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On the heritability of blue-green eggshell coloration

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Abstract

Avian blue-green eggshell coloration has been proposed as a female signal of genetic or phenotypic quality to males. However, little is known about the relative importance of additive genetic and environmental effects as sources of eggshell colour variation in natural populations. Using 5 years of data and animal models, we explored these effects in a free-living population of pied flycatchers. Permanent environmental and year effects were negligible, although year environmental variance (V_{Year}) was significant for all but one of the traits. However, we found high–moderate narrow-sense heritabilities for some colour parameters. Within-clutch colour variability showed the highest coefficient of additive genetic variation (i.e. evolvability). Previous evidence suggests that eggshell colour is sexually selected in this species, males enhancing parental effort in clutches with higher colour variability and peak values. Eggshell colour could be driven by good-genes selection in pied flycatchers although further genetic studies should confirm this possibility.

Introduction

Evolutionary change within a population requires a genetic basis of phenotypic variation. Thanks to quantitative genetic approaches, we have gained valuable insight into the genetic variation of fitness and nonfitness traits (see reviews by Merilä & Sheldon, 1999, 2001; Charmantier & Sheldon, 2006; Kruuk et al., 2008). However, empirical knowledge on the heritability of sexually selected traits lags behind that about other traits (Qvarnström, 1999; Kruuk et al., 2002). This is surprising because some influential models of sexual selection such as good-genes or fisherian processes require that sexual ornaments are heritable (Andersson, 1994; Jones & Ratterman, 2009). This relatively poor understanding is even more evident in the case of secondary sexual traits in females. Good examples on the inheritance of female ornaments come from studies on wild bird populations. These were initially based on parent–offspring comparisons (Møller, 1993; Potti, 1993), and subsequently, also on cross-fostering manipulations to break environmental correlations between parents and offspring (Roulin et al., 2000, 2001; Roulin & Dijkstra, 2003; Bize et al., 2006; Gasparini et al., 2009; Quesada & Senar, 2009). Still, we need a better empirical understanding of the genetics underlying the evolution of female ornaments and of sexual traits in general (Garant et al., 2004).

The blue-green biliverdin pigment is used by female birds to colour their eggshells green and blue (Kennedy & Vevers, 1976). Blue-green eggshell coloration has been very recently incorporated into the sexual selection framework, as it has been proposed as a signal of female phenotypic or genetic quality to males in species with biparental care (Moreno & Osorno, 2003). The offspring of high-quality females would merit more effort according to the differential allocation hypothesis (Burley, 1986), as applied to female traits. This post-mating character has been shown to mirror some aspects of female quality in most species studied to date (Moreno et al., 2005, 2006a; Morales et al., 2006; Siefferman et al., 2006; Krist & Grim, 2007; Hanley et al., 2008; Hargitai et al., 2008; López-Rull et al., 2008; Soler et al., 2008; Morales et al., 2010; but see Hanley & Doucet, 2009).

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Also, it has received support that males respond to egg colour by enhancing parental contribution to their offspring in some studies (see Moreno et al., 2004, 2006b, 2008; Hanley et al., 2008; Soler et al., 2008) however not in others (Krist & Grim, 2007; Hanley & Doucet, 2009). Besides, blue-green eggshell colour intensity is positively associated with the duration of nestling care period across species (Soler et al., 2005). Apparently, hole-nesting breeders are able to detect experimental variations in eggshell appearance and respond by enhancing parental care (e.g. Moreno et al., 2006b; Soler et al., 2008; Avilés et al., 2009), despite the fact that their retinal dark adaptation has been suggested to be slow (Cassey, 2009).

Investigations into the genetic architecture of eggshell coloration go back to Punnett & Bailey (1920). Subsequent studies, mainly on poultry, have supported that blue-green eggshell coloration was under genetic control (Punnett, 1933; Yang et al., 2003; reviewed by Stevens, 1991 and Washburn, 1990; see also Collias, 1993 on a sub-Saharan passerine). However, most research to date has been restricted to captive populations (but see studies on the inheritance of porphyrins-based pigmentation, e.g. Gosler et al., 2000 and Mahler et al., 2008). The controlled and constant conditions in the laboratory are more homogeneous than natural variation, and this may lead systematically to reduced environmental variance and increased estimates of heritabilities (for a review, see Charmantier & Garant, 2005). Thus, although the existence of a genetic basis seems well established, little is known about the relative importance of additive genetic and environmental effects as sources of blue-green eggshell colour variation in natural populations. Estimating genetic variability in the wild can be tricky, as environmental variation may obscure underlying evolutionary patterns (Kruuk, 2004). Thus, quantitative genetic approaches applied to natural populations should allow the observed phenotypic variation to be separated into genetic and environmental components. Animal models are mixed models that use all information available in a pedigree to estimate additive genetic variance (i.e. covariance across all possible pairs of relatives; Kruuk, 2004) and may include additional variance components for specific environmental effects (Postma, 2006; Wilson et al., 2010).

Here, we explored the heritable variation of blue-green eggshell coloration in a wild population of pied flycatchers (*Ficedula hypoleuca*), using animal models. Although tests exploring male investment decisions according to egg colour have yielded diverse results (reviewed by Reynolds et al., 2009), some pieces of evidence suggest that egg colour is sexually selected in this species (Moreno et al., 2004, 2006b, 2008). Egg pigmentation in pied flycatchers reflects female immunological quality (Moreno et al., 2005; Morales et al., 2006), is subject to varying environmental conditions, such as food availability (Moreno et al., 2006a) and is costly to produce for laying females (Morales et al., 2008). Furthermore, egg colour predicts fledgling condition (Moreno et al., 2008) and reproductive success (Morales et al., 2006, 2008), which may ultimately affect fitness. We explored the additive genetic, year and permanent environmental (repeated measures) effects on blue-green eggshell colour over a period of 5 years, while controlling for other known sources of variation of egg colour in this species (e.g. manipulated early maternal effects).

