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Multiple sexual ornaments signal heterozygosity in male blue tits

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Higher individual genetic quality has been hypothesized to be associated with the expression of conspicuous ornaments. However, the relationship between multicomponent sexual signals and heterozygosity is poorly understood. In this study, we examined whether different ornaments, including song (repertoire size and bout length) and plumage coloration (yellow breast and blue crown), reflect individual genetic diversity in male blue tits (Aves: *Cyanistes caeruleus*). We estimated genetic diversity using 26 microsatellite markers that were classified as putatively functional (12 loci) and neutral (14 loci). We found that yellow breast carotenoid chroma, blue crown brightness, bout length and body condition were positively associated with heterozygosity at functional loci, but not with genetic diversity estimated at all typed loci or the subset of neutral markers. The lack of strong single-locus effects and the presence of identity disequilibrium in our population suggest that the observed heterozygosity-phenotype associations are driven by loci widely distributed across the genome. The predominant role of putatively functional loci evidences that the expression of secondary sexual characters is more tightly reflected by heterozygosity at genomic regions containing coding genes that are being actively expressed, a fact that may make ornamental traits more reliable indicators of the genetic quality of individuals. Overall, this study shows that multiple secondary sexual characters reflect male genetic diversity and lends support to the good-genes-as-heterozygosity hypothesis. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **115**, 362–375.

ADDITIONAL KEYWORDS: back-up signal – *Cyanistes caeruleus* – functional markers – good genes – honest signalling ornaments – plumage coloration.

INTRODUCTION

Mate choice based on elaborated sexual ornaments is an important focus of study in behavioral and evolutionary research. The expression of secondary sexual traits often entails high costs, which implies that individuals (generally males) face a trade-off between investing in these ornaments and allocating resources towards other necessary physiological processes (Andersson, 1994). Thus, only superior males will be able to develop and maintain these conspicuous traits without jeopardizing their viability and, as a result, ornaments become reliable and honest signals of

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individual quality (Zahavi, 1975; Getty, 1998). Female preferences for ornamented males are maintained as a result of the benefits derived from such selective behavior. Females may choose attractive males for direct benefits in terms of either increased parental care (Hoelzer, 1989; Kokko, 1998; Senar, Figuerola & Pascual, 2002) or enhanced fertility (Sheldon, 1994; Helfenstein *et al*., 2010). Such a preference for more ornamented males may also result in indirect additive genetic benefits if they are able to produce offspring of superior genetic quality through the transmission of good alleles or fewer deleterious alleles (Von Schantz *et al*., 1996; Fromhage, Kokko & Reid, 2009; Cutrera, Fanjul & Zenuto, 2012). Another possibility is that ornaments reflect male heterozygosity ('good-genes-as-heterozygosity hypothesis'; Brown, 1997), a genetic trait that has often been found to positively affect fitness due to overdominance and a reduced chance that deleterious recessive alleles will be expressed (reviewed in Chapman *et al*., 2009; Szulkin, Bierne & David, 2010). Selection on highly ornamented and heterozygous males may increase female fitness directly, e.g. via increased provisioning effort of more heterozygous partners (e.g. García-Navas, Ortego & Sanz, 2009), or indirectly, via non-additive genetics benefits such as the production of more heterozygous descendants (reviewed in Kempenaers, 2007). The latter can be possible when allele frequencies are asymmetric (Mitton *et al*., 1993; Reid, Arcese & Keller, 2006; Roberts, Hale & Petrie, 2006; Ortego *et al*., 2009). Under this circumstance, the most common in multi-allelic loci, more heterozygous parents produce more heterozygous offspring (i.e. heterozygosity becomes 'heritable' *sensu* Mitton *et al*., 1993).

Information conveyed by different ornaments can be complementary ('multiple messages' hypothesis) or redundant ('back-up signal' hypothesis) (reviewed in Candolin, 2003). According to the 'multiple messages' hypothesis, different ornaments can provide information about different aspects of mate quality and, evaluated together, these traits reflect overall quality (Møller & Pomiankowski, 1993). Meanwhile, multiple back-up cues (i.e. traits that reflect the same quality with some error) may facilitate mate assessment and/or make it more difficult for mates to misrepresent their quality (Johnstone, 1996, 1997). Back-up signals are thought to be less common than multiple messages as the majority of studies have found multiple traits to be uncorrelated (e.g. Marchetti, 1998; but see Hegyi *et al*., 2015). However, there is little available information about the relationship between the expression of secondary sexual traits and individual genetic diversity and most studies on this topic have focused only on one or few traits (e.g. Foerster *et al*., 2003; Marshall, Buchanan & Catchpole, 2003; Reid *et al*., 2005; but see Bolund *et al*., 2010; Leclaire *et al*., 2011 for exceptions). Thus, more studies testing the good-genes-as-heterozygosity hypothesis across multiple secondary sexual traits can help to elucidate whether a single ('multiple messages' hypothesis) or several ('back-up signal' hypothesis) ornaments are signalling individual genetic diversity.

