Behav Ecol Sociobiol (2007) 61:863–876
DOI 10.1007/s00265-006-0315-9

ORIGINAL PAPER

Are blue eggs a sexually selected signal of female collared flycatchers? A cross-fostering experiment

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Received: 1 June 2006 / Revised: 29 August 2006 / Accepted: 15 November 2006 / Published online: 10 January 2007

Abstract Impressive variation in egg colouration among birds has puzzled evolutionary biologists for a long time. The most frequently studied selective forces moulding egg colouration—predation and brood parasitism—have either received little empirical support or may play a role in only a minority of species. A novel hypothesis has suggested that egg colour may be significantly influenced by sexual selection. Females may deposit a blue-green pigment biliverdin into eggshells instead of using it for themselves as a powerful antioxidant. By this handicap, females may signal their quality to males, which are then hypothesized to increase their paternal effort. We experimentally tested the hypothesis in the collared flycatcher (*Ficedula albicollis*), a species laying blue-green eggs. We cross-fostered clutches between nests to disentangle effects of female/territory quality and egg colour on paternal effort and nestling quality. The results supported two assumptions of sexual signalling through egg colour hypothesis: Blue pigment seems to be a limited resource for females, and female quality is positively correlated with the intensity of the blue-green colour. However, we did not find support for the main prediction of the hypothesis, as male parental effort parameters (feeding frequencies to nestlings and intensity of nest defence) were unrelated to egg colour. We discuss possible reasons for the discrepancy between our results and previous correlative analyses that supported the hypothesis that blue egg colour may be a postmating, sexually selected signal in females.

Keywords Egg colour · Differential allocation · Female signalling · Immunity · Parental investment

Introduction

Egg colouration has been attracting the attention of biologists for decades, and various hypotheses have been generated to explain the variation in this trait (Underwood and Sealy 2002). Recent research indicates that pigments may physically strengthen the eggshell (Gosler et al. 2005). However, the majority of suggested explanations propose that egg colouration primarily has a signalling function to either heterospecifics or conspecifics. On the heterospecific level, most attention has been paid to nest predators and brood parasites. Eggs may have cryptic colours to preclude predators from locating eggs/nests (Tinbergen et al. 1962). Brood parasitism may select for small intraclutch variation but large interclutch variability in host egg colouration (Øien et al. 1995; Soler and Møller 1996), and hosts may select for mimetic eggs in parasites by rejecting eggs differing from their own (Davies and Brooke 1988; Grim and Honza 2001). On the intraspecific level, egg colouration may be important for the recognition of ones own eggs in colonial birds (Birkhead 1978). By laying a pale egg last in a clutch, females may signal that they have started to incubate, which would diminish the opportunities for successful conspecific brood parasitism (Yom-Tov 1980; Ruxton et al. 2001).

Communicated by M. Soler

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The support from observations and experiments for each of these hypotheses is equivocal (Underwood and Sealy 2002). Thus, the large amount of variation in egg colour remains unexplained. For example, in a carefully designed study by Weidinger (2001), open-cup nests baited with different coloured (white, blue and cryptic) eggs had equal survival rates despite the fact that the study was conducted in a population where most nests were eventually destroyed by nest predators and where one could therefore expect strong selection for egg crypsis.

One of the most striking—and most puzzling—egg colours is blue (Underwood and Sealy 2002). The evolutionary significance of blue eggs was previously studied in the context of predation and brood parasitism. Götmark (1992) and Weidinger (2001) found no support for a hypothesized cryptic function of blue eggs (blending with specific nest micro-environment). On the other hand, there is some evidence that the blue egg morph in the common cuckoo (*Cuculus canorus*) is a counter-adaptation against host egg discrimination in host species laying bluish eggs (Moksnes et al. 1995). However, this does not explain why the hosts themselves lay blue eggs in the first place. Consequently, until recently, blue-green colouration of eggs was considered to be a mystery (Underwood and Sealy 2002).