Methods

Study species and general procedures

The pied flycatcher is an insectivorous, migratory, hole-nesting passerine that breeds in European woodlands (Lundberg & Alatalo, 1992). It is a summer visitor that adapts readily to breeding in nestboxes. The study population breeds in a deciduous forest located in Valsaín, central Spain (Segovia, 40°53′74N, 4°01′W, 1200 m a.s.l.), where there are 300 nestboxes monitored since 1991 (between 75 and 125 are occupied by pied flycatchers depending on the year). Egg laying typically begins in late May, and clutch size ranges from 4 to 7 eggs with a mode of six eggs. The most common pattern in this species is to start full incubation with the penultimate or last egg in the clutch (Potti, 1998a). Males in the study population visit their nestbox frequently during the laying period, when females have not yet started full incubation, and spend some time inside the nest cavity (Moreno et al., 2005). Hence, males have ample opportunities to observe freshly laid eggs (see a recent study on this male behaviour in another hole-nesting breeder, the blue tit, *Cyanistes caeruleus*; Holveck et al., 2010).

In all breeding seasons, adults are captured at the nest with nestbox traps when nestlings are about to fledge (day 11 or 12; hatching day = day 0). All birds are individually marked with numbered aluminium rings (Dirección General de Medio Natural, ringing permit by regional authorities), and their reproductive success and local survival between years are known (female local survival probability is very high in this population, reaching 90% at 3–4 years of age; Sanz & Moreno, 2000). We assume for all recruited adult females that were not raised in the study area a minimum age of two (Sanz & Moreno, 2000). We used all data available for eggshell colour, which was measured in 346 pied flycatcher nests of 215 individual females during five consecutive breeding seasons, from 2003 to 2007. In 2003, we experimentally manipulated the size of the sexually selected white forehead patch of males on arrival to the breeding grounds (see Osorno et al., 2006 for a full description). Females laid smaller eggs when paired with less attractive (patch reduced) mates (Osorno et al., 2006). In 2005, females that were supplemented with mealworms before and during laying laid bluer and heavier clutches (Moreno et al., 2006a). To account for

potential maternal effects on eggshell colour derived from these two experiments, we included all nests but controlled for experimental group (see statistical analyses).

Eggshell colour measurement

Eggshell colour was measured with a portable spectrophotometer MINOLTA CM-2600d (Minolta Co. Ltd., Osaka, Japan) on the day eggs were laid or on the following day. Eggs were placed directly with their broad pole on a target mask with a diameter of 8 mm, so that eggs completely filled the space of the mask. Reference calibrations against zero and a white standard tablet (Minolta Co. Ltd.) were performed periodically according to the apparatus instructions. The reflectance spectra are automatically obtained as means of three sequential measures of each egg by changing the position of the egg with respect to the apparatus. The SPECTRAMAGIC software (Minolta Co. Ltd.) was used to obtain the reflectance spectra from 360 nm in intervals of 10 nm. From the reflectance spectra, we calculated blue-green chroma (BGC) as the proportion of total reflectance that is in the blue-green region of the spectrum ($R_{400–570}/R_{360–700}$). This corresponds to the region with least absorbance (and therefore greatest reflectance) of biliverdin (Falchuk et al., 2002) and pied flycatcher eggs reflect maximally in it (Moreno et al., 2005). There is a highly positive significant correlation of BGC values estimated with the MINOLTA spectrophotometer and with an OCEAN OPTICS spectrophotometer covering also the UV range (Moreno et al., 2006a). In 2003, we only recorded Lightness and Chroma in CIELAB colour space (CIEL*a*b*, Commission Internationale de l'Eclairage, 1976) (see Moreno et al., 2004) and not the reflectance spectra of eggs. Measures in CIELAB are highly correlated with BGC, so that higher values of BGC are positively associated with Chroma and negatively with Lightness (Moreno et al., 2006a). Although they may be redundant, these measures describe different aspects of colour, and birds may show different detection rates for different colour attributes (Cazetta et al., 2009). While Lightness represents achromatic properties of an object on a scale from 0 = black to 100 = white, Chroma, often used as a synonym of purity or saturation, characterizes colour by the a* (red to green) and b* (yellow to blue) values. It is calculated according to the formula: Chroma ¼ $\sqrt{(a^{*2} + b^{*2})}$, which indicates that the further the value is away from zero, the more saturated is the colour. Finally, BGC measures reflectance in the blue-green part of the spectrum and thus includes both chromatic and achromatic aspects. We estimated variance components for the three colour parameters: Lightness, Chroma (n = 346 nests) and BGC (n = 282), which are associated with biliverdin amount in the eggshell (Moreno et al., 2006a). Pied flycatcher males in our population invest more parental effort in clutches more variable in colour and with higher peak values rather than with higher means (Moreno et al., 2006b, 2008). Therefore, we have used within-clutch means, maxima and standard deviations of Lightness, Chroma and BGC for statistical analyses.

