Extra-pair paternity, breeding density and synchrony in natural cavities versus nestboxes in two passerine birds

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Abstract

Most of what is known about extra-pair paternity in hole-nesting birds derives from studies using artificial nesting sites, such as nestboxes. However, it has rarely been investigated whether inference drawn from breeding events taking place in nestboxes matches what would be observed under natural conditions, i.e. in natural cavities. We here report on a variation in promiscuity in blue tits and great tits nesting in natural cavities and nestboxes in an urban forest in Warsaw, Poland. Specifically, we tested whether breeding density, breeding synchrony and extra-pair paternity (inferred from SNP data generated with a high-throughput genotyping by sequencing method) differed between birds nesting in natural cavities and nestboxes. In both blue and great tits, the frequency of extra-pair paternity was similar between the two cavity types. Breeding density and synchrony was higher in nestboxes than in natural cavities in blue tits, but not in great tits. Moreover, the occurrence of extra-pair paternity in the nest in great tits increased with the number of neighbours and the number of synchronously breeding neighbours within a 120 m radius from the focal nest, regardless of cavity type. In blue tits, the proportion of extra-pair offspring per nest increased with neighbour density within 160 m of the focal nest, both in natural cavities and nestboxes. Our results indicate that providing nestboxes can alter the distribution of individuals in space and time, which potentially influences mating outcomes among breeders outside the social bond. Whilst these parameters should be carefully considered when comparing studies and/or sites, we highlight that conclusions drawn from nestbox studies on natural variation in extra-pair matings might be adequate in some species, and/or sites, but not in others.

Keywords: natural cavity, nestbox, extra-pair paternity, cavity-nesting birds, SNP data, genotyping by sequencing method

Introduction

Mating systems are influenced by the spatio-temporal distribution of individuals, which impacts encounter rates and the timing of mating opportunities (Westneat & Stewart, 2003). An important attribute of avian mating systems is extra-pair paternity, a behaviour reported for the first time c. four decades ago: the application of molecular tools to avian ecology revealed actual mating patterns and true genetic parentage of offspring (Griffith et al., 2002). While social bonds between mates are widespread in birds (Cockburn, 2006), there is now pervasive evidence of mating outside the social bond in 76% of sampled socially monogamous species with biparental care (but note the phylogenetic and geographical bias: only <4% of all avian biodiversity and 47% of passerine families have been sampled; Brouwer & Griffith, 2019). Interestingly, there is great variation in genetic polyandry among species, populations and individuals of the same species (Petrie & Kempenaers, 1998; Griffith et al., 2002; Cornwallis et al., 2010). Several adaptive and non-adaptive hypotheses have been suggested to explain extra-pair paternity (see Brouwer & Griffith, 2019 for an overview). Briefly, females might engage in extra-pair copulations to ensure the fertilization of their clutch in case of infertility of their social mate, maximize the genetic diversity among their offspring, obtain “good genes'' for their offspring, maximize the genetic compatibility with the genetic mate, seek direct benefits and resources, or avoid male harassment (Sheldon, 1994; Wetton & Parkin, 1991; Westneat et al., 1990; Foerster et al., 2003; Birkhead & Møller, 1992; Hamilton, 1990; Møller, 1988; Burke et al., 1989; Colwell & Oring, 1989; Wolf, 1975). In addition, ecological drivers such as breeding density, breeding synchrony, latitude, life-history traits and predation rates have been identified to influence extra-pair paternity rates and suggested to explain variation among and within species (Westneat et al., 1990; Charmantier & Perret, 2004; Stutchbury & Morton, 1995; Wink & Dyrcz, 1999; Yuta & Koizumi, 2016).

Extra-pair paternity in secondary cavity nesters has been widely investigated in studies that involve breeding in artificial cavities (i.e. nestboxes) (Kempenaers et al., 1992; Dunn & Robertson, 1993; Gowaty & Bridges, 1991). In general, nestboxes have become the standard reference in ecology and evolution because of the convenience of sampling and experimental manipulation (Lambrechts et al., 2010; Wesołowski, 2011) and therefore knowledge stemming from these studies is perceived as the reference state. However, nestboxes might represent a source of bias for studies investigating cavity nesters, whose original nesting place is a tree hollow. Tree hollows are cavities naturally occurring in old-growth stands or excavated by primary cavity nesters, such as woodpeckers. A handful of studies contrasted reproductive performance between birds breeding in natural cavities and nestboxes at the same location and uncovered biological and ecological differences between the two types of cavities (East & Perrins, 1988; Johnson & Kermott, 1994; Miller, 2002; Czeszczewik, 2004; Llambías & Fernández, 2009; Robertson & Rendell, 1990; Sudyka et al., 2022a). Made of plywood or woodcrete (a mixture of timber and concrete), with standard dimensions and wall thickness, nestboxes differ from tree hollows in several aspects. Compared to natural cavities, they provide a far less stable microclimate in terms of temperature and humidity, they have lower buffering capabilities against ambient conditions (Maziarz et al., 2017; Sudyka et al., 2022b) but provide protection from nest soaking or flooding, which in contrast is very common in natural cavities (Wesołowski et al., 2002; Sudyka et al., 2022a). Moreover, nestboxes and natural cavities differ in terms of ectoparasite loads (Wesołowski & Stańska, 2001), with high levels observed in wooden nestboxes. Predation pressures have been shown to differ between natural cavities and nestboxes, which typically provide better protection from a variety of nest predators (Miller, 2002; Czeszczewik, 2004), possibly leading to increased productivity and nesting success (Norris et al., 2018 but see Johnson & Kermott, 1994). There is also evidence that nestboxes may create artificial breeding densities (Perrins, 1979; Tiainen et al., 1984) and increase proximity among individuals, with consequences for reproductive outputs (Pöysä and Pöysä, 2002). Higher breeding density may also increase encounter rates and mating opportunities outside the social bond since most extra-pair matings occur between close neighbours (Møller, 1991; Canal et al., 2012; Mayer & Pasinelli, 2013; Schlicht et al., 2015). For instance, manipulating nestbox availability increased the likelihood of extra-pair paternity in some species (Gowaty & Bridges, 1991; Charmantier & Perret, 2004; Stewart et al., 2010), despite the fact that other studies failed to find any relationship between breeding density and frequency of extra-pair paternity in other species (Dunn et al., 1994; Tarof et al., 1998). Since extra-pair paternity is the result of females and males interacting in space and time, both the spatial distribution of individuals (i.e. breeding density) and the temporal concentration of fertile females (i.e. breeding synchrony) create opportunities for extra-pair copulations. Contrasting hypotheses have been suggested regarding breeding synchrony, thereby synchronous or asynchrounous breeding might favour extra-pair matings (see for example Stutchbury & Morton, 1995 and Arlt et al., 2004). Under synchronous conditions, females can compare the quality of many males simultaneously (Kempenaers et al., 1992) or alternatively males have the opportunity to seek extra-pair copulations with many fertile females (Stutchbury & Morton, 1995). In contrast, asynchronous breeding might allow males to seek extra-pair copulations when their own mate is no longer fertile, once they are free from mate-guarding (Neudorf, 2004). Artificial breeding densities created by nestboxes might therefore interact with breeding synchrony to affect extra-pair paternity.

Given how much nestboxes differ from natural cavities, criticism has been raised over the ecological validity of results derived from nestbox studies (Møller, 1992; Wesołowski, 2007; Lambrechts et al., 2010; Wesołowski, 2011; but see Koenig et al., 1992). It is therefore important to establish whether trait variation observed in nestboxes is representative of the natural variation occurring in tree hollows and assess the general significance of the conclusions drawn from nestbox studies. This is true for quantifying the baseline promiscuity levels in both types of cavities and also to infer possible consequences generated by those levels, such as offspring body condition, physiology, survival or lifetime reproductive success, which were shown to differ between extra-pair and within-pair offspring (Magrath et al., 2009; Schmoll et al., 2009; Sardell et al., 2012; Bowers et al., 2015). Here, we investigated variation in extra-pair paternity levels in blue tits and great tits (*Cyanistes caeruleus* and *Parus major* respectively) breeding in natural cavities and nestboxes in the same urban forest in Warsaw, Poland. Blue tits and great tits are small passerine cavity nesters, breeding readily in nestboxes and equally widespread in natural and urban environments. They are socially monogamous with biparental care and varying levels of extra-pair paternity among populations (Cramp & Perrins, 1993; Gullberg et al., 1992; see Brouwer & Griffith, 2019 for an overview of rates of extra-pair paternity). In this study, we tested whether providing nestboxes influenced the spatial and temporal component of extra-pair mating behavior by contrasting rates of extra-pair paternity between two environmentally homogenous plots within the same urban forest: one plot had natural cavities without any nestboxes, while the other plot was supplemented with nestboxes. We predicted that in both species the nestbox plot would have a higher local density of breeding pairs and higher levels of extra-pair paternity relative to the natural cavity plot. We further predicted that this would translate into a larger number of broods with extra-pair offspring in the nestbox plot and a higher proportion of extra-pair offspring in nestboxes.

Materials and methods

Study site and field methods

Detailed information on the study site and field methods is given in Sudyka et al., 2022a. In short, the study was carried out on wild blue tits and great tits breeding in natural cavities and nestboxes in Bielany Forest, a natural reserve within the city limits of Warsaw (52°17'37.0"N 20°57'22.6"E) over two consecutive seasons (2018 and 2019). Bielany Forest (ca. 150 ha) is a remnant of the Mazovian Primeval forest, with multi-species and uneven-aged stands, and protected under the Natura 2000 scheme. Naturally occurring and excavated cavities were monitored in the SE part of the reserve in a 50 ha area plot, where study birds mainly inhabit cavities in hornbeams (*Carpinus betulus*) and common oaks (*Quercus robur*). In late January 2018, 65 woodcrete Schwegler 1b nestboxes, with a 32 mm diameter entrance, were set up 50 meters apart from each other in the NW part of the forest, in an overall area of 15 ha. Nestboxes were hanged at approximately 2.91 m height with a random orientation, without any protective device against predation (Sudyka et al., 2022a). The minimum distance between the edges of the two plots was 200 m. The two plots share the same environment and are functionally homogeneous: food availability (assessed in the same study years as frass fall collection) is similar between plots, as well as ambient temperature and humidity and anthropogenic variables, such as sound and air pollution (Wesołowski & Rowiński, 2014; Sudyka et al., 2022a; Sudyka et al., 2022b). At the start of the field season, baseline conditions were alike between the two cavity types, as nest material naturally degrades in natural cavities (Wesołowski, 2000; Sudyka et al., 2022a) and nestboxes were cleaned to remove old nesting material and/or winter roosts. From the end of March/beginning of April, nest searches started in the natural cavity plot to locate as many active nests as possible at the nest building stage. After 2-3 weeks, search efforts were reduced and individual nest monitoring followed. In May, nest searches were temporarily resumed in order to locate nests of parents that failed their first clutch. In 2019 we also performed a nest search in the nestbox plot, during which 16 nests in natural cavities were discovered. Since we were only able to sample nestlings in 3 of these nests, due to their limited accessibility and high number of nest failures before the nests were accessed (see details in Sudyka et al., 2022a), neither parental nor nestling genetic data were included in the main analyses. Each cavity was accessed (whenever possible) with ladders or by climbing on trees (except for ground-level cavities) and inspected using portable led lights, mirrors and an NTS200 Digital Inspection Camera (NovoTech Industries Ltd.), equipped with a 8.2 mm camera head at the end of a 5 m long probe. Nestboxes were inspected weekly from the beginning of April to record all breeding events. From the onset of incubation onwards, each nest was monitored individually. Laying date, clutch size, number of hatchlings and number of fledglings were recorded for each nest. During ringing, blood samples were collected from adults and nestlings by puncturing the brachial vein with a sterile needle into heparin-free capillary tubes and preserved in 99% ethanol at +4 °C until DNA isolation (when blood sampling was unsuccessful, a tail feather was collected to ensure that genetic material from all offspring was available for analyses). We sampled nestlings at the age of 14 days (hatching day = 0) to make sampling time uniform between cavity types, as in natural cavities it is not possible to extract nestlings safely from the nest at a younger age (see Appendix A for information on the proportion of the brood sampled between cavity types). Blood and tissue (feather or muscle) samples were also collected from dead nestlings and preserved in the same way. Only first clutches were included in the analyses, as second clutches (i.e. a second nesting attempt after successfully fledging young in an earlier nesting attempt) were very rare: five clutches out of a total of 282 across 2 years (1.8%). Nests were marked for coordinates with a GPSMAP® 64 (Garmin).