In the present study, we use Mediterranean blue tit (*Cyanistes caeruleus*) as a model system to investigate whether different ornaments reflect male heterozygosity. In particular, we used a total of 26 microsatellite markers to estimate individual genetic diversity and analyse its association with male physical condition, body size and the expression of multiple secondary sexual traits (yellow breast coloration, blue crown coloration and song characteristics). Further, we employed two different arrays of markers classified as neutral (14 loci) or functional (12 loci) by considering whether the genomic region where the markers are located is transcribed to RNA (*sensu* Olano-Marín, Mueller & Kempenaers, 2011a, b; see also Da Silva *et al*., 2009; Küpper *et al*., 2010; Laine *et al*., 2012). This allowed us to test for the first time potential differences in the relationships between the above described traits and these subsets of markers, which may reflect different biological processes (Olano-Marín *et al*., 2011a, b; Szulkin & David, 2011; Ferrer *et al*., 2014). The specific goals of this study are to: (1) analyse the relationship between heterozygosity and the expression of secondary sexual traits and determine whether individual genetic diversity is reflected by a single ('multiple messages' hypothesis) or several ('back-up signal' hypothesis) ornaments (Candolin, 2003); (2) test if this relationship varies depending on whether functional or neutral loci are considered. Furthermore, (3) we examined whether the observed associations between phenotype and heterozygosity reflect a genome-wide effect ('general effect hypothesis'; Weir & Cockerham, 1973; David, 1998) or strong linkage disequilibrium between the employed loci and genes involved in the expression of the studied traits ('local effect hypothesis'; David, 1998; Hansson *et al*., 2001; Hansson & Westerberg, 2002). In particular, we expect neutral markers to cause these associations either by general effects (Weir & Cockerham, 1973; David, 1998) or local effects if they happen to be linked to functional loci (David, 1998; Hansson *et al*., 2001; Hansson & Westerberg, 2002; Balloux, Amos & Coulson, 2004), but we hypothesize that direct or strong local effects are more likely to be caused by functional markers (Olano-Marín *et al*., 2011a, b; Laine *et al*., 2012).

MATERIAL AND METHODS

Study site and general field methods

The study area is located in San Pablo de los Montes, Toledo province (central Spain;39°31′N, 4°21′W), and comprises two nearby (< 2 km) forest patches ('Majadillas' and 'Arroyo del Marchés') dominated by Pyrenean oak (*Quercus pyrenaica*). During the 2012 breeding season, we obtained basic reproductive parameters from 50 breeding pairs. Parents were captured by means of spring traps when feeding nestlings 8–9 days old. All adults birds were identified with metal rings, sexed and aged according to Svensson (1992) as juveniles (yearlings) or experienced breeders (second-year and older birds). Birds were weighed to the nearest 0.1 g using an electronic portable balance, and their wing length was measured to the nearest 1 mm using a top-ruler. Blood samples (≤ 25 μL) were taken from the brachial vein of adults and stored on Flinders Technology Associates reagent loaded cards (Whatman Bioscience, Florham Park, NJ, USA) until needed for genetic analyses.

Microsatellite genotyping and basic genetic statistics

We genotyped a total of 50 male blue tits using a panel of 26 polymorphic microsatellite markers (see Supporting Information Table S1). These markers were classified as presumably functional or neutral as described by Olano-Marín *et al*.(2011a, b) (Table S1). DNA extraction, microsatellite amplification and genotyping and tests for linkage disequilibrium (ID) between each pair of loci and deviations from Hardy–Weinberg equilibrium (HWE) were performed as described in Ferrer *et al*., (2014). We investigated genetic differentiation between the two sampling locations by calculating the pair-wise F_{ST}-value and testing its significance with a Fisher's exact test after 10 000 permutations as implemented in ARLEQUIN 3.1 (Excoffier, Laval & Schneider, 2005).

Heterozygosity estimates and identity disequilibrium

We used homozygosity by locus (*HL*) to estimate individual genetic diversity (Aparicio, Ortego & Cordero, 2006). The *HL* index represents homozygosity instead of heterozygosity, and we used the inverse of *HL* (i.e. 1-*HL*) as an estimate of individual heterozygosity. *HL* values were calculated using CERNICALIN, an EXCEL spreadsheet available on request. We used two methods to analyse the presence of identity disequilibrium (ID) and test whether heterozygosity measured at our set of microsatellite loci was representative of genome-wide inbreeding. We calculated heterozygosity–heterozygosity correlations (HHC) following Balloux *et al*.(2004). We used the R package 'RHH' to run 1000 randomizations of the markers and estimate the average HHC coefficient (r) and the 95% confidence intervals (Alho, Valimaki & Merila, 2010). Moreover, we calculated the parameter g^2, a central measure of identity disequilibrium that quantifies the excess of double heterozygotes at two loci relative to the expectation under random association (David *et al*., 2007). This estimate is constant for any pair of loci considered and only depends on the mean and variance of inbreeding in the population (David *et al*., 2007; Szulkin *et al*., 2010). We used the RMES software to calculate g^2 and test whether this parameter differed significantly from zero (David *et al*., 2007).

Song data

We recorded 50 male blue tits at dawn chorus using Song Meter SMS2 (Wildlife Acoustics Inc., Maynard, MA, USA) and Olympus DM-650 (Olympus Corp., Beijing, China) digital recording devices. Males were recorded during their female's fertile period (two days before egg laying until one day before the last egg was laid). Audio recording devices were set up in close proximity (< 1 m) to the focal nestbox and programmed to record between 04:30 h and 09:00 h during 2 consecutive days in order to reduce the possibility of obtaining inaccurate recordings. Even so, we did not get any clear dawn chorus recording for eight individuals and they were not considered for further analyses. Dawn chorus was considered finished when the male did not sing for more than 5 min (Poesel, Foerster & Kempenaers, 2001). All recordings were analysed by two observers (ESF, JBE) using the same criteria. We used AUDACITY 2.0.0 (http://audacity.sourceforge.net) to filter and remove background noise and RAVEN PRO 1.5 (http://www.birds.cornell.edu/raven) to measure song variables. A total of 43 different song types (strophes repeated and constituting a bout) were identified in this population, of which one was sung by 40 males (i.e. 95% of the analysed individuals). The length of this song type, the most common one in the study population, was measured using RAVEN PRO 1.5 (Dreiss *et al*., 2006; Murphy *et al*., 2008). We also calculated individual repertoire size. In this case, we only considered chorus that contained more than 70 strophes, the number of strophes required to achieve 95 % confidence that the complete individual repertoire was recorded (Dreiss *et al*., 2006). Song recordings from 39 males met such criteria and were selected to examine repertoire size.

