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# Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice

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The general hypothesis of mate choice based on non-additive genetic traits suggests that individuals would gain important benefits by choosing genetically dissimilar mates (compatible mate hypothesis) and/or more heterozygous mates (heterozygous mate hypothesis). In this study, we test these hypotheses in a socially monogamous bird, the blue tit (*Cyanistes caeruleus*). We found no evidence for a relatedness-based mating pattern, but heterozygosity was positively correlated between social mates, suggesting that blue tits may base their mating preferences on partner's heterozygosity. We found evidence that the observed heterozygosity-based assortative mating could be maintained by both direct and indirect benefits. Heterozygosity reflected individual quality in both sexes: egg production and quality increased with female heterozygosity while more heterozygous males showed higher feeding rates during the brood-rearing period. Further, estimated offspring heterozygosity correlated with both paternal and maternal heterozygosity, suggesting that mating with heterozygous individuals can increase offspring genetic quality. Finally, plumage crown coloration was associated with male heterozygosity, and this could explain unanimous mate preferences for highly heterozygous and more ornamented individuals. Overall, this study suggests that non-additive genetic traits may play an important role in the evolution of mating preferences and offers empirical support to the resolution of the lek paradox from the perspective of the heterozygous mate hypothesis.

**Keywords:** assortative mating; blue tit; genetic diversity; heterozygosity; relatedness

## 1. INTRODUCTION

Individuals can gain direct or indirect fitness benefits for their offspring through mate choice (Andersson 1994). Direct benefits can be obtained in the form of resources such as food, shelter or parental care provided by the partner. In both resource and non-resource mating systems, preferences for particular mates can also evolve via the indirect benefits associated with the alleles passed on to their offspring (Kempenaers 2007). This could particularly help to explain the existence of mating preferences when the chooser does not receive any benefit other than the 'genes' from their mate (Mays & Hill 2004). The evolution of mate choice based on genetic benefits has prompted a large number of studies, most of them focused on the dichotomy between additive ('good genes', or more properly 'good alleles', *sensu* Kempenaers 2007) and non-additive ('complementary genes' or 'complementary alleles', *sensu* Kempenaers 2007) effects. However, there is growing evidence that the two models are not mutually exclusive, so that mate choice could have evolved through both mechanisms (Colegrave *et al.* 2002; Roberts & Gosling 2003; Mays & Hill 2004; Reid 2007).

The good alleles hypothesis proposes that some individuals have superior alleles or combinations of alleles, which are revealed by condition-dependent traits, as is the case of certain ornaments (Hamilton & Zuk 1982; Møller & Alatalo 1999; Neff & Pitcher 2005). According to this model, individuals with more exaggerated ornaments are always preferred ('universally attractive' individuals), leading to directional selection on these traits. Therefore, the good alleles involved in the expression of more elaborated sexual traits are expected to spread rapidly throughout the population, depleting the additive genetic variation of the trait and, thus, the benefit of the choice (the so-called lek paradox; Kirkpatrick & Ryan 1991; reviewed in Kotiaho *et al.* 2008). In contrast to the good alleles model of sexual selection, the genetic compatibility hypothesis does not assume that the broad genetic quality of some individuals is intrinsically superior, but rather proposes that mate attractiveness depends on the interaction between male and female genotypes (Trivers 1972; Zeh & Zeh 1996, 1997). That is, the non-additive genetic benefit lies in acquiring a mate with a genome compatible with one's own. By mating with genetically unrelated individuals or with those dissimilar at certain fitness-related genes (e.g. genes of the major histocompatibility complex, MHC), individuals can produce more heterozygous offspring at genes experiencing some form of balancing selection while reducing the risk of expression of deleterious or partly deleterious recessive

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alleles in their progeny (Falconer & Mackay 1996; Charlesworth & Charlesworth 1999). This can ultimately result in increased offspring fitness as suggested by a number of studies finding associations between individual genetic diversity and fecundity (e.g. Foerster *et al.* 2003; Ortego *et al.* 2007*a*), immunocompetence (e.g. Hawley *et al.* 2005), parasite resistance (Acevedo-Whitehouse *et al.* 2003; Ortego *et al.* 2007*b*) and survival probability (e.g. Kruuk *et al.* 2002; Van de Casteele *et al.* 2003). However, several aspects of mate choice based on self-referential genetic characteristics are still unresolved: individuals will not show preferences for the same partner, and the mechanism by which individuals can identify genetically complementary mates and, more importantly, how this mechanism could have evolved are not clear (Colegrave *et al.* 2002; Wheelwright *et al.* 2006).