Genetic analysis, parentage and sex assignment

Genomic DNA was extracted using the Blood Mini kit from 1112 blood samples and the Genomic Mini kit from 71 feather and tissue samples (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s protocol, with the modification of overnight incubation at 37 °C. DNA concentration and purity was assessed with a DeNovix DS-11 spectrophotometer. DNA sequencing was outsourced to Diversity Arrays Technology Pty, Ltd (Canberra, AU) and performed using DArTseqLD, a high-throughput genotyping by sequencing method that employs genomic complexity reduction using restriction enzyme pairs (Kilian et al., 2012). Details on the DArT sequencing technology can be found in Appendix B. All subsequent analyses were performed in R (version 4.1.2) (R Core Team, 2021) and run separately for each species. We filtered out individuals and loci with call rate below 70% using *dartR* (version 1.9.9.1) (Gruber et al., 2018). Genetic relationships among pooled individuals from natural cavity and nestbox plots were estimated using the function *snpgdsGRM* with the method GCTA (Yang et al., 2011) implemented in *SNPRelate* (version 1.26.0) (Zheng et al., 2012) and represented with a histogram (Figure 1 shows the distribution of relatedness zoomed in on related individuals). The resulting Genomewide Relatedness Matrix (GRM) was compared with a social pedigree of all individuals ringed in the field, created using *ggroups* (version 2.1.0) (Nilforooshan & Saavedra-Jiménez, 2020).

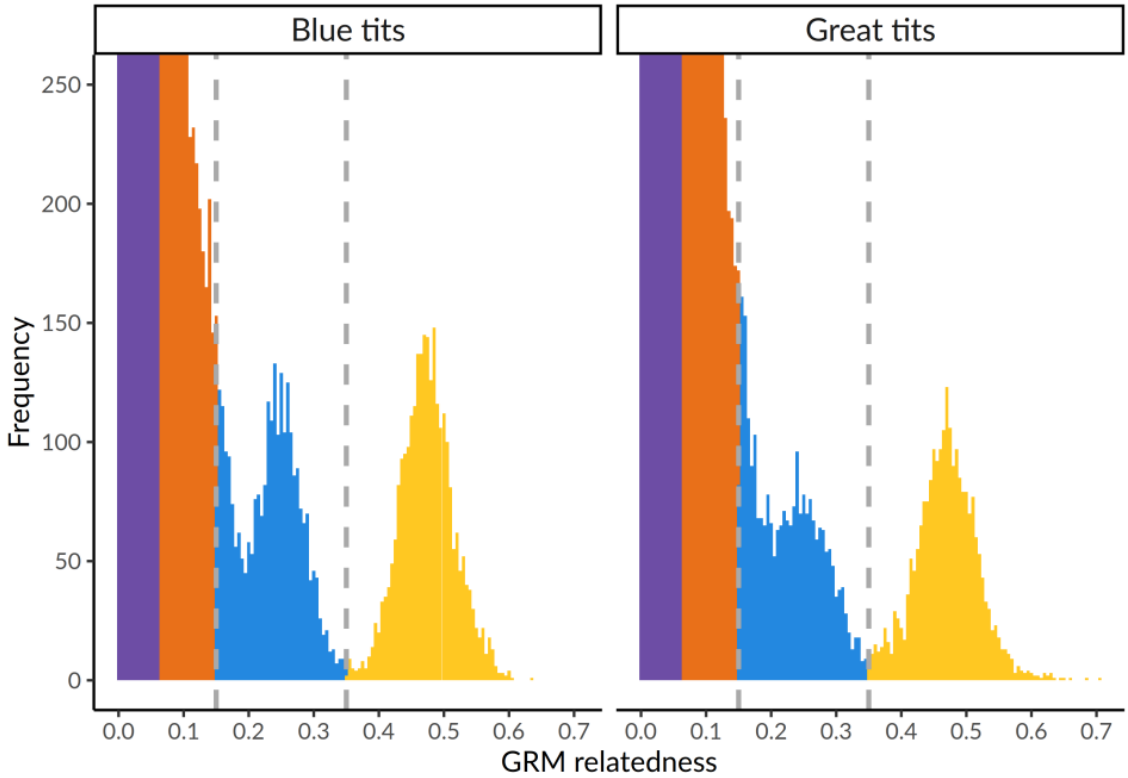

Figure 1 – Distribution of GRM values zoomed in on related individuals. Color intervals are indicative of different coefficients of relatedness: purple indicates 1/16; orange 1/8; blue 1/4 (reflecting half-sibling relationships) and yellow 1/2 (full sibling and parent-offspring relationships). Vertical dotted lines indicate cut-off values used to identify half-siblings, full-siblings and extra-pair paternities.

Aligning the GRM against the social pedigree (Figure 2) allowed to detect cases of erroneous pedigree relationships (due to observational errors or brood parasitism) and extra-pair paternities. Cuckolded fathers, extra-pair fathers, extra-pair offspring and broods with unknown parents containing half-siblings were identified based on discrepancies between the GRM and the social pedigree following Perrier et al. (2018), as described here. Father – offspring pairs (social relatedness = 0.5) showing GRM relatedness estimates above 0.35 were classified as within-pair paternities (falling within the yellow band in Figure 2). Father – offspring pairs (social relatedness = 0.5) showing GRM relatedness estimates below 0.15 were classified as instances of extra-pair paternity (falling within the orange/purple band in Figure 2). Adult males with GRM relatedness estimates above 0.35 with offspring from other nests (social relatedness = 0) were identified as extra-pair fathers (falling within the yellow band in Figure 2).

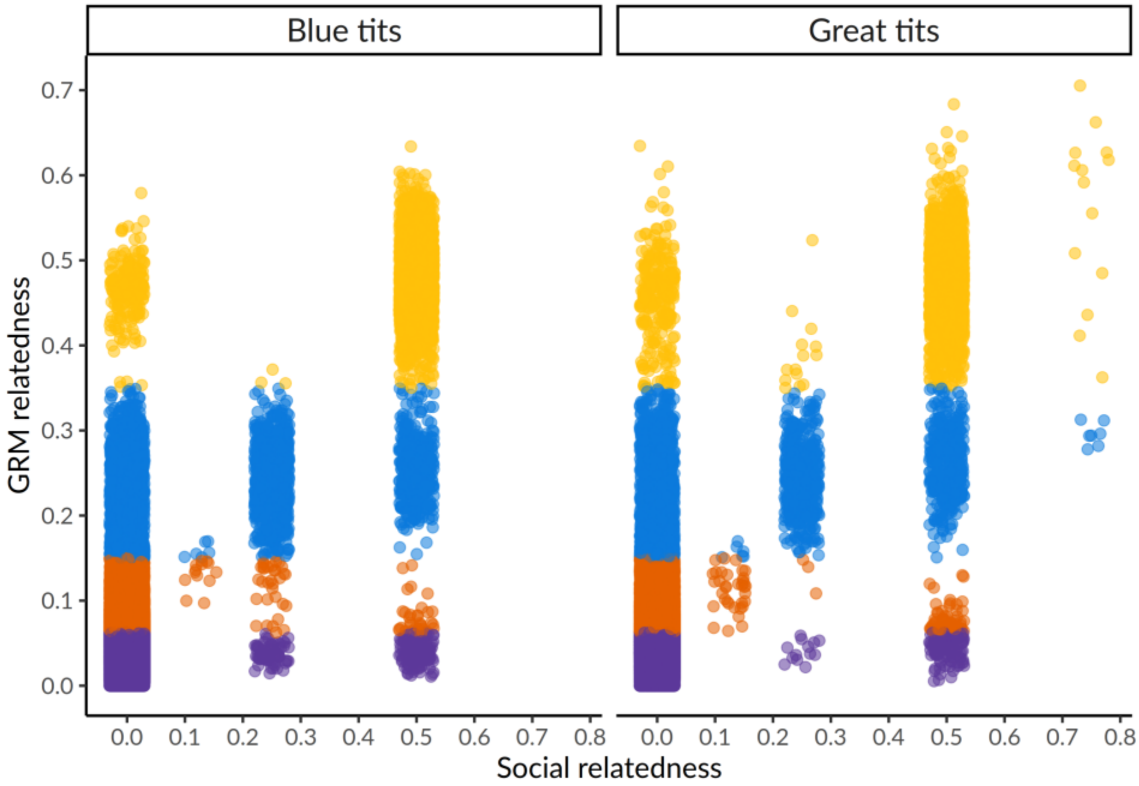

Figure 2 - Biplot of GRM and social relatedness values among all individuals illustrating the concordances and discrepancies between the two. GRM and social information are not always concordant and GRM gives in-depth information on relatedness that is often not represented by social pedigrees. Color intervals are indicative of different coefficients of relatedness: purple indicates 1/16; orange 1/8; blue 1/4 (reflecting half-sibling relationships) and yellow 1/2 (reflecting full sibling and parent-offspring relationships).

False positives between extra-pair father and offspring relationships (namely male full siblings from previous years) were identified by checking against field records. It was not possible to identify which particular nestlings were extra-pair in nests where the social father was not sampled, but we established whether the brood contained full or half-siblings. Pairs of siblings within a given nest (social relatedness = 0.5) with GRM estimates between 0.15 and 0.35 were classified as half-siblings (falling within the blue band in Figure 2) and above 0.35 as full siblings (falling within the yellow band in Figure 2). Nestlings with GRM relatedness estimates below 0.1 to both social parents and social siblings (social relatedness = 0.5) were classified as instances of brood parasitism (falling within the orange/purple band in Figure 2). Sex in nestlings was determined using a machine-learning population assignment approach. First, we identified SNPs that diverged between known females and males by looking for markers with sex differences in heterozygosity and showing high FST and by using BayeScan 2.1 (Brelsford et al., 2017; Trenkel et al., 2020; Foll & Gaggiotti, 2008). BayeScan was run with default parameter options and outlier SNPs were identified with a q-value below 0.05. The identified markers (11 in blue tits and 7 in great tits) were then used to assign sex to 939 nestlings with *assignPOP* (version 1.2.2) (Chen et al., 2018). Figure S1 shows the assignment accuracy of Monte-Carlo cross-validation.