Recently, a new hypothesis based on intraspecific signalling has been proposed to explain variation in egg colours (Moreno and Osorno 2003). According to this hypothesis, females colour their eggs using costly pigments to signal their quality and, consequently, the quality of their progeny to their mates. The costs of such extended phenotype should be outweighed by benefits from increased paternal care into the current brood. Such an increase of paternal care in high quality offspring is in turn predicted by the differential allocation hypothesis (Burley 1986; Sheldon 2000). Moreno and Osorno (2003) paid special attention to blue-green eggs when formulating their hypothesis because biliverdin, which causes the blue colour of eggs (Mikšík et al. 1996), has also a strong antioxidant activity (McDonagh 2001; Kaur et al. 2003). Thus, the deposition of biliverdin into eggs may signal female capacity to control free radicals despite the handicap (Moreno and Osorno 2003). Such a signal would be in principle similar to colouration of plumage with other strong antioxidants such as carotenoids. At present, the latter topic has received considerable attention in respect to male signalling (e.g. Hill 2002; McNett and Marchetti 2005).

Female signalling through ornamentation of plumage has been neglected both in studies of mate choice (Amundsen 2000; Hill 2002) and differential allocation (Sheldon 2000). An interest in this topic is currently growing, and some evidence has already been found to suggest that female plumage ornaments are important for male mate choice (Hill 2002; Griggio et al. 2005), paternal effort (Hill 2002; Pilastro et al. 2003) and even sperm allocation (Pizzari et al. 2003). Although female signalling through colour of plumage and eggs is similar in principle, colour of eggs may be under stronger sexual selection than that of plumage, as the first signal might be more informative than the latter. Although many types of pigments appearing in plumage may have antioxidant capacities similar to the egg pigment biliverdin (McGraw 2005), they may be indicative of either health or pigment availability at the time of molting. However, molting is usually separated from breeding by considerable time periods. Consequently, at the time of breeding, males can assess only genetic or persistent environmental quality from female plumage. In contrast to plumage ornaments, egg colour may be indicative of the females' current physiological state, condition and immunity.

To date, five correlational and one experimental study have tested the sexual selection hypothesis of evolution of the blue colouration of eggs (SSEC hypothesis). Three of the correlative and the one experimental study tested the validity of the assumption of the hypothesis, namely whether egg colour was correlated with female and/or offspring quality. In the pied flycatcher (*Ficedula hypoleuca*), a species laying blue eggs, it has been found that the saturation of egg colour was positively correlated with female immunocompetence (Moreno et al. 2005) and amount of maternal yolk antibodies (Morales et al. 2006). Blue-green chroma of eggs was also positively correlated with female condition in another species laying blue eggs, the bluebird *Sialia sialis* (Siefferman et al. 2006). Food supplementation before and during egg laying enabled female pied flycatchers to lay more saturated eggs, which suggests that egg colour depends on female current nutritional state (Moreno et al. 2006).

So far, only two correlative studies tested the main prediction of the sexually selected egg colour (SSEC) hypothesis, i.e. whether egg colour predicts paternal effort. Moreno et al. (2004) found that males of the pied flycatcher fed broods hatched from eggs with more saturated colour with greater frequency. Soler et al. (2005), in a comparative study, found positive correlations between blue colour of eggs, mating system and duration of nestling period. The two latter variables were used by Soler et al. (2005) as surrogates of intraspecific variation in paternal effort, a variable that should affect the evolution of female signalling.

Thus, all studies performed so far have given results that are consistent with the SSEC hypothesis. However, the causality of the observed relationships is unclear because of the correlative nature of most of these studies. Moreover, in three studies on the pied flycatcher (Morales et al. 2006; Moreno et al. 2004, 2005), the reflectance spectra of eggs were measured at wavelengths above 360 or 400 nm. As most passerines are able to see even shorter wavelengths (Cuthill et al. 2000), further studies that include the UV part of the spectrum when measuring egg colour are particularly needed.

The main aim of this study was to experimentally test for a causal link between egg colour and paternal effort. We performed the study on the collared flycatcher (*Ficedula albicollis*), which lays, similarly to its sister species, the pied flycatcher, blue-green eggs. As the collared flycatcher is a hole-nester, the potential confounding effects of both predation and interspecific brood parasitism on egg colour can be rejected a priori. To test for causality between egg colour and paternal effort, we cross-fostered freshly laid eggs between pairs of nests. Consequently, egg colour was randomized (in the extent of 92%, see "Materials and Methods") with respect to parental and territory quality. The UV range was included in the measuring of egg spectra to deal with the presumed UV vision of flycatchers. Three measures of paternal effort were scored (feeding frequencies to young and old nestlings and nest defence against a nest predator) to enable a more detailed estimate to be obtained. In addition, we tested for relationships between egg colour and offspring survival, morphology and T cell-mediated immunity. We also paid attention to female parents to test whether the egg colour is correlated with any other female traits.

