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Extra-pair offspring are less heterozygous than within-pair offspring in American redstarts (*Setophaga ruticilla*)

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Abstract

The vast majority of bird species are socially monogamous; however, extra-pair paternity is nearly ubiquitous and a number of theories have been proposed to explain the prevalence of this mixed mating strategy. Here, we test the genetic compatibility hypothesis—the idea that females that are genetically similar to their social partners will mate with extra-pair males that are genetically dissimilar to produce offspring that are more heterozygous. For this study, we examined eight years of paternity data (2004-2011) from a Nearctic-Neotropical migratory bird, the American Redstart (*Setophaga ruticilla*), breeding in southeastern Ontario, Canada. We predicted that females paired with genetically similar males (higher relatedness) would be more likely to produce extra-pair offspring and that extra-pair offspring would have higher levels of heterozygosity than within-pair offspring. Alternatively, because this population experiences high levels of immigration, females may produce extra-pair offspring with more genetically similar males because of the potential for outbreeding depression. Using five highly variable microsatellite markers, we examined patterns of relatedness among social pairs as well as measures of offspring heterozygosity. In contrast to our predictions, we found no difference in relatedness between social pairs where the females produced extra-pair offspring and social pairs where the females produced only within-pair offspring. However, extra-pair offspring were significantly less heterozygous than within-pair offspring. Together, these findings suggest that females a) are not engaging in extra-pair fertilizations based on relatedness to their social mate and b) appear to be mating with extra-pair males that are more genetically similar to themselves. We suggest there may be benefits for females to mate with genetically similar extra-pair males in highly outbred populations with high rates of immigration, such as for maintaining co-adapted gene complexes or genes coding for local adaptations.

Keywords: Heterozygosity, extra-pair paternity, American redstart, *Setophaga ruticilla*, microsatellite

Introduction

In many bird species, females form monogamous social bonds with their mates; however, in over 75% of these species, females mate with extra-pair males as part of a mixed-mating strategy (Griffith et al. 2002). Because of the frequency and ubiquity of extra-pair mating, many hypotheses have been developed to explain why females may engage in extra-pair paternity, including fertility insurance (Kroken et al. 1998), healthy mates (Reynolds and Gross 1990), good genes (Hamilton 1990, Zeh and Zeh 2001), sexy sons (Weatherhead and Robertson 1979), and genetic compatibility (Kempemaers et al. 1999). According to the genetic compatibility hypothesis, females may seek extra-pair copulations with males whose genes are more compatible with her own. One way mate incompatibility can arise is through inbreeding, which can lead to the loss of heterozygosity (genetic diversity) and the accumulation of deleterious homozygous recessive alleles (Charlesworth and Charlesworth 1987). Even in populations that are not inbred, maintaining heterozygosity has been positively linked to various fitness-related traits (reviewed in Reed and Frankham 2003). Therefore, it may be beneficial for females to mate with extra-pair males that are more genetically dissimilar to themselves to increase the heterozygosity of their offspring. For example, Splendid Fairy Wren (*Malurus splendens*) females who were more genetically similar to their social mates were more likely to have extra-pair offspring (EPO), and these offspring were more heterozygous than within-pair offspring (WPO) (Tarvin et al. 2005). Similarly, in Blue Tits (*Parus caeruleus*) extra-pair offspring were more heterozygous than within-pair offspring, and also had higher fitness (Foerster et al. 2003).

Alternatively, in open, outbred populations it may be more beneficial to females to mate with more genetically similar males, preserving locally-adapted genes or co-adapted gene complexes (Price and Waser 1979, Frankham 1995). Several studies in open, potentially outbred populations such as in Tree Swallows (*Tachycineta bicolor*; Barber et al. 2005), Pied Flycatchers (*Ficedula hypoleuca*; Rätti et al. 1995), and Waved Albatross (*Phoebastria irrorata*; Huyvaert and Parker 2010) have provided evidence for females in less closely related pairs producing extra-pair offspring with more closely related males.