Spatio-temporal parameters

To study the effects of breeding density and breeding synchrony in natural cavities and nestboxes at the local level, for each given nest we estimated: (i) nearest neighbour distance (distance in meters to the nearest conspecific occupied nest); (ii) neighbour density (number of conspecific nests within three different radii around each nest) and (iii) synchronous neighbour density (number of conspecific neighbouring females whose fertile period overlapped with that of the focal female within each of the three chosen radii; the fertile period was calculated from 2 days prior of onset of laying for blue tits and from 5 days for great tits until the penultimate day of laying; Møller, 1991; Yezerinac & Weatherhead, 1997; Johnson et al., 2002). The three radii used to estimate neighbour density and synchronous neighbour density were chosen as follows. We calculated the median distance between the nest of extra-pair fathers identified in the population and the nest where they sired extra-pair offspring: 160 m in blue tits and 120 m in great tits. As extra-pair fathers were identified in only a limited number of nests (n = 8 blue tit and n = 5 great tit nests), we additionally repeated the analysis with radii two and three times larger than the original estimate: 320 m and 480 m in blue tits and 240 m and 360 m in great tits. Nearest neighbour distance is the distance to the nearest possible extra-pair partner, while the other two measures within each radius reflect the number of extra-pair mating opportunities within the neighbourhood of a focal nest. Results for the smallest radius are presented in the main text, while those for the other two are presented in the Appendix. Additionally, neighbour density and synchronous neighbour density estimation was repeated after including data from natural cavities monitored in the nestbox plot in 2019 (see Appendix). All calculations were run for each year and each species separately.

Statistical analysis

At the plot level, to compare promiscuity between cavity types (natural cavity vs nestbox), we used chisquare tests on the proportion of nests with extra-pair paternity relative to the overall number of nests and on the proportion of extra-pair offspring relative to the overall number of nestlings. We also performed a chi-square test to determine whether the proportion of extra-pair nests and extra-pair offspring differed between study years. We used Mann-Whitney U-tests on the three spatio-temporal parameters to test for differences between cavity types (also including natural cavities in the nestbox plot found in 2019) and between years. In addition, we used Mann-Whitney U-tests on the proportion of extra-pair young per nest in nests with extra-pair paternity between the natural cavity and nestbox plot. For the analyses at the nest level, we employed generalized linear mixed-effects models with a binomial error distribution and a logit link function to test for the effects of cavity type (binary response: natural cavity vs. nestbox) and the three spatio-temporal parameters on two response variables: occurrence of extra pair paternity in each nest and proportion of extra-pair young per nest. Occurrence of extra pair paternity for each brood was coded as 0 (no extra-pair offspring) or 1 (at least one extra-pair offspring). The three spatio-temporal parameters showed moderate correlations (Table S2) and were therefore separately included as covariates in three models. Year was included as a fixed factor, laying date, squared laying date and clutch size as covariates and mother identity as random effect. Squared laying date was removed from final models whenever nonsignificant. Because of model convergence failure, when testing the occurrence of extra-pair paternity in great tits we discarded the random term and used generalized linear models. When testing the proportion of extra-pair offspring we excluded nests where the social father was not sampled, as well as 2 blue tit nests and 3 great tits nests where less than 50% of the clutch was sampled. Additionally, at the individual level, we employed a generalized linear mixed model with a binomial error distribution and a logit link function to investigate the effect of cavity type and sex on being an extra-pair offspring. The response variable was offspring status as within-pair or extra-pair (0/1). A similar model was used to test for the effect of being extra-pair on a proxy of fitness (i.e. successful fledging of nestlings sampled at day 14) in the two cavity types (natural cavity vs nestbox); offspring were coded as 0 (not fledged) or 1 (fledged) as response variable. In both these models, cavity type, year and sex were included as fixed factors, and body index, laying date and clutch size were covariates. Body index was calculated as 'scaled mass index' following Peig & Green (2009). In the model for fledging success, offspring status as within-pair or extra-pair was also included as a fixed factor. Nest identity was introduced as a random effect in both models. In all the above models, two-way interactions between cavity type and other predictors were initially tested and removed whenever not significant. All models were checked for dispersion, zero inflation and multicollinearity (VIF scores in each model never exceeded 4). All statistical analyses were performed in R 4.1.3 (R Core Team, 2021), separately for each species.

Results

Genetic paternity

For blue tits, a total of 118 adults and 497 nestlings were successfully sequenced, together with 114 great tit adults and 402 nestlings. In each species, the distribution of GRM values among individuals from the two plots combined together (Figure 1) showed the presence of parent-offspring (yellow), full sibling (yellow) and half-sibling relationships (blue). Males with 0.5 social relatedness and GRM relatedness below 0.15 with their social offspring represented cuckolded fathers (n = 25 in blue tits and n = 25 in great tits; Figure 2); males with 0 social relatedness and GRM relatedness above 0.35 with offspring from other nests were identified as extra-pair fathers (n = 7 blue tits and n = 4 great tits;

Figure 2); extra-pair offspring were identified by having 0.5 social relatedness and GRM relatedness below 0.1 with their social father and 0 social relatedness and GRM relatedness above 0.35 with males from other nest (n = 44 in blue tits and n = 50 in great tits; Figure 2); in nests with unknown father identity, nestlings with 0.5 social relatedness and GRM relatedness between 0.15 and 0.35 with each other were identified as half-siblings (n = 3 nests in blue tits and n = 3 nests in great tits; Figure 2). One blue tit nestling with GRM relatedness estimates below 0.1 to the other nestlings in the nest and to both the social parents (0.5 social relatedness) was classified as an instance of brood parasitism (Figure 2).

No clear effect of cavity type on extra-pair paternity at the plot level

Figure 3 shows the nest distribution within Bielany Forest with information on extra-pair paternity. In blue tits, 37% of broods in the natural cavity plot (n = 11 of 30) and 46% in the nestbox plot (n = 17 of 37) contained at least one extra-pair offspring. 8% of blue tit nestlings in the natural cavity plot (n = 16 of 197) and 12% in the nestbox plot (n = 28 of 237) were the result of extra-pair fertilizations (Figure 4). In great tits, 38% of broods in the natural cavity plot (n = 12 of 32) and 62% in the nestbox

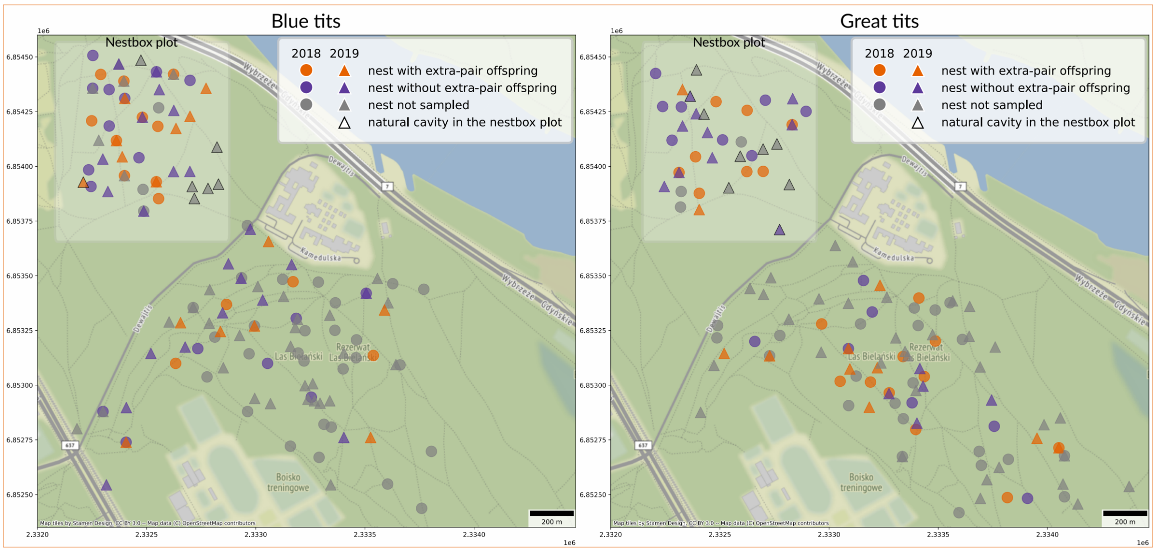

Figure 3 – Map of blue tit and great tit nests from Bielany Forest in 2018 and 2019 with information on extra-pair paternity. Grey symbols represent clutches that failed before day 14 and inaccessible nests in natural cavities.

plot (n = 16 of 26) contained at least one extra-pair offspring, and 11% of nestlings in the natural cavity plot (n = 20 of 179) and 16% in the nestbox plot (n = 30 of 189) were sired by males other than their social father (Figure 4).

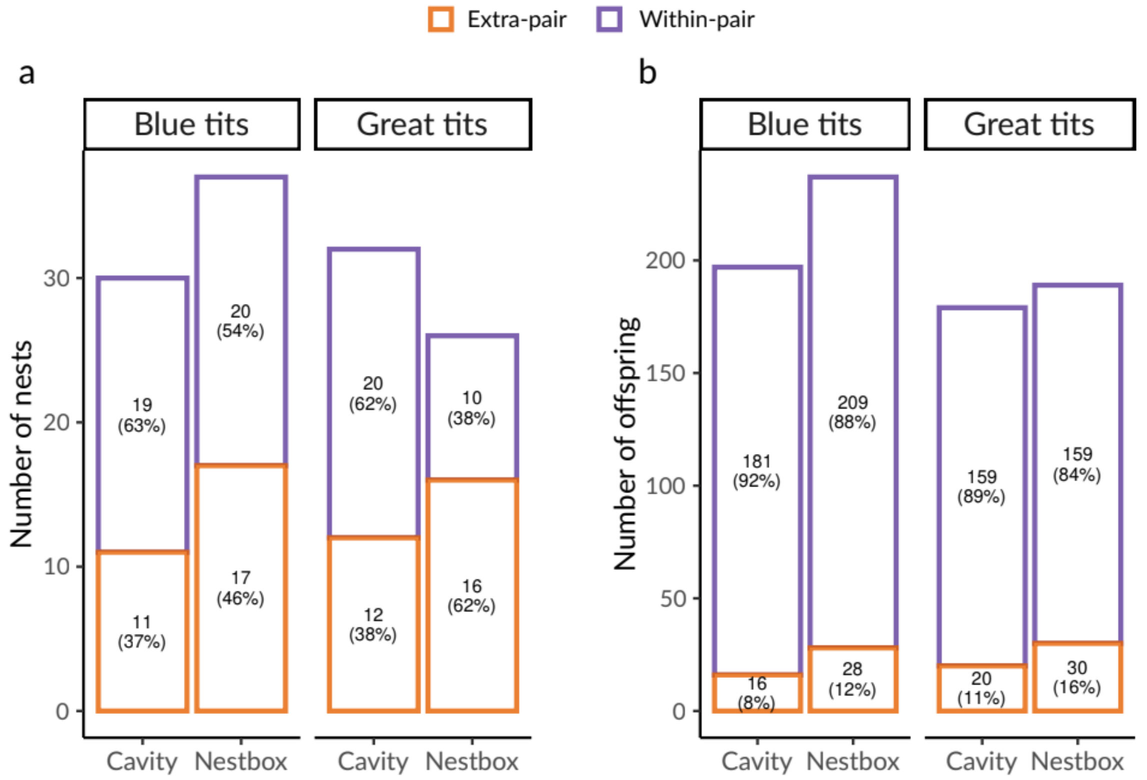

Figure 4 - Number of extra-pair and within-pair (a) nests and (b) offspring in blue tits and great tits by cavity type (percentages in parentheses).