Colour data

Plumage reflectance measurements were taken from the blue crown and yellow breast of 49 male blue tits.

However, some spectral measurements failed and some individuals showed little or no blue plumage on the crown probably due to fights with other conspecifics. As a result, blue crown and yellow breast coloration data from 20 and 13 individuals, respectively, could not be used in subsequent analyses. Colour data were collected in the field using an Ocean Optics USB2000 (Ocean Optics Inc., Dunedin, FL, USA) 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 in a reading area of about 1 mm^2. The measurements were taken at a 90° angle to the sample. All measurements were relative to a white WS-1-SS Spectralon tablet (Ocean Optics) and the system was frequently calibrated. For each individual, we took three different measurements of yellow breast and blue crown coloration and averaged the values obtained from the three readings. Reflectance curves were determined by calculating the median of the percentage reflectance in 10 nm intervals, from 320–700 nm, the full spectral range that can be perceived by birds (Cuthill *et al*., 2000). We calculated three standard colourimetrics variables for breast: yellow breast carotenoid chroma, calculated as the difference in reflectance (R) at the wavelengths of the two main carotenoids, lutein and zeaxanthin ($(R_{700}-R_{450})/R_{700}$) (Andersson & Prager, 2006); yellow breast brightness, calculated as total reflectance in the range 320–700 nm; and yellow breast hue, calculated as wavelength of peak reflectance λ (R_{max}). In addition to the last two variables, we also calculated chroma ($(R_{max} - R_{min})/R_{average}$) and UV-chroma ($R_{320–400}/R_{320–700}$) for the blue crown. Analyses for blue crown chroma are not presented because this variable was highly correlated with blue crown UV-chroma ($r > 0.93$). Further, analyses for hue are not presented because hue was highly correlated with brightness for both the yellow breast and blue crown ($r > 0.98$). We obtained qualitatively identical results for these parameters and those with which they were correlated (data not shown).

Statistical analyses: multilocus effects

We used an information-theoretic model-selection approach to analyse the association between individual heterozygosity and song and plumage coloration parameters described above (Burnham & Anderson, 1998). For each dependent variable we constructed two separate general linear models (GLMs), one including as predictor variable individual heterozygosity (i.e. 1-*HL*) calculated for all loci (HL_{Total}) and another including as predictor variables heterozygosity estimated for the subsets of neutral ($HL_{Neutral}$) and functional ($HL_{Functional}$) markers. Note that heterozygosity estimated at the subset of neutral markers was not correlated with heterozygosity at the subset of functional markers ($r = 0.10$, $P = 0.478$; see also Olano-Marín *et al*., 2011a for a similar result). Study plot and male age were included as fixed factors in all the models. Given that the expression of some ornaments is condition dependent (e.g. Scheuber, Jacot & Brinkhof, 2003; Peters *et al*., 2008; Griggio *et al*., 2009), we included body condition (estimated as the residuals of a linear regression of body mass on wing length) as a covariate in the models for all the studied secondary sexual traits. The model for bout length included the time an individual had been singing before switching to the common song, as this could influence bout length due to fatigue. Models for both repertoire size and bout length also included recording date as a covariate because habitat structure differs between early and late spring due to the development of tree foliage and this could potentially influence the transmission of sound and the singing strategy of individuals (Boncoraglio & Saino, 2007). We ranked the resulting models following a model-selection approach on the basis of the Akaike's information criterion corrected for small sample size (AICc; Burnham & Anderson, 1998). AICc values for each model were rescaled (ΔAICc) calculating the difference between the AICc value of each model and the minimum AICc obtained among all competing models (i.e. the best model has ΔAICc = 0). Models with ΔAICc ≤ 2 were considered equivalent (Burnham & Anderson, 1998). In cases where model selection as a function of AICc did not give a single model, we performed an averaging of equivalent models (i.e. models with ΔAICc ≤ 2; Burnham & Anderson, 2002). We calculated the mean of the predictor estimators, their unconditional standard errors (USE) and confidence intervals (CI), and the relative importance of each variable in the final averaged model (Σ ωi, the sum of Akaike weights of models with ΔAICc ≤ 2 in which the variable was included). Parameter estimates were considered significant when their 95% CI did not span zero (Burnham & Anderson, 2002). Model selection and averaging was performed using the R package LME4 and AICCMODAVG (R Core Team, 2012). Finally, we examined correlations between all the studied secondary sexual characters and body condition using Pearson rank correlations. Basic statistics (mean ± standard error (SE) and range) for the studied phenotypic traits are summarized in Table S2 (see Supporting Information).

