Behav Ecol Sociobiol (2010) 64:463–473
DOI 10.1007/s00265-009-0862-y

ORIGINAL PAPER

Extra-pair paternity in eastern bluebirds: effects of manipulated density and natural patterns of breeding synchrony

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Received: 27 April 2009 / Revised: 7 September 2009 / Accepted: 9 September 2009 / Published online: 30 September 2009

Abstract The causes of variation in rates of extra-pair paternity among avian populations remain unclear, but could include environmental factors such as breeding density and synchrony. By experimentally manipulating nest site availability, we tested the effects of breeding density on the frequency of extra-pair paternity in eastern bluebirds (*Sialia sialis*). We also examined the role of breeding synchrony on extra-pair paternity using natural timing of nests. Microsatellite analysis revealed 34 of 305 nestlings (11.2%) were the result of extra-pair fertilizations; and 21 of 79 broods (26.6%) had at least one extra-pair nestling. Several measures of breeding density had independent effects on extra-pair paternity. First, experimental plot type affected extra-pair paternity, with 28 of 34 (82.4%) extra-pair young from nests in high density areas, and only six (17.6%) from nests in low density areas. Independently of plot type, the number of breeding neighbors within a 320-m radius was a significant predictor of the likelihood of extra-pair paternity at the nest. Extra-pair paternity was associated with temporal factors such as absolute timing of breeding and natural levels of local breeding synchrony, but only in bivariate comparisons. We found a positive interaction between density of neighbors within a 320-m radius and local breeding synchrony; this term reduced the main effects of synchrony and number of neighbors, but not experimental treatment. Our results demonstrate the importance of utilizing multiple aspects of proximity in breeding density analyses and testing for interactions between ecological factors that can influence the behavioral events leading to extra-pair fertilizations.

Keywords Breeding ecology · Mating system · Nest box density · Extra-pair copulation · *Sialia sialis*

Introduction

Most birds are socially monogamous, and both parents care for their young (Lack 1968; Kempenaers et al. 1998). However, many socially monogamous birds copulate outside their social pair bond resulting in genetic promiscuity (e.g., Westneat 1987). These extra-pair copulations often translate into extra-pair paternity. The frequency of extra-pair paternity (EPP) in natural populations of birds varies considerably, from a low of 0% in species such as common loons (*Gavia immer*; Piper et al. 1997) to a high of 76% in the superb fairy wren (*Malurus cyaneus*; Mulder et al. 1994). Likewise, intraspecific studies between populations have revealed variation in the prevalence of EPP, suggesting notable differences in behavior at varying spatial scales (Griffith et al. 2002). The reasons, however, for such variation in EPP within and among species remain unclear.

The frequency of extra-pair paternity likely depends on factors affecting the rate of encounters between females and extra-pair males and the timing of extra-pair copulations relative to when eggs are fertilized. The tendency for a

Communicated by S. Pruett-Jones

Electronic supplementary material The online version of this article (doi:10.1007/s00265-009-0862-y) contains supplementary material, which is available to authorized users.

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female to move away from her social mate, by nearby extra-pair males to seek out females on other territories, or by the pair male to stay close to the female (mate-guarding) could each influence the frequency of EPP in a population (Westneat et al. 1990; Westneat and Stewart 2003). What remains uncertain is how specific ecological factors influence these tendencies.

An early hypothesis addressing this issue was that increased density should increase EPP (Birkhead 1978). This idea assumes that increasing density increases proximity between extra-pair males and females and therefore increases encounter rate. However, comparative studies have revealed relatively little influence of density on EPP (e.g., Westneat and Sherman 1997). Analyses of data from within species have sometimes produced a positive correlation between natural breeding densities and levels of extra-pair paternity in American robins (*Turdus migratorius*; Rowe and Weatherhead 2007), Bullock's orioles (*Icterus bullockii*; Richardson and Burke 2001), and barn swallows (*Hirundo rustica*; Moller 1991). In other cases, investigators have found no relationship between breeding density and EPP rates, e.g., hooded warblers (*Wilsonia citrina*; Tarof et al. 1998), and black-throated blue warblers (*Dendroica caerulescens*; Chuang et al. 1999). At least one study found a negative relationship, with more EPP in less dense areas (tree swallows, *Tachycineta bicolor*; Conrad et al. 2001). These results suggest that the effects of density may depend on other attributes of the organism, such as territoriality or variation in individual quality. Density could also be linked to other socioecological factors such as the level of breeding synchrony (Petrie and Kempenaers 1998; Thusius et al. 2001). Finally, another possibility is that density has different effects depending on how it is measured. This is conceptually important because it implies that density is not a single variable but several related ones, and these may have differing effects.

Most analyses of density have used natural variation in the proximity of individuals and a correlative approach. A few have attempted to experimentally manipulate density to separate its effects from possible confounding variables, usually by manipulating nest sites. These experimental studies have also revealed variable results; EPP and breeding density were positively correlated in two studies (Gowaty and Bridges 1991; Charmantier and Perret 2004), uncorrelated in two other studies (Dunn et al. 1994; Vaclav and Hoi 2002), and negatively correlated in one study (Ratti et al. 2001).

