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Individual Heterozygosity Influences Arrival Times and Mating Success of Male Red-Breasted Flycatchers *Ficedula parva*

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We examined the relationship between individual heterozygosity of male Red-breasted Flycatchers (*Ficedula parva*; a small long-distance migratory, socially monogamous bird species) and their mating success, arrival time and age. Using eight polymorphic microsatellite loci, we found that male heterozygosity is related to both mating success and arrival time, but not to age. Mated and earlier arriving males had higher heterozygosity than later arrivals and bachelors, but we did not find a relationship between age and individual heterozygosity of males. To our knowledge, this is the first study to provide evidence about the relationship between individual genetic diversity and arrival time, thus arrival time could be used as a signal of individual heterozygosity and quality.

Key words: Multi-locus heterozygosity, Mate choice, Arrival time, Birds.

BACKGROUND

Heterozygosity is considered as individual genetic diversity of individuals and can be used as a predictor of male quality (Brown 1997; Kempenaers 2007). Females should mate with males with high heterozygosity and such a choice should be dictated by the relationship between fitness and heterozygous advantage. Heterozygosity-fitness correlation (HFC) is explained by three main hypotheses (Kempenaers 2007): general effect, direct effect and local effect hypotheses. The general effect hypothesis states that heterozygosity in many loci is related to heterozygosity of the entire genome and marker loci and fitness loci are in identity disequilibrium (David 1998; Lynch and Walsh 1998; Hansson and Westerberg 2002). The direct effect hypothesis assumes that heterozygosity is more advantageous than homozygosity due to relative functional dominance (David 1998; Hansson and Westerberg 2002; Li et al. 2004). The third explains heterozygosity-fitness correlations as an effect of linkage between neutral loci and the loci affecting fitness (Lynch and Walsh 1998; Hansson and Westerberg 2002; Tiira et al. 2006, Malo and Coulson 2009).

Heterozygosity-fitness correlations are often reported (Szulkin et al. 2010) and are considered to influence various aspects of fitness including offspring number and survival, parasite resistance, reproductive success and characteristics of bird song (Daniels and Walters 2000; Kruuk et al. 2002; Slate et al. 2004; Whiteman et al. 2006; Reid et al. 2007; Cohas et al. 2009; Mainguy et al. 2009; Ryder et al. 2009; Harrison et al. 2011). On the other hand, there are a number of studies indicating weak or no correlation (Hoffman et al. 2006; Ortego et al. 2007 2010; Boerner et al. 2013; Zeng et al. 2013). Moreover, there is also the possibility

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that publication bias has falsely inflated the importance of HFCs in animals (Balloux et al. 2004; Chapman et al. 2009), as many negative results may not be published.

In migrating bird species, arrival time can be a good indicator of male quality. Patterns of arrival are often related to physical condition or age. Males that are higher quality, in better condition, and older usually arrive earlier (Stolt and Franson 1995; Møller 1994a; Arvidson and Neergaard 1991; Marra et al. 1998; Ninni et al. 2004; Mitrus 2007). To date, no reports about the relationship between the arrival time of migratory birds and individual heterozygosity have been published. To test for such a correlation, a long-distance migratory bird species can be used as a model.

The main aim of this study was to answer the question: is individual heterozygosity of male Red-breasted Flycatchers (*Ficedula parva*) related to their mating success, arrival time and/or age? We analysed a group of highly polymorphic microsatellite loci of a free-living population of this species breeding in natural conditions.

MATERIALS AND METHODS

Study area

The data were collected in the Białowieża Forest, northeast Poland (52°41'N, 23°52'E). The study was conducted over seven breeding seasons (2004–2011, excluding 2007) in three study plots (total area 79.5 ha) and along roads leading to those plots. The study plots were similar in floral composition and covered by old-growth lime-oak-hornbeam *Tilio Querco-Carpinetum* stands characterised mainly by Hornbeam *Carpinus betulus*, Small-leaved Lime *Tilia cordata*, Pendunculate Oak *Quercus robur*, Norway Maple *Acer platanoides* and Norway Spruce *Picea abies.* The Białowieża Forest is old-growth, deciduous temperate forest (Tomiałojć 1991; Wesołowski et al. 2003) characterised by a rich bird and mammal community (Wesołowski et al. 2003; Jędrzejewska and Jędrzejewski 1998). There are standing and fallen dead trees (Tomiałojć 1991; Wesołowski et al. 2003; Jaroszewicz et al. 2019).