Individuals may also benefit by choosing heterozygous mates irrespective of genetic complementarity (the heterozygous mate hypothesis; Brown 1997; reviewed in Kempenaers 2007). Because heterozygosity is generally linked to individual quality, choosing heterozygous partners may be beneficial as such mates are likely to provide direct benefits in the form of increased territory size, fertility, fecundity or parental care (reviewed in Kempenaers 2007). Besides, heterozygote mating advantages may also accrue through indirect benefits because individual genetic diversity is expected to be correlated between parents and their offspring, so that the fitness differentials associated with heterozygosity are likely to be partly heritable (Mitton *et al.* 1993; Roberts *et al.* 2006). There is also certain evidence that ornaments may reflect individual heterozygosity (e.g. Scribner *et al.* 1989; Aparicio *et al.* 2001; Foerster *et al.* 2003; Van Oosterhout *et al.* 2003), which offers a relatively simple mechanism by which individuals could assess and choose more heterozygous individuals as social partners (Brown 1997; Kempenaers 2007). Yet studies testing mate choice for heterozygous individuals are scarce and only recently has this hypothesis gained certain empirical support (Reusch *et al.* 2001; Bonneaud *et al.* 2006; Hoffman *et al.* 2007).

In this study, we test the ‘compatible’ and ‘heterozygous mate’ hypotheses in a socially monogamous bird, the blue tit (*Cyanistes caeruleus*). In particular, we first study whether blue tits avoid mating with genetically related mates and/or show a heterozygosity-based mating pattern. Second, we test the expected benefits of the observed mating pattern, analysing whether heterozygosity is associated with mate quality (fecundity, egg quality, parental effort; i.e. direct benefits) and whether parental heterozygosity predicts offspring genetic diversity (i.e. indirect benefits). Finally, we analyse the association between heterozygosity and blue crown coloration, a sexually selected trait influencing social mating decisions in the blue tit (Andersson *et al.* 1998; Hunt *et al.* 1998, 1999).

## 2. MATERIAL AND METHODS

### (a) *Study site and general methods*

The breeding performance of blue tits was studied in two nearby localities of central Spain: the Cabañeros National Park (Ciudad Real and Toledo provinces, 39°24′ N, 3°35′ W) and Quintos de Mora (Toledo province, 39°25′ N, 4°04′ W). Both study areas comprise mainly oak-dominated woodlands with Mediterranean scrublands. A total of 250 and 200 nest-boxes in Cabañeros and Quintos de Mora, respectively, were monitored from April to June in 2007 and 2008. Basic data on reproduction were obtained by frequent checks of nest-boxes starting during nest building and continuing until fledging. Adult birds (Cabañeros: 59 females and 62 males; Quintos de Mora: 79 females and 69 males) were captured when feeding 8-day-old chicks by means of a spring trap shutting the entrance hole as the bird entered the nest. At capture, we measured tarsus length (digital caliper, $\pm 0.01$ mm) and body mass (electronic balance, $\pm 0.1$ g). Birds were sexed by the presence/absence of a brood patch and aged according to Svensson (1992) as juveniles (yearlings) or experienced breeders (second-year and older birds). Blood samples ($\leq 25$ μl) for genetic analyses were obtained by brachial venipuncture and stored in ethanol (96%). We equipped 60 females and 62 males with a passive integrated transponder (PIT) tag glued to two colour bands on one of their legs to monitor feeding activity (see below). All birds handled were individually marked with aluminium rings for further identification.

### (b) *Speckling assessments*

We used eggshell pigmentation pattern as an indicator of egg quality because this parameter is strongly associated with hatching success in the study population (Sanz & García-Navas 2009). To assess this trait, we took a digital photograph of each egg (749 eggs belonging to 103 nests) after clutch completion. All eggs were scored by the same observer (V.G.-N.) on the basis of the degree of spotting distribution (ranked in 0.5 increments from 1 for eggs whose spots are all on the broad end to 5 for eggs with specklings evenly distributed over the shell surface; see Gosler *et al.* 2000).

### (c) *Parental feeding effort*

Parental investment during the brood-rearing period (11–12 days post-hatching) was estimated as the number of visits to the nest during a 24 h period. For this purpose, individuals were fitted with PIT tags (Trovan ID 103). These tags transmit a unique code in the presence of an electromagnetic field. On day 10, we attached an antenna to the inside of the entrance hole of the focal nest-box. The antenna was then connected to a battery and a data-logging module placed in a plastic box camouflaged with leafy branches under the nest-box. By means of this system, we obtained a dataset reporting the time when a tagged bird entered or left the nest and individual identity. From these recordings, we extracted feeding rates following a procedure similar to that described by Johnsen *et al.* (2005). We first calculated the time difference between each record and removed all the zero time differences because an individual in the entrance hole causes multiple recordings on the same visit. We then sorted the data for each sex and again calculated the time difference between each record. We excluded all time differences that amounted to 6 s or less, which are not likely to represent actual feeding rates but are probably due to repeated entering on the same visit. We chose 6 s as cut-off point because it is unlikely that an individual can obtain a prey in such short time intervals. Finally, we counted the remaining records per day and divided this number by two to reach a daily rate of feeding. We also filmed 13 nests during the brood-rearing period to compare the above method with a direct assessment of feeding rates. We found a strong correlation between parental feeding rates estimated using videotape recording and those obtained by means of PIT tags (males: $r = 0.985$, $p < 0.001$; females: $r = 0.990$, $p < 0.001$).