Eggs were weighed on the day of colour measurement with a portable electronic balance (accuracy 0.1 g) in all years except 2003, when eggs were measured with a digital calliper (accuracy 0.01 mm) and their volume estimated in mm^3 with the formula volume =)0.042 + 0.4976 (length - $(width)^2$) (Ojanen et al., 1978). Using data from another study population of pied flycatchers (see Morales et al., 2006, for a description), we performed the regression of mass on volume ($r = 0.99$, $P < 0.001$, $n = 38$) and estimated egg mass in 2003 from the resulting equation (egg mass = 0.0819 + 0.001 - egg volume). There are no appreciable differences in egg shape between both populations. After colour measurement, eggs were marked for identification according to their laying order and placed back in the nest.

Statistical analyses

Exploration of fixed effects

Prior to the quantitative genetic analyses using animal models, we first explored the effects of potential covariates on each egg colour trait, using linear mixed effect models (LME) with female identity as a random effect: female age, egg mass and experimental treatment [four-level factor: control or unmanipulated nest (i), male's forehead patch either enlarged (ii) or reduced (iii) and nest supplemented with mealworms (iv)]. As we used pooled data from all years, treatment had an unbalanced structure because of a large amount of unmanipulated nests (all nests in 2004, 2006 and 2007) and thus, no clear conclusion can be drawn from its effects on egg colour. Effects of the food supplementation experiment on eggshell colour were already presented and discussed by Moreno et al. (2006a), when they were appropriately tested. Therefore, here we only accounted for them to ensure that at least part of the variation in egg colour because of manipulated early maternal effects is taken into account. Initially, an LME with all explanatory variables as fixed effects and individual identity as random effect was fitted for each egg colour trait using a maximum-likelihood (ML) algorithm (full model). Then nonsignificant terms were dropped sequentially to simplify the model. The significance of the removal of each term was tested using a likelihood ratio test (Crawley, 2007). This model simplification method proceeded until we obtained minimum adequate LMEs that included only terms significant at the $P < 0.05$ level. The minimum adequate models were then rerun using restricted maximum-likelihood (REML) methods (Crawley, 2007). Although BGC is a proportion, its normal approximation is good (Kolmogorov–Smirnov tests for mean, SD and maximum values of BGC: all $P > 0.20$). All other colour parameters and laying date

were normally distributed as well (K–S test; all $P > 0.20$). None of the variables were thus transformed. Analyses were carried out using R version 2.7.2 (R Development Core Team, 2008).

The relevant fixed effects found for each colour parameter (see Appendix 1) were taken into account in the quantitative genetic analyses (see the following section). Note that laying date was not included as a fixed effect in the univariate models, as this life-history trait is expected to show substantial additive genetic variance (e.g. Sheldon *et al.*, 2003; in the related collared flycatcher, *Ficedula albicollis*). Instead, we explored the heritability of laying date with another univariate model. In contrast, egg mass, which is expected to be mainly determined by maternal effects and environmental conditions (e.g. Moreno *et al.*, 2006a; Osorno *et al.*, 2006), was included as a fixed effect in subsequent quantitative genetic analyses where significant.

Animal model

Animal model is a form of mixed model that partitions individual phenotypic variations for a quantitative character into different variance components and includes an individual's genetic merit as a random effect (Lynch & Walsh, 1998; Kruuk, 2004). We fitted univariate animal models using a REML procedure. ASReml v2 (VSN International; Gilmour *et al.*, 2006) was used to fit animal models and calculate variance ratios and standard errors (see Lynch & Walsh, 1998).

Univariate animal models were fitted to the pedigree for each egg colour trait and laying date, including the significant fixed effects determined from the LME analyses (see Appendix 1). The pedigree was formed by 242 identities, incorporating 27 fathers and 28 mothers. Note that extra-pair paternity is moderately low in our population (7.5% of nestlings), and intraspecific brood parasitism is extremely rare (Moreno *et al.*, 2010). Thus, our estimates of genetic parameters are conservative. Among the 346 clutches included in the animal models, there were 112 belonging to females with at least one known relative (i.e. with known mother, or at least one known daughter or sister). The rest of the clutches included in the analyses did not belong to females with known relatives, but they contributed to estimating more accurately the total phenotypic variance of eggshell colour in the study population. In the univariate model for each egg colour trait and laying date, we included the additive genetic, year-specific and permanent environmental (individual-specific) effects as random factors. The permanent environmental effect, estimated using repeated measures, includes individual sources of variance that are conserved across repeated records on individuals but are not because of additive genetic effects (Kruuk, 2004). There were 88 females that bred in various years (54 bred twice, 27 bred in 3 years, six in 4 years and one in 5 years) and 127 that bred once. The total phenotypic variance (V_P) is the sum of all the variance components of each random effect and was calculated as $V_P = V_A + V_{Year} + V_{PE} + V_R$, where V_A is the variance explained by additive genetic effects, V_{Year} is the environmental variance because of year, V_{PE} is the permanent environmental variance (i.e. individual identity), and V_R is the residual variance (Falconer & Mackay, 1996). Narrow-sense heritability ($h^2 = V_A/V_P$), the effects of year ($y^2 = V_{Year}/V_P$) and permanent environmental effects ($pe^2 = V_{PE}/V_P$) were calculated as the proportion of the relevant variance component to the total phenotypic variance for each trait. We also calculated the coefficient of additive genetic variance as $CV_A = 100 \sqrt{V_A}/\bar{X}$, for which the additive genetic variance was scaled by the trait mean rather than the total variance (Houle, 1992). The statistical significance of each variance component was assessed using likelihood ratio tests that compare models based on -2 times the difference in REML log-likelihood scores distributed as χ^2 where the number of degrees of freedom equalled the number of variance terms removed. The significance of variance ratios (h^2, y^2 and pe^2) was assessed using two-tailed t-tests.