One methodological problem with most manipulative studies is that they have only included one replicate plot and, in some cases, have manipulated density to extreme levels. For example, Gowaty and Bridges (1991) placed 51 nest boxes for eastern bluebirds (*Sialia sialis*) an average of 33 m apart in their single high density site; yet 57% of the boxes were unused presumably due to their extreme proximity. An exception was the study of Vaclav and Hoi (2002), which created several replicate plots for house sparrows (*Passer domesticus*) containing three, five, and ten nest boxes each, allowing for a completely randomized design. They found no influence of this treatment on extra-pair paternity.

One conceptual problem with studies of density to date is that most researchers assume that density is a single variable. For example, Charmantier and Perret (2004) found that a manipulated plot supplemented with extra nest boxes had both a greater number of birds and a reduced nearest neighbor distance. Both independently influenced EPP at the nest, but did so in a complex way; nearest neighbor distance affected EPP only when the number of neighbors was zero or one, but not when two or more neighbors were present. Vaclav and Hoi (2002) contended that the lack of density effects in their experimental study was due to changes in colony size, but not breeding density in their plots containing five versus ten nest boxes. Finally, increased density of breeding individuals could have two other unexpected effects; they could attract more floater individuals who are involved in EPCs, or they could change the ways individuals move through space, thereby increasing (or decreasing) encounters nonlinearly.

Density may also interact with other socioecological variables, such as breeding synchrony, to influence extra-pair paternity. The synchrony hypothesis (Stutchbury and Morton 1995) predicts that EPP will increase with synchrony because synchrony allows females to compare males more effectively. Alternatively, asynchronous breeding could skew the operational sex ratio toward males, thereby increasing the frequency of EPP (Birkhead and Biggins 1987). Moreover, if male mate-guarding conflicts with the pursuit of extra-pair copulations (EPCs) whereas parental care during incubation does not, most males should be able to pursue EPCs more often during periods of asynchrony (Westneat et al. 1990; Westneat and Gray 1998). Although a phylogenetically controlled comparative analysis between species found a higher incidence of EPP in species with greater breeding synchrony (Stutchbury 1998), most intraspecific studies have either failed to find a positive relationship between the two (Dunn et al. 1994; Chuang et al. 1999; Griffith et al. 1999; Arlt et al. 2004), or have found a negative relationship (Conrad et al. 1998; Saino et al. 1999).

Evidence is growing that breeding density and synchrony have not been adequately examined in previous studies; with only a few investigators examining the two factors simultaneously (e.g., Dunn et al. 1994; Vaclav and Hoi 2002). For example, in common yellowthroats (*Geothlypis trichas*), EPP decreased with increasing synchrony, but the magnitude of this relationship became less negative as density increased (Thusius et al. 2001). An interaction between these two factors may arise because males breeding in low density areas where territories are larger could have trouble guarding their mate effectively, especially during periods of asynchrony when many neighboring males are pursuing EPCs.

To better understand how socioecological factors affect the prevalence of EPP, we manipulated breeding density and assessed its impact and that of synchrony on EPP in a population of eastern bluebirds. Bluebirds are socially monogamous, but are known to exhibit moderate rates of extra-pair paternity (~20%, Gowaty and Plissner 1998). Little is known about how extra-pair copulations occur in this species, but we expected both density and synchrony to influence how male and female eastern bluebirds encounter extra-pair partners. Bluebirds are secondary cavity nesters and readily use nest boxes placed in suitable habitat. Thus, breeding densities can be manipulated by varying the density of nest boxes. Gowaty and Bridges' (1991) manipulation of density in this species provided strong support for the breeding density hypothesis with significantly more extra-pair young at the high density site (44%) versus the standard (17%) or low density (8.3%) sites. However, their high density treatment contained only one replicate plot and they used only one measure of breeding density, so there remain questions about the ways in which density affects extra-pair paternity. We manipulated nest box availability in multiple plots. We tested four predictions of the general hypothesis that spatial and temporal variables affect extra-pair paternity: (1) that extra-pair paternity would be higher in high density plots, (2) that nearest neighbor distance and local density would also affect EPP, (3) that EPP would be negatively related to breeding synchrony, but (4) that the negative effect of synchrony would be stronger at low densities.

Material and methods

Field site and methods

We studied eastern bluebirds at the Blue Grass Army Depot (BGAD) in Madison County, Kentucky from 15 April to 31 August 2005. The BGAD is a 6,070 ha munitions storage facility that consists of rows of earthen bunkers and is characterized by old field pastures with scattered trees. Because of cattle grazing, much of the area has sparse ground cover that bluebirds prefer for foraging. To examine the possible effect of breeding density on rates of extra-pair copulations, nest boxes were arranged into high and low density areas approximately 2 months prior to the beginning of the study. In two study plots chosen arbitrarily, nest boxes (*N*=35 per plot) were placed an average of 200 m apart to create high-density breeding areas. The distance between the two high density plots was 1,860 m. Five other study plots, including one linear roadside plot, were designated as low-density breeding areas, with nest boxes (*N*=60 total) an average of 500 m apart. The closest any two plots were to each other was 610 m (See supplementary Table 1 for additional information about breeding densities of individual plots). The creation of high and low density plots resulted in a notable difference in actual breeding densities of nesting pairs (Table 1).

