Early arrival is not associated with more extra-pair fertilisations in a long-distance migratory bird

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Abstract

When assessing the benefits of early arrival date of migratory birds, a hidden and often ignored component of males' fitness is the higher chance of early-arriving birds to obtain extra-pair fertilisations. Here we investigated how extra-pair paternity might affect the relationship between male arrival date and number of fertilisations in a model study system, the European pied flycatcher (*Ficedula hypoleuca*). For this purpose, we sampled and genotyped breeding pairs, unpaired males and offspring (including embryos from unhatched eggs when possible) of a Dutch pied flycatcher population. Detailed information on arrival date of males, egg-laying date of their social mates and nest success was also recorded. Early-arriving males had early-laying females and males with early-laying females had a higher probability of siring extra-pair eggs and obtain more fertilisations. However, male arrival date alone did not correlate with the probability to gain extra-pair paternity and neither to the amount of fertilised eggs. Both early- and late-arriving males had a higher probability of losing paternity in their own nest compared to birds with an intermediate arrival date. Finally, late-arriving males were more likely to remain unpaired but, interestingly, a few of these birds obtained paternity via extra-pair copulations. Because earlier arrival date did not lead to more extra-pair fertilisations and because such relationship seems to be driven mainly by the female's laying date, we conclude that the contribution of extra-pair paternity to the overall fitness benefits of early male arrival date is relatively small.

Introduction

Migratory birds need to time the different stages of their complex annual cycle to take advantage of the distinct favourable conditions in their wintering and breeding environments (Alerstam et al. 2003; Buehler & Piersma 2008). The timing of migration from the wintering to the breeding grounds has been a topic of particular interest to evolutionary biologists, as the arrival time on the breeding grounds is considered a trait with major fitness consequences (Møller 1994; Velmala et al. 2015; Visser et al. 2015). Both males and females may suffer the costs of arriving too early or too late. Because males usually arrive earlier, they are more likely to be affected by the costs of early arrival than females, and also due to their conspicuousness upon arrival, they are easier study subjects and thus the vast majority of studies have focused on male arrival phenology (e.g., Møller et al. 2003; Møller 2004; Reudink et al. 2009; Canal et al. 2012a; Velmala et al. 2015). An excessively early arrival may mean facing very harsh early spring conditions en route or at breeding territories (Møller 1994; Brown & Brown 2000), but these risks have to be traded off against the potential fitness benefits of early male arrival. These include the claim of better territories (Alatalo et al. 1984; Slagsvold 1986; Potti & Montalvo 1991; Hasselquist 1998; Canal et al. 2012a), earlier breeding (Cooper et al. 2010; Canal et
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al. 2012a; Velmala et al. 2015), more opportunities to find a mate (Alatalo et al. 1984; Møller 1994; Lozano et al. 1996; Canal et al. 2012a) and/or attract additional ones (Alatalo et al. 1984; Reudink et al. 2009; Cooper et al. 2010; Canal et al. 2012a), and more chances of second breeding attempts in case of failure of the first nest (Cooper et al. 2010). Ultimately, this would lead to higher breeding success for early-arriving males.

Early-arriving males might also have further opportunities to achieve extra-pair mating (Langefors et al. 1998; Møller et al. 2003; Reudink et al. 2009; Cooper et al. 2010; Canal et al. 2011, 2012a, 2012b). Extra-pair paternity (henceforth EPP) is an important fitness component that can increase reproductive success in males, as long as this do not come with the cost of paternity loss in their own nest (Webster et al. 1995). As the number of arriving females is limited and the number of competing males in a breeding site increases throughout the season, males that arrive early (and thus mate early) would have more time and chances to achieve extra-pair fertilisations.

When assessing fitness in wild species, often only the number of recruiting offspring from the social brood to the breeding population is used (Perrins 1965; Visser & Lessells 2001), overlooking EPP. If there is a relationship between arrival date and the probability of obtaining fertilisations outside the social nest, considering only the recruits from the social nest might provide a misleading picture of the actual fitness benefits of early arrival (Albrecht et al. 2007). Correct fitness estimations are needed for an unbiased estimate of selection on arrival date. This is especially true as seasonal timing is currently shifting due to anthropogenic alterations (e.g., climate change), which calls for studies aiming to understand how quickly species can respond and adapt to rapid environmental changes (Parmesan & Yohe 2003; Visser 2008). A number of studies have described either advancement trends or unchanging migration dates (Both & Visser 2001; Lehikoinen et al. 2004; Gill et al. 2014) and to understand such (lack of) responses, there is also an increasing interest on the fitness consequences of arrival date in long-distance migrants (Velmala et al. 2015; Visser et al. 2015).

EPP has been shown to be affected by many factors, including secondary sex features (Møller et al. 2003; Lehtonen et al. 2009; Canal et al. 2011), body size (Canal et al. 2011), age (Moreno et al. 2010; Canal et al. 2012b), polygyny (Lubjuhn et al. 2000), and timing of breeding (Canal et al. 2012a, 2012b). However, to date, the number of studies that have investigated how male arrival date associates with paternity (gain or loss) is small (Table 5.1). Such studies can be divided in cases in which early males gained paternity by having a higher probability of obtaining EPP (Table 5.1; Langefors et al. 1998; Cooper et al. 2010) and cases in which early males had a lower probability of losing paternity in their own nest (Table 5.1; Møller et al. 2003; Cooper et al. 2010). In any case, both situations lead to clear fitness benefits for early-arriving males.