### (d) *Colour measurements*

The spectral reflectance of the crown of blue tits (36 females and 42 males when feeding young in 2008) was measured in the Cabañeros study area. Data were collected in the field using an Ocean Optics USB2000 spectrophotometer (range 250–800 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 μm fibre-optic probe. The fibre-optic probe both provided illumination and obtained light reflected from the sample, and had a reading area of approximately 1 mm$^2$. The measurements were taken at a 90° angle to the sample. All measurements were relative to a white 'Spectralon' tablet (WS-1-SS, Ocean Optics), and reference measurements were frequently made. Three readings were obtained on different points of the crowns of the birds. Because of their small size, these readings were recorded at the same point but by removing the probe after each measure. For each individual, we averaged these three readings. Reflectance curves were determined by calculating the median of the percentage reflectance in 10 nm intervals, beginning at 320 nm and ending at 700 nm, to cover the full spectral range that can be detected by birds (Cuthill *et al.* 2000). We calculated parameters of the three dimensions of colour perception: brightness (total reflectance, $R_{320-700}$), chroma (spectral purity, $(R_{\max}-R_{\min})/R_{\text{average}}$) and hue (wavelength of peak reflectance, lambda ($R_{\max}$)). We used chroma and brightness in further analyses since these indices have been identified in previous studies as important predictors of mate attractiveness in blue tits (e.g. Andersson *et al.* 1998; Hunt *et al.* 1998, 1999).

### (e) *Genotyping and genetic diversity estimates*

We genotyped 212 blue tits (Cabañeros $n = 107$; Quintos de Mora $n = 105$) across 14 polymorphic microsatellite markers (see table 1 in the electronic supplementary material). We used NucleoSpin Tissue Kits (Macherey-Nagel) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10 μl reaction volumes containing 1× reaction buffer (67 mM Tris–HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf) thermal cycler. The PCR programme used was 9 min denaturing at 95°C followed by 35 cycles of 30 s at 94°C, 45 s at the annealing temperature (see the electronic supplementary material) and 45 s at 72°C, ending with a 5 min final elongation stage at 72°C. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems). Tests for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between markers were calculated following Weir (1996). We used two metrics to estimate individual genetic diversity: (i) uncorrected heterozygosity ($H_O$), calculated as the proportion of loci at which an individual is heterozygous; (ii) homozygosity by loci (*HL*), a microsatellite-derived measure that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability (Aparicio *et al.* 2006). *HL* is calculated as follows: $HL = (\Sigma E_h)/(\Sigma E_h + \Sigma E_j)$, where $E_h$ and $E_j$ are the expected heterozygosities of the loci that an individual bears in homozygosis ($h$) and in heterozygosis ($j$), respectively (Aparicio et al. 2006). $H_O$ and *HL* were calculated using Cernicalin, an Excel spreadsheet available on request.

### (f) *Spatial genetic structure*

To eliminate the possibility that spurious associations between the studied parameters and multilocus heterozygosity arise from population stratification (Slate *et al.* 2004; Slate & Pemberton 2006), we assessed evidence for genetic subdivision between and within Cabañeros and Quintos de Mora subpopulations using a Bayesian model-based clustering method (structure 2.1; Pritchard *et al.* 2000). We ran structure assuming correlated allele frequencies and admixture, and conducted five independent runs of $K = 1–5$ to estimate the true number of clusters with $10^6$ Markov chain Monte Carlo (MCMC) cycles, following a burn-in period of 100 000 iterations (Pritchard *et al.* 2000). Although we found no evidence for genetic subdivision, we followed the conservative criterion of fitting subpopulation identity as a random effect into the models (see below).

### (g) *Statistical analyses*

#### (i) *Relatedness-based mating pattern: a test of the compatible mate hypothesis*

We explored whether blue tits avoid mating with genetically similar individuals by comparing the observed distribution of mate relatedness with a distribution obtained if individuals would have mated randomly with any breeding opposite-sex individual in the same subpopulation (Kempenaers 2007). We calculated relatedness between individuals using the similarity index described by Li *et al.* (1993) as implemented by the program STORM (Frasier 2008). This index has been proved to be an adequate marker-based estimator of relatedness in natural populations and often outperforms other estimators (Van de Casteele *et al.* 2001). We generated 1000 iterations to obtain the average relatedness of randomly generated mating pairs, and compared observed and expected relatedness values using paired *t*-tests.

