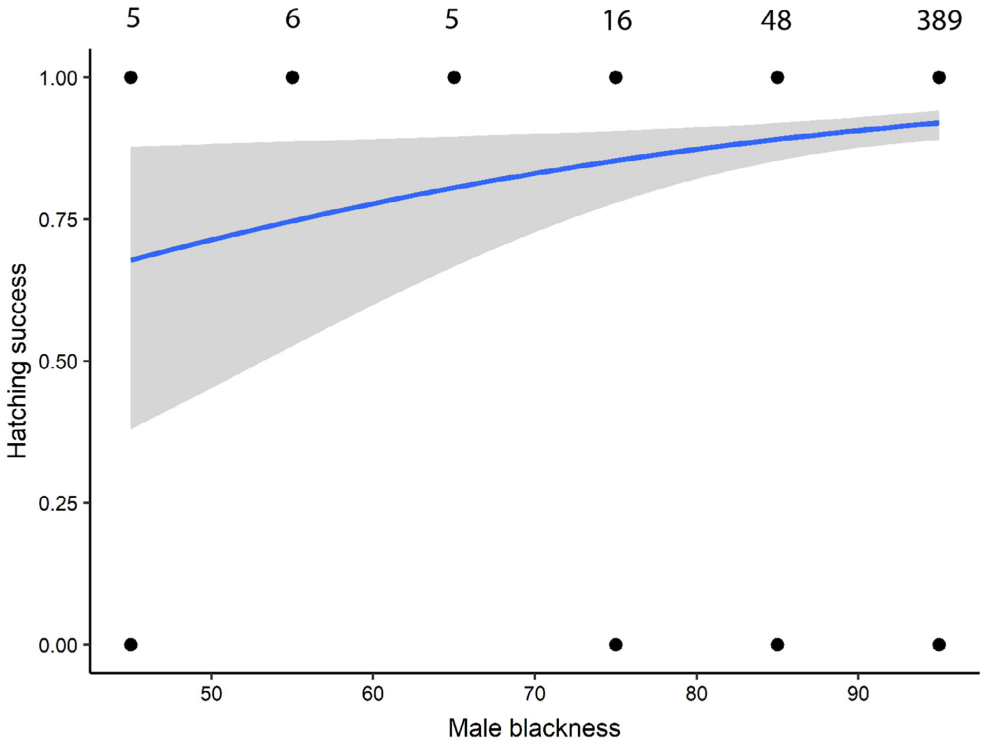

Figure 5. Predicted hatching success in relation to male blackness estimated via the generalized linear mixed models. Regression line and standard error are shown. The numbers above correspond to the number of eggs in the analyses belonging to the males of different dorsal blackness category.

species. Differences in the methods used to obtain egg measurements (mean egg volume of 1.70 mm^3 versus 1.59 mm^3 reported in Potti and Merino (1996) for the same species) or local effects on egg morphological traits may also have compromised detection of effects.

We found that females paired with males with more dorsal blackness achieved higher hatching success. Male dorsal coloration has been described as a sexually selected and reliable signal of individual quality in male Pied Flycatchers (Siitari & Huhta 2002). Darker plumages are preferentially selected by females and are positively associated with aggressiveness and territoriality (Sirkiä & Qvarnström 2021). Hence, dark males might have a competitive advantage over nest-sites and mates. Females paired with darker males are known to suffer less oxidative damage and show higher incubation attendance (Moreno *et al.* 2013), which may explain their higher hatching success. In contrast, females breeding with dull males (lower quality) may incur hatching failure. Given that male phenotype also predicts extra-pair paternity in this species (males with more dorsal blackness are considered to be more attractive; Canal *et al.* 2011), further studies are necessary to address whether embryonic mortality differs between embryos resulting from intra- and extra-pair copulations.

Egg dimensions showed consistent quadratic patterns of variation with laying order, with relatively large volumes and sizes in the first and last positions of the clutch compared with those in intermediate positions. The decrease in egg size throughout the laying sequence is a phenomenon already reported in other bird species (Cabezas-Díaz & Virgós 2007, Kozlowski & Ricklefs 2010, Monclús *et al.* 2017), a phenomenon whose mechanistic explanation has been attributed to the gradual depletion of the resources necessary for egg production. Egg dimensions have been correlated with body mass and skeletal size of the emerging chick (Krist 2011). Through a decrease in the relative size of the final eggs in a clutch, females could be facilitating a brood reduction strategy. The beginning of incubation before the clutch is complete may lead to an increase in the degree of hatching asynchrony such that the last chick is delayed compared with its siblings (Enemar & Arheimer 1989, Forbes *et al.* 1997, Johnson *et al.* 2009, Lord *et al.* 2011). This scenario, however, is not applicable to the Pied Flycatcher, where our results show that females adaptively allocate more resources to a given position in the laying order (last-laid egg) to avoid such competitive disadvantages (Ostreiher 1997, 2001, Jeon 2008, Węgrzyn 2012). However, the strategy does not seem to be sufficient as we demonstrate there is a greater embryonic mortality in the last eggs despite their larger size.

Although hatching failures have been extensively investigated, limited attention has been paid to the precise causes of embryonic mortality, other than as a function of infertility. Furthermore, little attention has been paid to identifying critical periods of vulnerability during embryonic development in wild birds. The ability to identify the specific stages at which embryos die during incubation would allow embryonic mortality to be correlated with local or other environmental factors. This study could, in turn, help to identify potential factors contributing to embryonic mortality. In conclusion, the position of the eggs in the laying sequence is related to hatching failure, with the first and last positions being more susceptible. Laying order also predicted embryo mortality in late development. More studies should be conducted to investigate possible natural causes of why embryos die during early and middle stages of development.

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AUTHOR CONTRIBUTIONS

Manuel Fuertes-Recuero: Methodology; formal analysis; writing – original draft. **Davide Baldan:** Validation; writing – review and editing; formal analysis; data curation; resources; software. **Alejandro Cantarero:** Writing – review and editing; data curation; formal analysis; supervision; methodology; conceptualization; funding acquisition; validation; project administration; resources.

CONFLICT OF INTEREST

We declare we have no competing interests.

ETHICAL NOTE

Permissions for handling birds were provided by Consejería de Medio Ambiente de Castilla y León (protocol number AUES/SG/17/2023 – SG_2022_299). The work was approved by Consejería de Medio Ambiente de la Comunidad de Madrid (approval ref. PROEX 125.1/23).

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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