Due to the potential relevance of extra-pair fertilisations when accounting for the total
fitness benefits of early arrivals, here we investigated the contribution of this “hidden” fitness component to the selection for early arrival. To this end, we collected data on arrival and laying dates and we sampled and assigned paternity for eggs and chicks of a Dutch pied flycatcher population. We then explored how the probability of gaining or losing paternity is associated with arrival date and tested whether the relationship between fertilisations and arrival date is significantly affected by EPP. Moreover, we not only focused on sampling the surviving chicks but attempted to sample any fertilised egg that was produced by our breeding population, gaining additional information on the fertilisation attempts of the males in our population.

Table 5.1. Review of studies that correlated arrival dates with paternity gain or loss in different bird species. The symbols show the direction of the pattern in relation to early-arriving birds.

| Species                  | Percentage of broods with EPP | EPP gain | Paternity loss | Fertilized eggs/offspring | Authors, year of publication |
|--------------------------|-------------------------------|----------|----------------|---------------------------|-----------------------------|
| Sedge warbler (Acrocephalus schoenobaenus) | 23%                           | +        |                |                           | Langebors et al., 1998       |
| Barn swallow (Hirundo rustica)       | 32.4%                         | -        |                |                           | Møller et al., 2003         |
| American redstart (Setophaga ruticilla) | 43%                           | -        | +              |                           | Reudink et al., 2009        |
| Eastern kingbird (Tyrannus tyrannus)  | 58-70%                        | +        |                |                           | Cooper et al., 2010         |
| Pied flycatcher (Ficedula hypoleuca)  | 19.8%                         | 0        | +              | 0                         | Only via female lay date    |
|                                         |                               |          | (quadratic relation) |                           | Present study               |

Methods

Study system and study area

European pied flycatchers Ficedula hypoleuca ([Pallas], 1764), Muscicapidae, are long-distance migrants that breed in Europe and winter in Africa. They breed in tree cavities but readily accept nest boxes, which makes the species suitable for field research. Pied flycatcher males are usually easy to distinguish from females by the presence of a wider white wing patch, a white forehead patch and the presence of black or grey plumage, while females are brown. In our study area in the Netherlands, however, some males have a female-like appearance, but they can be distinguished from females by their conspicuous singing behaviour. Voucher material of this population was deposited in the ornithology collection of the Naturalis Biodiversity Centre (Leiden, The Netherlands) under the inventory numbers RMNH 592347, RMNH 592348 and RMNH 592349.
Our study was conducted between early April and late June 2014, in the National Park de Hoge Veluwe (The Netherlands; 52°02’07”N, 5°51’32”E). Forested areas in the park are dominated by pedunculate oaks *Quercus robur*, northern red oaks *Quercus rubra*, Scots pines *Pinus sylvestris*, European larches *Larix* spp. and birches *Betula* spp. We provide around 400 nest boxes year-round, in an area of 171 ha, which are occupied in spring by cavity-nesters such as pied flycatchers, great tits (*Parus major*), blue tits (*Cyanistes caeruleus*), nuthatches (*Sitta europaea*) and coal tits (*Periparus ater*).

**Field sampling and data collection**

Arrival time was assessed by daily scoring newly arriving males in our study area from early April using a protocol very similar to the one described in Potti (1998), Visser *et al.* (2015) and Both *et al.* (2016). Birds choose a territory upon arrival and advertise their cavity or nest box to the females by singing continuously at or close to the potential nest site. Two or three trained observers walked independently pre-established routes covering the whole study area and visiting all boxes. Routes and direction of the routes were alternated daily among observers in order to prevent any potential bias among them. Detected birds were described in terms of plumage and aluminium/colour ring combinations. Male pied flycatchers display relatively large individual variation in plumage features which, combined with colour ring combinations, allows an initial recognition in the field without the need of capturing the birds. In our study site, plumage colouration varied from female-brown or light grey to almost entirely black (Drost 1936). The forehead white patch also varied in size, from absent to a large patch covering most of the forehead, and also in shape from two distinct dots to a rectangular-shaped patch. We associated singing males to the closest nest-box in the vicinity. During chick-rearing phase (see below), those males were caught and described again in terms of plumage characteristics and ring combinations. This allowed us to link the original identifications to an individual ring number and assign individual arrival dates. Any bird still singing by the 1st of May (around one week after the first egg was found) was considered a “bachelor” and was captured to be identified and blood sampled. Some of those bachelors became breeding birds later in the season.

Upon arrival of females and start of nest building, nests were checked daily to assess the date of first egg laid, clutch size, date of egg hatching and number of hatched chicks. As part of an experiment running in parallel, some eggs were also marked and swapped (see also the assignment of paternity section). Both adults were then captured when their chicks were seven days old with a spring door trap or a wire door trap installed in the nest box entrance. These breeding birds were identified, measured and blood sampled (10 µl) by brachial venepuncture for paternity analysis. When 12 days old, chicks were also measured and a 10 µL blood sample was taken for paternity analysis. Blood samples were stored in 1 ml of Cell lysis Buffer (Qiagen, Venlo, The Netherlands). Any
unhatched egg or dead chick found before blood sampling were collected and stored at -20°C. Tissue samples from unhatched embryos, dead adults (all found dead inside nest-boxes as a consequence of agonistic interactions with great or blue tits) and dead chicks were taken for DNA analysis. In the few cases in which blood samples were missing but feather samples were available, DNA was isolated from the proximal tip of the feather (i.e., calamus).