#### (ii) *Heterozygosity-based mating pattern: a test of the heterozygous mate hypothesis*

We compared individual genetic diversity between social mates using two-tailed Pearson's correlation analyses. To avoid individuals captured in consecutive years contributing data in the same analysis, we only selected a single datum for an individual and a social pair. We also explored the possibility of assortative mating with respect to other variables that may affect mating decisions in blue tits. In particular, we compared age, tarsus length, body weight and physical condition (residuals of body mass on tarsus length) between social mates. Finally, we used generalized linear mixed models (GLMMs) implemented with the GLIMMIX macro of SAS (SAS Institute 2004) to analyse the association between these variables and individual genetic diversity. GLMMs with a normal error structure and an identity link function were constructed fitting individual genetic diversity as covariate. Year, subpopulation and individual identity were included as random effects in all these analyses in the manner of a randomized complete block design to avoid pseudoreplication.

#### (iii) *Heterozygosity and mate quality: direct benefits*

We analysed the effects of individual genetic diversity on different parameters that may ultimately have important consequences on mate reproductive performance. In females, we analysed the effects of heterozygosity on both clutch size (Foerster *et al.* 2003; Ortego *et al.* 2007*a*) and eggshell pigmentation pattern (Sanz & García-Navas 2009). In both sexes, we analysed the effects of heterozygosity on provisioning rates, an estimate of parental investment on reproduction. We performed a GLMM with a Poisson error structure and log link function to analyse clutch size, whereas a normal error and identity link function was used to analyse all other dependent variables. Initially, each GLMM was constructed with all explanatory terms fitted, including first-order interactions and quadratic effects to account for potential nonlinear relationships. Final models were selected following a backward procedure, by progressively eliminating non-significant variables and fitting year, subpopulation and individual identity as random effects.

#### (iv) *'Heritability' of heterozygosity: indirect benefits*

We analysed the association between expected offspring heterozygosity and both maternal and paternal heterozygosities. For this purpose, we used parental genotype data to calculate expected offspring heterozygosity. For each mating pair, we calculated average *HL* of 100 simulated offspring over 1000 iterations using the program STORM (Frasier 2008). Then, we analysed the association between estimated average offspring *HL* and both maternal and paternal *HL* using linear regression analyses. In these analyses, we selected a single datum for an individual and a social pair to avoid pseudoreplication ($n = 78$ breeding pairs). We also used the observed genotypes in our population to calculate the heritability of heterozygosity under random mating. For this purpose, we designed an EXCEL macro to perform Monte Carlo simulations and generate the distribution of expected correlations between parent–offspring heterozygosities in our dataset, assuming that mating is random with respect to heterozygosity. We performed 1000 iterations of 78 mating pairs each to create a distribution of expected correlations coefficients ($r$) between parent–offspring heterozygosities and compare them with those actually observed in our population.

#### (v) *Heterozygosity and ornamentation*

We analysed the effects of individual heterozygosity on blue crown coloration, including brightness and chroma as dependent variables in different GLMs, individual heterozygosity (estimated as *HL* or $H_O$) and physical condition as covariates and age as fixed factor. We also tested the possibility of assortative mating with respect to these traits using two-tailed Pearson's correlation analyses.

## 3. RESULTS

### (a) *Genetic diversity and spatial genetic structure*

The number of alleles per locus ranged from 10 to 51, the mean being 19.4 (see the electronic supplementary material). After applying sequential Bonferroni corrections to compensate for multiple statistical tests, no locus deviated from HWE. There was no evidence of genotypic linkage disequilibrium at any pair of loci (all $p > 0.05$). The measures *HL* and $H_O$ were highly correlated ($p < 0.0001$; $r = 0.95$). Under a wide range of simulated scenarios, *HL* has been proved to be a better predictor of genome-wide heterozygosity than $H_O$ in open populations (Aparicio *et al.* 2006). Furthermore, *HL* generally provides a better fit of the data than $H_O$ in both this and previous genetic studies (e.g. Ortego *et al.* 2008*a*). For these reasons, statistical analyses and graphical outputs are only presented for *HL* analyses. $H_O$ data provided analogous results and are available from the authors. Results of STRUCTURE analyses showed a maximum $\Pr(X|K)$ for $K = 1$, indicating no genetic subdivision within the study area.

### (b) *Relatedness-based mating pattern: a test of the compatible mate hypothesis*

Relatedness values of breeding pairs ranged from 0.38 to $-0.44$ (mean $= 0.02$), suggesting that some pairs are constituted by genetically related individuals. This is also supported by some pedigree data: we have detected two cases of mating between close relatives: a mother–offspring pair and a full-sib pair. We found no difference between observed and expected relatedness values under random mating (paired *t*-test; $n = 78$, $t = 0.036$, $p = 0.972$), suggesting no relatedness-based mate choice. Neither observed nor expected relatedness values differed when they were compared for each study subpopulation separately (Cabañeros: $n = 44$, $t = 1.174$, $p = 0.247$; Quintos de Mora: $n = 34$, $t = 1.238$, $p = 0.224$).