To examine patterns of genetic compatibility, we studied the American Redstart (*Setophaga ruticilla*), a socially monogamous Nearctic-Neotropical migratory bird with relatively high rates of extra-pair paternity (43% of nests, 23% of offspring; Reudink et al. 2009a). Previous work on extra-pair paternity in our study population in southeastern Ontario, Canada, has revealed that males arriving early on the breeding

Accepted Article

grounds are much less likely to lose paternity, which provides possible support for the good genes hypothesis (Reudink et al. 2009a). Based on the genetic compatibility hypothesis, we predicted females that were more closely related to their social partners would be more likely to produce EPO compared to those less closely related to their social partners. We also predicted that within broods, EPO would be more heterozygous than WPO. Alternatively, because this population has high rates of immigration (Wilson et al. 2017), outbreeding may represent a higher cost than inbreeding. Under this scenario, females less closely related to their social partner would be more likely to produce EPO and that, within broods, EPO should be less heterozygous than WPO. Finally, we asked whether body size and timing of arrival and breeding were associated with adult heterozygosity. We determined relatedness of social parents and heterozygosity of offspring by analyzing blood samples of parents and offspring over eight years.

Methods

We studied a population of American redstarts from May-July 2004-2011 at the Queen's University Biological Station in southeastern Ontario, Canada ($n = 106$ nests, 414 nestlings; mean nestlings per nest = 3.8 ± 1.2 SD). Details of all field methods are described in Reudink et al. (2009a). Briefly, birds were monitored and territories mapped throughout the breeding season; plots were monitored throughout the arrival period and adults were captured in mist-nets using decoys and conspecific playback, at which time we took a 15-20ul blood sample by piercing the brachial vein. Each individual was banded with a Canadian Wildlife Service-issued aluminum band and 2-3 colour bands and we took standard measurements of tarsus length, tail length, unflattened wing chord length, and body mass. For nestlings, blood samples were taken on day 5 after hatching or on the day of fledging. No pairs were the same in multiple years; however, we accounted for the fact that some females ($n = 13$) and males ($n = 20$) were present in two years; no females were present in more than two years, but four males were present in more than two years.

Paternity analysis was conducted using five highly variable microsatellite loci (*Dpm01*, *Dpm03*, *Dpm05*, *Dpm15* and *Dpm16*; 13-29 alleles per locus; see Table 1 in Reudink et al. 2009a) originally isolated from Yellow Warblers (*Setophaga petechia*; Dawson et al. 1997). For details on paternity analysis and assignment see Reudink et al. (2009a). To determine relatedness between all social pairs, we used the R package *related* (Pew et al. 2015). Using this package's simulation function, we compared four relatedness estimators by using the allele frequencies of our population to generate 100 simulated pairs in each of the

following categories: parent-offspring, full-siblings, half-siblings and unrelated individuals. We determined that Wang's relatedness estimate (Wang 2002) provided the highest correlation coefficient between observed and expected relatedness estimates and thus was used as our measure of relatedness. Note that due to the low success of assigning paternity to specific extra-pair males and the presence of multiple extra-pair sires at nests (see Reudink et al. 2009b), we were not able to directly compare relatedness of extra-pair sires to within-pair males. However, because social pair relatedness was strongly associated with the heterozygosity of within-pair young ($r^2_{94} = 0.30$, $p < 0.0001$), we felt that extra-pair offspring heterozygosity could act as a useful proxy for inferring relatedness of females and extra-pair sires.

We used the R package *Rhh* (Alho et al. 2010) to analyze individual genotype data to determine three parameters describing offspring heterozygosity: internal relatedness (IR), standardized heterozygosity (SH) and homozygosity by loci (HL). IR is a measure of heterozygosity that allows the frequency of each allele to contribute to the calculated value so that rare shared alleles will produce higher values than shared common alleles (Amos et al. 2001). This measure also provides an estimate of relatedness between an individual's parents (Bean et al. 2004). High levels of heterozygosity are indicated by negative IR values while high levels of homozygosity are indicated by positive values. SH provides a standard scale to measure heterozygosity for all individuals, and is calculated by dividing the proportion of heterozygous loci by the average heterozygosity of that locus (Coltman et al. 1999). Unlike IR, all loci equally contribute to the calculated SH value despite their allelic frequencies, and higher SH values indicate higher heterozygosity. HL determines the weight given to each locus by considering the allelic variability at each locus, so loci with less variability and more alleles contribute more to the calculated value (Aparicio et al. 2006). Higher HL values indicate lower heterozygosity and vice versa. This index was developed to accurately predict heterozygosity in open populations with high levels of immigration; because IR gives more weight to rare shared alleles and thus may underestimate the heterozygosity of offspring of a native and an immigrant individual carrying rare alleles, (Aparicio et al. 2006).