**Genotyping**
FavorPrep 96-well Genomic DNA kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan) was used to isolate the DNA from blood and tissues, following the manufacturer’s protocol and using 200 µL of blood-Cell lysis buffer mixture. In the case of tissue samples, around 25 mg were processed like proposed in the FavorGen protocol with 2 hours of lysis incubation. PCR was performed by using five microsatellite DNA loci: Fhyu336, Fhyu234, Fhyu304, Fhyu453, Fhyu448 (Leder et al. 2008). Separation of the PCR fragments took place using an ABI 3130 Genetic Analyser (Thermo Fisher Scientific, Waltham, USA). The capillary electrophorese results of the ABI were analysed with the software GeneMapper 5.0 (Thermo Fisher Scientific, Waltham, USA) that determined the sizes of the amplification products.

**Assignment of paternity**
The paternity of offspring was analysed with Cervus version 3.0.7. (Field Genetics Ltd., London, UK; Kalinowski et al. 2007). Cervus calculates a likelihood ratio for each father–offspring pair using genotype data from young and adults as candidate parents. It assigns paternity according to the difference in the likelihood ratio scores between the most likely and second-most-likely fathers. Five microsatellite loci were used to determine genotype and assign parentage with one mismatch allowed. Confidence was set at 95% level. Based on that information, we determined whether a male sired an extra-pair young or not, whether it lost paternity in its own nest or not and, whether it was able to father any egg at all or not, finally, we determined how many eggs and 12 days-old chicks each male fathered in total. The probability of assignment was 0.99 and mismatches between known pairs of parent and offspring were also re-visualised for accuracy (see Supplementary material for more details).

Not all complete families were caught, so, for the nests in which only one of the parents was caught, the genetics of the offspring were used to determine the other parent. This reconstructed genotype was then matched with all other genotypes that we generated to either identify the parent among the captured birds or infer that the parent was another, not captured, bird. If the father could not be identified (which was the case for only 5 out of 208 birds), it was still possible to determine if the brood had any extra-pair eggs.
When an unknown male had fathered an egg, it was counted as a paternity lost for the social male, but no paternity gain for another.

The data collection for extra-pair-paternity analysis was conducted in the same year in which a field experiment was also carried out. The manipulations involved in this experiment had the purpose of advancing or delaying hatch dates, combined with cross-fostering, and then evaluating the consequences related to the timing of breeding. Chicks from Advanced and Delayed groups hatched with four days of difference from Controls and eight days from each other. Purposes and results of this experiment will be presented elsewhere (Chapters 6 and 8). This experiment had two potential implications for our analysis. First, the time spent by females in incubation was shortened or lengthened in Advanced or Delayed females, respectively. Due to the longer incubation time, Delayed females could offer more opportunities for their pairs to achieve extra-pair paternity than Advanced ones. However, when such incubation effects were tested in relation to the variables analysed in the present work, there were no significant effects in any aspect. This is also supported by other studies with pied flycatchers that did not find an effect of brood manipulations on EPP (Moreno *et al.* 2013). Therefore, all nests were treated equally, regardless of any experimental manipulation, in all analysis presented here. Second, when females laid a seventh egg, these were often stored or put under non-experimental birds and as a consequence they were significantly more likely to be lost, therefore not assigned to any male, than eggs number one to six (Fisher’s Exact Test, *p*-value <0.001). However, out of those eggs which were assigned, there was no significant difference of being extra-pair between eggs one to six and egg seven (Fisher’s Exact Test, *p*-value = 0.15). Therefore for the purpose of extra-pair paternity analysis, eggs were treated in the same way, independent of their lay order (see Table S5.2 for more details).

Unassigned eggs (not genotyped and thus without known paternity) needed to be considered differently depending on the analysis. When we analysed whether a father gained or lost the paternity of any extra-pair egg (see below in the data analysis part), any unassigned eggs had to be excluded. When we calculated the number of eggs a given male fathered, we took into account the probability of losing paternity of an egg in our population (9% based on all own and extra-pair eggs laid in 2014). Eggs not previously assigned to a male were then given to the social father with a 91% of probability of being his own. All the males in the population received an extra paternity value which consisted on a 9% fraction of all eggs not assigned (72 eggs) divided by all males of the population (109 males). Four of the eggs that we genotyped did not match with any male of the population (within or extra-pair), they were also divided by all males of the population and added to the total number of fathered eggs as a fixed value. Therefore, for the final number of fathered/fertilised eggs we considered: own genetically-identified social eggs + identified eggs fathered in other nests (including polygyny and extra-pair) +
\[ 0.91 \times \text{(social eggs not assigned)} + 0.09 \times \text{(all eggs not assigned/all males in the population)} + \text{(eggs with no match in the population/all males in the population)}. \]

**Data analysis**

Analyses were performed in R version 3.2.1 (18-Jun-2015; R Core Team 2015). We used generalised linear models in R (logit-link and Binomial error-distribution) to test if the probabilities to gain extra-pair paternity or lose paternity could be explained by the arrival date of the male (as linear and quadratic term) or the laying date of the female. We performed backwards model selection, dropping non-significant terms in each step. Because the inclusion of the laying date of the female forced the model to only include males that had a nest (and no bachelors), we also tested in simple regression analysis the probability to gain paternity in relation to the arrival date of all males, in relation to the arrival date of only males that had a female and in relation to the female laying date. We also tested whether the probability of fertilizing an egg at all was related to arrival date of the male or laying date of the female, however, it was not possible to include both terms in the same multiple regression analysis since males with known arrival date that failed to fertilise eggs were all bachelors, so we only tested these probabilities in separate simple regressions (arrival date of the male including bachelors or laying date of the female). Using multiple linear regressions we tested whether or not the arrival date of the male correlated with the laying date of their female and whether or not the number of fathered eggs and chicks was related to arrival date of the male or the laying date of the female. For this analysis, we only included data from males that had a female (no bachelors) and tested both fathered eggs with EPP or only the social eggs. Finally, to shed some light on the pattern of fertile female availability, we calculated for each male the difference between the laying date of his social female and the laying date of his extra-pair female. The difference was then correlated with the arrival date of the male and the laying date of its social female. For polygynous males, we only considered the laying dates of the primary brood as secondary broods could have a higher incidence of paternity loss due to differences in behaviour of the male (Canal et al. 2011). Moreover, the incidence of polygyny was very low in our dataset (only three males).