### (c) *Heterozygosity-based mating pattern: a test of the heterozygous mate hypothesis*

*HL* was positively correlated between social mates ($r = 0.341$, $n = 78$, $p = 0.002$; figure 1). We explored the possibility that the observed correlation of intra-pair male and female heterozygosities is a consequence of differences in genetic diversity between study areas and plots, which may have resulted in a passive heterozygosity-assortative mating independent of individual preferences (*sensu* Ferrer & Penteriani 2003). We found that *HL* did not differ between study areas (one-way ANOVA: males, $F_{1,95} = 2.06$, $p = 0.154$; females, $F_{1,111} = 0.52$, $p = 0.472$). To further confirm our results, we also compared individual genetic diversity between social mates in each subpopulation separately: we found that intra-pair male and female heterozygosities were positively correlated in both Cabañeros ($r = 0.362$, $n = 44$, $p = 0.016$) and Quintos de Mora ($r = 0.345$, $n = 34$, $p = 0.046$), indicating that the observed heterozygosity-based assortative mating was consistent across the study areas. By contrast, individual size (tarsus length: $r = 0.174$, $n = 107$, $p = 0.073$; weight: $r = 0.131$, $n = 107$, $p = 0.178$), physical condition ($r = 0.126$, $n = 107$, $p = 0.200$) or age (binomial test: $\chi^2 = 1.09$, $n = 107$, $p = 0.290$) were not correlated between social mates and GLMMs showed that these traits are not associated with *HL* in any sex (all $p > 0.05$). Values for all individual measurements are provided in table 2 in the electronic supplementary material.

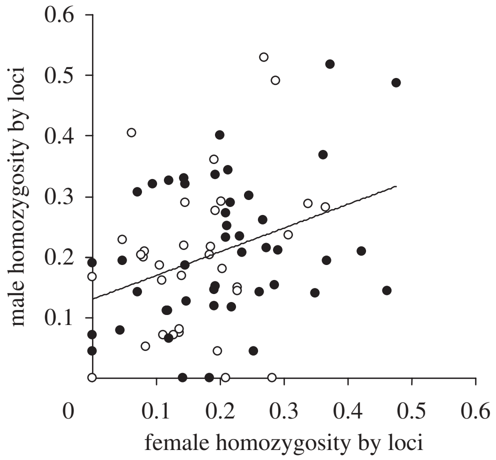

Figure 1. Male homozygosity by loci plotted against female homozygosity by loci for 78 breeding blue tit pairs. Cabañeros breeding pairs, filled circles; Quintos de Mora breeding pairs, open circles.

### (d) *Heterozygosity and mate quality: direct benefits*

After controlling for other influential variables (table 1), clutch size was negatively associated with female *HL* (table 1, figure 2*a*). Further, spotting distribution was negatively associated with squared female *HL* (table 1, figure 2*b*), i.e. heterozygous females laid eggs with pigment spots evenly distributed over the egg surface and this effect was more pronounced at low heterozygosity levels. Male feeding rates were positively associated with brood size and negatively associated with their *HL* (table 1, figure 3). However, we did not detect any effect of *HL* on female feeding rates (table 1). After controlling for subpopulation, year and male and female identities, we found that the total number of fledged chicks (Poisson error and log link) was positively associated with clutch size ($F_{1,62} = 40.91$, $p < 0.001$) and eggshell spotting distribution ($F_{1,62} = 4.56$, $p = 0.037$), but we found no direct effect of male ($F_{1,61} = 0.06$, $p = 0.810$) or female ($F_{1,61} = 0.10$, $p = 0.759$) *HL*. Values for all breeding performance parameters are provided in table 2 in the electronic supplementary material.

### (e) *Heritability of heterozygosity: indirect benefits*

Linear regression analyses showed that estimated offspring *HL* is associated with both paternal ($\beta = 0.241$, $n = 78$, $p = 0.033$) and maternal ($\beta = 0.326$, $n = 78$, $p = 0.004$) *HL*, indicating that genetically diverse parents produce on average more heterozygous offspring. The heritability of heterozygosity (estimated using calculated correlation coefficients, *r*) under random mating (mean ± s.e.; males: $0.204 \pm 0.103$; females: $0.144 \pm 0.111$) was smaller than that observed in the study population (males: $r = 0.241$; females: $r = 0.326$). The heritability of heterozygosity observed in the study population only marginally differed from the distribution obtained under random mating in females ($p = 0.056$) but not in males ($p = 0.369$).

### (f) *Heterozygosity and ornamentation*

Crown brightness was negatively associated with male ($F_{1,39} = 6.23$, $p = 0.017$; figure 4) but not with female ($F_{1,33} = 0.01$, $p = 0.984$) *HL*. However, there was no significant relationship between crown chroma and male ($F_{1,39} = 0.84$, $p = 0.366$) or female ($F_{1,33} = 0.50$, $p = 0.484$) *HL*. Physical condition and age were not associated with crown brightness or chroma in any sex (all $p > 0.05$). Finally, crown plumage coloration was not significantly associated between social mates (brightness: $r = -0.097$, $n = 26$, $p = 0.637$; chroma: $r = 0.352$, $n = 26$, $p = 0.078$).