To ask whether the relatedness of social pairs whose nests contained extra-pair offspring differed from those that contained only within pair offspring, we constructed a linear mixed effects model using the Wang relatedness value as a response, presence or absence of extra-pair offspring as a main effect, and female ID as a random effect. We also asked whether relatedness predicted the proportion of within-pair offspring in

a nest by constructing a model with proportion of extra-pair offspring as a response, Wang relatedness as a main effect, and female ID as a random effect. Next, we asked whether relatedness of social pairs predicted reproductive success by creating three generalized linear models with Poisson error distributions and a log link functions, using number of eggs, number of nestlings, or number of fledglings as a response variable, Wang relatedness as a main effect, and female ID as a random effect. Finally, to ask whether the heterozygosity of extra-pair offspring differed from within-pair offspring, we constructed three linear mixed models using heterozygosity (IR, SH or HL) as a response variable, extra-pair/within-pair as a main effect and nest ID nested within female ID as a random effect. We asked whether adult heterozygosity (IR, SH, or HL) was associated with body size (mass, wing, tarsus, tail) and arrival date (males) and first-egg date (females) using Spearman's ρ . To avoid pseudoreplication, each individual was only included once in the analysis (most recent occurrence).

Results

Overall, we detected extra-pair offspring in 47/106 (44.3%) nests, which accounted for 116/414 (28%) offspring in the study. In nests containing EPO, the number of EPO ranged from 1-4 (mean $\pm$ SD = 1.97 ± 1.10 EPO). We found no difference in the relatedness of social pairs that produced only within-pair offspring ($n = 59$ pairs; relatedness: 0.05 ± 0.03 SD) compared to social pairs whose nests contained extra-pair offspring ($n = 47$ pairs; relatedness: 0.09 ± 0.03 SD) ($F_{1,87.8} = 1.72$, $p = 0.19$). Similarly, there was no relationship between proportion of within-pair offspring in a nest and relatedness of the social pair ($F_{1,95.2} = 0.08$, $p = 0.77$). In addition, there was no relationship between the relatedness of the social pair and number of eggs laid ($n = 80$ nests, $z = 0.55$, $p = 0.58$), number of nestlings ($n = 103$, $z = 1.21$, $p = 0.23$), or number of fledglings ($n = 103$, $z = 0.85$, $p = 0.40$).

We found that EPO had higher internal relatedness (IR), lower standardized heterozygosity (SH), and higher homozygosity (HL) than WPO (Table 1). These results suggest that females were more genetically similar to extra-pair than within-pair mates, resulting in less heterozygous EPO.

We found no associations between any measure of male heterozygosity and tarsus length (all $p > 0.37$), tail length (all $p > 0.33$), wing length (all $p > 0.88$), mass (all $p > 0.12$) or arrival date on the breeding

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grounds (all $p > 0.25$). Similarly, no measures of female heterozygosity were associated with body size: tarsus length (all $p > 0.36$), tail length (all $p > 0.33$), wing length (all $p > 0.55$), mass (all $p > 0.44$), first-egg date (all $p > 0.54$).