**Results**

In total, 555 eggs were produced by the sampled breeding population. We could assign 87% (481) of those to a male (see Table S5.3 for more details). The unassigned eggs were either broken, non-viable, predated, discarded by the female, had poor quality DNA or, if hatched, the chick had died and was not in the nest anymore by the time we could sample or collect it. 9% of the assigned eggs were fertilised by an extra-pair male; however, the proportion of clutches containing an extra-pair egg was close to 20%
(Table 5.1). Out of 109 males analysed in total, their spring arrival dates and laying date of their social females were obtained for two overlapping (but not the same) sets of 87 birds, with 72 males having both arrival and laying date known.

The probability of losing paternity showed a quadratic relation with the arrival date of the male, with both early and late-arriving birds showing a higher probability to have an egg fertilised in their own social nest by other males than males arriving on intermediate dates (quadratic term: $\chi^2 = 5.27$, $p$-value = 0.02, estimate ($\pm$ s.e.) = 0.03 ($\pm$0.01) (Fig. 5.1a).

**Figure 5.1.** Correlation and model predictions of **a)** the probability that a male loses paternity in his social nest in relation to his arrival date (given in April days: number of days since 31st of March). Points correspond to the average probability of losing paternity of all males that arrived in each of the 2 day-bins starting from April day 7 (e.g., between days 7 and 8, 9 and 10, 11 and 12, etc.). The size of the symbols indicate sample size (largest = 15, smallest = 1). **b)** The laying date of the female and the arrival date of the male (both given in April days). **c)** The probability of a male obtain extra-pair paternity in relation to the laying date of his social female (points correspond to the average probability of losing paternity of all males that had females laying in each of the 4 day-bins starting from April day 23; point sizes reflect sample sizes: largest = 26, smallest = 1). **d)** The number of eggs male fathered and the laying date of his social female. From top to bottom: Dark grey circles represent the number of fathered eggs taking extra-pair eggs gained into account; the black line is the prediction of this model. Grey circles represent the number of fathered eggs without taking extra-pair eggs gained into account; the medium grey line is the prediction of this model. White circles represent the number of number of social eggs, the light grey line is the prediction of this model.
Late-arriving males were significantly more likely to not father any egg throughout the season than early-arriving males ($\chi^2 = 6.90$, $p$-value = 0.01, estimate (±s.e.) = -0.19 (±0.08); Fig. 5.2). The majority of those males were bachelors (no social nest), although they were captured performing song displays near nest-boxes. A few bachelor birds were able to obtain paternity via extra-pair copulations (Fig. 5.2).

Early-arriving males did not have a significantly different probability of gaining EPP compared to late-arriving males. Arrival date of the male was not significant when considered as the only term in a simple regression and also not when analysed in conjunction with the laying date of the female (Table S5.4). The pattern of the simple regression was the same regardless of the inclusion of bachelor males or not. However the probability to gain EPP significantly declined with the laying date of a male’s social female ($\chi^2 = 4.39$, $p$-value = 0.04, estimate (±s.e.) = -0.07 (±0.04); Fig. 5.1c). Also, early-arriving males had earlier laying females than late-arriving males ($F_{1,70} = 16.37$, $p$-value <0.01, estimate (±s.e.) = 0.81 (±0.2); Fig. 5.1b).

Male arrival date either alone or in conjunction with egg-laying date of his female did not explain the total number of eggs that the male was able to father, independently on the EPP (Table S5.5). Laying date of a female was significantly related to the total number of eggs fertilised by her social mate throughout the season, also independently on whether EPP was taken into account or not (with EPP $F_{1,85} = 14.75$, $p$-value <0.01, estimate (±s.e.) = -0.09 (±0.02); only EPP lost $F_{1,85} = 13.69$, $p$-value <0.01, estimate (±s.e.) = -0.07 (±0.02); only social eggs $F_{1,85} = 9.71$, $p$-value <0.01, estimate (±s.e.) = -0.05 (±0.02); Fig. 5.1d) since clutch size normally declines over the season. As expected, the number of fathered eggs with EPP was significantly correlated to the number of social fathered eggs (without taking EPP into account) ($F_{1,85} = 47.87$, $p$-value <0.01, estimate (±s.e.) = 0.84 (±0.12)).
Males with early-laying females first fathered eggs in their own social nest and then eggs in other nests. With the progress of the season, though, the pattern reversed, with males with late-laying females first fathering eggs in another nest and then eggs in their own nest ($F_{1,13} = 17.18$, $p$-value <0.01, estimate (±s.e.) = -1.36 (±0.32; Fig. 5.3). This pattern was not observed in relation to the arrival date of the males (Table S5.5).

**Discussion**

Our study supports the well-known pattern that early arrival date leads to higher breeding success by increasing the chances of finding a mate and/or promoting early breeding, which is associated with larger clutch sizes. However, we did not find evidence supporting the hypotheses that early arrival date is associated with more EPP or less loss of paternity in the own social nest (Table 5.1). We observed a non-linear relationship between arrival date and paternity loss and a significant relationship between female laying date and the probability to gain EPP. This last result fully agrees with observations from pied flycatcher populations breeding in the south-western edge of the distributional range of the species (Canal *et al.* 2012a, 2012b).