Despite recording both a larger number of broods with extra-pair offspring and more extra-pair offspring in nestboxes in both species, the trend was not significant, either in terms of proportion of extra-pair broods (blue tits: $\chi^2 = 0.267$, df = 1, P = 0.605; great tits: $\chi^2 = 2.427$, df = 1, P = 0.119 but note the tendency for a higher proportion in the nestbox plot) or in terms of proportion of extra-pair offspring (blue tits: $\chi^2 = 1.230$, df = 1, P = 0.267; great tits: $\chi^2 = 1.352$, df = 1, P = 0.245). There was no year effect on the proportion of broods containing at least one extra-pair offspring (blue tits: $\chi^2 = 0.073$, df = 1, P = 0.787, n = 67; great tits: $\chi^2 = 0.238$, df = 1, P = 0.626, n = 58) or on the proportion of extra-pair offspring in the population (blue tits: $\chi^2 = 0.319$, df = 1, P = 0.572, n = 434; great tits: $\chi^2 = 2.801$, df = 1, P = 0.094, n = 368, but note the tendency for a higher proportion in 2019). No brood consisted entirely of extra-pair young either in blue or great tits over the 2 years in either plot. Table S3 shows the number of broods with the number of extra-pair offspring per cavity type. There was no difference in the proportion of extra-pair young per nest between natural cavity and nestbox plot in nests with extra-pair offspring neither in blue tits (W = 71.5, P = 0.867, n = 23) nor in great tits (W = 90.5, P = 0.403, n = 22) [2 blue tit nests and 3 great tits nests were excluded because less than 50% of the clutch was sampled] (Figure 5). No difference was also observed between study years (blue tits: W = 74, P = 0.596; great tits: W = 44, P = 0.347).

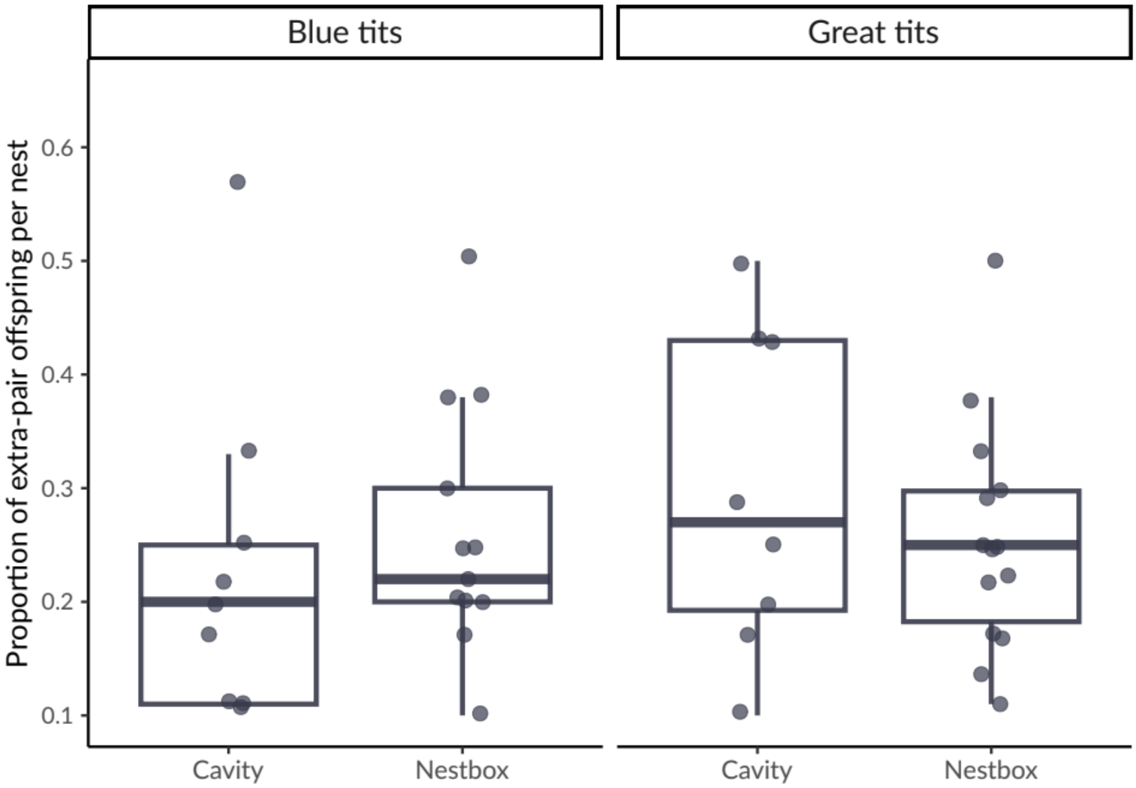

Figure 5 - Distribution of the proportion of extra-pair offspring relative to within-pair offspring per nest in blue tits and great tits, by cavity type. Nests where the social father was sampled were included.

Spatio-temporal parameters differ between cavity types in blue tits but not in great tits

In blue tits, the average breeding density for the two years was 11.8 pairs/10 ha in the natural cavity plot and 17.7 pairs/10 ha in the nestbox plot, while in great tits there were 10 and 12.2 breeding pairs in the natural cavity and nestbox plot, respectively. At the plot level, nearest neighbour distance was significantly shorter in the nestbox plot than in the natural cavity plot in blue tits (W = 2424, P = 0.024, n = 131; median nearest neighbour distance for natural cavities: 95 m and nestboxes: 79 m), but not in great tits (W = 1024, P = 0.131, n = 116, despite a tendency for longer nearest neighbour distance in nestboxes; median nearest neighbour distance for natural cavities: 82 m and nestboxes: 88 m). In blue tits, neighbour density and synchronous neighbour density were significantly lower in natural cavities than in nestboxes within a radius of 160 m and 320 m (n = 131; Table 1). In great tits, synchronous neighbour density within 240 m was lower in natural cavities than in nestboxes and neighbour density within 360 m was higher in natural cavities than in nestboxes (n = 116; Table 1).

Table 1 – Mann-Whitney U-tests on neighbour density and synchronous neighbour density in the three investigated radii for each species. Significant differences (P < 0.05) are in bold, trends (P < 0.2) in italics.

	Radius	**Neighbour density**				**Synchronous neighbour density**			
		W	**P**	**Median number in the cavity plot (range)**	**Median number in the nestbox plot (range)**	**W**	**P**	**Median number in the cavity plot (range)**	**Median number in the nestbox plot (range)**
Blue tits	**160 m**	1374	**0.005**	2 (0-7)	3 (1-6)	1259	**<0.001**	1 (0-5)	2 (0-6)
	320 m	1363.5	**0.004**	9 (1-17)	11 (6-18)	1290	**0.001**	4 (0-13)	7 (0-13)
	480 m	1926	0.891	18 (2-31)	18 (13-22)	1560	*0.057*	9 (1-20)	12 (1-18)
Great tits	**120 m**	1163	0.509	1 (0-4)	1 (0-3)	1091.5	0.240	0 (0-3)	1 (0-2)
	240 m	1367	0.500	5 (0-11)	5 (1-8)	862.5	**0.010**	2 (0-7)	4 (0-7)
	360 m	1598.5	**0.032**	10 (2-21)	8 (1-15)	995.5	*0.089*	5 (0-12)	6 (0-13)

In blue tits, when including natural cavities found in the nestbox plot in 2019, spatio-temporal parameters between cavity types differed in the same way as without those nests (Table S4). In great tits no difference between cavity types emerged with natural cavities found in the nestbox plot (but note the tendency for lower synchronous neighbour density in the natural cavity plot within 240 m; Table S4). Density parameters were uniform between the two study years in blue tits (Table S5). In contrast, in great tits neighbour density within 240 m and synchronous neighbour density within 360 were lower in 2019 compared to 2018 (Table S5). Qualitatively identical results were found when testing for differences among cavity types separately for each year (see Appendix C).

Relationship between spatio-temporal parameters and extra-pair paternity

At the nest level, nearest neighbour distance had no effect on the occurrence of extra-pair paternity in blue tits (χ^2 = 0.819, P = 0.365) or great tits (LR χ^2 = 0.502, P = 0.479). In blue tits, none of the remaining investigated variables affected the occurrence of extra-pair paternity in the brood (but note the tendency for a positive relationship with neighbour density within 160 m; Table 2; Table S6).

Table 2 - Generalized linear mixed models for blue tits and generalized linear models for great tits with presence of extra-pair paternity in the nest (0/1) as the dependent variable. Cavity type, year, laying date, squared laying date, clutch size, neighbour density (model a) and synchronous neighbour density (model b) were included as predictors. Social mother identity was introduced as random effect in models for blue tits. Squared laying date was removed from final models whenever nonsignificant. Nonsignificant interactions between cavity type and other main factors were removed. Reference levels were: natural cavity (cavity type) and 2018 (year). Significant differences (P < 0.05) are in bold, trends (P < 0.2) in italics.

Occurrence of extra-pair paternity in the nest						
	Blue tits (n = 52) **160 m**			**Great tits (n = 48)** **120 m**		
		Estimate (SE)	**Pr(>χ^2)**		**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	0.349 (0.236)	*0.139*	**Neighbour density**	0.832 (0.411)	**0.030**
	Cavity type	0.291 (0.653)	0.655	**Cavity type**	1.140 (0.793)	*0.141*
	Year	0.133 (0.634)	0.834	**Year**	0.782 (0.926)	0.392
	Laying date	-0.094 (0.414)	0.820	**Laying date**	1.781 (0.683)	**0.002**
	Clutch size	-0.138 (0.212)	0.514	**Clutch size**	-0.397 (0.265)	*0.117*
	Laying date^2			**Laying date^2**	0.798 (0.388)	**0.028**
Model b	**Synchronous neighbour density**	0.127 (0.268)	0.634	**Synchronous neighbour density**	1.036 (0.497)	**0.031**
	Cavity type	0.402 (0.710)	0.571	**Cavity type**	0.636 (0.761)	0.404
	Year	0.207 (0.637)	0.745	**Year**	0.729 (0.905)	0.415
	Laying date	0.135 (0.383)	0.724	**Laying date**	1.915 (0.701)	**0.001**
	Clutch size	-0.061 (0.210)	0.771	**Clutch size**	-0.400 (0.256)	*0.104*
	Laying date^2			**Laying date^2**	0.884 (0.403)	**0.018**

In great tits, the occurrence of extra-pair paternity in the nest was higher in early and late broods, in smaller clutches and with increasing neighbour density and synchronous breeding density within 120 m (Table 2; Figure 6).