## 4. DISCUSSION

We have found that individual heterozygosity of blue tits is positively correlated between social mates, suggesting that this species may base their mating preferences on partner's heterozygosity. Other variables such as individual size, weight or physical condition did not correlate between social mates and were not associated with individual genetic diversity, suggesting that the observed heterozygosity-based assortative mating is not mediated by a third association between heterozygosity and other variables related to individual quality. To the best of our knowledge, and with the exception of a single MHC-based study (Bonneaud *et al.* 2006), our study provides the first evidence of heterozygosity-based assortative mating in any animal population (Kempenaers 2007). It should be noted that blue tits show high rates of extra-pair paternity and so an interesting question to be addressed in the future would be also analysing the correlation between the heterozygosities of mates actually producing offspring through extra-pair fertilizations (Foerster *et al.* 2003, 2006). Although genetic similarity as a target for mate choice has received more attention than the effects of heterozygosity *per se*, we have found no evidence for relatedness-based mate choice in blue tits, suggesting that active inbreeding avoidance after settlement does not determine mating decisions in this species. This is not particularly noteworthy, as most previous studies on birds have also generally not found any evidence for relatedness-based mate choice (e.g. Van Tienderen & Van Noordwijk 1988; Keller & Arcese 1998; Van de Casteele & Matthysen 2006; Hansson *et al.* 2007; see also Wheelwright *et al.* 2006), which suggests that natal dispersal may be a sufficient mechanism to avoid close inbreeding or reach a certain dissimilarity threshold in most situations (Van de Casteele & Matthysen 2006; Ortego *et al.* 2008*b*; Szulkin & Sheldon 2008).

A possibility for the role of heterozygosity on the observed mating pattern is that genetic diversity is associated with individual quality and this ultimately results in increased mate fitness (i.e. individuals obtain direct benefits from choosing more heterozygous mates; Brown 1997; Kempenaers 2007). The overwhelming majority of studies on mating preferences so far have tended to focus predominantly on female choice. However, in monogamous species with biparental care, such as the blue tit, mutual choosiness is expected and males may also prefer heterozygous females as social partners (Kempenaers 2007; Clutton-Brock 2009). In the studied blue tit population, we have certain evidence that the observed assortative mating for heterozygosity could be explained by the fact that heterozygosity seems to reflect individual quality in both sexes. We have found a positive association between individual genetic diversity and

Table 1. GLMMs for clutch size (Poisson error and log link function), eggshell spotting distribution (normal error and identity link function) and feeding rates (normal error and identity link function) in relation to homozygosity by loci (*HL*) and non-genetic terms. Parameter estimates ± s.e. are only indicated for variables included in the models and were calculated considering a reference value of zero for second-year individuals in the variable 'age'.

| | independent variables | estimate ± s.e. | test | *p* |
|---|---|---|---|---|
| *females* | | | | |
| clutch size | intercept | 2.299 ± 0.084 | | |
| | laying date | −0.011 ± 0.001 | $F_{1,143} = 88.08$ | <0.001 |
| | female age | −0.072 ± 0.024 | $F_{1,143} = 7.26$ | 0.008 |
| | female *HL* | −0.379 ± 0.161 | $F_{1,143} = 5.54$ | 0.019 |
| | individual identity | 0.018 ± 0.005 | $Z = 3.45$ | <0.001 |
| | year | 0.012 ± 0.012 | $Z = 0.95$ | 0.171 |
| | subpopulation | 0.001 ± 0.004 | $Z = 0.13$ | 0.447 |
| spotting distribution | intercept | 3.072 ± 0.312 | | |
| | clutch size | 0.064 ± 0.031 | $F_{1,119} = 4.36$ | 0.039 |
| | female $HL^2$ | −2.945 ± 1.416 | $F_{1,119} = 4.32$ | 0.039 |
| | laying date | | $F_{1,118} = 0.80$ | 0.372 |
| | female age | | $F_{1,118} = 0.98$ | 0.324 |
| | individual identity | 0.277 ± 0.065 | $Z = 4.24$ | <0.001 |
| | year | 0.083 ± 0.091 | $Z = 0.91$ | 0.182 |
| | subpopulation | 0 | — | — |
| feeding rates | intercept | 61.112 ± 34.903 | | |
| | brood size | 12.432 ± 4.490 | $F_{1,55} = 7.67$ | 0.008 |
| | female *HL* | | $F_{1,54} = 0.88$ | 0.353 |
| | laying date | | $F_{1,54} = 1.40$ | 0.241 |
| | female age | | $F_{1,54} = 0.35$ | 0.559 |
| | female identity | 0 | — | — |
| | year | 363.30 ± 898.94 | $Z = 0.40$ | 0.343 |
| | subpopulation | 0 | — | — |
| *males* | | | | |
| feeding rates | intercept | 76.778 ± 74.358 | | |
| | brood size | 16.875 ± 5.991 | $F_{1,39} = 7.93$ | 0.008 |
| | male *HL* | −216.34 ± 88.22 | $F_{1,39} = 6.01$ | 0.019 |
| | laying date | | $F_{1,38} = 0.37$ | 0.547 |
| | male age | | $F_{1,38} = 0.33$ | 0.568 |
| | male identity | 0 | — | — |
| | year | 2210.70 ± 4013.52 | $Z = 0.55$ | 0.291 |
| | subpopulation | 2616.34 ± 7439.97 | $Z = 0.35$ | 0.363 |