Because bluebirds are multibrooded and typically renest after nest failure and after successfully fledging young, actual breeding densities varied throughout the breeding season. Therefore, we recorded breeding density (the number of nest boxes with active nests per unit area) weekly using the land mapping GPS-based software Maptech® Terrain Navigator Pro (Maptech, Inc., Amesbury, MA, USA). We also calculated local (neighborhood) density, or the number of active bluebird nests within a 320-m radius of each nest. Our rationale for this distance criterion was based on several factors. Eastern bluebird territory sizes typically encompass about 2 ha (165-m radius), while their home ranges often cover larger areas around 8 ha in size (320-m radius; Gowaty and Plissner 1998). Additionally, a previous study of paternity in eastern bluebirds identified an extra-pair sire approximately 300 m from the focal nest (Meek et al. 1994). Thus, we chose to measure breeding density within a 320-m radius since this likely encompassed the majority of birds that routinely interacted. We also calculated the distance from each active bluebird nest to the nearest active nest box. We surveyed the study site for evidence of bluebirds nesting in natural cavities, and were only aware of one pair that did so.

Beginning in mid-April, we checked nest boxes twice a week and, for boxes with nesting eastern bluebirds, recorded the nesting stage as nest building, prelaying, laying, incubating, nestling, or postfledging. Because eastern bluebirds may have up to three clutches in a single breeding season (Tucker 1990), nest boxes were monitored through the end of August. Adult bluebirds were captured using mist nets during the nestling stage. Birds were lured into the net using playback of a nestling distress call from a speaker placed below the net. During the study, we captured and marked 72% of box holders, and, because unbanded males settled at boxes throughout the season, we suspected there was a sizeable population of birds not associated with our boxes. For each captured bluebird, we obtained standard morphological measurements, including mass, tarsus length, tail length, and wing chord. We also plucked the two outermost rectrices for ptilochronology (growth bar width) analysis (Grubb 1989). The age of captured bluebirds was determined (second year [SY] or after second year [ASY]) by examining the shape and color

Table 1 Density and extra-pair paternity in low density and high density plots of eastern bluebirds (*Sialia sialis*). All values in parentheses represent standard deviation

	Low density plots	High density plots
Mean plot size (ha)	150.7 (47.3)	169.9 (26.4)
Mean nest box density (per ha)	0.07 (0.05)	0.2 (0.02)
Mean breeding density (number of breeding pairs/ha)	0.05 (0.03)	0.12 (0.02)
Mean number of neighbors (within 320 m)	0.4 (0.7)	2.7 (1.7)
Mean distance to nearest neighbor (m)	516 (207)	219 (104)
Number of offspring genotyped	157	148
Number of broods genotyped	42	37
Broods containing EPY	4	17
Mean proportion of EPY in broods with EPY	0.4 (0.1)	0.5 (0.2)
Overall percent EPY	2.5	18.9

EPY extra-pair young

of the tenth primary covert (Pitts 1985). Adults were banded with a numbered U.S. Geological Survey aluminum band and a unique combination of three-colored plastic bands.

Breast and rump feathers from eastern bluebird adult males were analyzed for melanin pigmentation and ultraviolet (UV) structural blue coloration, respectively. Approximately eight–10 feathers were taped on a black background, with feathers overlapping as they might on a bird. Reflectance data were generated using a 200-micron fiber optic spectrometer (Model S2000, Ocean Optics, Inc., Dunedin, FL, USA) held perpendicular to the surface of the feathers. Three readings were taken from each feather sample at different areas on the feather surface and were later averaged for analysis. Percent reflection and wavelength data were collected for each feather sample. ColoR© v 1.7, a software program, was used to analyze eastern bluebird feather reflectance data. For each individual male, UV chroma (300–400/300–700 nm), blue chroma (400–500/300–700 nm), total brightness (300–700 nm), and hue (maximum λ) were calculated for the rump (structural blue coloration). In addition, total brightness and red chroma (500–700/300–700 nm) were calculated for the breast (melanin pigmentation; Siefferman and Hill 2003).

Timing of breeding was calculated using the date of laying of the first egg of each nest. We assumed that a female's fertile period covered the 3 days before the first egg was laid through to the day the penultimate egg was laid. Breeding synchrony was measured both locally and population-wide. Local breeding synchrony was defined as the number of females within a given study plot (N=2 high density plots, five low density plots) whose fertile period overlapped the focal female's fertile period by one or more days. The local synchrony index was this number divided by the number of females occupying boxes within the plot. Population-wide synchrony was determined by counting the number of females which overlapped the focal female's fertile period from the entire BGAD population.

We collected a blood sample from each captured adult and from nestlings when they were 8–14 days old. We did not collect DNA from unhatched eggs or dead nestlings. Before analyzing paternity, we assigned a female and a male as social parents at a nest. We identified the social mother of a brood by observing her enter the box during incubation. Social fathers were identified based on one or more of the following observations: territory defense during the incubation period, mate guarding (Gowaty and Bridges 1991), or provisioning nestlings. We obtained about 50–75 µl of blood from the brachial vein of each bluebird. Blood was collected in microhematocrit capillary tubes and placed in 1.5–mL vials containing 100 µl of TNE buffer (0.01 M Trizma base: 0.001 M Na Cl: 0.002 M EDTA). Vials were kept on ice in the field and later stored in a −20°C freezer.