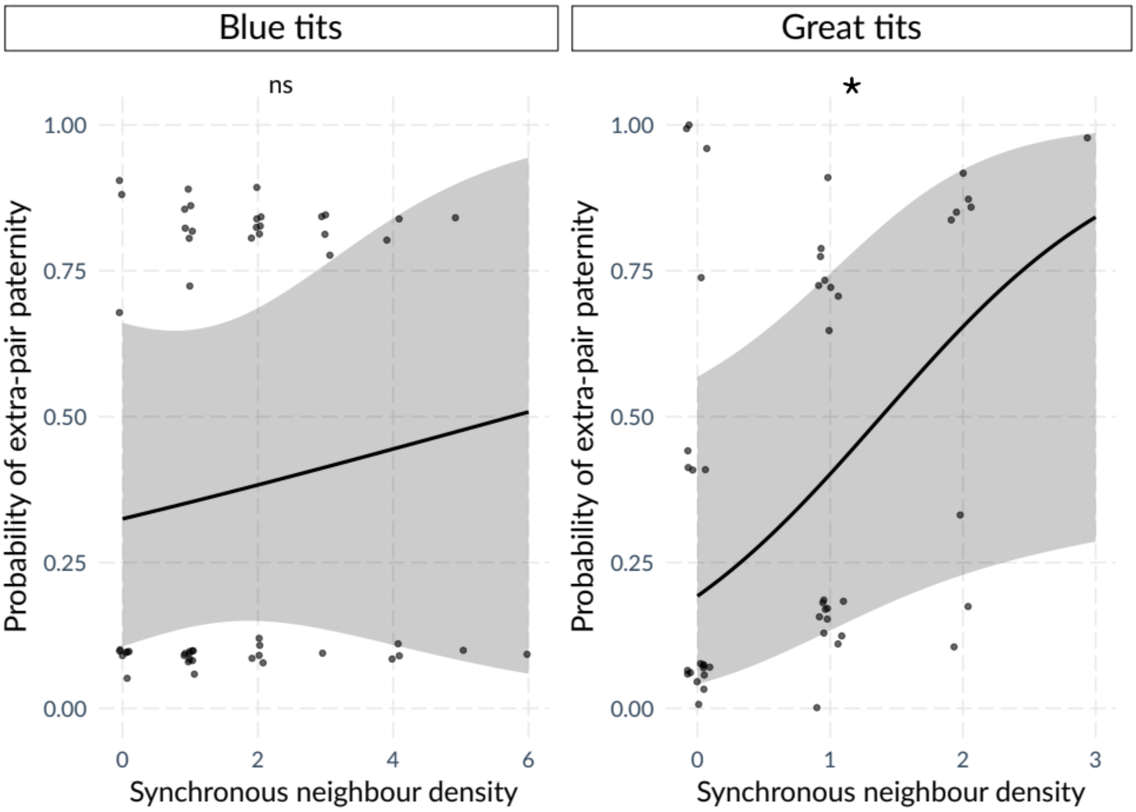

Figure 6 – Occurrence of extra-pair paternity at the nest level relative to the number of synchronous neighbours within 160 m in blue tits and 120 m in great tits (based on values reported in Table 2). Predicted values with 95% confidence interval are shown. ns = non significant; * denotes 0.01≤p≤0.05.

Within 240 and 360 m, great tit neighbour density and synchronous neighbour density were not associated with occurrence of extra-pair paternity (Table S6). In blue tits, the proportion of extra-pair offspring per nest increased with increasing neighbour density within 160 m (Table 3; Figure 7). It also tended to increase within 480 m and to decrease with increasing clutch size (Table 3; Table S7). There was also a tendency for a lower proportion of extra-pair offspring with increasing nearest neighbour distance (χ^2 = 2.340, P = 0.126). Cavity type had an effect when in interaction with synchronous neighbour density within 160 m (Table 3): the proportion of extra-pair offspring increased with increasing synchronous neighbour density in natural cavities. In great tits, the proportion of extra-pair offspring per nest was explained by a significant interaction between cavity type and year (from the minimal model: $\chi^2 = 4.29$, P = 0.038). In nestboxes it was higher in 2019 compared to 2018 and in 2019 it was higher in nestboxes compared to natural cavities. Nearest neighbour distance had no effect on the proportion of extra-pair offspring per nest ($\chi^2 = 0.464$, P = 0.496), similarly to the other spatio-temporal parameters in all 3 radii and to the other investigated variables (Table 3; Table S7).

Table 3 - Generalized linear mixed models with proportion of extra-pair offspring per nest as the dependent variable. Cavity type, year, laying date, squared laying date, clutch size, neighbour density (model a) and synchronous neighbour density (model b) were included as predictors. Mother identity was introduced as a random effect. Squared laying date was removed from final models whenever nonsignificant. Reference levels were: natural cavity (cavity type) and 2018 (year). Significant differences ($P < 0.05$) are in bold, trends ($P < 0.2$) in italics.

Proportion of extra-pair offspring per nest						
	Blue tits (n = 50) **160 m**			**Great tits (n = 44)** **120 m**		
		Estimate (SE)	**Pr(>χ^2)**		**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	0.403 (0.154)	**0.009**	**Neighbour density**	0.038 (0.259)	0.884
	Cavity type	0.012 (0.514)	0.981	**Cavity type**	-0.310 (0.690)	0.448
	Year	-0.226 (0.494)	0.647	**Year**	-0.411 (0.777)	0.213
	Laying date	-0.131 (0.316)	0.678	**Laying date**	0.249 (0.277)	0.369
	Clutch size	-0.259 (0.149)	*0.082*	**Clutch size**	-0.024 (0.184)	0.896
				Cavity type x Year	1.984 (1.029)	*0.054*
Model b	**Synchronous neighbour density**	1.119 (0.523)	0.568	**Synchronous neighbour density**	0.136 (0.305)	0.656
	Cavity type	1.643 (0.885)	0.740	**Cavity type**	-0.368 (0.691)	0.512
	Year	-0.424 (0.514)	0.410	**Year**	-0.437 (0.766)	0.224
	Laying date	-0.156 (0.318)	0.625	**Laying date**	0.252 (0.272)	0.354
	Clutch size	-0.261 (0.157)	*0.096*	**Clutch size**	-0.032 (0.180)	0.860
	Cavity type x Synchronous neighbour density	-1.161 (0.560)	**0.038**	**Cavity type x Year**	1.982 (1.020)	*0.052*

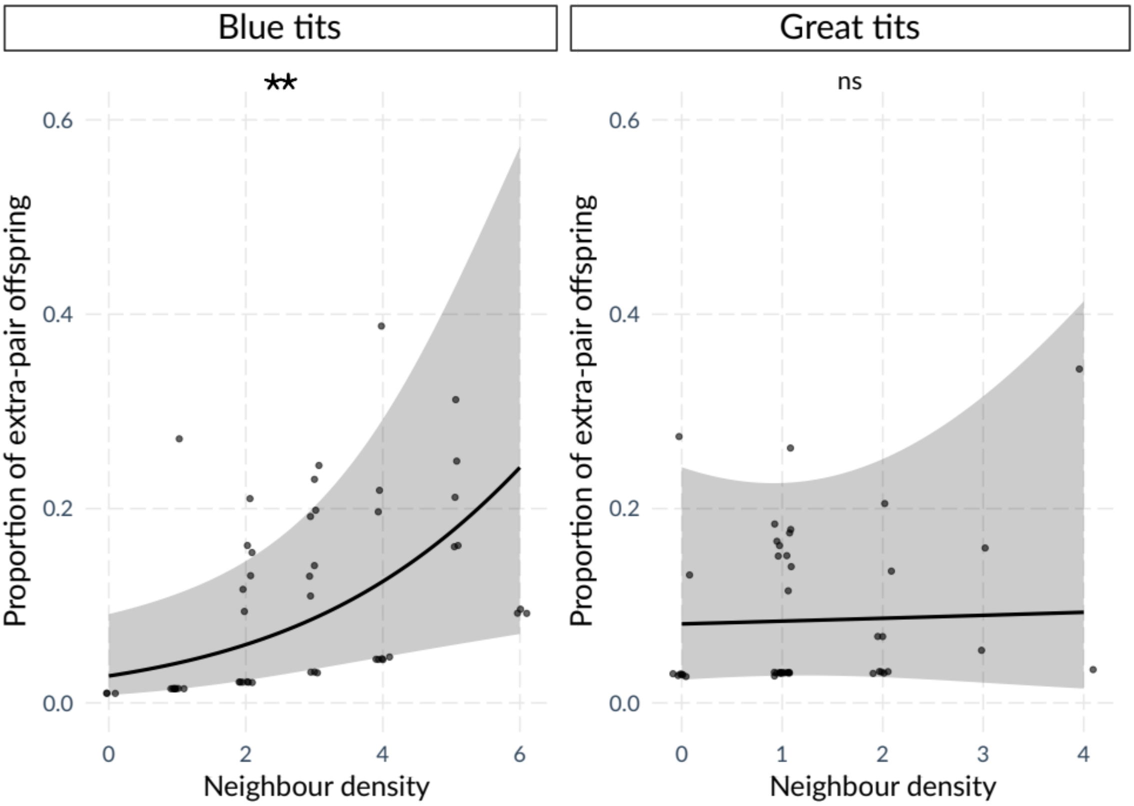

Figure 7 – Proportion of extra-pair offspring per nest (number of extra-pair offspring over total number of nestlings in the nest) in relation to the number of neighbours within 160 m in blue tits and 120 m in great tits (based on values reported in Table 3). Predicted values with 95% confidence interval are shown. ns = non significant; ** denotes $0.001 \leq p \leq 0.01$.

Extra-pair paternity and cavity types at the individual level

At the individual level, variation in being extra-pair in blue tits was explained by a significant interaction between cavity type and sex: in the nestbox plot, female nestlings had higher probability of being extra-pair than in the natural cavity plot (Table 4; Figure 8). In great tits, the probability of being extra-pair was not explained by any of the investigated variables (Table 4). In blue tits, late mortality rate did not differ between extra-pair and within-pair young ($\chi^2 = 0.041$, P = 0.841) or between sexes ($\chi^2 = 1.637$, P = 0.201). In great tits, low sample size led to model convergence issues so it was not possible to test for successful fledging.

Table 4 - Generalized linear mixed model testing the probability of nestlings of being within-pair or extra-pair (0/1; dependent variable), with cavity type, year, sex, clutch size and body index as predictors. Reference levels were: natural cavity (cavity type), 2018 (year) and female (sex). Significant differences ($P < 0.05$) are in bold, trends ($P < 0.2$) in italics. Interactions between nest type and other main factors were kept only if significant.