Additionally, we tried to fit a multivariate animal model to explore the heritability of eggshell colour when accounting for potential genetic and environmental correlations between the colour parameters and laying date. Unfortunately, a convergence problem was encountered when attempting to fit the individual-specific effect, possibly because the permanent environmental (co)variances were close to zero in the multivariate model. Note that dropping the permanent environmental effect from this type of model may result in overestimated heritabilities (see Kruuk & Hadfield, 2007) and, therefore, the multivariate model is not shown.

Results

Exploration of fixed effects for colour traits and laying date

Female age did not affect any of the colour traits, but did affect laying date, young breeders laying later in the season (Appendix 1). Egg mass affected maximum Lightness, clutches with smaller eggs showing higher peaks in Lightness (Appendix 1).

Univariate animal models

Estimates of additive genetic variance (V_A) were significant for SD Lightness, maximum Lightness, SD BGC and marginally nonsignificant ($P < 0.1$) for mean BGC (Table 1). Heritabilities (h^2) of blue-green eggshell colour traits ranged from 0.15 to 0.54 and were significant for the later four parameters and marginally nonsignificant for mean Lightness and maximum Chroma (Table 1). The highest significances (both for V_A and h^2 values) were found for SD BGC and maximum Lightness (Table 1). Coefficients of additive genetic variance

Table 1 Quantitative genetics for egg colour traits and laying date (univariate animal models). Estimates (SE) of variance components, total phenotypic variance (V_P), additive genetic variance (V_A), year environmental variance (V_{Year}), permanent environmental variance (V_{PE}), residual variance (V_R) and the variance ratios, narrow-sense heritability (h^2), year environmental effects (y^2), permanent environmental effects (pe^2) and coefficient of additive genetic variance (CV_A). These models included the fixed effects that were relevant for each colour trait (see Appendix 1).

Trait	V_P	V_A	V_{Year}	V_{PE}	V_R	h^2	y^2	pe^2	Mean	CV_A
Mean Lightness	2.297 (0.375)	0.961 (0.563)	0.457 (0.334)	0.484 (0.531)	0.396 (0.051)	0.42 (0.24)	0.20 (0.12)	0.21 (0.23)	72.27 (0.08)	1.36
		P = 0.111	P < 0.001	P = 0.328		P = 0.088	P = 0.092	P = 0.369		
SD Lightness	0.280 (0.026)	0.043 (0.022)	0.018 (0.016)	0	0.219 (0.025)	0.15 (0.08)	0.06 (0.05)	0	1.41 (0.03)	14.71
		P = 0.035	P = 0.007			P = 0.044	P = 0.243			
Max Lightness	2.527 (0.267)	1.018 (0.213)	0.224 (0.185)	0	1.286 (0.155)	0.40 (0.07)	0.09 (0.07)	0	74.24 (0.09)	1.36
		P < 0.001	P < 0.001			P < 0.001	P = 0.187			
Mean Chroma	3.193 (0.728)	1.150 (0.698)	0.964 (0.699)	0.514 (0.659)	0.565 (0.071)	0.36 (0.23)	0.30 (0.15)	0.16 (0.21)	11.38 (0.09)	9.42
		P = 0.144	P < 0.001	P = 0.431		P = 0.113	P = 0.051	P = 0.443		
SD Chroma	0.228 (0.019)	0.076 (0.060)	0.006 (0.007)	0.013 (0.056)	0.134 (0.016)	0.33 (0.26)	0.03 (0.03)	0.06 (0.25)	1.40 (0.03)	19.69
		P = 0.242	P = 0.090	P = 0.828		P = 0.193	P = 0.363	P = 0.823		
Max Chroma	3.588 (0.748)	1.398 (0.825)	0.973 (0.710)	0.519 (0.774)	0.698 (0.088)	0.39 (0.24)	0.27 (0.15)	0.14 (0.22)	12.82 (0.10)	9.22
		P = 0.155	P < 0.001	P = 0.509		P = 0.099	P = 0.063	P = 0.508		
Mean BGC*	1.062 (0.166)	0.573 (0.295)	0.164 (0.139)	0.130 (0.274)	0.196 (0.029)	0.54 (0.27)	0.15 (0.11)	0.12 (0.26)	0.589 (0.001)	1.29
		P = 0.091	P < 0.001	P = 0.639		P = 0.048	P = 0.168	P = 0.637		
SD BGC*	0.115 (0.014)	0.055 (0.011)	0.010 (0.010)	0	0.050 (0.007)	0.48 (0.08)	0.09 (0.07)	0	0.009 (0.000)	26.06
		P < 0.001	P < 0.001			P < 0.001	P = 0.242			
Max BGC*	1.275 (0.214)	0.481 (0.366)	0.219 (0.186)	0.301 (0.352)	0.274 (0.040)	0.38 (0.29)	0.17 (0.12)	0.24 (0.28)	0.598 (0.001)	1.16
		P = 0.244	P < 0.001	P = 0.383		P = 0.187	P = 0.160	P = 0.398		
Laying date	33.92 (9.83)	3.491 (5.398)	13.47 (9.678)	6.229 (5.502)	10.73 (1.323)	0.10 (0.16)	0.40 (0.17)	0.18 (0.17)	48.12 (0.31)	3.88
		P = 0.497	P < 0.001	P = 0.245		P = 0.525	P = 0.022	P = 0.278		

*Variance components and their SEs: e^{-4}.
BGC, blue-green chroma.