Bird data

Data were collected for the Red-breasted Flycatcher, which is a small migratory, insectivorous bird. After arrival males try to attract females by singing and defending territories. The earliest males arrive to the Białowieża Forest in Poland from late April to early May (Mitrus et al. 2005). Based on the colour of the breast plumage, we estimated male age using two categories: old (after-2nd-year, with orange badge on the breast) and young (2nd-year, nestlings from the previous season, without orange breast feathers) (Svensson 1992). The old males arrive significantly earlier than younger ones (Mitrus 2007). In our studied population, old males are more numerous (2/3 of all males) and achieve higher mating success than young males (Mitrus 2006 2018). Most males are socially monogamous (only one case of polygyny has been reported to date), and low extra-pair paternity has been recorded (Mitrus and Soćko 2005; Mitrus et al. 2014). The Red-breasted Flycatcher is a rare species in Europe, and occurs at low density in the Białowieża Forest, *i.e.*, in recent years, in optimum breeding habitat, up to 1.5 pairs/10 ha (Wesołowski et al. 2010 2015).

To determine the males' arrival dates (usually from the end of April to the middle of May), up to four people searched for newly arriving males every day. In the statistical analyses, the arrival date each year was standardised by considering the arrival date of the first male as day 1. The active territories were checked every day to determine mating success. Unmated males of the Red-breasted Flycatcher sing intensely; therefore, if a male was seen to have stopped singing and was observed with a female, or was seen copulating, then it was defined as mated (Mitrus et al. 2012).

Males were captured using a concealed tape-recorder with a broadcasted conspecific song to lure birds into a mist net (mesh – 16 mm, dimensions – 6 × 25 m). Each male was ringed using a unique combination of aluminum and colour-coded rings. Additionally, three feathers from the lesser coverts were collected for DNA isolation and analysis of paternity.

Molecular data

The feathers were stored in disposable, sterile tubes in 96% alcohol in the fridge at 4°C. Before DNA isolation was performed, the alcohol was evaporated and the quills (source of DNA, Morin et al. 1994; Kawka et al. 2012) were cut into fragments (0.5–1 cm long) using a sterile scalpel. The DNA was extracted using the Qiagen® QIAamp DNA Investigator Kit.

The nine most polymorphic loci described for other species in the genus *Ficedula* were used: Fhy336, Fhy350, Fhy224, Fhy458, Fhy429, Fhy452, Fhy428 (Leder et al. 2008) and FhU, FhU4 (Primmer et al. 1996). Selected microsatellites were characterized by high allelic diversity, the number of alleles at a single locus ranged from 4 to 26 (mean = 15.4) (Mitrus et al. 2013). Amplification of locus Fhy350 failed in some individuals, which we assumed was due to a null allele. Many authors recommend avoiding the use of loci with null alleles (Dakin and Avise 2004; Selkoe and Toonen 2006) and this locus was not considered for heterozygosity analysis. Most of the loci (except Fhy350) were in Hardy-Weinberg equilibrium (Mitrus et al. 2013).

Statistical analysis

To characterise multi-locus individual heterozygosity the Excel macro Cernicalin was used (Aparicio et al. 2006). Cernicalin calculates observed homozygosity per individual (HO). We used standardized heterozygosity (HST) (Coltman et al. 1999), which takes into account the proportion of heterozygous loci divided by the mean observed heterozygosity. This measure of heterozygosity is highly conservative and performs better than other measures when there is allele dropout or when individuals are genotyped at different numbers of loci (Coulon 2010).

We first examined if there were differences in heterozygosity between paired and unpaired males. Most genotyped young and older males did not come back to the study area the following year and most adults were new individuals that came from other places. Therefore, in many cases an individual was observed for only one year and no repeated measurement was used in the analysed; in this case, the data for each individual were dealt with as independent data.