parental investment in males, indicating that females will obtain better paternal care for their progeny by mating with highly heterozygous males. By contrast, we have found no association between heterozygosity and feeding rates in females. This may be expected, as in our study population feeding rates are much higher in males than in females (paired *t*-test: $n = 51$; $t = -3.433$; $p = 0.001$) and so the effects of heterozygosity would be particularly patent in the sex that invests more during this breeding phase. On the other hand, males would also obtain important benefits by preferring heterozygous females as social partners: genetically more diverse females laid larger clutches and produced eggs with a more optimal pigmentation pattern, parameters that are ultimately associated with breeding performance in our study population (Sanz & García-Navas 2009). The relationship between individual genetic diversity and female fecundity has been previously reported in blue tits (Foerster *et al.* 2003) and other bird species (Tomiuk *et al.* 2006; Ortego *et al.* 2007*a*), suggesting that egg production and quality, both depending on maternal allocation during the critical egg-laying period, are good candidates to reveal the effects of genetic diversity in comparison with other components of fitness that are not so strictly under female control (Ortego *et al.* 2007*a*).

The possible role of inbreeding in heterozygosity–fitness correlations has recently been put into question (Coltman & Slate 2003; Balloux *et al.* 2004; Pemberton 2004). However, the associations we have found between individual genetic diversity and the studied fitness-related traits could respond to the fact that the study system may show inbreeding variance higher enough as to be reflected by heterozygosity estimated using neutral markers (Balloux *et al.* 2004; Van de Casteele & Matthysen 2006). Accordingly, we have recorded two cases of close inbreeding and this may also be relatively frequent in other populations of blue tits (Van de Casteele & Matthysen 2006) and other related species (Szulkin & Sheldon 2008).

Another possibility for the role of heterozygosity on the observed mating pattern is that mate heterozygosity affects offspring genetic diversity, which can ultimately have important consequences on different components of offspring fitness, such as post-fledgling survival and future mating success and reproduction (see Kempenaers 2007 for a review). Accordingly, we have found an association between estimated offspring heterozygosity and

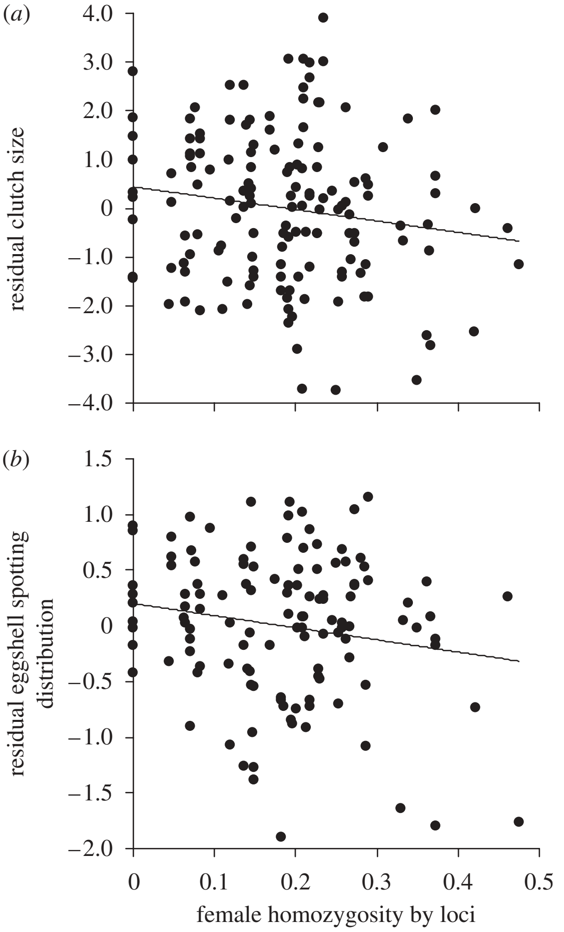

Figure 2. Relationship between homozygosity by loci of female blue tits and (*a*) clutch size and (*b*) eggshell spotting distribution. Clutch size and spotting distribution are both expressed as statistical residuals obtained after controlling for other influencing variables (see table 1).