(CV_A) ranged from 1.16 to 26.06; the highest values were found for parameters describing within-clutch colour variation (SD in Lightness, Chroma and BGC; Table 1). Surprisingly, although year environmental variance (V_{Year}) was significant for most colour parameters, year effects (y^2) were nonsignificant for all of them, because of high standard errors; note, however, that they were marginally nonsignificant for within-clutch mean Lightness, mean Chroma and maximum Chroma (Table 1). Also, permanent environmental effects (pe^2) were nonsignificant for all colour traits (Table 1). Laying date had nonsignificant V_A and h^2 but significant y^2 (Table 1).

Discussion

We found significant additive genetic variance on some eggshell colour parameters in a free-living population of pied flycatchers. The highest coefficients of additive genetic variance (CV_A) were found in traits describing within-clutch colour variation, suggesting that this trait shows the highest evolvability. On the other hand, year and permanent environmental effects did not explain a significant proportion of the variance. However, year environmental variance (V_{Year}) was significant for all but one of the traits.

The exploration of fixed effects indicated that within-clutch maximum Lightness was negatively associated with egg mass, supporting previously observed trends in the relationship between egg colour and size (Moreno *et al.*, 2004). As could be expected, young females laid later in the season, which is a common pattern in this species (Lundberg & Alatalo, 1992).

Our findings support a genetic basis of blue-green eggshell coloration, as suggested in previous studies (e.g. Collias, 1993 and references therein). The univariate animal models estimated high additive genetic variances (V_A), which were significant for maximum Lightness, SD Lightness and SD BGC, and marginally nonsignificant for mean BGC. Narrow-sense heritabilities (h^2) were significant for the later four parameters and marginally nonsignificant for another two. Heritabilities for all colour traits were moderately high and explained between 15% and 54% of the total phenotypic variance. Laying date showed very low and nonsignificant heritability ($h^2 = 0.10$), which may contrast with previous evidence in other avian species. For instance, in the closely related collared flycatcher, *Ficedula albicollis*, the heritability of laying date estimated with animal models was similarly low ($h^2 = 0.19$) but significant (Sheldon *et al.*, 2003). Nevertheless, previous pied flycatcher studies using parent–offspring regressions either found substantial heritability on laying date (Lundberg & Alatalo, 1992) or no evidence of additive genetic variation (see Potti, 1998b; in a close Iberian population). Note that there are moderate–low levels of extra-pair paternity in the study population (7.5% of nestlings; Moreno *et al.*,

2010), which may lead to downward bias of genetic variance and hence heritability (Wilson et al., 2010).

The CV_A may provide a more informative measure of genetic variability than heritability, as it scales the component of additive genetic variance by the trait mean instead of by the total variance, and so it is not confounded by the magnitude of other variance components (Houle, 1992; Kruuk et al., 2000). Interestingly, the three parameters describing within-clutch SD in colour showed the highest CV_A, suggesting that variation in colour within a clutch has the highest potential for evolutionary change under directional selection (Houle, 1992; Roff, 1997). Genetic variance for variation in colour may indicate genetic heterogeneity of environmental variance, which can arise from genetic differences in environmental sensitivity (Falconer & Mackay, 1996; Lynch & Walsh, 1998). The fact that genotypes may differ not only in mean, but also in environmental variance of the traits they affect, has been considered a rare biological phenomenon (discussed by Mulder et al., 2007); yet empirical studies on this idea are scarce.

Previous findings in the study species stressed the role of environmental effects on eggshell colour, as females laid colourful clutches when food availability was experimentally increased (Moreno et al., 2006a). Also in other species, blue-green eggshell colour seems to be influenced by spring environmental conditions (Avilés et al., 2007) and pollution (Jagannath et al., 2008). Accordingly, the variance because of year (V_y) was significant for all but one colour parameters. However, year effects (y^2) were non-significant (or almost significant) for all of them. One possibility is that the environmental variances because of year were partly confounded by treatment effects; although treatment was included as a fixed factor, the experiments were performed in two of the five study years. This may explain why V_y was significant for most traits, despite very high standard errors. On the other hand, the significance estimated by the likelihood ratio test for the year variance component may be more reliable than that obtained with the t-test for the year variance ratio, if the distribution of year effect estimates is asymmetric. Thus, the interpretation of the year variance component and its ratio should be taken with caution. Permanent environmental variance (V_{PE}) and effects (pe^2) were negligible for all colour parameters, indicating that between-individual differences (for instance because of mate or territory quality) were not persistent over time (Kruuk, 2004). Current environmental conditions could have played a more important role.

Evidence in the study species suggests that males respond to experimentally increased eggshell colour by enhancing parental contribution to their offspring (Moreno et al., 2006b, 2008). Additionally, egg colour reflects important aspects of female condition (e.g. Moreno et al., 2005) or the quality of maternal effects transferred to the eggs (Morales et al., 2006). Furthermore, it can be subject to trade-offs with crucial self-maintenance processes in females (Morales et al., 2008) that may ensure the honesty of the signal. Moreover, egg colour predicts fledgling condition (Moreno et al., 2008) and reproductive success (Morales et al., 2006, 2008), which may ultimately affect fitness. This study shows that egg colour is heritable. Therefore, blue-green egg colour in pied flycatchers may indicate heritable variation in female fitness and could have evolved via a good-genes process, as expected from theory (Kokko, 2001). In this case, it would add to one clear example of a signal of female genetic quality to males in barn owls (see Roulin et al., 2000, 2001). Nonetheless, to evidence selection by good genes in our pied flycatcher population, we still need to demonstrate that there are positive associations between eggshell colour and female lifetime fitness mediated by the mate's response to colour stimulation.