Our results suggest that a gain in EPP for an early-arriving male would occur indirectly via the laying date of his social female and not exclusively due to his own arrival date. The lack of significant association between the probability of EPP and the arrival date of the male could be explained by the very large variation in the relationship between arrival and laying dates. Despite this relationship being significantly positive, there is much residual variance (Supplementary material, Fig. S5.1), as has been also described previously in our study site (de la Hera *et al.* 2013) and for other geographically close pied flycatcher populations (Both *et al.* 2016). Arrival dates of males, although repeatable across years, are not necessarily related to their breeding date in different years (Both *et
If EPP attempts is male initiated, breeding synchrony among individuals of the population can affect the relationship between laying date and EPP (Canal et al. 2012b). Thus, more synchronous breeding is associated with reduced rates of EPP, and this could, in turn, increase the difference between early and late breeders (Canal et al. 2012b). In the year that our data were collected (see Supplementary material, Fig. S5.1, for more details), the breeding synchrony was below the average (although still similar to other populations, such as those studied by Canal et al. 2012b). According to this, it could be argued that our lack of association between arrival date and EPP might be year-specific phenomena, caused by the low breeding synchrony in 2014. However, the explanation of Canal et al. (2012b) might also not apply here because it requires that arrival and laying dates are highly correlated or that such correlation became stronger in years with high breeding synchrony. Since this was not the case in our population (Fig. S5.1c), we do not think the relationship between arrival date and EPP changes significantly under different degrees of breeding synchrony, but a more grounded statement would require the repetition of this data collection in several years with distinct breeding synchrony.

In our results, when EPP is considered in relation to laying date, males that mated early (i.e., early laying dates of their social female) obtained extra-pair fertilisations shortly after their social female initiated a clutch, while males that obtained a social female relatively late in the season had first fertilised an extra-pair female shortly before fertilizing their social female or even before obtaining a social nest (i.e., while still a “bachelor”). This result partially supports the findings from Canal et al. (2012b) and suggests that the middle of the breeding season is the period when more fertile females are available and more chances exist of being cuckolded (Fig. 5.1c). Interestingly, if a similar comparison is done with arrival dates, the pattern changes: early and late-arriving males had a higher probability of losing paternity in their own nests than intermediate males (Fig. 5.1a), again suggesting that egg-laying date is a better predictor of EPP than arrival date.

We also acknowledge that other factors could also play a role in explaining the observed variation in EPP. Thus, it is also possible that our observed variation in EPP paternity probability could be explained by female choice of other male traits, such as body condition or some secondary sexual characters (Møller et al. 2003; Reudink et al. 2009) that are already known to contribute to paternity gain or loss in pied flycatchers (Sirkiä & Laaksonen 2009; Moreno et al. 2010; Canal et al. 2011). Moreover, female traits could also be important in explaining variation in EPP (Moreno et al. 2015). For our population, we had data on male’s blackness and front patch size, which are signs of attractiveness in other populations (Sirkiä & Laaksonen 2009). Thus we tested whether those were related to EPP (Supplementary material, Table S5.6). However, we did not find any relationship between these secondary sexual traits and male arrival date. Interpopulational variation of female preference on male trait exists (Galván & Moreno al. 2016).
2009) and this relationship between EPP and male traits are not always straightforward as shown in Moreno et al. (2010, 2013).

It is important to take the sampling moment into account when evaluating the differences between the present study and others that evaluated the relationship between arrival date and EPP (Table 5.1). Here, we looked at the egg stage to define fertilisation probability in relation to arrival time instead of using only samples of the surviving chicks. Although the final fitness measure is not affected by evaluating eggs instead of chicks, our method would have the potential to reveal details, such as a different survival probability of genetic and extra-pair offspring (Sardell et al. 2011), or explain the differences in paternity loss in relation to other studies (Table 5.1).

Another factor that could contribute to the dissimilarity of our results when compared to earlier works is the relatively low proportion of extra-pair broods in our population (19.8%, Table 5.1), which would reduce the power of the tests associating arrival date and paternity. Our rate of extra-pair broods is consistent with the values obtained in other pied flycatcher populations: 10.4 to 18.9% in Germany (Lubjuhn et al. 2000); 15% (Lifjeld et al. 1991) or 16% (Slagsvold et al. 2001) in Norway; 13% (Lehtonen et al. 2009) or 22% (Rätti et al. 1995) in Finland; 22.4% (Moreno et al. 2010) or 28.8% (Moreno et al. 2013) in Spain. However, Canal et al. (2012b) and Moreno et al. (2015) reported higher rates of nests involved in EPP (between 33 and 40%) in Spanish populations. We supposedly had a higher than normal rate for our population in comparison to previous years due to the high breeding asynchrony (Canal et al. 2012b) and indeed a much lower proportion was reported in a previous year for our population (<10%, de la Hera et al. 2013). Thus, for the majority of the populations, rates of EPP are generally low and fitness benefits of EPP are not always that clear (e.g., Slagsvold et al. 2001; Moreno et al. 2013).