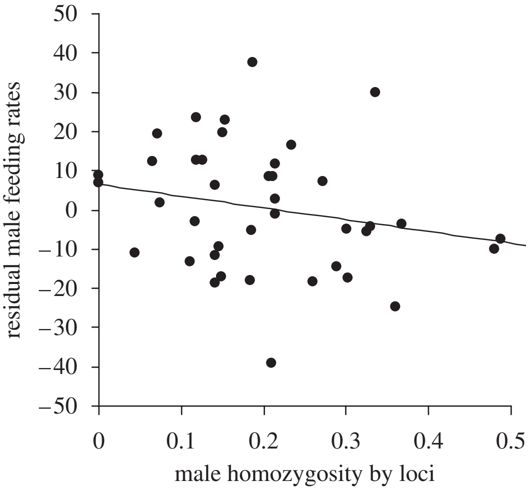

Figure 3. Relationship between homozygosity by loci and feeding rates in male blue tits. Feeding rates are expressed as statistical residuals obtained after controlling for other influencing variables (see table 1).

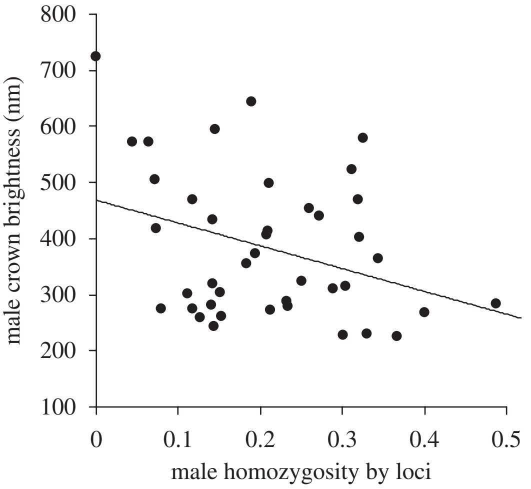

Figure 4. Relationship between homozygosity by loci and crown coloration brightness in male blue tits.

both maternal and paternal heterozygosities, suggesting that individuals may choose heterozygous mates not only to gain certain direct benefits but also to increase the likelihood of producing more heterozygous offspring (Hoffman *et al.* 2007). Such an association arises from the fact that, at most allelic frequencies, heterozygous parents produce higher proportions of heterozygous progeny than do homozygous parents (Mitton *et al.* 1993; Roberts *et al.* 2006; Hoffman *et al.* 2007). Our findings are in agreement with a recent study that has found strong evidence that female Antarctic fur seals (*Arctocephalus gazelle*) show active choice for heterozygous males and that such preferences increase offspring heterozygosity, because male (but not female) heterozygosity predicted offspring genetic diversity in their study population (Hoffman *et al.* 2007).

It is important to understand the way by which individuals may assess the genetic quality of their potential partners. In this respect, we have found that heterozygosity is positively correlated with crown brightness in male blue tits. However, we did not find any association between heterozygosity and female crown coloration. Although there is evidence that this plumage trait indicates sexual attractiveness in both sexes (Andersson *et al.* 1998; Hunt *et al.* 1998, 1999), sexual selection is likely to act more strongly in males than females (e.g. due to asymmetrical investment in gametes and high levels of extra-pair paternity) and this may explain the absence of association between female heterozygosity and the studied secondary sexual trait. According to our results, Foerster *et al.* (2003) also showed a similar correlation between male heterozygosity and crown coloration in this species. Thus, both our finding and previous findings suggest that the studied ornamental secondary sexual trait is also a reliable indicator of heterozygosity in this species and therefore individuals should tend to converge when choosing a social partner. This means that directional mating preferences favouring more ornamented individuals could be driven in part by non-additive genetic benefits. Evidence is accumulating that individual genetic diversity is associated with other sexually selected traits such as plumage ornamentation (Weatherhead *et al.* 1999; Aparicio *et al.* 2001), song characteristics (Marshall *et al.* 2003; Seddon *et al.* 2004; Reid *et al.* 2005; Reid 2007) and courtship display (Van Oosterhout *et al.* 2003). The existence of direct and indirect benefits of mating with heterozygous individuals could explain both unanimous mating preferences and the maintenance of the additive genetic variability in ornamental traits (Fromhage *et al.* 2008; Neff & Pitcher 2008). Thus, our findings are consistent with the suggestion that non-additive benefits may underlie mating patterns and this offers an alternative explanation that could help to resolve the lek paradox (Hoffman *et al.* 2007; Reid 2007).

In conclusion, we have found an example of heterozygosity-based assortative mating that can be explained by both direct and indirect benefits because genetic diversity predicts both mate quality (egg production and quality in females and parental effort in males) and offspring heterozygosity. Further, genetic quality in the form of higher heterozygosity is reflected by a secondary sexual trait that may ultimately result in unanimous directional mate preferences for more ornamented individuals. Overall, this study suggests that the expression of secondary sexual traits and mate preferences have a relevant non-additive genetic component, and offers empirical support to the resolution of the lek paradox from the perspective of the heterozygous mate hypothesis.

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