If the SSEC hypothesis (Moreno and Osorno 2003) holds, egg colour of cross-fostered, but not original eggs, should predict paternal effort. If the colour of original eggs predicted paternal effort, this would suggest one of the following: (1) Males adjust their effort according to some female trait that is correlated with the colour of her eggs; (2) high quality females laying eggs with more saturated colours mate assortatively with high quality males that are able to provide superior parental care; and (3) the relationship is driven by territory quality: On superior territories, it may be less costly both to lay eggs with saturated colours and feed young with greater frequency. If offspring quality was correlated with colour of cross-fostered eggs (i.e. eggs from which the young actually hatched), this would imply either genetic or environmental (i.e. egg composition) quality of eggs with saturated colour or differential male effort. In contrast, if colour of original eggs was predictive of offspring quality, this would imply that parental or territory quality is correlated with egg colour.

Materials and methods

Field methods

We conducted the study in the Velký Kosíř area (49°32′N, 17°04′E, 300–400 m a.s.l.) of the Czech Republic in 2005. In the study area, there were approximately 300 nest-boxes in oak (*Quercus petraea*) forest. We conducted the experiment with collared flycatcher, a small migratory passerine that easily adopts nest-boxes for breeding. Females usually lay one egg per day and solely incubate clutches of 4–8 eggs. Similar to the sister species, the pied flycatcher (Moreno et al. 2005), eggs are unspotted and brightly blue-green in colour. Both collared flycatcher parents feed nestlings with invertebrate food for about 15 days until fledging.

We conducted cross-fostering experiments among 70 occupied nest-boxes. We cross-fostered eggs between pairs of nests in which laying began on the same day ($n=30$ nest pairs) or which differed in laying date by 1 day ($n=5$ nest pairs). We cross-fostered eggs on the day they were laid with the exception of five nests in which the first eggs were moved the day after they were laid. We recorded the time of both the removal of the original egg and the addition of the cross-fostered egg. We performed the exchange of first eggs of the clutches within 2 h, during which, nest-box entrances were blocked by sticks to ensure that no parent was aware that its nest was empty. Subsequent exchanges were done within a more variable timeframe (0–10 h), during which, the nest-box entrances were not blocked. We continued with exchanges on daily basis until the clutches in both nests had been completed. Original and cross-fostered clutch size was the same in 16 pairs of experimental nests; they differed by one in 14 pairs, by two in four pairs and by three in one pair. Where appropriate, we controlled for the difference in original and cross-fostered clutch size in statistical analyses. No experimental nest was abandoned in the course of laying or incubation.

The experimental design ensured that in the incubation stage, there were only cross-fostered eggs in a focal nest. However, in the laying stage, there were both original and cross-fostered eggs in focal nests. Under the assumption that eggs were visible from 6 A.M. to 8 P.M. in the laying stage (before the last egg was laid), the number of egg-hours for which the original eggs were exposed in the nests was 41.7±9.9 (mean±SD), whereas the respective figure for cross-fostered eggs was 195.0±62.0. Some females began to incubate before their clutch was complete. As eggs are less visible when females are incubating, we also counted the number of egg-hours for which eggs were exposed in nests before females began continuous incubation. For original eggs, this figure was 35.7±10.9 and that for cross-fostered eggs was 143.8±77.6. Taken together, these data suggest that the colour of cross-fostered eggs should have an overwhelming effect on paternal behaviour when compared with that of original eggs. Moreover, we can reasonably expect males to examine egg colours after clutch completion to avoid any assessment bias resulting from assessing an only partially laid clutch. In another system where birds assess egg appearance (hosts of brood parasites), this happens as a rule after clutch completion (Davies and Brooke 1988).

We began to check nests 2 days before the presumed hatching to determine hatching date and hatching success. To estimate cell-mediated immune response of nestlings, we injected them with 0.1 mg phytohaemagglutinin in 20 μl of physiological saline solution into the right wing web when they were 12 days old. Before injection, we took two measurements of wing web thickness (to the nearest 0.01 mm) with a thickness gauge (Mitutoyo Quick-Mini) that was adjusted to push with constant pressure of 1 N. We re-measured wing web thickness 24 h (±2 h) after injection. Both measures were highly repeatable (before injection: $r=0.797$, $F_{251,252}=8.84$, $p<0.001$; after injection: $r=0.972$, $F_{249,250}=70.38$, $p<0.001$). Therefore, we calculated cell-mediated immune response for each chick as the difference in average thickness of wing web after injection minus the average thickness of wing web before injection. At age 13 days, we also weighed nestlings with Pesola spring balance (to the nearest 0.25 g), measured their tarsus with digital calliper (to the nearest 0.01 mm) and recorded whether ectoparasitic mites (*Dermanyssus gallinoides*) were present in the nest. We also captured adults while feeding nestlings and weighed and measured them in the same way as nestlings.