We also attempted to sample as many bachelor males as possible, which allowed the observation that late arriving males have a higher probability of not obtaining paternity at all (similar results were obtained by Potti & Montalvo 1991). However, since bachelors comprise a different category of males and could behave very differently from breeding birds, their inclusion in the paternity gain analysis could offer another explanation on why our results were not consistent with previous studies. However, even when we excluded the bachelors, the results remained the same (Table 5.1), and thus the observed pattern is not related to a different behaviour of bachelor males. A very interesting outcome of the inclusion of bachelor birds in the analysis is the fact that they seem to be able to achieve some paternity. Some studies report that the identity of a few extra-pair father remained unknown, even when a high proportion of the breeding birds are identified (e.g., Canal et al. 2011), these missing individuals could either be birds breeding outside of the study area or even floaters. Here we provide evidence that it is indeed possible for a male pied flycatcher to obtain paternity without a social nest.
In conclusion, our results suggest that the component of the male pied flycatcher phenology that affects the variation in EPP is the laying date of its female. Although arrival date is significantly related to laying date, the arrival date effect on EPP will largely depend on how strong is the relationship between arrival and laying dates in a given year or population. Thus, considering all fitness components that affect selection of arrival date (clutch size, probability to obtain a female, etc.), the final contribution of EPP will probably be fairly small. Therefore, we suggest that in this long-distance migrant, the estimation of fitness of arrival date should not be largely affected when information on EPP is not available.

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Supplementary material

Details on the paternity assignment

We used the program Cervus (Kalinowski et al. 2007) to analyse the paternity of the offspring. The results obtained from Cervus are summarised in Table S5.1. Our analysis started with a total of 691 samples (488 eggs, 110 males and 93 females), but not all loci were recognisable in all cases.

Table S5.1. Summary statistics for the five microsatellite loci used in this study. Number of birds screened (n), number of alleles (NA), observed heterozygosity (HO), expected heterozygosity (HE), Weir and Cockerham’s (1984) within-population inbreeding coefficient (FIS), frequency of null alleles (FNull), exclusion probability of the locus for the first parent (P(Ex1)), exclusion probability of the locus for the second parent with the first assigned (P(Ex2)), and the exact probability for deviation from Hardy-Weinberg equilibrium (PH-W).

| Locus  | n  | NA | HO  | HE  | FIS | FNull | P(Ex1) | P(Ex2) | PH-W |
|--------|----|----|-----|-----|-----|-------|--------|--------|------|
| Fhyu234| 688| 29 | 0.93| 0.92| 0.92| 0.005 | 0.27   | 0.16   | 0.95 |
| Fhyu304| 691| 10 | 0.83| 0.83| 0.81| 0.001 | 0.5    | 0.33   | 0.001|
| Fhyu336| 691| 19 | 0.87| 0.87| 0.86| 0.002 | 0.4    | 0.25   | 0.29 |
| Fhyu448| 691| 23 | 0.92| 0.88| 0.87| 0.02  | 0.37   | 0.23   | 0.002|
| Fhyu453| 687| 24 | 0.89| 0.91| 0.9  | 0.009 | 0.32   | 0.19   | 0.003|

We had three loci that deviated from the Hardy-Weinberg equilibrium, therefore we verified the accuracy of these by re-visualizing all mismatches between known pairs of parent and offspring to obtain the final and accurate paternity data (Marshall et al. 1998; Hoffman & Amos 2005; Casey et al. 2011).

We also calculated the combined exclusion power of our five loci with the program Cervus, obtaining the following results:

- Combined exclusion probability (first parent): 0.9936;
- Combined exclusion probability (second parent): 0.99945;
- Combined exclusion probability (parent pair): 0.9999976.
Contingency tables for Fisher’s exact test

Table S5.2. Contingency tables used for the Fisher’s exact test described in the Methods section. a) Table containing all eggs in the (experiment described above) and used to test for differences in assignment between eggs with lay order 1 to 6 from eggs with lay order 7. b) Table containing all assigned eggs from Table S5.2a and used to determine whether lay order could affect proportion of EPP.

|     | Eggs 7 | Eggs 1 to 6 | Row total |
|-----|--------|-------------|-----------|
| a.  |        |             |           |
| Assigned | 10    | 314         | 324       |
| Not assigned | 14    | 35          | 49        |
| Column total | 24    | 349         | 373       |

|     | Eggs 7 | Eggs 1 to 6 | Row total |
|-----|--------|-------------|-----------|
| b.  |        |             |           |
| EPP | 2      | 21          | 23        |
| not EPP | 8    | 293         | 301       |
| Column total | 10    | 314         | 324       |

Sample sizes

Table S5.3. Sample sizes at different moments of the data collection from the estimated number of birds based on the number of broods (94) to the actual sampled and assigned birds.

|       | Detected (brood) | Sampled (incl. bachelors) | Assigned | With Arrival date | With lay date | With arrival and laying date |
|-------|------------------|---------------------------|----------|-------------------|---------------|-----------------------------|
| Males | 94               | 110                       | 109      | 87                | 87            | 72                          |
| Females | 94            | 93                        | 93       | n.a.              | n.a.          | n.a.                        |
| Eggs  | 555              | 488                       | 481      | n.a.              | n.a.          | n.a.                        |
| Total | 743              | 691                       | 683      | 87                | 87            | 72                          |
**Additional tables**

**Table S5.4.** Model results of individual simple and multiple logistic regression analysis, explaining the probability of gain extra-pair paternity, lose paternity or father an egg. Statistics are given at the point of exclusion from the model. In the case of significant quadratic terms, statistics for the other terms are given in the presence of the quadratic term. The initial sample size was two overlapping (but not the same) sets of 87 birds with known arrival and/or laying date of the female (and, from these, 72 had both arrival and laying date known). Estimates and standard errors are only given in case of significant terms.