To conclude, our results from univariate animal models showed substantial additive genetic variation in blue-green eggshell colour in wild pied flycatchers, as predicted by the sexual signalling hypothesis (Moreno & Osorno, 2003). Within-clutch colour variation (SD in colour) showed the highest coefficient of additive genetic variance and thus, the highest potential for evolution. Interestingly, male pied flycatchers in the study population enhance their parental contribution in clutches with higher variation in colour and peak values rather than average values (Moreno et al., 2006b, 2008).

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References

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, NJ.

Avilés, J.M., Stokke, B.G., Moksnes, A., Røskaft, E. & Møller, A.P. 2007. Environmental conditions influence egg color of

red warblers Acrocephalus scirpaceus and their parasite, the common cuckoo Cuculus canorus. Behav. Ecol. Sociobiol. 61: 475–485.

Avilés, J.M., Pérez-Contreras, T., Navarro, C. & Soler, J.J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. Anim. Behav. 78: 993–999.

Bize, P., Gasparini, J., Klopfenstein, A., Altwegg, R. & Roulin, A. 2006. Melanin-based coloration is a non-directionally selected sex-specific signal of offspring development in the Alpine swift. Evolution 60: 2370–2380.

Burley, N. 1986. Sexual selection for aesthetic traits in species with biparental care. Am. Nat. 127: 415–445.

Cassey, P. 2009. Biological optics: seeing colours in the dark. Curr. Biol. 19: R1083–R1084.

Cazetta, E., Schaefer, H.M. & Galetti, M. 2009. Why are fruit colorful? The relative importance of achromatic and chromatic contrast for detection by birds. Evol. Ecol. 23: 233–244.

Charmantier, A. & Garant, D. 2005. Environmental quality and evolutionary potential: lessons from wild populations. Proc. R. Soc. Lond. B 272: 1415–1425.

Charmantier, A. & Sheldon, B.C. 2006. Testing genetic models of mate choice evolution in the wild. Trends Ecol. Evol. 21: 417–419.

Collias, E.C. 1993. Inheritance of egg-color polymorphism in the village weaver (Ploceus cucullatus). Auk 110: 683–692.

Crawley, M.J. 2007. The R Book. John Wiley & Sons, Chichester.

Falchuk, K.H., Contin, J.M., Dziedzic, T.S., Feng, Z.L., French, T.C., Heffron, G.J. & Montorzi, M. 2002. A role for biliverdin IX alpha in dorsal axis development of Xenopus laevis embryos. Proc. Natl. Acad. Sci. USA 99: 251–256.

Falconer, D.S. & Mackay, T.F.C. 1996. Introduction to Quantitative Genetics. Longman, Essex, U.K.

Garant, D., Sheldon, B.C. & Gustafsson, L. 2004. Climatic and temporal effects on the expression of secondary sexual characters: genetic and environmental components. Evolution 58: 634–644.

Gasparini, J., Bize, P., Piault, R., Wakamatsu, K., Blount, J., Ducrest, A.-L. & Roulin, A. 2009. Strength and cost of an induced immune response are associated with a heritable melanin-based colour trait in female tawny owls. J. Anim. Ecol. 78: 608–616.

Gilmour, A.R., Gogel, B.J., Cullis, B.R. & Thompson, R. 2006. ASReml User Guide Release 2.0. VSN International Ltd, Hemel Hempstead, UK.

Gosler, A.G., Barnett, P.R. & Reynolds, S.J. 2000. Inheritance and variation in eggshell patterning in the great tit Parus major. Proc. R. Soc. Lond. B 267: 2469–2473.

Hanley, D. & Doucet, S.M. 2009. Egg coloration in ring-billed gulls (Larus delawarensis): a test of the sexual signalling hypothesis. Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. & Dearborn, C.D. 2008. Testing and assumption of the signalling hypothesis: does blue-green egg color reflect maternal antioxidant capacity? Condor 110: 767–771.

Hargitai, R., Herénnyi, M. & Török, J. 2008. Eggshell coloration in relation to male ornamentation, female condition and egg quality in the collared flycatcher Ficedula albicollis. J. Avian Biol. 39: 413–422.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. & Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and modelling of egg visual discrimination in blue tits. Biol. Lett., doi: 10.1098/rsbl.2009.1044.

Houle, D. 1992. Comparing evolvability and variability of quantitative traits. Genetics 130: 195–204.

Jagannath, A., Shore, R.F., Walker, L.A., Ferns, P.N. & Gosler, A.G. 2008. Eggshell pigmentation indicates pesticide contamination. J. Appl. Ecol. 45: 133–140.

Jones, G.A. & Ratterman, N. 2009. Mate choice and sexual selection: what have learnt since Darwin? Proc. Natl Acad. Sci. USA 106: 10001–10008.

Kennedy, G.Y. & Vevers, H.G. 1976. A survey of avian eggshell pigments. Comp. Biochem. Physiol. 55B: 117–123.

Kokko, H. 2001. Fisherian and 'good genes' benefits of mate choice: how (not) to distinguish between them. Ecol. Lett. 4: 322–326.

Krist, M. & Grim, T. 2007. Are blue eggs a sexually selected signal of female collared flycatchers? a cross-fostering experiment. Behav. Ecol. Sociobiol. 61: 863–876.

Kruuk, L.E.B. 2004. Estimating genetic parameters in natural populations using the 'animal model'. Phil. Trans. R. Soc. Lond. B 359: 873–890.

Kruuk, L.E.B. & Hadfield, J.D. 2007. How to separate genetic and environmental causes of similarity between relatives. J. Evol. Biol. 20: 1890–1903.