Measures of parental investment

To investigate parental investment in the current brood, we recorded two principal types of investment. First, we recorded parental feeding frequencies of young at two different ages. We videotaped nest-boxes with cameras for 70 min on day 6 and 12 of the nestling period (hatching day = day 0). Feeding frequency was determined separately for each sex as the number of visits to the nest-box per hour starting 10 min after the beginning of the tape recording. We disregarded the first 10 min of recording to minimize the effect of disturbance due to installation of cameras. Collared flycatchers in our study area readily resume feeding regimes within minutes after nest-box checks (personal observations).

Second, we recorded parental nest defence against a predator of eggs and young. At a variable age of young (5–13 days), we simulated intrusion of the great spotted woodpecker (*Dendrocopos major*), which is a common nest predator on our study plots, in the close vicinity of flycatchers' nests. We chose this species because it is not a threat to parents themselves, which could confound results (we were interested in the parental defence of their nestlings, not of parents themselves). Natural interactions between our study species and the great spotted woodpecker are common in our study area (own observations) and confirm that woodpeckers are perceived as a threat by flycatchers. This is further confirmed by the fact that the frequency of contact attacks against the woodpecker dummy was much greater (approximately tenfold) than that against a control species used in a previous study which was done according to a similar methodology (Krist 2004). We used a stuffed specimen of female woodpecker in a posture simulating foraging on the bark. We attached the dummy to the bark of the tree at approximately 0.5 m below the nest-box of a tested pair. The dummy was oriented (looked) towards the nest-box. Before the dummy had been placed, a camera was installed that videotaped the vicinity of the nest-box. After the experiment began, the observer retreated into the shelter that was installed at least 20 m from the focal nest-box.

After a member of the pair approached close vicinity of the nest and so presumably noticed the dummy, observation of this individual began. Observations for the later-arriving individual began after its arrival. Observations lasted 5 min for each focal bird, which is the recommended length for this type of study (Sealy et al. 1998). During that time, we recorded for each individual the number of dive/contact attacks against a dummy and the latency from arrival to the first dive/contact attack. We focused our attention on dive/contact attacks because these are presumably the most risky behaviours with the greatest efficiency in deterring nest predators (see also Krist 2004; Grim 2005). Further, we scored the overall response of each individual on an ordinal scale: (1) normal feeding or silent watching on the dummy, (2) fluttering against a dummy and/or vocalization and (3) dive or contact attack against a dummy. We checked our field diary notes against the video recordings to ensure that no behaviour was overlooked when quantifying parental response for analyses.

Egg colour measurements

We measured the colour of the eggs on the day they were laid before they were transferred to foster nests. Egg colour was measured by Avantes spectrometer (AvaSpec-2048) which was configured for measurements in the UV-visible part of the spectrum. The light source for measurements was PX-2 pulsed xenon lamp. The spectrometer and the lamp were connected by a bifurcated fibre optic cable to a reflection probe, which consisted of seven optic fibres. Six of them transfer light from the PX-2 lamp to a measured surface, and the seventh one transfers reflected light to the spectrometer. The reflection probe was fixed in a probe holder at an angle of 45° and at a distance of approximately 1 cm from the measured surface. We placed eggs side by side on the measurement port (ellipse with axes 7 and 9 mm) in the probe holder. We then covered the eggs with a black cap to prevent ambient light from confusing measurements. To improve the signal/noise ratio, each spectrum was obtained as the mean of 100 readings with one reading over 10-ms integration time. We measured each egg twice (on the opposite sides), and the mean from the two measurements was used in subsequent analyses. All data were generated relative to a white standard (WS-2 Avantes).