Statistical analyses: single-locus effects

First, we analysed the effect of single-locus heterozygosity (*SLH*) by fitting one GLM per locus

and secondary sexual trait. Effect size was calculated for each locus as the partial correlation coefficient obtained from its respective model (Nakagawa & Cuthill, 2007). Second, we examined whether *multilocus heterozygosity (MLH)* explained more variance than *SLH* following the approach described in Szulkin *et al.*(2010). We performed *F*-test ratio tests to compare models including *MLH* with those in which we replaced *MLH* with 'normalized' *SLH* at all markers (Szulkin *et al.*, 2010). Finally, we used a GLM to analyse whether absolute effect sizes of single-locus heterozygosities were associated with marker variability (allelic richness and observed and expected heterozygosity, included as covariates in different models) and differed between neutral and putatively functional loci (marker category was included as a fixed factor) (e.g. Olano-Marín *et al.*, 2011a, b; Ruiz-López *et al.*, 2012; Ferrer *et al.*, 2014).

RESULTS

Basic genetic statistics, genetic differentiation and identity disequilibrium

Observed heterozygosity at each locus ranged from 0.34 to 0.97, with 3–26 alleles per locus (see Table S1). Neutral loci had higher allele richness than functional loci ($F_{1,\ 24} = 4.90$, $P = 0.036$), but the subsets of loci did not significantly differ in observed (H_O) ($F_{1,\ 24} = 2.09$, $P = 0.160$) or expected heterozygosity (H_E) (one-way ANOVA: $F_{1,\ 24} = 2.58$, $P = 0.120$). After applying sequential Bonferroni corrections to compensate for multiple statistical tests, only loci *Tgu07* and *CcaTgu14* showed significant deviations from HWE in one study plot ('Majadillas'). Significant linkage disequilibrium (LD) was detected for loci *Tgu07*/*PK12* and *Tgu07*/*Ase18* in 'Arroyo del Marchés' locality after sequential Bonferroni corrections. Pairwise F_{ST} values were not significant, indicating that individuals from the two studied localities are not genetically differentiated (all markers: $F_{ST} = 0.006$, $P = 0.099$; neutral markers: $F_{ST} = 0.008$, $P = 0.070$; functional markers: $F_{ST} = -0.000$, $P = 0.448$). We found significant (i.e. 95% quantiles did not cross zero) and positive HHC between different subsets of loci, suggesting that genetic diversity estimated at our set of markers is representative of genome-wide heterozygosity (all markers: $r = 0.356$, 95% CI = 0.185–0.547; neutral markers: $r = 0.209$, 95% CI = 0.034–0.362; functional markers: $r = 0.257$, 95% CI = 0.106–0.421). However, this was not supported by analyses based on the parameter g^2, which did not significantly differ from zero for all markers ($g^2 = -0.003$, $P = 0.765$) or when the subsets of neutral ($g^2 = 0.002$, $P = 0.348$) and functional markers ($g^2 = -0.007$, $P = 0.750$) were analysed separately.

Multilocus effects

Our most parsimonious models showed that repertoire size was higher in 'Arroyo del Marchés' than in 'Majadillas' locality, but it was not significantly associated with any heterozygosity estimate (Tables 1, S3–S5). Strophe bout length increased with recording date and was higher in 'Majadillas' than in 'Arroyo del Marchés' locality (Tables 1, S3–S5). We also found a positive relationship between bout length (Table 1; Fig. 1A), yellow breast carotenoid chroma (Table 1; Fig. 1B), and blue crown brightness (Table 1; Fig. 1C) and heterozygosity estimated at the subset of functional loci, but these variables were not significantly associated with heterozygosity estimated at the subset of neutral loci (Tables 1, S3) or at all typed markers (Tables S4, S5). Yellow breast brightness and blue crown UV-chroma were not associated with any estimate of individual genetic diversity (Tables 1, S3–S5). Blue crown UV-chroma was the only variable positively associated with body condition (Tables 1, S3–S5). Wing length was not associated with any estimate of individual genetic diversity (Tables 1, S3–S5). After correcting for wing length, body mass was also positively associated with heterozygosity estimated at the subset of functional markers (Tables 1, S3; Fig. 1D). However, body mass was not associated with heterozygosity calculated at all markers or the subset of neutral loci (Tables 1, S3–S5). When examining the interdependence of studied traits, we only found a significant relationship between blue crown brightness and yellow breast brightness (Table 2).

Single-locus effects

We did not find significant differences in the variance explained by the models including *MLH* compared to the models including *SLH* considering any subset of loci (all $Ps > 0.05$). For each trait, the direction of *SLH* effects did not differ significantly for the subsets of neutral and functional markers (all $Ps > 0.05$). Absolute effect sizes of *SLH* did not differ between the subsets of neutral and functional loci and were not associated with allelic richness or observed or expected heterozygosity in any trait (all $Ps > 0.05$) (see Fig. S1 and Table S6).

DISCUSSION

Our results suggest that more heterozygous individuals may be able to produce more conspicuous ornaments and support the hypothesis that secondary sexual traits can mirror the genetic quality of its bearer (Brown, 1997). The fact that ornamentation is associated with individual genetic diversity across

Table 1. General linear models (GLMs) for (a) repertoire size, (b) bout length, (c) yellow breast brightness, (d) yellow breast carotenoid chroma, (e) blue crown brightness, (f) blue crown UV-chroma, (g) wing length, and (h) body mass. A single model with $\Delta AICc \leq 2$ was obtained for bout length. For the rest of the studied variables we performed model averaging of the best ranked equivalent models ($\Delta AICc \leq 2$) to obtain parameter estimates and unconditional standard errors (USE) (see Supporting Information, Table S3). Variables are sorted according with their relative importance based on the sum of Akaike weights ($\Sigma\ \omega i$) of those models with $\Delta AICc \leq 2$ in which the variable was present. Bold type indicates significant variables, i.e. variables for which their unconditional 95% confidence interval (CI) did not cross zero