Paternity analysis

The Chelex 100® extraction method was used to extract DNA from blood (Walsh et al. 1991) followed by the polymerase chain reaction (PCR) to amplify DNA. For each tube prepared for PCR, the mix contained 10.8 µl of DNA-free water, 5 µl of ThermoPol buffer (New England BioLabs, Inc.), 1.6 µl each of primer (forward and reverse), 2 µl of dNTPs, 0.1 µl of Taq polymerase, and 2 µl of extracted DNA. We loaded samples in a thermal cycler machine (Eppendorf Mastercycler) set on a cycle of 94°C for 5 min, 72°C for 1 min, 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min for 33 cycles.

We chose three microsatellite loci (sections of hypervariable DNA) with good variability (>10 alleles) from a set of 15 loci that amplified bluebird DNA. The loci we used were *Pdo 05*, *Pca 08*, and *CM001E* (Table 2; Griffith et al. 1999; Dawson et al. 2000; Polakova et al. 2007).

Table 2 Four microsatellite loci used in paternity analysis, sequences, number of alleles, observed heterozygosity, and the probability of excluding an unrelated offspring assuming the first parent's genotype is known, from eastern bluebirds on the Blue Grass Army Depot, Kentucky

Locus	Sequence (5′→3′)	No. of alleles	Heterozygosity observed	Probability of exclusion
Pdo5	GATGTTGCAGTGACCTCTCTTG (F) GCTGTGTTAATGCTATGAAAATGG (R)	18	0.743	0.655
Pca8	ACTTCTGAAACAAAGATGAAATCA (F) TGCCATCAGTGTCAAACCTG (R)	19	0.868	0.711
CM001E	TATAAGGTCCTCACCGAGCC (F) CACTGCATCTTTCCAAAGCTAA (R)	16	0.676	0.548
Eabl007	CCTGCACAAAGTCACCTCCT (F) TGACACTAGGTGGGGATTGA (R)	11	0.528	0.528

However, *CM001E* yielded many homozygous genotypes, suggesting a high frequency of null alleles, which make accurate assignment of paternity difficult. When determination of paternity was not consistent for all three loci (N=26 broods), a fourth locus, *Eabl007* (Table 2; Balenger et al. 2009) was used to verify paternity.

Once the PCR process was completed, we added 5 μl of PAGE loading dye to each sample. Amplified samples and a ten base pair ladder (Invitrogen, Carlsbad, CA, USA) needed to size the amplified DNA fragments were run through a 4% polyacrylamide gel on an electrophoresis rig (Gibco BRL Sequencing System Model S2, Life Technologies, now Invitrogen, Carlsbad, CA, USA).

Gels used in determining paternity were developed using the silver-staining method (Bassam et al. 1991). Gels were scored by hand and scanned into a Macintosh computer for later comparison. All offspring of a pair of eastern bluebird adults were run on the same gel. We considered a nestling to be extra-pair if it mismatched its presumed father's genotype at two or more of the four loci used. If nestlings mismatched at a single locus, these were either considered mutations or null alleles, and thus were still considered to be within-pair. Paternity analysis was performed using CERVUS 2.0, a program that uses codominant loci to determine parentage (Marshall et al. 1998). Across all four loci, the total exclusionary power of detecting extra-pair offspring was 0.98 at a 95% confidence interval. We did not attempt to assign paternity at individual nests because the high percentage of unbanded males within the population made the probabilities of uniquely identifying the true father low.

Statistics

We assessed possible differences between high- and low-density areas in the distribution of pair types (ages of members of a pair, e.g., ASY male and ASY female or SY male and ASY female) using a chi-square test. A similar test was used to assess associations between pair types and the frequency of EPY. We used Spearman rank correlations to test the effect of breeding season on clutch size and brood size. Principal components analysis was used to combine rump color metrics (e.g., brightness, chroma, hue) and breast color metrics, respectively. We used the first principal component for each body region, which accounted for 57% of the variation in the rump and 74% of the variation in the breast. We used t tests to determine the possible relationship between plot type (high and low density) and the phenotypic traits of males. Because ten females renested during the season and multiple females settled within each replicate plot, we used a general linear mixed model (GLMM; Procedure GLIMMIX in SAS with logit link) to analyze the independent effects of multiple factors and their interactions on the frequency of EPY. The dependent variable was the number of EPY and was linked with a binomial error to the brood size (number of EPY/ number of chicks in the brood). We included the random effects of female and plot identity in all analyses containing our experimental treatment (plot type). Random effect terms were tested using the likelihood ratio test, in which −2 times the difference in log-likelihood between models differing in one term is distributed as a chi-square with 1 df (Crawley 2002). We analyzed a restricted set of fixed effects using Type III sums of squares and tested the sensitivity of the results by comparing models with and without key terms also using the likelihood ratio test. Denominator degrees of freedom for F-tests of fixed effects were calculated using the Satterthwaite approximation. All analyses were conducted using the Statistical Analysis System (SAS Institute 1989). All values are presented as means±standard error. All tests were considered significant at α=0.05.