We calculated three values from the reflectance spectra. (1) Brightness or total reflectance was obtained as the summed reflectance at each 1-nm interval from 301 to 700 nm. (2) Blue-green chroma (BGC hereafter) was calculated as the reflectance between 401–600 nm divided by the total reflectance. We focused on this part of the spectrum because biliverdin, the main eggshell pigment of blue-green eggs (Mikšík et al. 1996), absorbs light weakly at this spectral range but strongly at shorter (<400 nm) and longer (>600 nm) wavelengths (Falchuk et al. 2002), which causes the blue-green appearance of eggs. (3) Hue was calculated as $\arctan\{[(Q_G - Q_{UV})/Q_T]/[(Q_R - Q_B)/Q_T]\}$ where Q_T denotes brightness (summed reflectance between 301–700 nm), Q_{UV} is the summed reflectance in the UV area of the reflectance spectrum (301–400 nm), Q_B is the summed reflectance in blue area of the spectrum (401–500 nm), Q_G is the summed reflectance in green area of the spectrum (501–600 nm) and Q_R is the summed reflectance in the red area of the spectrum (601–700 nm). The method for calculating hue was basically the same as that used by Saks et al. (2003), with the exception that we used the whole spectral range visible to birds (i.e. 301–700 nm) in the calculations (for a similar approach see McNett and Marchetti 2005; Montgomerie 2006). Note that in such method, boundaries of segments differ from those which are used in human vision studies. This is because the segment method (Endler 1990) on which the calculation of our index of hue was based requires division of the whole visible spectrum into four segments of the same length regardless of the range of the spectrum (see also Montgomerie 2006). Eggs with greater values of the hue (i.e. less negative values) have their peak of reflectance at shorter wavelength than those with more negative values.

All three colorimetric variables described above should be affected by pigment concentration, however, in unsaturated colour such as that of flycatchers eggshells, chroma should be most indicative of pigment concentration (Andersson and Prager 2006). Indeed chroma has been found to predict concentration of carotenoids in the plumage of greenfinches *Carduelis chloris* (Saks et al. 2003) and biliverdin in eggshell of pied flycatcher (Moreno et al. 2006). Consequently, as BGC should be most closely linked to biliverdin content of eggshells, we would expect that it should be the main cue for male assessment of female quality and, therefore, the main predictor of paternal behaviour.

Mean brightness of eggs in a clutch was strongly correlated with mean BGC ($r=-0.835$, $n=70$, $p<0.001$), whereas mean hue was not correlated with either mean brightness ($r=0.009$, $n=70$, $p=0.944$) or mean BG chroma ($r=-0.179$, $n=70$, $p=0.138$). The strong negative correlation between mean brightness and mean BGC suggests that these two measures are largely from the same trait. Therefore, we did not use brightness for statistical testing. We focused our attention on BGC, as this measure should be more indicative of biliverdin content in eggshells (see above) and was used for the testing of the SSEC hypothesis in three recent studies (Morales et al. 2006; Moreno et al. 2006; Siefferman et al. 2006).

Statistical analyses

Separate models were fitted for effects of BGC and hue on paternal effort and nestling performance. In each of these models, the spectral quality in question of both original and cross-fostered eggs was the main factor of interest and was, therefore, always retained in the final models. To reduce unexplained variation and increase the power of the test, we added potentially important covariates as predictors in the initial models. These covariates were backward eliminated on the basis of their significance. Thus, only significant covariates remained in the final models. Different classes of covariates were entered into models with different response variables. In models where the response variable was feeding rate, we controlled for hour, ambient temperature and brood size when feeding frequency was recorded. We fitted separate models for paternal feeding frequency at the two ages of young, as these were only weakly correlated ($r=0.222$, $n=45$, $p=0.143$). In models where the response variable was nest defence, we controlled for offspring age and brood size on the day of the experiment. In models where the response variable was hatching/fledging success or nestling immunity, we controlled for laying date of original clutch (1st May=1), total feeding frequency at age 12 days, number of parents attending the nest, presence of mites (binary variable) and the difference between original and cross-fostered clutch size. We included the difference in clutch sizes in the model to control for a potential effect of enlargement or reduction of clutch size caused by our cross-fostering design. We also included the number of parents attending the nest in addition to total feeding frequency, as we assumed that a parent rearing the brood alone may reduce the quality or quantity of food delivered per visit. In models where the response variable was fledging weight or tarsus length, we included all covariates as in the former models, as well as midweight or midtarsus of genetic parents, respectively. The weight of parents was adjusted for nestling age when a parent was captured by including residuals from the regression of parental weight on nestling age, rather than entering the actual parental weight into the model ($female\ weight = 14.39 - 0.14 \times age$; $F_{1,53}$=19.27, p<0.001; $male\ weight = 13.66 - 0.09 \times age$; $F_{1,47}$=7.13, p=0.010). The reason for the inclusion of the parental characteristic into these models is that morphological traits are usually highly heritable (Merilä and Sheldon 2001), which means they would affect the response variable. If parental morphology was correlated with the colour of the original eggs, its inclusion among predictors would be needed to avoid spurious results (Krist and Remeš 2004). We used brood means in models investigating the effect of predictors on nestling performance (immunity, weight and tarsus length). We did not adjust nestling weight for nestling tarsus length, as these variables were only weakly correlated (r=0.205, n=50, p=0.152). Only the young that subsequently fledged were included in the computation of brood means.