Kruuk, L.E.B., Clutton-Brock, T., Slate, J., Pemberton, J.M., Brotherstone, S. & Guinness, F. 2000. Heritability of fitness in a wild animal population. Proc. Natl Acad. Sci. USA 97: 698–703.

Kruuk, L.E.B., Slate, J., Pemberton, J.M., Brotherstone, S., Guinness, F. & Clutton-Brock, T. 2002. Antler size in red deer: heritability and selection but no evolution. Evolution 56: 1683–1695.

Kruuk, L.E.B., Slate, J. & Wilson, A.J. 2008. New answers for old questions: the evolutionary quantitative genetics of wild animal populations. Annu. Rev. Ecol. Evol. Syst. 39: 525–548.

López-Rull, I., Miksik, I. & Gil, D. 2008. Egg pigmentation reflects female and egg quality in the spotless starling Sturnus unicolor. Behav. Ecol. Sociobiol. 62: 1877–1884.

Lundberg, A. & Alatalo, R.V. 1992. The Pied Flycatcher. Poyser, London.

Lynch, M. & Walsh, B. 1998. Genetics and Analysis of Quantitative Traits. Sinauer, Sunderland, USA.

Mahler, B., Confalonieri, V.A., Lovette, I.J. & Reboreda, J.C. 2008. Eggshell spotting in brood parasitic shiny cowbirds (Molothrus bonariensis) is not linked to the female sex chromosome. Behav. Ecol. Sociobiol. 62: 1193–1199.

Merilä, J. & Sheldon, B.C. 1999. Genetic architecture of fitness and nonfitness traits: empirical patterns and development of ideas. Heredity 83: 103–109.

Merilä, J. & Sheldon, B.C. 2001. Avian quantitative genetics. In: Current Ornithol. Vol. 16 (V. Nolan & C.F. Thompson, eds), pp. 179–255. Kluwer Academic Publishers, New York.

Møller, A.P. 1993. Sexual Selection in the Barn Swallow Hirundo rustica .3. Female Tail Ornaments. Evolution 47: 417–431.

Morales, J., Sanz, J.J. & Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. Biol. Lett. 2: 334–336.

Morales, J., Velando, A. & Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. Behav. Ecol. Sociobiol. 63: 227–233.

Morales, J., Torres, R. & Velando, A. 2010. Parental conflict and blue egg coloration in a seabird. Naturwissenschaften, 97: 173–180.

Moreno, J. & Osorno, J.L. 2003. Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? Ecol. Lett. 6: 803–806.

Moreno, J., Osorno, J.L., Morales, J., Merino, S. & Tomás, G. 2004. Egg colouration and male parental effort in the pied flycatcher Ficedula hypoleuca. J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. & Martínez-de la Puente, J. 2005. Evidence for the signalling function of egg color in the pied flycatcher Ficedula hypoleuca. Behav. Ecol. 16: 931–937.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-de la Puente, J., Sanz, J.J., Mateo, R. & Soler, J.J. 2006a. Experimental evidence that egg color indicates female condition at laying in a songbird. Behav. Ecol. 17: 651–655.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. & Martínez-de la Puente, J. 2006b. More colorful eggs induce a higher relative paternal investment in the pied flycatcher Ficedula hypoleuca: a cross-fostering experiment. J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Merino, S. & Martínez-de la Puente, J. 2008. Blue-green eggs in pied flycatchers: an experimental demonstration that a supernormal stimulus elicits improved nestling condition. Ethology 114: 1078–1083.

Moreno, J., Martínez, J.G., Morales, J., Lobato, E., Merino, S., Tomás, G., Vásquez, R.A., Möstl, E. & Osorno, J.L. 2010. Cuckolded males are young, stressed and reactive the pied flycatcher. Ethology 116: 76–84.

Mulder, H.A., Bijma, P. & Hill, W.G. 2007. Prediction of breeding values and selection responses with genetic heterogeneity of environmental variance. Genetics 175: 1895–1910.

Ojanen, M., Orell, M. & Väisänen, R. A. 1978. Egg and clutch sizes in four passerine species in northern Finland. Ornis Scand. 55: 60–68.

Osorno, J.L., Morales, J., Moreno, J., Merino, S., Tomás, G. & Vásquez, R.A. 2006. Evidence for differential allocation to eggs in relation to manipulated male attractiveness in the pied flycatcher (Ficedula hypoleuca). J. Ornithol. 147: 605–611.

Postma, E. 2006. Implications of the difference between true and predicted breeding values for the study of natural selection and micro-evolution. J. Evol. Biol. 19: 309–320.

Potti, J. 1993. A male trait expressed in female pied flycatchers, Ficedula hypoleuca: the white forehead patch. Anim. Behav. 45: 1245–1247.

Potti, J. 1998a. Variation in the onset of incubation in the pied flycatcher (Ficedula hypoleuca): fitness consequences and constraints. J. Zool. (Lond.) 245: 335–344.

Potti, J. 1998b. Arrival time from spring migration in male pied flycatcher: individual consistency and familial resemblance. Condor 100: 702–708.

Punnett, R.C. 1933. Genetic studies in poultry. IX. The blue egg. J. Genet. 27: 465–470.

Punnett, R.C. & Bailey, P.G. 1920. Genetic studies in poultry. II. Inheritance of colour and broodiness. J. Genet. 10: 277–292.

Quesada, J. & Senar, J.C. 2009. Cross-fostering experiments to compare carotenoid and melanin-based plumage traits and long-term parental effects in post-moulted great tits. Behaviour 146: 1235–1251.

Qvarnström, A. 1999. Genotype-by-environmental interactions in the determination of the size of a secondary sexual character in the collared flycatcher (Ficedula albicolis). Evolution 53: 1564–1572.