Results

Patterns of reproduction

We analyzed data on 136 adults and 305 offspring from 79 broods. The mean clutch size (N=79) for eastern bluebirds was 4.2±0.1 eggs, whereas the mean brood size (N=79) was 3.8±0.1. Although mean clutch size did not differ among breeding pair types (e.g., ASY female and ASY male, ASY female and SY male, SY female and ASY male, SY female and SY male; $F_{3,\ 66}=0.51$, $P=0.68$), pairs consisting of an SY male and ASY female had significantly smaller broods than pairs consisting of an ASY male and ASY female (3.0±0.3 v. 4.0±0.2; $F_{3,\ 66}=3.2$, $P=0.031$). Egg hatching failure may have contributed to this difference, with SY males having higher rates of suspected infertility (one or more unhatched eggs in their nests; seven of 14, or 50%, of nests of SY males) than ASY males (12 of 65, or 18% of nests; $\chi_1^2 = 4.7$, $P=0.03$). There was no difference in distribution of pair types between high and low breeding density areas ($\chi_3^2 = 1.4$, $P=0.7$). In addition, there was no difference among pair types and frequency of the surviving young that were extra-pair (EPY; $\chi_2^3 = 2.1$, $P=0.55$).

Extra-pair paternity and breeding density

Microsatellite analysis revealed that 34 of 305 eastern bluebird nestlings (11.2%) were the result extra-pair fertilizations, with a mean of 0.4±0.1 EPY per nest (range 0–3 EPY/nest). We found no EPY in 58 of 79 broods (73%), whereas 21 nests (27%) had at least one EPY (mean=1.61±0.13, range 1–3). For broods with EPY, nine had one EPY, 11 had two, and one had three. We found no instances of intraspecific brood parasitism.

Seventeen of 37 (46%) nests contained at least one EPY in high density areas; whereas four of 42 (9.5%) nests contained at least one EPY in low density areas (Table 1). High-density areas averaged 0.7±0.2 extra-pair young per nest whereas nests in low density areas averaged 0.2±0.1, a significant difference (GLMM, $F_{1,71}=11.9$, $P=0.001$) in a GLMM that included plot and female identity as random effects. Overall, 19% (28 of 148) of nestlings genotyped from the high density areas were the result of EPP, whereas only 3% (4 of 157) of nestlings genotyped were the result of EPP in low-density areas (Table 1).

The local spatial distribution of pairs also had an effect on extra-pair paternity. First, we analyzed nearest neighbor distance and density of neighbors within a 320-m radius using separate bivariate GLMMs. Both density measures were significant predictors of EPP at the nest (nearest neighbor distance: effect=−0.004, $F_{1,49.3}=5.0$, $P=0.03$; density 320 m radius: effect=0.35, $F_{1,39.2}=7.8$, $P=0.008$; Fig. 1). However, nearest neighbor distance and breeding density within a 320-m radius were negatively correlated ($N=79$, $r=-0.74$, $P<0.0001$); therefore, we examined the independent effects on the proportion of EPP in a nest of all three density measures (experimental plot type as a categorical fixed effect and nearest neighbor distance and number of nests within 320 m as continuous fixed effects) using GLMM with female identity and plot identity as random effects. Experimental plot type remained significant ($F_{1,72.4}=7.8$, $P=0.007$), as did the number of neighbors within 320 m ($F_{1,66}=6.2$, $P=0.02$). Nearest neighbor distance was not significant ($F_{1,71.1}=1.3$, $P=0.25$). In a model without nearest neighbor distance, the interaction between plot type and density 320 was not significant ($F_{1,73.3}=0.01$, $P=0.98$).

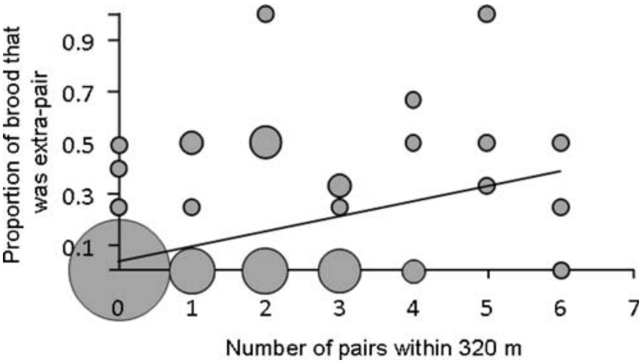

Fig. 1 Proportion of extra-pair young (EPY) and number of neighbors within a 320-m radius in eastern bluebirds. *Point size* indicates the number of coincident values

The effect of plot type on EPP could be due to higher quality males settling in low density areas. However, morphological attributes of male eastern bluebirds breeding in high and low density plots did not differ, with no significant differences in mass ($t=0.70$, $df=69$, $P=0.49$), tarsus ($t=0.38$, $df=74$, $P=0.71$), or tail length ($t=1.9$, $df=77$, $P=0.07$) between high density and low density areas. We also found no significant differences in mean growth bar width (an index of condition at time of molt) between males in high density and low density plots ($t=1.01$, $df=65$, $P=0.32$). Structural blue coloration (rump) was not significantly different between males in high density plots (mean PC score=0.21±0.18) versus males in low density plots (mean PC score=−0.20±0.17; $t=1.6$, $P=0.11$). Melanin pigmentation (breast) also did not differ between high density plots (mean PC score=−0.07±0.22) and low density plots (mean PC score=0.06±0.15; $t=-0.5$, $P=0.6$).