To determine factors affecting the heterozygosity of males General Linear Models (GLM) were constructed: heterozygosity was used as the dependent variable, age and matting success as categorical factors and arrival time as a continuous independent variable (covariate). Statistica for Windows v. 13.0 was used for statistical calculations

RESULTS

Individual standardised heterozygosity within males ranged from 0.26 to 1.03 (Me = 0.883) and was non-normal in distribution (Kolmogorov-Smirnov test; d = 0.19, $p < 0.05$). The lower quartile (Q1) was 0.773, the upper quartile (Q3) was 0.903 (Fig. 1). In the GLM ($R^2 = 0.21$, $F_{3.38} = 9.43$, $p < 0.01$), mating success was related to the heterozygosity of males (Table 1) and the median heterozygosity of paired males was higher than that of unmated males (Fig. 2). The heterozygosity of males was related to their time of arrival (Table 1):

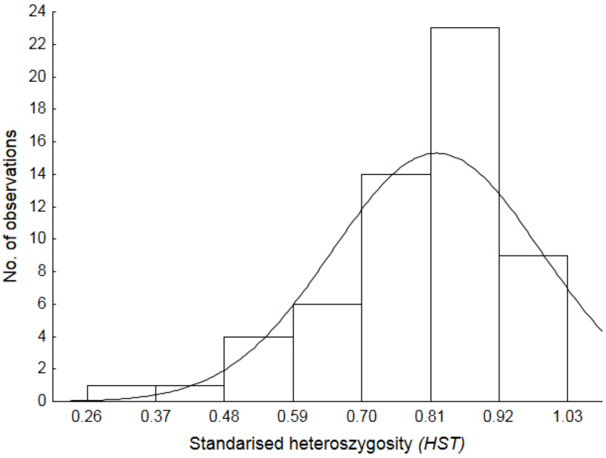

Fig. 1. Distribution of individual heterozygosity of male Red-breasted Flycatchers.

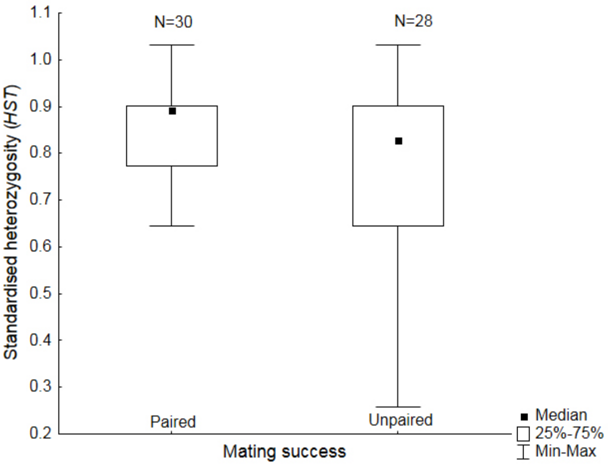

Fig. 2. Individual heterozygosity in relation to the mating success of male Red-breasted Flycatchers.

Table 1. Results of General Linear Model testing the relationships between heterozygosity and arrival time, mating success and male age

Variable	Degr. of freedom	MS	F	*p*-value
Arrival time	1	0.286	14.873	< 0.01
Mating success	1	0.225	8.314	< 0.01
Age	1	0.013	0.503	0.48
Error	37	0.027		

and the earlier arriving males had a higher individual standardised heterozygosity than later arriving ones (Fig. 3). The age of males was not related to their individual heterozygosity (Table 1.)