| Response variable | Explanatory variables | Estimates | s.e. | df | Sample size | $\chi^2$ | $p$-value |
|-------------------|-----------------------|-----------|------|----|-------------|--------|-----------|
| Probability to gain extra-pair paternity | (Arrival date of male)$^2$ | 0.10 | 0.01 | 1 | 72 | 5.27 | 0.02* |
| Probability to gain extra-pair paternity | Arrival date of male | -0.12 | 0.48 | 1 | 72 | 5.84 | 0.02* |
| Probability to gain extra-pair paternity (simple regressions) | Lay date of female | -0.07 | 0.04 | 1 | 72 | 4.39 | 0.10 |
| Probability to lose paternity | (Arrival date of male)$^2$ | 0.03 | 0.01 | 1 | 72 | 5.27 | 0.02* |
| Probability to lose paternity | Arrival date of male | -1.12 | 0.48 | 1 | 72 | 5.84 | 0.02* |
| Probability to lose paternity | Lay date of female | -0.07 | 0.04 | 1 | 72 | 4.39 | 0.04* |
| Probability to father an egg (simple regressions) | Arrival date of male | -0.19 | 0.08 | 1 | 87 | 6.90 | 0.01* |
| Probability to father an egg (simple regressions) | Lay date of female | -0.07 | 0.04 | 1 | 87 | 4.39 | 0.10 |
Table S5.5. Model results of individual simple and multiple regression analysis, explaining the laying date of the female, the number of fathered eggs and the difference in laying date of social and extra-pair female. Statistics are given at the point of exclusion from the model. Estimates and standard errors are only given in case of significant terms.

| Response variable | Explanatory variables | Estimate | s.e. | ndf | ddf | $R^2$ (adjusted) | $F$-test | $p$-value |
|-------------------|-----------------------|----------|------|-----|-----|-----------------|---------|-----------|
| Lay date of the female | Arrival date of male | 0.81 | 0.20 | 1 | 70 | 0.18 | 16.37 | <0.01* |
| Number of fathered eggs (with EPP) | (Arrival date of male)^2 | 1 | 68 | 0.18 | 3.58 | 0.06 |
| | Arrival date of male | 1 | 69 | 0.15 | 0.48 | 0.49 |
| | Lay date of female | -0.09 | 0.02 | 1 | 85 | 0.14 | 14.75 | <0.01* |
| Number of fathered eggs (with EPP) (simple regressions) | Arrival date of male | 1 | 70 | -0.002 | 0.88 | 0.35 |
| | Lay date of female | -0.09 | 0.02 | 1 | 85 | 0.14 | 14.75 | <0.01* |
| Number of fathered eggs (only EPP lost) | (Arrival date of male)^2 | 1 | 68 | 0.18 | 4.07 | 0.05 |
| | Arrival date of male | 1 | 69 | 0.14 | 0.02 | 0.90 |
| | Lay date of female | -0.07 | 0.02 | 1 | 85 | 0.13 | 13.69 | <0.01* |
| Number of fathered eggs (only EPP lost) (simple regressions) | Arrival date of male | 1 | 70 | 0.01 | 1.91 | 0.17 |
| | Lay date of female | -0.07 | 0.02 | 1 | 85 | 0.13 | 13.69 | <0.01* |
| Number of social eggs | (Arrival date of male)^2 | 1 | 68 | 0.04 | 0.57 | 0.45 |
| | Arrival date of male | 1 | 69 | 0.04 | 1.29 | 0.26 |
| | Lay date of female | -0.05 | 0.02 | 1 | 85 | 0.09 | 9.71 | <0.01* |
| Number of social eggs (simple regressions) | Arrival date of male | 1 | 70 | -0.01 | 0.02 | 0.88 |
| | Lay date of female | -0.05 | 0.02 | 1 | 85 | 0.09 | 9.71 | <0.01* |
| Number of fathered eggs (with EPP) | Number of social eggs | 0.84 | 0.12 | 1 | 85 | 0.35 | 47.87 | <0.01* |
| Number of fathered day 12 chicks (with EPP) | (Arrival date of male)^2 | 1 | 68 | 0.05 | 0.97 | 0.33 |
| | Arrival date of male | 1 | 69 | 0.05 | 2.38 | 0.13 |
| | Lay date of female | -0.06 | 0.02 | 1 | 85 | 0.05 | 5.31 | 0.02* |
| Number of fathered day 12 chicks (with EPP) (simple regressions) | Arrival date of male | 1 | 70 | -0.01 | 0.37 | 0.55 |
| | Lay date of female | -0.06 | 0.02 | 1 | 85 | 0.05 | 5.31 | 0.02* |
| Number of fathered day 12 chicks (no gained EPP) | (Arrival date of male)^2 | 1 | 67 | 0.04 | 1.05 | 0.31 |
| | Arrival date of male | 1 | 68 | 0.04 | 1.64 | 0.20 |
| | Lay date of female | -0.05 | 0.02 | 1 | 84 | 0.04 | 4.39 | 0.04* |
| Number of fathered day 12 chicks (no gained EPP) (simple regressions) | Arrival date of male | 1 | 69 | -0.01 | 0.16 | 0.69 |
| | Lay date of female | -0.05 | 0.02 | 1 | 84 | 0.04 | 4.39 | 0.04* |
| Difference (lay date social - EP nest) | (Arrival date of male)^2 | 1 | 9 | 0.51 | 0.02 | 0.89 |
| | Arrival date of male | 1 | 10 | 0.56 | 1.59 | 0.24 |
| | Lay date of female | -1.36 | 0.32 | 1 | 13 | 0.54 | 17.18 | <0.01* |
| Difference (lay date social - EP nest) (simple regressions) | Arrival date of male | 1 | 11 | -0.08 | 0.11 | 0.75 |
| | Lay date of female | -1.36 | 0.32 | 1 | 13 | 0.54 | 17.18 | <0.01* |
Arrival and breeding synchrony

Because breeding synchrony could be an important factor in determining the amount of extra-pair paternity in a given year (Canal et al. 2012b), we calculated the breeding synchrony for all years we had laying date data in our population using the formula from Kempenaers (1993). We assumed that the fertile period of the female ranged from 5 days before the first egg was laid until the egg-laying date of the penultimate egg. The higher the index, the more synchronous the population. The year 2014 had one of the lowest values of breeding synchrony in our population, which would predict a large amount of extra-pair paternity (Fig. S5.1a). For comparison, we also calculated the synchrony in arrival dates showing that 2014 was an average year in terms of spread of arrival dates (Fig. S5.1b).