Timing of breeding and synchrony

Seasonal timing affected several reproductive variables. Clutch size was significantly larger early in the breeding season than later ($r=-0.42$, $df=78$, $P=0.0001$). Brood size was also largest early in the season ($r=-0.28$, $df=78$, $P=0.01$). However, breeding density at 320 m ($r=0.08$, $df=78$, $P=0.48$) and mean distance to the nearest active nest ($r=0.08$, $df=78$, $P=0.46$) were not related to date in the breeding season.

Local and population level synchrony indices were significantly correlated ($r=0.53$, $P<0.0001$). Nests were

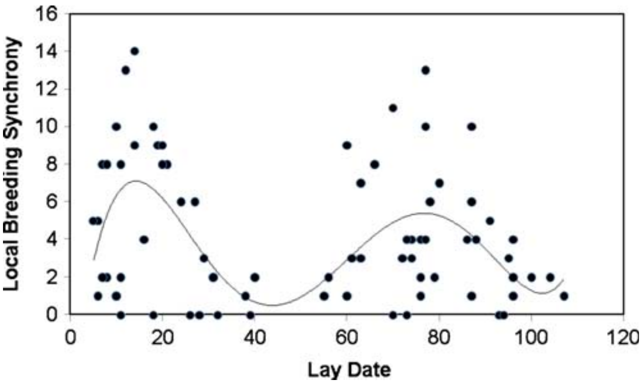

Fig. 2 Local breeding synchrony of eastern bluebirds across the population related to the date the first egg was laid in the nest (lay date 1=1 April). Trend line is displayed as fifth order polynomial with $R^2=0.23$

started in two distinct peaks during the breeding season, and both population-level and local-level indices of breeding synchrony revealed this pattern (Fig. 2). The number of females breeding simultaneously was significantly associated with experimental treatment (mixed model ANOVA with plot identity as random effect; *effect*=4.5±1.5, $F_{1,7.4}=8.6$, $P=0.02$) but not the number of pairs within 320 m (*effect*=0.3±0.2, $F_{1,72.7}=2.2$, $P=0.14$). The local synchrony index, which controls for number of breeders within plots, was not associated with either measure of density (plot type, *effect*=0.02±0.09, $F_{1,6}=0.05$, $P=0.83$; density within 320 m, *effect*=0.01±0.02, $F_{1,74.7}=0.1$, $P=0.74$).

Bivariate analyses revealed that the proportion of each brood that was extra-pair declined significantly through the breeding season (*effect*=−0.03±0.004, $F_{1,27.3}=47.0$, $P=0.001$) but increased with population-wide synchrony (*effect*=0.07±0.04, $F_{1,58.3}=4.0$, $P=0.051$). The proportion of EPP increased significantly with the local synchrony index (*effect*=2.7±1.0, $F_{1,46.5}=7.2$, $P=0.01$).

We used a mixed model to test the independent effects of density and synchrony and their interactions on the frequency of EPP. In a model with plot type and density within 320 m, date in the season was not significant (*effect*=0.01±0.03, $F_{1,65}=0.5$, $P=0.48$), nor were interactions between date and either density measure (both p values>0.08). We therefore dropped date from any further analyses. Because of limited sample sizes, we restricted our final analysis to a model of plot type, density within 320 m, local synchrony index, and the interactions between the synchrony index and both density measures while including female identity and plot identity as random effects. In the full model, neither interaction term was significant (F-values<1.7, p values>0.20). A model with the interaction term between density within 320 m and the synchrony index had a significantly better fit than either the full model (*difference in log-likelihood*=−3.0, $X^2=6$, $df=2$, $P<0.05$) or one with no interaction terms (*difference in log-likelihood*=−4.6, $X^2=8.4$, $df=2$, $P<0.02$). A model with an interaction between plot type and synchrony had a smaller log-likelihood than either the full model or the model with no interactions. The best model included a significant positive interaction between density at 320 m and the synchrony index (Fig. 3), and replicated the significant positive effect of plot type that we found in earlier models lacking synchrony (Table 3). In this model, neither the number of neighbors within 320 m nor the synchrony index was significant (Table 3).

Discussion

Our results indicate that variation in breeding density, natural levels of breeding synchrony, and their interaction influenced the mating strategies of eastern bluebirds. Experimental manipulation of nest-boxes influenced density in replicate plots and produced significantly higher EPP in those locations. Our analyses also revealed that EPP was independently positively associated with the number of box holders within 320 m of the focal nest, although this result appears sensitive to local breeding synchrony. By contrast, the proximity of the closest neighbor had no effect on EPP. We did not manipulate timing of breeding; natural breeding synchrony contributed to EPP but only in concert with higher local density. In a multivariate analysis of breeding density and synchrony, we found a significant interaction between breeding density of neighbors within a 320-m radius and local breeding synchrony, which together positively influences EPP (Fig. 3).