R Development Core Team 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Reynolds, S.J., Martin, G.R. & Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? Anim. Behav. 78: 209–215.

Roff, D.A. 1997. Evolutionary Quantitative Genetics. Chapman & Hall, New York.

Roulin, A. & Dijkstra, C. 2003. Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl. Heredity 90: 359–364.

Roulin, A., Jungi, T.W., Pfister, H. & Dijkstra, C. 2000. Female barn owls (Tyto alba) advertise good genes. Proc. R. Soc. Lond. B 267: 937–941.

Roulin, A., Riols, C., Dijkstra, C. & Ducrest, A.-L. 2001. Female plumage spottiness and parasite resistance in the barn owl (Tyto alba). Behav. Ecol. 12: 103–110.

Sanz, J.J. & Moreno, J. 2000. Delayed senescence in a southern population of the Pied Flycatcher Ficedula hypoleuca. Ecoscience 7: 25–31.

Sheldon, B.C., Kruuk, L.E.B. & Merilä , J. 2003. Natural selection and inheritance of breeding time and clutch size in the collared flycatcher. Evolution 57: 406–420.

Siefferman, L., Navara, K.J. & Hill, G.E. 2006. Egg colouration is associated with female condition in eastern bluebirds (Sialia sialis). Behav. Ecol. Sociobiol. 59: 651–656.

Soler, J.J., Moreno, J., Avilés, J.M. & Møller, A.P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. Evolution 59: 636–644.

Soler, J.J., Navarro, C., Pérez-Contreras, T., Avilés, J.M. & Cuervo, J.J. 2008. Sexually selected egg coloration in spotless starling. Am. Nat. 171: 183–194.

Stevens, L. 1991. Genetics and Evolution of the Domestic Fowl. Cambridge Univ. Press, Cambridge.

Washburn, K. W. 1990. Genetic variation in egg composition. In: Poultry Breeding and Genetics (R.D. Crawford, ed), pp. 781–804. Elsevier Publishing Co, Amsterdam.

Wilson, A.J., Réale, D., Clements, M.N., Morrisey, M.M., Postma, E., Walling, C.A., Kruuk, L.E.B. & Nussey, D.H. 2010. An ecologist's guide to the animal model. J. Anim. Ecol. 79: 13–26.

Yang, N., Zhao, R., Li, X.Y., Xu, G.Y., Yang, C.S. & Yan, H.X. 2003. Study of molecular markers related to blue-shelled gene in chicken. In: Proc. 11th Natl. Poult. Symp., pp. 37–39. Chinese Assoc. Anim. Sci. Vet. Med., Qingdao, China.

Appendix 1

Summary of minimum adequate linear mixed effect models (LMEs) using REML (random effect: individual identity), exploring fixed effects for nine egg colour traits and laying date in pied flycatchers. Full model for colour traits: female age + experimental treatment + egg mass. Full model for laying date: female age + experimental treatment. The significance of each fixed term is based on marginal Wald-type statistics.

Term	Coefficient ± SE	Test	P
(a) Mean lightness	(n = 346)		
Intercept	71.72 ± 0.35		
Treatment			
enlarged patch size	0	$F_{3,127}$ = 21.60	< 0.001
reduced patch size	0.38 ± 0.47		
food supplementation	)0.67 ± 0.37		
control	0.71 ± 0.35		
(b) SD lightness	(n = 346)		
Intercept	1.48 ± 0.15		
Treatment			
enlarged patch size	0	$F_{3,127}$ = 5.47	0.001
reduced patch size	0.22 ± 0.21		
food supplementation	0.18 ± 0.17		
control	)0.11 ± 0.15		
(c) Max lightness	(n = 346)		
Intercept	77.92 ± 1.43		
Egg mass	)2.24 ± 0.78	$F_{1,126}$ = 8.30	0.005
Treatment			
enlarged patch size	0	$F_{3,126}$ = 4.70	0.004
reduced patch size	0.13 ± 0.60		
food supplementation	)0.60 ± 0.47		
control	0.16 ± 0.44		
(d) Mean chroma	(n = 346)		
Intercept	10.12 ± 0.41		
Treatment			
enlarged patch size	0	$F_{3,127}$ = 8.55	< 0.001
reduced patch size	0.16 ± 0.56		
food supplementation	1.12 ± 0.44		
control	1.49 ± 0.41		
(e) SD chroma	(n = 346)		
Intercept	1.43 ± 0.13		
Treatment			
enlarged patch size	0	$F_{3,127}$ = 4.88	0.003
reduced patch size	0.15 ± 0.18		
food supplementation	0.19 ± 0.14		
control	)0.06 ± 0.13		
(f) Max chroma	(n = 346)		
Intercept	11.66 ± 0.43		
Treatment			
enlarged patch size	0	$F_{3,127}$ = 3.74	0.013
reduced patch size	0.82 ± 0.58		
food supplementation	1.36 ± 0.46		
control	1.32 ± 0.43		
(g) Mean BGC	(n = 282)		
Intercept	0.589 ± 0.001		
(h) SD BGC	(n = 282)		
Intercept	0.011 ± 0.0005		
Treatment			
food supplementation	0	$F_{1,93}$ = 23.20	< 0.001
control	)0.002 ± 0.0005		
(i) Max BGC	(n = 282)		
Intercept	0.598 ± 0.001		
(j) Laying date	(n = 346)		
Intercept	50.41 ± 0.69		
Age	)0.80 ± 0.22	$F_{1,129}$ = 13.32	< 0.001

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