Probability of being extra-pair				
	Blue tits (n = 401)		**Great tits (n = 333)**	
	Estimate (SE)	**Pr(>χ^2)**	**Estimate (SE)**	**Pr(>χ^2)**
Cavity type	1.442 (0.658)	0.302	0.617 (0.551)	0.262
Year	-0.323 (0.500)	0.516	0.842 (0.582)	*0.148*
Clutch size	-0.109 (0.147)	0.456	-0.030 (0.184)	0.870
Laying date	0.028 (0.268)	0.918	0.266 (0.259)	0.304
Sex	1.046 (0.647)	0.858	0.489 (0.373)	*0.190*
Body index	-0.387 (0.216)	*0.074*	-0.038 (0.230)	0.870
Cavity type*Sex	-1.703 (0.795)	**0.032**		

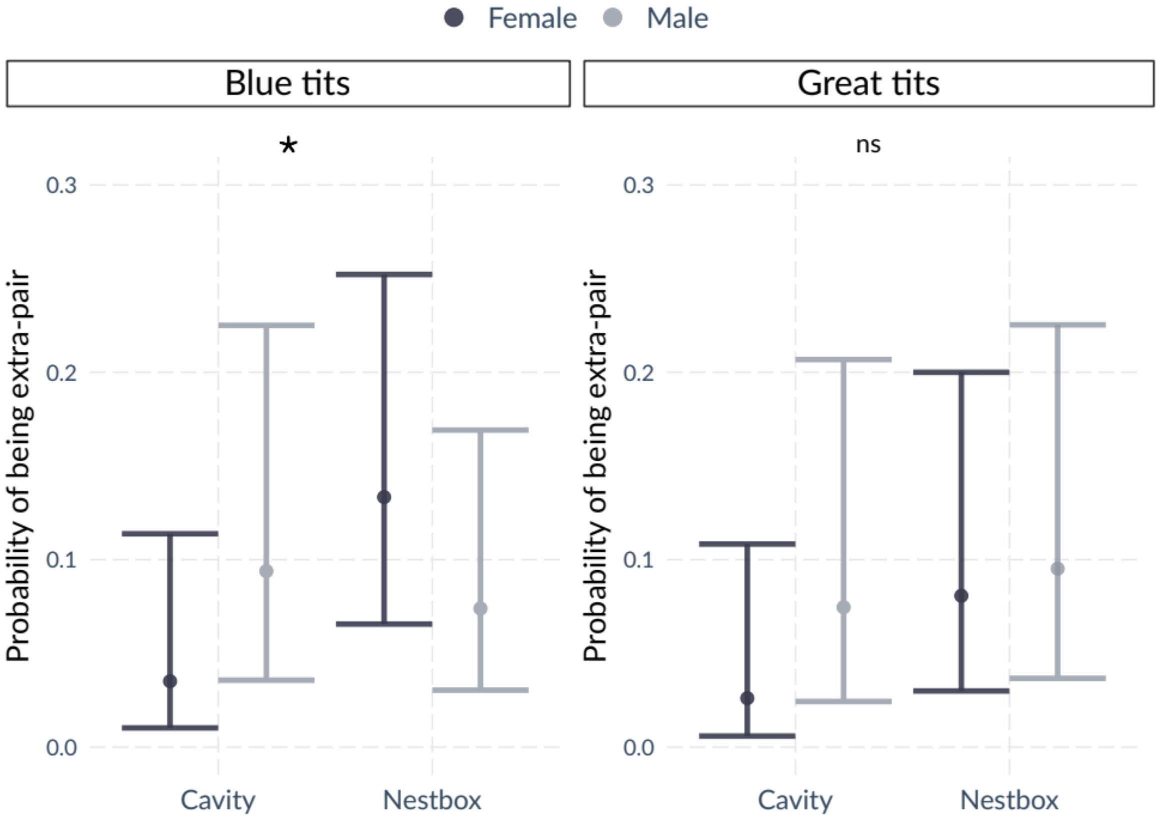

Figure 8 – Probability of being extra-pair for blue tit and great tit offspring with respect to cavity type and sex. Predicted values with 95% confidence interval are shown. ns = non significant; * denotes $0.01 \le p \le 0.05$.

Discussion

Similar and statistically identical rates of extra-pair paternity were detected at the plot level in blue tits and great tits breeding in natural cavities and nestboxes within the same urban forest. Nearest neighbour distance, neighbour density and synchronous neighbour density differed between cavity types in blue tits, with nestboxes reducing the distance between closest neighbours and increasing the total number of neighbours and the number of fertile neighbours around the nest. Furthermore, the occurrence of extra-pair paternity in the nest in great tits was predicted by the number of neighbours and the number of synchronously fertile neighbours within 120 m of the focal nest. In blue tits, the proportion of extra-pair offspring per nest was positively related to neighbour density within 160 m. These results suggest that the distribution of nestboxes may alter the spatio-temporal distribution of individuals, which potentially affects variation in extra-pair paternity.

Extra-pair paternity and cavity type

Rates of extra-pair paternity for blue tits and great tits breeding in nestboxes were similar to those observed in natural cavities. In both species, no differences between cavity types emerged in terms of proportion of mixed broods, overall proportion of extra-pair offspring or proportion of extra-pair offspring per nest. This contrasts with our predictions of higher extra-pair paternity levels in the nestbox plot. To date, several studies have compared and uncovered differences in breeding phenology and reproductive success between birds breeding in natural cavities and nestboxes. However, very few studies have investigated whether mating systems might be affected by cavity type (Barber et al., 1996; Llambías & Fernández, 2009, Kaluthota & Rendall, 2017). Our results confirm previous observations of similar extra-pair paternity levels in tree swallows breeding in natural cavities and nestboxes (Barber et al., 1996), but do not match patterns found in house wrens, where nestbox studies reported two to three times higher rates of polygyny in nestboxes compared to natural cavities (Kaluthota & Rendall, 2017 and references within). This might indicate a species-specific pattern, since this study reports that the type of cavity (natural vs artificial) does not correlate with extra-pair paternity in blue tits and great tits (although we found a tendency for a higher proportion of extra-pair broods in nestboxes in great tits). Therefore, inference drawn from nestbox studies might adequately represent the natural variation of traits in some species but not in others.

While blue tits were found to be more sensitive than great tits in terms of reproductive success when breeding in nestboxes relative to natural cavities (thereby exhibiting lower fledging success and fledging number, longer time spent in the nest and later fledging date in nestboxes; Sudyka et al., 2022a), this sensitivity was not reflected by variation in promiscuity, as reported here. In great tits, the probability for a brood of containing extra-pair offspring decreased with clutch size. Interestingly, previous reports uncovered that larger clutches included a lower proportion of extra-pair offspring in blue tits (Vedder et al., 2012). It has been hypothesized that a declining likelihood of extra-pair offspring over the laying order might be a general relationship in all species, as females cease to engage in extra-pair copulations at the onset of laying (Magrath et al., 2009; Vedder et al., 2012). The occurrence of extra-pair paternity in the nest in great tits was also associated with timing of breeding in a quadratic manner, independently of cavity type (no difference in laying date between cavity types was observed in our populations; Sudyka et al., 2022a). Several studies showed that extra-paternity increased as the season progressed (Lubjuhn et al., 2001; Beheler & Rhodes, 2003; Major & Barber, 2004; but see Stewart et al., 2010). Females might gain extra-pair copulations more easily later in the season when their mates are caring for fledglings. Alternatively, females that paired later in the season might be in poor-quality habitats or mated with poor-quality mates, and therefore more likely to engage in extra-pair copulations (Møller, 1992). Higher extra-pair paternity in early broods has been suggested to occur in migratory species, when mates are not able to accurately assess their partner quality, resulting in hasty pairings with poor-quality individuals (Spottiswoode & Møller, 2004). However, this explanation does not likely apply to blue tits and great tits from Bielany Forest, which are mostly residents (or short-space migrants).

At the individual level, we found an association between offspring sex and cavity type in blue tits: extra-pair offspring were female-biased in the nestbox plot. It is not easy to explain this observation, which is opposite to the expected outcome of sex allocation theory predicting that females should produce more sons than daughters among extra-pair offspring, since male offspring yield greater fitness benefits (Westneat et al., 1995; Sheldon & Ellegren, 1996). It is possible that the observed difference is not related to paternity but to a measure of environmental or parental quality that co-varies with extra-pair paternity, which we did not assess. For instance, females in poor condition are predicted to produce more often female than male offspring (Cockburn et al., 2002; Dietrich-Bischoff et al., 2006). However, we assessed food availability in the same study years as frass fall collection and feather colouration of breeding adults as an indicator of parental condition and we found no difference between the two cavity types (Sudyka et al., 2022a; Janas et al., 2022 preprint). Alternatively, since blue tits produced fewer fledgings in nestboxes in the same forest (Sudyka et al., 2022a), this result might stem from early selective mortality of male offspring, which require a bigger investment than females.

Extra-pair paternity, breeding density and breeding synchrony

Overall nest density was comparable between the nestbox and the natural cavity plot. Breeding densities were higher than those observed in a natural habitat such as Białowieża National Park (average 4.0 pairs/10 ha for blue tits and 4.9 pairs/10 ha for great tits; Wesołowski et al., 2010), but comparable to those found from nestbox studies in other urban and forest habitats (Dhondt, 2010). At the local scale, nearest neighbour distance in blue tits was lower in natural cavities than in nestboxes, similarly to neighbour density and synchronous neigbour breeding density within a radius of 160 m and 320 m of the focal nest. This result was further corroborated when natural cavities found in the nestbox plot in 2019 were included (Table S4). In great tits there was no difference in breeding density and synchrony between cavity types, with the exception of synchronous neighbour density within 240 m and neighbour density within 360 m. However, these differences were not fully confirmed when including natural cavities found in the nestbox plot (Table S4). Our results for blue tits are in line with previous observations of nestboxes creating artificially high breeding densities compared to natural populations, but this pattern might not be general across species as it was not apparent in great tits. In blue tits the proportion of extra-pair offspring per nest increased with neighbour density within 160 m and in great tits neighbour density and synchronous neighbour density were predictors of extra-pair paternity at the nest within a 120 m radius independently of cavity type. There was also a tendency for occurrence of extra-pair paternity to increase with neighbour density within 160 m in blue tits.

Previous studies have shown a positive effect of breeding density and synchrony on extra-pair paternity and even a positive interaction term between the two variables (Thusius et al., 2001; Westneat & Mays, 2005; Stewart et al., 2010; Mayer & Pasinelli, 2013). At greater breeding densities, potential extra-pair mates might be more accessible and searching costs for them might be low (Birkhead and Møller, 1992; Westneat et al., 1990). At the same time, breeding synchrony may facilitate extra-pair mate choice by females (Stutchbury & Morton, 1995). However, there is also evidence of a negative effect of breeding synchrony on extra-pair paternity, which may derive from increased risks of paternity loss or male harassment (Dunn et al., 1994; Westneat & Gray, 1998; Stewart et al., 2006; Olsen et al., 2008). This difference among studies and species is likely linked to species-specific differences pertaining to which sex initiates or controls extra-pair copulations and differences in mate-guarding behavior. In our study, the distance to the nearest neighbour was smaller in the nestbox plot than in the natural cavity plot in blue tits. However, the proximity of the nearest neighbour did not affect the occurrence of extra-pair paternity in either species. Nearest neighbours have been identified as sires of extra-pair offspring in some species, where nearest neighbour distance influences the frequency of extra-pair paternity (Bollinger & Gavin, 1991; Gray, 1997; Perreault et al., 1997). However there is also evidence that extra-pair sires travel longer distances (Rowe et al., 2001; Westneat & Mays, 2005; Balenger et al., 2009; Meek et al., 1994). These contrasting results have also been found within species: for instance, in blue tits Charmantier and Perret (2004) found that nearest neighbour distance influenced extra-pair paternity, but nearest neighbours accounted only for 39.3% of extra-pair paternities, confirming previous results showing that extra-pair sires were not always closest neighbours (Kempenaers et al., 1997). Similarly, six of the seven extra-pair fathers identified in our population sired offspring farther away than the closest neighbouring nest, at 149, 157, 158, 164, 230 and 742 m. Likewise in great tits three over four identified extra-pair fathers sired offspring at 117, 118 and 133 m, farther away than the closest neighbouring nest.