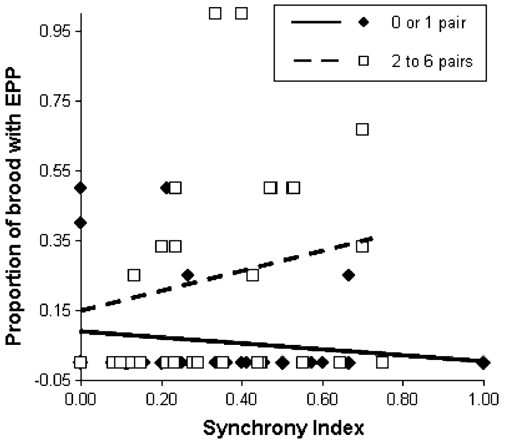

Fig. 3 Relationship between the proportion of other females on a plot that were fertilizable and the proportion of the brood that were extra-pair. *Solid diamonds* indicate nests with 0 or 1 pairs nesting within 320 m and *open squares* indicate broods having two–six pairs within 320 m. Lines are least-squares regression lines to these data; the difference in slopes was significant in a mixed model suitable for binomial data and controlling for the random effects of female identity and plot (see text and Table 2)

Table 3 Results of a general linear mixed model of the relative frequency of EPP (extra-pair offspring over brood size) and two density and one synchrony measure from 79 eastern bluebird nests

Variables	Effect±SE	*F*-value (den. df)	*P* value
Plot type (high vs. low)	3.1±1.1	6.9 (8.1)	0.025
Density 320 m	−0.6±0.4	3.1 (70.6)	0.085
Synchrony index	−2.4±2.4	1.0 (68.8)	0.322
Density 320 m×synchrony index	0.18±0.8	4.8 (70.8)	0.031

The model that best fits our data contained an interaction between synchrony and local density. Our result is similar to that found in common yellowthroats (Thusius et al. 2001) in which local density and local synchrony also interacted with a positive coefficient to affect EPP. A key difference is that Thusius et al. (2001) found synchrony to negatively affect EPP at low density, in contrast to our result of it positively affecting EPP at high densities. A positive effect of synchrony only at high densities could arise for several reasons. One possibility, slightly modified from the mechanism proposed by Stutchbury and Morton (1995), is that synchrony may allow females opportunities to compare males in similar states, and if such comparisons are somewhat costly, the closer distances between pairs as density increases might reduce those costs and increase the number of females engaging in extra-pair copulations. Alternatively, increased synchrony and higher density may combine to increase the rate at which females who are foraging to produce eggs, along with their attending mates, arrive in resource-rich areas simultaneously. A common use of space at the same time could lead to more between-pair interactions and the increased likelihood of EPP. Testing either of these hypotheses would require detailed behavioral observations as well as assignment of paternity.

As with many other hypothesized factors affecting EPP, a positive interaction term between synchrony and density has not been found in all species where it has been tested. For example, Dunn et al. (1994), Stewart et al. (2006), and Olsen et al. (2008) found no interaction between density and synchrony and, interestingly, also found no evidence of a main effect of either variable. Vaclav and Hoi (2007) found an effect of timing on EPP in replicate colonies of house sparrows where synchrony was manipulated. Synchrony did not affect colony-wide levels of EPP, but nests that were delayed compared to others within the colony had higher EPP. However, because density was similar in all colonies, no effect of a possible interaction could be tested. Thus, a clear explanation for the difference in results between studies may be elusive. The manner in which breeding density and synchrony could potentially interact to affect extra-pair behavior is likely intertwined with species-specific differences in mate-guarding behavior and which sex initiates extra-pair copulations. This complex combination of ecological and behavioral factors will result in various interspecific patterns of extra-pair paternity. Unfortunately, too little is known about the behavioral aspects of extra-pair paternity to do more than speculate how the above factors might interact. The next challenge in understanding exactly how socioecological factors influence EPP will be to manipulate both density and synchrony in systems where the impact of these variables on behavior can also be detected.

Our study confirms, with a replicated design, the influence of density found by Gowaty and Bridges (1991) on the same species. In addition, we also tested the effects on EPP of several correlates of density. Gowaty and Bridges (1991) claimed that nearest neighbor distance was important in their study, but provided no statistical analysis, and did not state whether they controlled for the effect of treatment. We found no evidence that the proximity of the nearest neighbor affected EPP in our study. Because nearest neighbors are often the sires of EPY in some species (e.g., bobolinks, *Dolichonyx oryzivorus*; Bollinger and Gavin 1991; red-winged blackbirds, *Agelaius phoeniceus*; Gray 1997; American redstarts, *Setophaga ruticilla*; Perreault et al. 1997), the proximity of the nearest neighbor is a likely influence on EPP. Indeed, Charmantier and Perret (2004) found that experimental plot, local density, and nearest neighbor distance each influenced the frequency of EPP independently. However, other studies have found that extra-pair sires come from farther away (Kempenaers et al. 1997; Rowe et al. 2001; Westneat and Mays 2005), and in a recent study of mountain bluebirds (*Sialia currucoides*), EPP was not associated with nearest neighbor distance and surprisingly, nearest neighbors were never extra-pair sires (Balenger et al. 2009). In eastern bluebirds, Meek et al. (1994) found an extra-pair sire that was located 300 m away from the focal nest, suggesting that interactions within the local neighborhood may create more opportunities for extra-pair copulations than encounters with nearest neighbors. Our results thus join others in suggesting that other factors, such as the location of resources, heterogeneity of the landscape, and/or species specific differences in how far individuals of either sex will travel for EPCs might influence the extent to which nearest neighbors are involved in extra-pair copulations. More studies that tease apart the effect of different aspects of density will be necessary to test this idea.