	Estimate ± USE	$\Sigma\ \omega i$	Lower 95% CI	Upper 95% CI
(a) Repertoire size				
Study plot	−2.25 ± 0.79	0.57	**−3.79**	**−0.71**
Body condition	−1.00 ± 0.69	0.23	−2.35	0.35
$HL_{Neutral}$	−3.51 ± 2.76	0.15	−8.92	1.89
Recording date	−0.03 ± 0.03	0.12	−0.09	0.03
$HL_{Functional}$	−3.24 ± 2.71	0.08	−8.56	2.08
(b) Bout length				
$HL_{Functional}$	200.61 ± 81.59	0.25	**40.69**	**360.54**
Study plot	56.33 ± 21.15	0.25	**14.87**	**97.78**
Recording date	1.87 ± 0.80	0.25	**0.30**	**3.44**
Body condition	17.79 ± 19.19	0.25	−19.82	55.4
(c) Yellow breast brightness				
$HL_{Functional}$	26.51 ± 149.64	0.53	−266.77	319.80
Age	42.01 ± 37.35	0.15	−31–19	115.21
Body condition	−34.53 ± 43.35	0.11	−119.50	50.44
(d) Yellow breast carotenoid chroma				
$HL_{Functional}$	0.53 ± 0.27	0.26	**0.01**	**1.06**
Body condition	0.11 ± 0.07	0.26	−0.03	0.25
Age	−0.10 ± 0.07	0.25	−0.23	0.03
Study plot	−0.08 ± 0.08	0.08	−0.24	0.07
$HL_{Neutral}$	−0.31 ± 0.28	0.06	−0.85	0.23
(e) Blue crown brightness				
$HL_{Functional}$	363.83 ± 155.86	0.37	**58.34**	**669.32**
Body condition	−89.61 ± 49.07	0.22	−185.79	6.57
(f) Blue crown UV-chroma				
Body condition	0.01 ± 0.01	0.50	**0.01**	**0.01**
$HL_{Functional}$	−0.01 ± 0.01	0.35	−0.01	0.01
Age	0.01 ± 0.01	0.10	−0.01	0.01
(g) Wing length				
Age	0.95 ± 0.41	0.75	**0.14**	**1.76**
$HL_{Functional}$	−1.32 ± 1.65	0.75	−4.56	1.92
$HL_{Neutral}$	3.57 ± 1.80	0.52	−0.01	7.10
(h) Body mass				
$HL_{Functional}$	1.14 ± 0.55	0.57	**0.06**	**2.22**
Wing length	0.11 ± 0.04	0.57	**0.03**	**0.20**
$HL_{Neutral}$	−0.65 ± 0.59	0.16	−1.80	0.50

multiple secondary sexual traits can also explain the evolution of directional mate preferences as suggested by the good-genes-as-heterozygosity hypothesis (Brown, 1997; Kempenaers, 2007). Our results support the 'back-up signal' hypothesis and suggest that different ornaments indicate redundant information about an aspect of individual quality, in our case individual genetic diversity, that may allow a more accurate assessment of mate quality based on the same aspect (Candolin, 2003). Several previous studies have found a positive relationship between heterozygosity and the expression of a single sexual ornament (Aparicio, Cordero & Veiga, 2001; Foerster *et al*., 2003; Marshall *et al*., 2003; Seddon *et al*., 2004; Reid *et al*., 2005; Araya-Ajoy *et al*., 2009; Pérez-González *et al*., 2010), but only a few have simultaneously considered multiple secondary sexual traits (Bolund *et al*., 2010; Zajitschek & Brooks, 2010; Leclaire *et al*., 2011), and none of these studies analysed whether associations between ornamentation