Observed low breeding synchrony in 2014 not only predicts a large amount of EPP but also a weaker relationship between EPP and female laying date (Canal et al. 2012b). Thus, sampling in years with higher breeding synchrony could lead to a stronger relationship between EPP and laying dates. A similar effect on male arrival date, however, would only happen if the association between arrival and laying dates is strong (i.e., large R-squared). Or if the strength of this association increases in a context of high breeding synchrony. Since neither scenario was supported by our data (Fig. S5.1c), it is unlikely that sampling another year with a higher breeding synchrony would have led to a stronger/significant relationship between arrival date and EPP. Likewise, a decrease in the standard deviation in arrival dates does not lead to a stronger association between arrival and laying dates (Fig. S5.1d).

Figure S5.1. a) Breeding synchrony index from 1964 to 2015; b) Standard deviation of arrival dates from 2005 to 2015; c) R-squared of the relation between arrival and laying dates in relation to the breeding synchrony index from 2005 to 2015 (p-value = 0.69); d) R-squared of the relation between arrival and laying dates in relation to the standard deviation of arrival dates from 2005 to 2015 (p-value = 0.39). The black dot represents 2014, the year in which this data collection took place. The dashed line represent the average value for the breeding synchrony index and standard deviation of arrival dates in plots a and b, respectively.
Extra-pair paternity and male characteristics

Since other characteristics could also be correlated to EPP, we tested using our dataset whether male blackness and front patch size could be correlated to probability of gain and lose paternity, father eggs and also on the number of fathered eggs and chicks. In 2014 for our population, there was not effect of male characteristics on these components (Table S5.6).

Table S5.6. Model results of individual multiple (logistic) regression analysis explaining the probability of gain extra-pair paternity and lose paternity and the number of fathered eggs and chicks in relation to male blackness and front patch size. Statistics are given at the point of exclusion of the term from the model. Estimates and standard errors are only given in case of significant terms.

| Probability to gain extra-pair paternity | Estimates | s.e. | df | Sample size | χ² | p-value |
|-----------------------------------------|-----------|------|----|-------------|----|---------|
| Arrival date of the male : Blackness    | 1         | 97   |    | 0.30        | 0.58 |
| Arrival date of the male : Front patch size | 1       | 97   |    | 0.12        | 0.73 |
| Lay date of the female : Blackness      | 1         | 97   |    | 0.23        | 0.63 |
| Lay date of the female : Front patch size | 1       | 97   |    | 0.92        | 0.34 |
| Arrival date of the male                | 1         | 97   |    | 0.41        | 0.52 |
| Lay date of the female                  | -0.07     | 0.04 | 1  | 4.39        | 0.04*|
| Blackness                               | 1         | 97   |    | 0.73        | 0.39 |
| Front patch size                        | 1         | 97   |    | 0.04        | 0.84 |

| Probability to lose paternity            | Estimates | s.e. | df | Sample size | χ² | p-value |
|------------------------------------------|-----------|------|----|-------------|----|---------|
| Arrival date of the male : Blackness     | 1         | 97   |    | 0.12        | 0.73 |
| Arrival date of the male : Front patch size | 1       | 97   |    | 0.55        | 0.46 |
| Lay date of the female : Blackness       | 1         | 97   |    | 0.99        | 0.32 |
| Lay date of the female : Front patch size | 1       | 97   |    | 0.02        | 0.88 |
| Arrival date of the male                 | 1         | 97   |    | 0.64        | 0.43 |
| Lay date of the female                   | 1         | 97   |    | 0.54        | 0.46 |
| Blackness                                | 1         | 97   |    | 2.03        | 0.15 |
| Front patch size                         | 1         | 97   |    | 1.39        | 0.24 |

| Probability to father an egg              | Estimates | s.e. | df | Sample size | χ² | p-value |
|------------------------------------------|-----------|------|----|-------------|----|---------|
| Arrival date of the male : Blackness     | 1         | 97   |    | 0.04        | 0.84 |
| Arrival date of the male : Front patch size | 1       | 97   |    | 0.06        | 0.80 |
| Arrival date of the male                 | -0.19     | 0.08 | 1  | 6.90        | 0.01*|
| Blackness                                | 1         | 97   |    | 0.10        | 0.76 |
| Front patch size                         | 1         | 97   |    | 0.12        | 0.73 |

| Number of fathered eggs (with EPP)        | Estimates | s.e. | ndf | ddf | F-test | p-value |
|------------------------------------------|-----------|------|-----|-----|--------|---------|
| Arrival date of the male : Blackness     | 1         | 56   | 0.67| 0.42|
| Arrival date of the male : Front patch size | 1       | 54   | 0.01| 0.94|
| Lay date of the female : Blackness       | 1         | 55   | 0.51| 0.48|
| Lay date of the female : Front patch size | 1       | 73   | 1.31| 0.26|
| Arrival date of the male                 | 1         | 57   | 0.33| 0.57|
| Lay date of the female                   | -0.09     | 0.02 | 87 | -3.84| <0.01*|
| Blackness                                | 1         | 74   | 0.02| 0.88|
| Front patch size                         | 1         | 75   | 0.81| 0.37|
Part III.

Causes and consequences: breeding, moult and departure