Our experiment increased the number of breeding pairs within an area, which increased the number of individuals within a given distance of a focal pair. We are thus confident that our manipulation of nest sites increased the density of breeding pairs. However, because we could not control which subjects settled in the plots, there could be biases in the attributes of individuals settling in high density areas that make EPP more likely. We cannot eliminate this as a possibility since we did not measure all possible attributes of our subjects, but we found no differences in several measures of body size, condition, and plumage ornamentation of males among the plot types, so we conclude this potential bias is unlikely to explain our results.

Our manipulation did not influence density uniformly throughout a plot. We found that variation in the number of neighbors within 320 m increased EPP independently of either plot type or nearest neighbor distance. We could not, however, determine if this effect was due to females having an easier time selecting extra-pair mates when neighborhoods were dense (Birkhead and Moller 1992; Charmantier and Perret 2004), because there were more extra-pair males able to take advantage of opportunities to approach females (Westneat and Sherman 1997), or to some effect of density on the ability of males to guard their social mates. Regardless, our results should serve to focus attention on neighborhoods of pairs and the means by which interactions between individuals occur within those neighborhoods.

We found an independent effect of plot type on EPP, with high density plots having higher rates of EPP even after controlling for the increased number of local neighbors and synchrony. There are several possible explanations for this result. One is that plot type influenced the quality of individuals settling there, but we found no evidence that this was the case. A second possible explanation for the plot effect is that high density areas may have attracted more floaters who then occasionally obtained EPCs. Because we did not conduct standardized surveys of birds away from nest boxes, we cannot assess this idea. A third possibility is that the spatial distribution of nest boxes in high density areas may have reduced encounters with nearby individuals while increasing encounters with more distant ones. Due to the unique landscape on the BGAD, nest boxes were arranged linearly in rows and adjacent to bunkers. This arrangement reduced visibility between adjacent neighbors, perhaps limiting interactions between them. We frequently observed bluebirds foraging in fields behind or in front of bunkers, typically moving through space where encounters with more distant neighbors might be more likely. Such encounters could produce increased EPP at high breeding densities independent of nearest neighbor distance and of the number of neighbors within 320 m. Complex interactions between habitat structure and the location of investigator-controlled nest sites are a potential problem but also produce an opportunity to test explicitly the interplay between landscape features and density. Finally, it is possible that high density plots increased the tendency of females to seek out EPCs. To explain the independent effect of plot, this requires that females have an easier time finding suitable extra-pair mates by traveling farther than 320 m and only in high density plots. Because we did not track female movements, we could not assess this idea directly.

We found evidence of temporal factors influencing EPP with extra-pair paternity declining through the breeding season and, in a separate analysis, with breeding synchrony. In contrast, several studies have found that EPP increases with season (Lubjuhn et al. 2001; Behler and Rhodes 2003; Major and Barber 2004). Because other variables such as synchrony also change with date, a multivariate analysis is required and few researchers have done this. Unfortunately, our dataset was not large enough to allow us to tease apart possible seasonal effects from those of relative timing and density.

In conclusion, our results indicate that extra-pair paternity in eastern bluebirds is influenced by several socioecological variables including two correlates of breeding density and one aspect of timing. Nests in high density areas and surrounded by a greater number of neighbors within a 320-m radius were significantly more likely to have extra-pair young. Our results support previous studies on this and other species but highlight the fact that increases in density change several aspects of spatial relationships and that each of these could have effects on EPP. Density apparently alters the frequency and nature of encounters between females and males at several levels within our population. Mating decisions, however, were also influenced by local breeding synchrony and timing of breeding. Indeed, there was a significant interaction between neighbor density within 320 m and local breeding synchrony on the likelihood of EPP. Thus, our data do not support a simple relationship between breeding density and extra-pair paternity, but, rather, an intricate network of multiple spatial and temporal factors acting upon individual behavioral decisions. Such complexity in effects makes understanding the ecological basis for mating patterns both compelling and challenging.

Acknowledgements We thank the University of Kentucky for use of laboratory facilities for paternity analysis, Jackie Bennett, Emily Clemons, and Barb Kieffer for help in the field, and Diana Torres and Ian Stewart for help in the lab; all of these individuals contributed significantly to the success of this project. We also thank Jackie Augustine, Radovan Vaclav, and two anonymous reviewers for providing helpful comments on previous versions of the manuscript.

Ethical standards The authors declare that all experiments comply with current United States laws.

Conflict of interest The authors declare that they have no conflict of interest.

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