DISCUSSION

We used a set of polymorphic microsatellite markers to estimate individual heterozygosity and then evaluated correlation between individual genetic diversity and fitness of Red-breasted Flycatcher males. Microsatellite markers are widely applied in studies of HFC (*e.g.*, Wetzel et al. 2012; Forstmeier et al. 2012; Canal et al. 2014); however, their usefulness in such investigations remain a hotly debated issue (Väli et al. 2008; Ljungqvist et al. 2010). First, they are abundant in genomes, highly polymorphic and easy to analyse, allowing for a direct estimation of heterozygosity. On the other hand, complex mutational processes occurring within microsatellite loci lead to homoplasy which, in turn, can cause an underestimation of heterozygosity (Hansson and Westerberg 2002). Another problem is a connection between heterozygosity in microsatellite loci and genome-wide heterozygosity (Szulkin et al. 2010). As highly polymorphic microsatellites are usually desirable for population genetic studies, isolation and characterization of novel markers often lead to ascertainment bias and an upwardly skewed estimate of genome-wide diversity (Brandström and Ellegren 2008). However, some experimental and simulation studies indicated that microsatellites are good indicators of a genome-wide heterozygosity and are more informative than bi-allelic markers (*e.g.*, SNPs) because they have a large number of variants per locus (Ljungqvist et al. 2010; Miller et al. 2014).

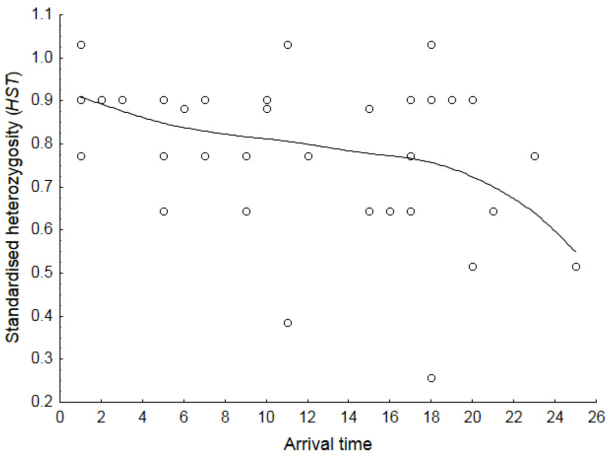

Fig. 3. Relationship between arrival time and heterozygosity of male Red-breasted Flycatchers.

Another factor affecting the reliability of heterozygosity-fitness analyses is sample size and the number of markers used (David 1998). The basic assumption underlying the HFC concept is that the level of individual heterozygosity is associated with performance traits. This suggests that individuals with low heterozygosity are inbred, and thus present low genome-wide diversity. However, the idea that a handful of neutral markers reflects genome-wide heterozygosity has often been criticized (Balloux et al. 2004; Taylor et al. 2010). Undoubtedly, in our study both the sample size and the number of microsatellite loci are low. Low sample size generated weak correlations in HFC studies (David 1998). Similarly, it was shown that a large number of markers are needed to increase the strength of correlation between heterozygosity and inbreeding, whereas a number of markers below 20 should result in a weak correlation (Balloux et al. 2004; Taylor et al. 2010; Miller et al. 2014). Slate et al. (2004) showed that the strength of the correlation between heterozygosity and inbreeding is a function of inbreeding prevalence, as well as the number of loci used. Hence, relationships between heterozygosity and fitness should be hard to detect if there are a low number of markers employed or the sample size is low. However, a low number of markers will usually indicate a significant correlation in the most extreme cases, when associations should be especially strong, *e.g.*, in highly inbred populations (Hedrick et al. 2001; Balloux et al. 2004; Slate et al. 2004). Hence, we can state that our results, despite having some flaws connected with sample size and number of markers, suggest that there are biologically important associations between individual heterozygosity and performance traits.

One finding of this study is that earlier arriving males of the Red-breasted Flycatcher had higher heterozygosity than later arriving males. To our knowledge, these results are the first documentation of a relationship between male heterozygosity and arrival time in a socially monogamous bird species. Protandry is often observed in migratory bird species, with males arriving before females (Kokko et al. 2006; Newton 2008; Harnos et al. 2014), and this is the case in Red-breasted Flycatchers. After arrival, males occupy and defend territories. The time that males arrive to the breeding areas can also be treated as an indicator of male quality. Arrival date is often correlated with size, condition and age of males (Mitrus 2007; Arvidson and Neergaard 1991; Marra et al. 1998; Forstmeier 2002; Stewart et al. 2002; Ninni et al. 2004; Risely et al. 2013). Individuals that arrive earlier may obtain higher quality territories (Potti and Montalvo 1991; Aebischer et al. 1996; Johnson 1997; Smith and Moore 2005) and can increase their probability of mating success

(Møller 1994b; Lozano et al. 1996; Kokko 1999). Also, in the Red-breasted Flycatcher, earlier-arriving males are older, possess a larger plumage ornament and have higher mating success (Mitrus 2007; Mitrus et al. 2012), all of which suggest that arrival time can be used as a good indicator of male quality.