Conclusions and outlook

There was no apparent difference in extra-pair paternity levels between natural cavities and nestboxes in our study site. We observed shorter nearest neighbour distance, higher neighbour density and higher synchronous neighbour density in the nestbox plot compared to the natural cavity plot in blue tits but not in great tits. Furthermore, the proportion of extra-pair offspring per nest increased with density of closest neighbours in blue tits and the presence of extra-pair paternity in the nest in great tits was positively associated with neighbour density and synchronous neighbour density within closest neighbours, independently of cavity type. The differences in spatio-temporal parameters observed in blue tits between cavity types complement previous knowledge of the differences introduced by nestboxes. Given that extra-pair paternity is the result of multiple ecological factors acting on individual decisions, from which variation at the population level stems, we encourage further comparisons of cavity nesters breeding in natural nesting sites and nestboxes. Additionally, we encourage authors to follow the recommendations given by Lambrechts et al. (2010) and Wesołowski (2011) to ensure adequate reporting of nestbox setups, in terms of nestbox dimension, their distribution, frequency of old material removal, presence of anti-predator devices, frequency of inspections of nestboxes and characteristics of the study site. Following these recommendations would promote comparison among studies and allow for easier assessment of the validity of their conclusions relative to the reference state.

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Data Accessibility Statement

The data that support the findings of this study are available in FigShare at https://doi.org/10.6084/m9.figshare.21905166.v1.

Competing Interests Statement

We declare none.

Author Contributions

Irene Di Lecce: conceptualization (equal); data curation (lead); formal analysis (lead); investigation (equal); methodology (lead); writing – original draft (lead); writing – review & editing (equal). Charles Perrier: conceptualization (supporting); formal analysis (supporting); methodology (supporting); writing – review & editing (supporting). Marta Szulkin: conceptualization (equal); formal analysis (supporting); investigation (supporting); methodology (supporting); funding acquisition (lead); writing – review & editing (equal). Joanna Sudyka: conceptualization (equal); data curation (supporting); investigation (equal); methodology (supporting); writing – review & editing (equal).

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Ethics statement

The research was conducted under the permits of RDOŚ, for the natural cavity plot: WPN-I.6401.80.2017. ŁM, WPN-I.6205.53.2017.AS, and for the nest-box plot: WPN-I.6401.515.2017.KZ and WPN-I.6205.227.2017.AS, as well as Lasy Miejskie – Warszawa – permit no. LM-W.LO.400.88.2017. DC1460.

APPENDIX

Appendix A. Proportion of the brood sampled between cavity types

We tested whether the proportion of sampled nestlings (number of nestlings which we were able to sample over total number of nestlings that hatched successfully in the nest) differed between cavity types (Table S1). There was no difference between cavity types in blue tits but there was a tendency in great tits for a lower proportion of sampled nestlings in natural cavities. After excluding the four natural cavities where we were not able to sample all nestlings in the nest, a weaker tendency persisted (W = 326, P = 0.134), indicating that this result was not due to a differential sampling effort between cavity types. We therefore retained these nests in subsequent analyses. Very few dead chicks were sampled in both natural cavities and nestboxes and in both cases at d14. This is because, usually prior to our visits, parents removed dead chicks small enough to be carried out of the nests. Since we did not sample nestlings at hatching, we were not able to investigate whether this difference among the two cavity types stemmed from differential mortality between extra-pair and within-pair young or between sexes at early nestling stages. The tendency to sample larger proportion of great tit nestlings in nestboxes was effected by a general tendency in great tits to produce more fledglings in nestboxes compared to natural cavities, reported in Sudyka et al., 2022a.

Table S1 – Proportion of sampled nestlings per nest between cavity types in the two study species

	Mean (SD) in natural cavities	**Mean (SD) in nestboxes**	**Wilcoxon rank sum test**		
			W	**P**	**n**
Blue tits	0.897 (0.140)	0.806 (0.247)	244	0.295	53
Great tits	0.778 (0.227)	0.875 (0.196)	371.5	*0.071*	48

Appendix B. Genotyping

DNA sequencing was outsourced to Diversity Arrays Technology Pty, Ltd and performed using DArTseqLD, a high-throughput genotyping by sequencing method that employs genomic complexity reduction using restriction enzyme pairs (Kilian et al., 2012). DArTseq™ represents a combination of DArT complexity reduction methods and next generation sequencing platforms (Sansaloni et al., 2011; Kilian et al., 2012; Courtois et al., 2013; Raman et al., 2014; Cruz et al., 2013). Therefore, DArTseq™ represents a new implementation of sequencing of complexity reduced representations (Altshuler et al., 2000) and more recent applications of this concept on next generation sequencing platforms (Baird et al., 2008; Elshire et al., 2011). Similarly to DArT methods based on array hybridization, the technology is optimized for each organism and application by selecting the most appropriate complexity reduction method (both the size of the representation and the fraction of a genome selected for assays). Based on testing several enzyme combinations for complexity reduction, Diversity Arrays Technology Pty Ltd selected the SbfI-HpaII method for the two species of interest – the great tit *Parus major* and the blue tit *Cyanistes caeruleus*. DNA samples were processed in digestion/ligation reactions principally following Kilian et al. (2012) but replacing a single SbfI-compatible adaptor with two different adaptors corresponding to two different Restriction Enzyme (RE) overhangs. The SbfI-compatible adapter was designed to include Illumina flowcell attachment sequence, sequencing primer sequence and "staggered", varying length barcode region, similar to the sequence reported by Elshire et al., 2011). Reverse adapter contained flowcell attachment region and HpaII-compatible overhang sequence. Only "mixed fragments" (SbfI-HpaII) were effectively amplified in 30 rounds of PCR using the following reaction conditions:

1. 94°C for 1 min
2. 30 cycles of: 94°C for 20 sec
   58°C for 30 sec
   72°C for 45 sec
3. 72°C for 7 min

After PCR equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2500. The sequencing (single read) was run for 77 cycles. Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline, the *fastq* files were first processed to filter away poor quality sequences, applying more stringent selection criteria to the barcode region compared to the rest of the sequence. In that way, the assignments of the sequences to specific samples carried in the "barcode split" step were highly reliable (Kilian et al., 2012).

Filtering was performed on the raw sequences using the following parameters:

Filter	Filter Parameters
Barcode region	Min Phred pass score 30, Min pass percentage 75
Whole read	Min Phred pass score 10, Min pass percentage 50

Approximately 1,410,000 sequences per sample were identified and used in marker calling. Finally, identical sequences were collapsed into "fastqcoll files". The fastqcoll files were "groomed" using DArT PL's proprietary algorithm which corrects low quality base from singleton tag into a correct base using collapsed tags with multiple members as a template. The "groomed" fastqcoll files were used in the secondary pipeline for DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation) calling algorithms (DArTsoft14). For SNP calling, all tags from all libraries included in the DArTsoft14 analysis were clustered using DArT PL's C++ algorithm at the threshold distance of 3, followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. Additional selection criteria were added to the algorithm based on the analysis of approximately 1,000 controlled cross populations. Testing for Mendelian distribution of alleles in these populations facilitated selection of technical parameters discriminating true allelic variants from paralogous sequences. In addition, multiple samples were processed from DNA to allelic calls as technical replicates and scoring consistency was used as the main selection criteria for high quality/low error rate markers. Calling quality was assured by high average read depth per locus (Average across all markers was over 20 reads/locus).

Figure S1 - Sex assignment accuracy of Monte-Carlo cross-validation using 50%, 70%, and 90% of individuals from each population (overall: all individuals, F: females, M: males) randomly chosen for the training. Models were run with 100 iterations. Support Vector Machine was used as a classification method for the prediction in blue tits and Random forest in great tits. The dataset used to infer sex included all nestlings sampled within Warsaw as part of a larger study on extra-pair paternity (n=3031).

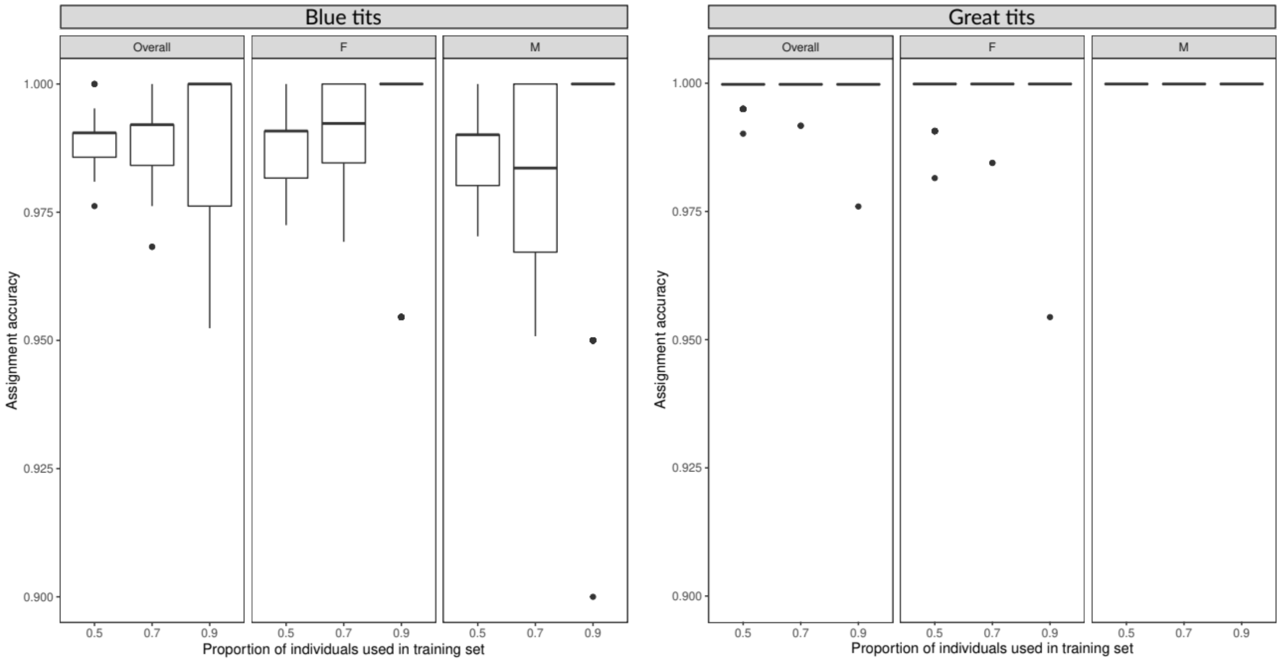

Table S2 – Correlation table of spatio-temporal variables for each radius in blue tits and great tits.