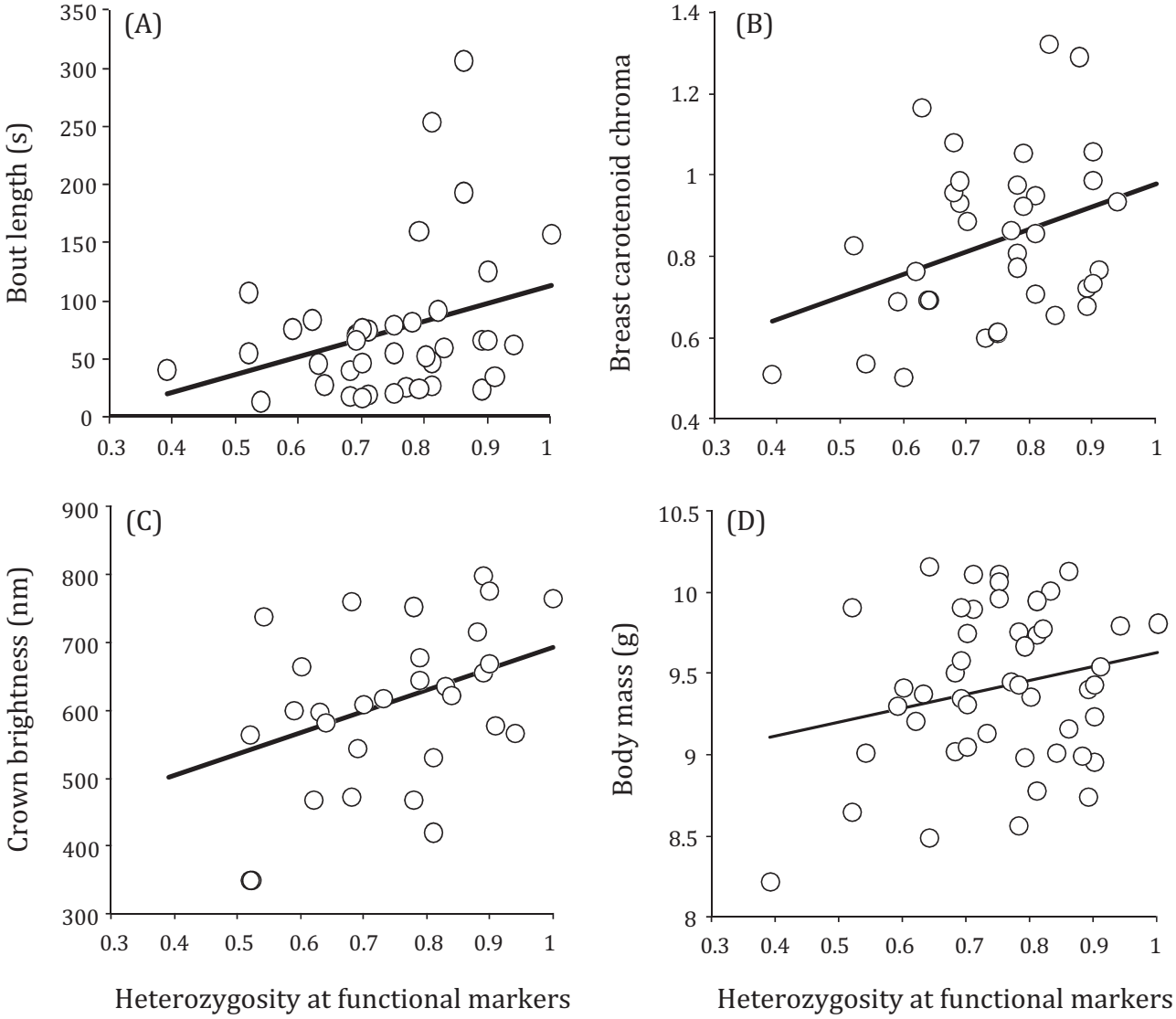

Figure 1. Relationship between multilocus heterozygosity at functional loci (1-$HL_{Functional}$) and (A) bout length, (B) yellow breast carotenoid chroma, (C) blue crown brightness, and (D) body mass.

Table 2. Pearson rank correlations between the studied secondary sexual characters and body condition in male blue tits. Correlation coefficients (below the diagonal) and significance values (above the diagonal) are shown. Asterisks denote variables statistically significant after sequential Bonferroni correction

Trait	Repertoire size	Bout length	Yellow brightness	Yellow chroma	Blue brightness	Blue UV-chroma	Body condition
Repertoire size	–	0.021	0.598	0.893	0.707	0.598	0.268
Bout length	−0.384	–	0.053	0.338	0.672	0.344	0.119
Yellow brightness	0.109	−0.376	–	0.662	0.001*	0.059	0.687
Yellow carotenoid chroma	0.028	0.192	−0.075	–	0.960	0.409	0.135
Blue brightness	0.085	−0.098	0.987	0.010	–	0.030	0.358
Blue UV-chroma	−0.119	−0.217	−0.375	0.169	−0.404	–	0.037
Body condition	−0.182	−0.254	−0.069	0.254	−0.177	0.389	–

and heterozygosity differed between neutral versus putatively functional markers.

Heterozygosity and ornamentation

Previous studies have found an association between different song parameters and individual genetic diversity or inbreeding (Marshall *et al*., 2003; Seddon *et al*., 2004; Reid *et al*., 2005; Bolund *et al*., 2010). Marshall *et al*. (2003) and Reid *et al*. (2005) reported a link between song complexity and heterozygosity in sedge warblers (*Acrocephalus schoenobaenus*) and song sparrows (*Melospiza melodia*), respectively. They interpreted their results as indicating that learning and brain capacity are affected by inbreeding and this may cause a reduced ability to memorize song. Seddon *et al*. (2004) showed that more heterozygous males of the subdesert mesite (*Monias benschi*) produce trills of longer duration and lower pitch, while Bolund *et al*. (2010) found that song rate was negatively affected by inbreeding in zebra finches (*Taeniopygia guttata*). We found that repertoire size was not associated with heterozygosity, suggesting that this parameter could be only influenced by morphometric, environmental, and social conditions in our study species (Johannessen, Slagsvold & Hansen, 2006; Doutrelant *et al*., 2000). However, more heterozygous male blue tits sang longer bouts than homozygous ones. Thus, bout length may be a reliable indicator of genetic diversity that could be used by females in mate choice decisions as suggested in a previous study on this species (Dreiss *et al*., 2006).