Our data show that mating success of Red-breasted Flycatcher males was related to their heterozygosity and that the mated males had higher individual heterozygosity than unmated ones. It is not clear how female preference for heterozygous mates could confer an indirect genetic benefit for her and/or her offspring. Heterozygosity is not a visible mark and females are unlikely to be able to distinguish it directly, but it is often related to condition-dependent phenotypic traits (Ditchkoff et al. 2001; Hansson and Westerberg 2002; Foerster et al. 2003; Roberts et al. 2005; Tiira et al. 2006; Malo and Coulson 2009). A male's heterozygosity may also be correlated with characteristics related to male quality, such as song structure, reproductive and fledging success (Foerster et al. 2003; Seddon et al. 2004). Thus, female Red-breasted Flycatchers are predicted to value heterozygosity in their mates. The expression of vigour, condition-sensitive ornaments and symmetry in males may be a direct reflection not of "good genes" but of individual heterozygosity at key loci or at many loci.

The third finding of this study is that there were no differences in heterozygosity of males in relation to their age. Consistent with our finding, some other studies of wild populations of animals have also observed no differences in individual heterozygosity between various age classes (Diehl and Koehn 1985; Patarnello et al. 1991; Banks et al. 2010). However, a positive correlation between heterozygosity and survival is usually reported in various groups of animals, individuals with higher heterozygosity having a greater chance of survival (Coulson et al. 1998; Hansson et al. 2001; Lesbarrères et al. 2005; Acevedo-Whitehouse et al. 2003; Cohas et al. 2009). The difference in heterozygosity between younger and older males can be explained by higher selection pressure according to heterozygosity level early in life (Charlesworth and Hughes 1996; Keller et al. 2008) and differences in the rate of mortality and probability of survival among individuals in various stages of life. In birds, the highest mortality is observed in early life stages (Naef-Daenzer and Grüebler 2016), during which time individuals in bad condition and of low quality are likely to be eliminated (Newton 2008). In migratory birds, additional mortality occurs during the migration period, and may comprise 85% of apparent annual mortality and is at least 15 times higher than during the resident periods (Sillett and Holmes 2002). The Red-breasted Flycatcher is a long-distance migratory species; its one-way migration distance may reach 5000 km (Mitrus et al. 2005; Erciyas-Yavuz et al. 2015). Therefore, it should be expected that migration is an important factor causing high mortality in birds, especially young, first time migrating individuals (Owen and Black 1989; Newton 2008). Young birds that arrived on the breeding grounds have survived two long-distance migrations and their first winter, thus there is strong selection pressure for first time breeders to be high quality individuals. In a closely related species, the Pied Flycatcher *Ficedula hypoleca*—also a migratory species—the highest mortality is in young birds and fledgling recruitment is low, whereas mean survival probabilities of adult birds are much higher (Canal et al. 2014). In our studied population of the Red-breasted Flycatcher, older males arrived earlier (Mitrus et al. 2005 2012). Thus we expected that older, earlier-arriving birds would have higher heterozygosity than young males. However, within first arrivals, there were also young males that were in good condition (Mitrus 2006 2007) and had high heterozygosity. On the other hand, within older males we can distinguish only one class. However, this group includes birds in their third year of life that arrive later and this can influence the lower heterozygosity in this group of older males.

CONCLUSIONS

Our results show that individual heterozygosity in a migratory bird species is correlated with arrival time and mating success, but not to the age of males. Studies of heterozygosity-fitness correlations carried out under natural conditions in long distance migratory bird species are scarce. Migration is a very important part of bird life; it is also a factor influencing survival and applies strong selection pressure on individuals. In the future, more studies should be conducted on the relationships between heterozygosity, condition and survival during migration.

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