	Blue tits						Great tits					
	160 m		320 m		480 m		120 m		240 m		360 m	
	r	p	r	p	r	p	r	p	r	p	r	p
Nearest neighbour distance vs Neighbour density	-0.614	<0.001	-0.432	<0.001	-0.395	<0.001	-0.716	<0.001	-0.378	<0.001	-0.278	0.003
Nearest neighbour distance vs Synchronous neighbour density	-0.513	<0.001	-0.469	<0.001	-0.425	<0.001	-0.473	<0.001	-0.255	0.006	-0.20	0.030
Neighbour density vs Synchronous neighbour density	0.705	<0.001	0.699	<0.001	0.656	<0.001	0.670	<0.001	0.681	<0.001	0.724	<0.001

Table S3 – Number of broods with number of extra-pair offspring per species and cavity type. Only broods whose parents were caught were included, as the number of extra-pair offspring can be identified comparing relatedness of each nestling to the social father (1 natural cavity and 2 nestboxes were excluded in blue tits and 2 natural cavities and 1 nestbox in great tits).

	Blue tits		**Great tits**	
Number of extra-pair chick per brood	**Natural cavity**	**Nestbox**	**Natural cavity**	**Nestbox**
0	19	20	20	10
1	6	7	4	5
2	3	4	3	6
3	0	3	2	3
4	1	1	1	1
Total	**29**	**35**	**30**	**25**

Table S4 – Spatio-temporal parameters among cavity types when including natural cavities found in the nestbox plot, in blue tits and great tits. In blue tits two cavities and in great tits one cavity outside the core area were removed.

	Nearest neighbour distance					**Neighbour density**				**Synchronous neighbour density**			
	Wilcoxon rank sum test					**Wilcoxon rank sum test**				**Wilcoxon rank sum test**			
	W	**P**	**Median in the cavity plot (m)**	**Median in the nestbox plot (m)**	**Radius (m)**	**W**	**P**	**Median number in the cavity plot (range)**	**Median number in the nestbox plot (range)**	**W**	**P**	**Median number in the cavity plot (range)**	**Median number in the nestbox plot (range)**
Blue tits (n = 138)	2787	**0.019**	95	79	**160**	1391	**<0.001**	2 (0-7)	4 (0-6)	1496.5	**<0.001**	1 (0-5)	2 (0-6)
					320	1427.5	**<0.001**	9 (1-17)	11 (6-19)	1567.5	**0.003**	4 (0-13)	7 (0-14)
					480	1834	*0.070*	19 (2-31)	19 (12-29)	1823.5	*0.060*	9 (1-20)	12 (1-18)
Great tits (n = 126)	1552	0.447	82	86	**120**	1448.5	0.170	1 (0-4)	1 (0-4)	1514.5	0.298	0 (0-3)	1 (0-3)
					240	1574.5	0.518	5 (0-11)	5 (2-8)	1332.5	*0.052*	2 (0-7)	3 (0-7)
					360	1879.5	0.334	10 (2-21)	10 (4-15)	1470	0.230	5 (0-12)	6 (0-13)

Table S5 – Density parameters for blue and great tits nests among the two study years.

	Nearest neighbour distance					**Neighbour density**				**Synchronous neighbour density**			
	Wilcoxon rank sum test					**Wilcoxon rank sum test**				**Wilcoxon rank sum test**			
	W	**P**	**Median in 2018 (m)**	**Median in 2019 (m)**	**Radius (m)**	**W**	**P**	**Median number in 2018 (range)**	**Median number in 2019 (range)**	**W**	**P**	**Median number in 2018 (range)**	**Median number in 2019 (range)**
Blue tits (n = 131)	2404	0.223	87	82	**160**	1809.5	0.124	2 (0-6)	3 (0-7)	1881.5	0.221	1 (0-6)	1 (0-5)
					320	1861	0.200	9 (1-16)	10 (2-18)	2038.5	0.643	4.5 (0-13)	5 (0-13)
					480	1810.5	0.130	17 (2-27)	19 (4-31)	2056.5	0.705	9 (1-18)	10 (1-20)
Great tits (n = 116)	1500	0.318	83	93	**120**	1885.5	0.235	1 (0-4)	1 (0-4)	1718.5	0.827	1 (0-3)	1 (0-3)
					240 m	2058	**0.036**	5 (2-11)	4 (0-11)	2027.5	*0.053*	3 (0-7)	2 (0-7)
					360 m	2023	*0.059*	10 (4-21)	8 (1-19)	2048.5	**0.042**	6 (1-13)	5 (0-12)

Appendix C. Spatio-temporal parameters among cavity types separately by year

For each year separately, we tested for differences among cavity types in those parameters differing between years in great tits. Results were similar to those combining the two years. Within a 240 m radius, neighbour density was uniform between cavity types both in 2018 (W = 400, p-value = 0.750) and 2019 (W = 299.5, p-value = 0.175), while synchronous neighbour density was significantly lower in natural cavities relative to nestboxes in 2018 (W = 241, p-value = 0.023) but there was no difference in 2019 (W = 185, p-value = 0.289). Within 360 m, neighbour density was uniform between cavity types in 2018 (W = 414.5, p-value = 0.580) but significantly higher in natural cavities than in nestboxes in 2019 (W = 390, p-value = 0.001), while synchronous neighbour density was significantly lower in natural cavities than in nestboxes in 2018 (W = 174.5, p-value = 0.001) but uniform in 2019 (W = 306.5, p-value = 0.133).

Table S6 - Generalized linear mixed models for blue tits and generalized linear models for great tits with presence of extra-pair paternity (0/1) in the nest as the dependent variable. Cavity type, year, laying date, squared laying date, clutch size, neighbour density (model a) and synchronous neighbour density (model b) were included as predictors. Mother identity was introduced as random effect in models for blue tits. Squared laying date was initially introduced in the models and removed from final models whenever nonsignificant. Nonsignificant interactions between cavity type and other main factors were removed. Reference levels were: natural cavity (cavity type) and 2018 (year). Significant differences ($P < 0.05$) are in bold, trends ($P < 0.2$) in italics.

Occurrence of extra-pair paternity					
Blue tits (n = 52)		**320 m**		**480 m**	
		Estimate (SE)	**Pr(>χ^2)**	**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	0.053 (0.086)	0.538	0.048 (0.061)	0.430
	Cavity type	0.403 (0.660)	0.541	0.450 (0.626)	0.473
	Year	0.110 (0.612)	0.858	0.061 (0.622)	0.922
	Laying date	0.054 (0.392)	0.891	0.039 (0.390)	0.921
	Clutch size	-0.061 (0.199)	0.758	-0.061 (0.197)	0.756
Model b	**Synchronous neighbour density**	0.101 (0.130)	0.438	0.043 (0.100)	0.669
	Cavity type	0.123 (0.833)	0.883	0.322 (0.836)	0.700
	Year	0.243 (0.634)	0.702	0.224 (0.644)	0.728
	Laying date	0.239 (0.408)	0.559	0.226 (0.447)	0.612
	Clutch size	-0.084 (0.209)	0.688	-0.051 (0.202)	0.802
Great tits (n = 48)		**240 m**		**360 m**	
		Estimate (SE)	**Pr(>χ^2)**	**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	0.111 (0.164)	0.494	-0.031 (0.092)	0.738
	Cavity type	1.003 (0.774)	*0.188*	0.851 (0.771)	0.264
	Year	0.482(0.858)	0.573	0.472 (0.874)	0.589
	Laying date	1.499 (0.636)	**0.005**	0.883 (0.453)	**0.031**
	Clutch size	-0.419 (0.263)	*0.092*	-0.332 (0.260)	*0.182*
	Laying date^2	0.694 (0.377)	**0.049**		
Model b	**Synchronous neighbour density**	0.203 (0.201)	0.303	0.003 (0.110)	0.980
	Cavity type	0.684 (0.718)	0.340	0.975 (0.688)	*0.153*
	Year	0.435 (0.850)	0.608	0.586 (0.862)	0.496
	Laying date	1.482 (0.635)	**0.005**	0.898 (0.453)	**0.027**
	Clutch size	-0.438 (0.264)	*0.078*	-0.332 (0.259)	*0.180*
	Laying date^2	0.720 (0.383)	**0.042**		

Table S7 - Generalized linear mixed models with proportion of extra-pair offspring per nest as the dependent variable. Cavity type, year, laying date, squared laying date, clutch size, neighbour density and synchronous neighbour density were included as predictors. Mother identity was introduced as a random effect. Squared laying date was initially introduced in the models and removed whenever nonsignificant. Nonsignificant interactions between cavity type and other main factors were removed. Reference levels were: natural cavity (cavity type) and 2018 (year). Significant differences (P < 0.05) are in bold, trends (P < 0.2) in italics.

Proportion of extra-pair offspring per nest					
Blue tits (n = 50)		**320 m**		**480 m**	
		Estimate (SE)	**Pr(>χ^2)**	**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	0.083 (0.072)	0.246	0.068 (0.051)	*0.179*
	Cavity type	0.179 (0.554)	0.747	0.314 (0.510)	0.537
	Year	-0.185 (0.515)	0.720	-0.232 (0.514)	0.652
	Laying date	-0.071 (0.343)	0.837	-0.080 (0.338)	0.812
	Clutch size	-0.221 (0.161)	*0.171*	-0.215 (0.157)	*0.171*
Model b	**Synchronous neighbour density**	0.072 (0.101)	0.474	0.036 (0.081)	0.657
	Cavity type	0.123 (0.685)	0.858	0.239 (0.693)	0.730
	Year	-0.038 (0.524)	0.943	-0.021 (0.541)	0.969
	Laying date	0.108 (0.355)	0.761	0.115 (0.385)	0.765
	Clutch size	-0.217 (0.171)	0.205	-0.195 (0.169)	0.249
Great tits (n = 44)		**240 m**		**360 m**	
		Estimate (SE)	**Pr(>χ^2)**	**Estimate (SE)**	**Pr(>χ^2)**
Model a	**Neighbour density**	-0.068 (0.102)	0.503	-0.068 (0.064)	0.286
	Cavity type	0.418 (0.561)	0.457	0.281 (0.588)	0.633
	Year	0.708 (0.521)	*0.174*	0.580 (0.537)	0.280
	Laying date	0.198 (0.258)	0.444	0.167 (0.263)	0.525
	Clutch size	-0.068 (0.169)	0.690	-0.056 (0.174)	0.746
Model b	**Synchronous neighbour density**	0.087 (0.141)	0.539	-0.056 (0.077)	0.462
	Cavity type	-0.427 (0.726)	0.470	0.606 (0.562)	0.281
	Year	-0.531 (0.815)	0.206	0.627 (0.543)	0.248
	Laying date	0.243 (0.283)	0.390	0.179 (0.265)	0.4996
	Clutch size	-0.025 (0.186)	0.892	-0.061 (0.176)	0.729
	Cavity type x Year	2.221 (1.130)	**0.049**		