Regarding plumage coloration, previous studies on blue tits suggest a relationship between crown coloration and individual attractiveness (e.g. Andersson, Ornborg & Andersson, 1998; Sheldon *et al*., 1999). We found that crown brightness is positively associated with heterozygosity, a pattern that has been consistently reported by studies performed in different populations of blue tits (Foerster *et al*., 2003; García-Navas *et al*., 2009). Our study has shown for the first time that male blue tits with higher yellow breast carotenoid chroma values have higher heterozygosity levels than less chromatic individuals. Past research indicates that carotenoid-based plumage reflects individual quality in a variety of birds (e.g. Jawor & Breitwisch, 2004; Senar *et al*., 2008) and is subjected to sexual selection (Badyaev & Hill, 2002; Jawor *et al*., 2003). Although some have argued that colour traits based on carotenoids reflect foraging ability and territory quality rather than genetic quality (Hõrak *et al*. 2000; Pagani-Núñez *et al*., 2014), recent studies have shown that carotenoid-pigmented ornaments have a heritable component (Evans & Sheldon, 2012; Vergara, Fargallo & Martínez-Padilla, 2015). In blue tits, yellow breast coloration reflects individual health and parasitism status (del Cerro *et al*., 2010) and has been associated with provisioning ability (García-Navas, Ferrer & Sanz, 2012) and foraging capacity (Senar & Quesada, 2006). Male heterozygosity is positively associated with nestling feeding rates in blue tits (García-Navas *et al*., 2009), suggesting that the higher performance of more pigmented individuals could be reflecting the greater foraging capacity and/or ability to acquire a better territory and assimilate resources of more heterozygous individuals. Previous studies have also shown a relationship between carotenoid-based coloration and heterozygosity in other species, suggesting that these ornaments can also be reliable signals to assess the genetic quality of potential partners (e.g. van Oosterhout *et al*., 2003; Bolund *et al*., 2010; Leclaire *et al*., 2011; Herdegen, Dudka & Radwan, 2014).

Body condition was positively associated with individual genetic diversity, a relationship that has been previously reported in other organisms and suggests that heterozygosity influences the capacity to obtain and assimilate resources (Lens *et al*., 2000; Pujolar *et al*., 2005; Bolund *et al*., 2010; Herdegen *et al*., 2013). However, the ornamental traits associated with heterozygosity were not correlated with either the age or the physical condition of individuals. The latter may be consequence of the index used for determining body condition is a poor estimate of general physical condition or it might only reflect some aspects of the individual's physiological state. Alternatively, if secondary sexual characters associated with individual heterozygosity mostly convey information about overall genetic quality, then, they may not be strongly influenced by environment or the physical condition of individuals (Scheuber *et al*., 2003; Freeman-Gallant *et al*., 2010). Thus, different proximate mechanisms can explain the observed associations between individual genetic diversity and the expression of secondary sexual characters. Highly heterozygous individuals could display more conspicuous ornaments if genes directly involved in their development exhibit overdominance or are affected by deleterious or partly deleterious recessive alleles that have a reduced chance of being expressed in genetically more diverse individuals (Charlesworth & Charlesworth, 1987; Falconer & Mackay, 1996). However, this would require that many genes are involved in the expression of secondary sexual characters so that they can collectively capture the effects of genome-wide heterozygosity (Aparicio, Ortego & Cordero, 2007). Another possibility is that more heterozygous individuals show a higher resistance to parasites and diseases (Acevedo-Whitehouse *et al*., 2003), superior physiological response to stress and/or

increased cellular homeostasis (Mitton & Grant, 1984), aspects that might have not been captured by our index of physical body condition and that are likely to reduce the costs of producing elaborated secondary sexual characters (Van Oosterhout *et al*., 2003).

Identity disequilibrium, functional verus neutral markers and local effects

Correlations between heterozygosity and phenotype or fitness-related traits are expected to be detected in populations that experience genetic drift, bottlenecks, non-random mating or population admixture, processes that cause variance in inbreeding and increase identity disequilibrium (ID) (Szulkin *et al*., 2010). Although we failed to detect significant g^2 values, we found positive heterozygosity–heterozygosity correlations (HHCs), suggesting that genetic diversity estimated at our different sets of markers may be representative of genome-wide heterozygosity in this population (Balloux *et al*., 2004; see also Kardos, Allendorf & Luikart, 2014). The very limited power to detect ID when variance in inbreeding is low and the number of employed loci is relatively small (< 100 markers), the typical situation in most studies in natural populations, may have resulted in we have been able to detect ID with one method but not with the other (Kardos *et al*., 2014; Miller & Coltman, 2014). Accordingly, a recent meta-analysis by Miller & Coltman (2014) showed that only ~20% of microsatellite-based studies found significant g^2 values. However, it should be considered that non-significant g^2 values (or HHCs) do not necessarily imply that the detection of correlations between heterozygosity and fitness or phenotypic traits are not due inbreeding (or a genome-wide effect), given that the studied traits are likely to capture the effect of potentially many more loci than the number of typed markers (see Szulkin *et al*., 2010).