Discussion

We found no evidence that females were more likely to produce extra-pair offspring based on relatedness to their social mate. However, all three measures of heterozygosity indicated that extra-pair offspring were significantly less heterozygous than within pair offspring (Table 1). This finding suggests that female American Redstarts may be mating with extra-pair males that are more genetically similar to themselves than their social mates. These results contrast with several studies demonstrating that extra-pair offspring are more heterozygous than within-pair offspring: e.g., Blue Tits (*Cyanistes caeruleus*; Foerster et al. 2003), Superb Starlings (*Lamprotornis superbus*, Rubenstein 2007), Tree Swallows (*Tachycineta bicolor*; Stapleton et al. 2007), and Reed Buntings (*Emberiza schoeniclus*; Suter et al. 2007). However, this pattern is not universal as other studies on Reed Buntings (*Emberiza schoeniclus*; Kleven and Lifjeld 2005), Blue-footed Boobies (*Sula nebouxi*; Kiere et al. 2016), Black-throated Blue Warblers (*Dendroica caerulescens*; Smith et al. 2005) and White-rumped Swallows (*Tachycineta leucorrhoa*; Ferretti et al. 2011) have found no difference in heterozygosity among extra-pair and within pair offspring. Fewer studies have shared our findings of lower heterozygosity in extra-pair offspring; however, Kleven et al. (2005) found North American Barn Swallow (*Hirundo rustica erythrogaster*) females were more genetically similar to their extra-pair partners than social mates and that extra-pair young were marginally significantly less heterozygous than within pair young. These inconsistent findings may be partially due to differences in methodology, as a meta-analysis of 43 studies found an overall positive relationship between genetic similarity of social pairs and probability of extra-pair paternity, but that only studies using microsatellite markers produced a positive effect size (Arct et al. 2015). The authors suggested that the level of outbreeding in a population could be an important confounding variable in studies on pair relatedness and extra-pair paternity.

One reason females may mate with more genetically similar extra-pair males could be to avoid outbreeding depression (reviewed in Pusey and Wolf 1996). Negative effects of outbreeding can arise due to the displacement of co-adapted gene complexes or genes coding for local adaptations (Price and Waser 1979,

Frankham 1995). In general, the optimal level of outbreeding is achieved by mating with distant relatives and avoiding mating with the closest relatives (Bateson 1978); for instance, female Japanese quail (*Coturnix japonica*) prefer to mate with cousins over brothers or unrelated males (Bateson 1982). As such, in highly outbred populations, we may predict that females paired to highly dissimilar males may be more likely to produce extra-pair offspring and do so with more genetically similar males, resulting in extra-pair offspring having lower heterozygosity.

Our population of American redstarts has extremely high immigration rates, especially among males (Wilson et al. 2017), suggesting the possibility of an outbred population. One prediction that arises from studies on potentially outbred populations that similarly found evidence of females in less closely related pairs producing extra-pair offspring with more closely related males (e.g., Rätti et al. 1995, Barber et al. 2005, Huyvaert and Parker 2010) is that less related social pairs should also experience reduced reproductive success (i.e., a cost associated with outbred pairings). We found no evidence of this effect: relatedness of pairs was not associated with number of eggs, nestlings, or fledglings. Similarly, we found no relationship between adult heterozygosity and body size or arrival date/first-egg date, suggesting that, at least in adults, any potential costs associated with high heterozygosity in this population are more subtle than these coarse metrics. Overall, we demonstrated that extra-pair offspring in our population of American redstarts are significantly more homozygous than within-pair offspring, suggesting that females are producing extra-pair offspring with more genetically similar extra-pair partners, possibly to prevent outbreeding depression.

Declarations

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- Accepted Article
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Table Legend

Table 1. Results from linear mixed models comparing heterozygosity of extra-pair offspring (EPO, n = 116) to within-pair offspring (WPO, n = 298). Nest ID nested within female ID was used as a random effect in all models.

Difference in Internal Relatedness (IR) between within-pair and extra-pair offspring				
Variable	Estimate	SE	<i>t</i>	<i>p</i>
Intercept	0.15	0.02	9.19	<0.0001
EPO vs. WPO	0.04	0.01	2.69	0.008
Difference in Standardized Heterozygosity (SH) between within-pair and extra-pair offspring				
Variable	Estimate	SE	<i>z</i>	<i>p</i>
Intercept	0.95	0.02	49.7	<0.0001
EPO vs. WPO	-0.04	0.02	-2.42	0.02
Difference in Homozygosity by Loci (HL) between within-pair and extra-pair offspring				
Variable	Estimate	SE	<i>z</i>	<i>p</i>
Intercept	0.25	0.01	17.56	<0.0001
EPO vs. WPO	0.03	0.01	2.68	0.0008