Most studies in natural populations have employed neutral markers to analyse the association between heterozygosity and fitness or phenotype, as their higher polymorphism is expected to better capture the effects of genome-wide inbreeding (Slate *et al*., 2004). However, we only detected significant associations between heterozygosity and the expression of ornaments across the panel of functional markers, despite the fact that our functional markers showed slightly lower polymorphism than our neutral markers (see also Olano-Marín *et al*., 2011a; Ferrer *et al*., 2014). This suggests that reduced heterozygosity at functional regions of the genome may be more relevant in the expression of secondary sexual characters, which may make these ornamental traits more reliable indicators of the genetic quality of individuals given that only functional genomic regions are translated into phenotypic differences. Further, we did not detect significant single-locus effects and the employed functional loci are distributed across nine chromosomes and are located within or in close vicinity to coding genes involved in different physiological processes (see Table 1 in Olano-Marín *et al*., 2011a). Different genes are also expected to be involved in the expression of the different studied ornaments (e.g. related to plumage coloration or song elaboration), which suggests that the observed associations between heterozygosity at functional loci and the expression of secondary sexual traits are driven by loci widely distributed across the genome and not due to the particular set of markers chosen or their specific functions. Our results contrast with previous microsatellite-based studies that have found different roles of neutral and putatively functional markers in observed correlations between heterozygosity and fitness or phenotype (e.g. Olano-Marín *et al*., 2011a, b; Laine *et al*., 2012; Ferrer *et al*., 2014). Several authors have reported stronger correlations with specific microsatellite loci, suggesting the presence of strong local effects (Da Silva *et al*., 2009; Küpper *et al*., 2010; Olano-Marín *et al*., 2011a, b; Laine *et al*., 2012; García-Navas *et al*., 2014), whereas others have found that heterozygosity at neutral markers is more strongly associated with the studied traits than heterozygosity at functional markers in absence of relevant single-locus effects (Olano-Marín *et al*., 2011a; Ferrer *et al*., 2014). Finally, some studies have found a different contribution of functional/neutral markers and general/local effects depending on the studied trait (Küpper *et al*., 2010; Olano-Marín *et al*., 2011b; Laine *et al*., 2012). It should also be considered that heterozygosity at neutral markers was not correlated with heterozygosity estimated at functional markers, a result reported in previous studies that may reflect the fact that the two sets of markers are impacted by selective processes in a different manner (Olano-Marín *et al*., 2011b; Szulkin & David, 2011; Ferrer *et al*., 2014). Natural selection across different life stages acting against individuals genetically less diverse at functional loci could contribute to partially decoupling levels of genetic diversity in selectively neutral and functional genomic regions. Mate choice could also play a role in these differences, for instance if individuals select mates more different (compatible) from themselves at multiple functional but not neutral loci (Yamazaki & Beauchamp, 2007). In this case, neutral loci would be expected to more accurately reflect inbreeding. However, functional loci are also likely to reflect genome-wide inbreeding to some extent and they could develop further identity disequilibrium due to variance among individuals in mate choice decisions that can be context-dependent

and influenced by different factors such as the availability of potential mates, age or the phenotypic or genotypic quality of individuals (Lie, Simmons & Rhodes, 2010). Thus, contrasting influences of sexual and natural selection on neutral vs. functional loci may cause these loci to show different associations with phenotype and fitness-related traits, even in the absence of strong local effects, potentially explaining the discrepancy between our study and some past research (Olano-Marín *et al*., 2011b; see also Hansson & Westerberg, 2008). Overall, this and previous work indicate that the expected association between phenotype or fitness-related traits and heterozygosity at functional/neutral markers is difficult to predict, highly dependent on the studied trait and, when the association is mostly driven by variability at putatively functional markers, does not necessarily have to be the result of local effects (Szulkin & David, 2011).

CONCLUSIONS

In summary, we found that more heterozygous males showed increased expression of secondary sexual traits and body condition. Males with a higher level of carotenoid chroma on the yellow breast, a brighter blue crown, longer song bouts, and higher body condition were more heterozygous, indicating that genetic diversity can be reflected across multiple traits that are likely to be used by females during mate choice decisions. The strength of selection may increase if mate choice based on traits that reflect the same attribute facilitates mate assessment and skews mate choice toward males that express high levels of multiple types of ornamentation. In our study population, both song and different colour attributes reflect male heterozygosity, which may increase female's ability to accurately identify a high-quality partner, thus reducing the costs of mate choice in accordance with the 'back-up signal' hypothesis (Candolin, 2003). However, we did not find correlations between most ornaments, which may be due to our relatively small sample sizes or because the studied traits being produced in different parts of the annual cycle (e.g. plumage moult in summer-autumn and singing in spring), reacting to other influential factors at different rates (fast response for singing vs. slow for coloration; Birkhead, Fletcher & Pellatt, 1998) or being involved in different processes (e.g. female choice vs. intrasexual competition; Candolin, 2003; e.g. Andersson *et al*., 2002; Freeman-Gallant *et al*., 2010). The lack of strong local effects and the presence of identity disequilibrium in our population suggest that genome-wide heterozygosity is the most likely mechanism behind the observed heterozygosity-phenotype associations, whereas the predominant role of putatively functional loci indicates that the expression of secondary sexual characters is more tightly reflected by heterozygosity at genomic regions containing coding genes that are being actively expressed. The implementation of candidate-gene approaches, considering loci with functions related with the trait of interest, and the application of high-throughput sequencing technology to get accurate estimates of genome-wide inbreeding based on thousands of loci will help to greatly increase our understanding of the role of genetic diversity in the expression of secondary sexual characters and disentangle the underlying mechanisms (Fitzpatrick *et al*., 2005; Walsh *et al*., 2011; Hoffman *et al*., 2014; Zuk & Balenger, 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Effect sizes and 95% confidence intervals of single-locus heterozygosity (SLH) for the studied phenotypic traits.
Table S1. Panel of 26 microsatellite markers used to genotype blue tits.
Table S2. Basic statistics (mean ± S.E and range) for the phenotypic traits analysed in the present study.
Table S3. Model selection to assess the association of the studied phenotypic traits with heterozygosity estimated at the subset of neutral (HL_{Neutral}) and functional loci ($HL_{\text{Functional}}$) and different non-genetic terms.
Table S4. Model selection to assess the association of the studied phenotypic traits with heterozygosity estimated at all the typed loci (HL_{Total}) and different non-genetic terms.
Table S5. General linear models (GLMs) for the studied phenotypic traits considering heterozygosity estimated at all the typed loci (HL_{Total}) and different non-genetic terms.
Table S6. Tests for the effects of single-locus heterozygosity (SLH) on the studied phenotypic traits.