To investigate the relationship between female characteristics and the colour of the eggs she lays, we computed correlations between spectral qualities of original eggs and female age, morphology (tarsus length and condition) and some reproductive parameters (egg size, clutch size and laying date). We knew the exact age of 37 females that we had ringed as nestlings in previous years. As this variable was not normally distributed, we assessed the relationship between female age and egg colour by non-parametric Spearman rank correlation. We decided to use female condition rather than weight for analyses, as the latter was correlated with tarsus length (r=0.371, n=54, p<0.001). Thus, condition was determined as residuals from the regression of female weight on tarsus length and age of young at female capture ($weight = -2.748 + 0.857 \times tarsus - 0.155 \times age$; $F_{2,51}$=20.89, p<0.001). To test for a potential association between egg colour and maternal effort (intensity of nest defence and feeding frequency), we fitted models where the colour of the original eggs was an independent factor of interest, and maternal effort was the response variable. We fitted models instead of computing correlations because it was easier to control for covariates in the former statistical design. In females, feeding frequencies recorded at the two ages of young were correlated (r=0.465, n=45, p=0.001). Therefore, we performed a principal components analysis (PCA) and used the first principal component (73.3% of variation explained) as a measure of maternal feeding frequency. Consequently, we included in initial models, hour (daytime), ambient temperature and brood size recorded on both days of measuring the feeding frequency. In the model for female intensity of nest defence, we included the same class of covariates as for male nest defence.

To investigate the effect of laying order on the two spectral measures, we first subtracted clutch mean from the actual value of the spectral measure (e.g. actual BGC of the sixth egg minus average BGC of eggs in that nest). By this method (centering), we received values that were more comparable between clutches and, consequently, the power of statistical tests relating to intraclutch variation in egg colour was increased. We fitted two types of models. In the first group, we used actual laying order as the predictor variable. In the second group, we used relative laying order, which may be more appropriate when different clutch sizes are pooled into a single analysis. In our sample, clutch size varied between 4–8. We therefore categorized eggs into the following categories: last, penultimate, pre-penultimate and precedent eggs. In the precedent category, one to four eggs were pooled depending on clutch size.

We fitted models with continuous response variables in JMP (SAS Institute 1995) and models with categorical or ordinal response in PROC GENMOD (SAS Institute 1999). We determined repeatabilities from variance components (PROC VARCOMP; SAS Institute 1999); the accompanying F-tests are the results of one-way analysis of variance. We determined prospective power of our tests in PROC POWER (SAS Institute 1999). When hatching and fledging success were the response variables, these were included in the models in event/trial syntax where the number of eggs/hatchlings in individual nests were trials, and the number of hatchlings/fledglings in individual nests were events in models for hatching and fledging success, respectively. These models had binomial error structure, logit link function and statistics corrected for overdispersion by Pearson chi-square/df. We assessed the paternal effort in nest defence in two models: (1) The response variable was the overall intensity of nest defence (ordinal variable; multinomial error structure and cumulative logit link function). (2) In the subgroup that attacked the dummy by dive or contact, the number of attacks and the latency to the first attack were correlated (r=−0.431, n=23, p=0.040). Therefore, we performed a PCA and used the first principal component (71.6% variability explained) for subsequent analyses. Greater values of PC1 indicate more attacks and a shorter latency to the first attack.

The colour of original and cross-fostered eggs was correlated within individual nests (BGC: r=0.278, n=70, p=0.020; hue: r=0.175, n=70, p=0.147). This might be caused by the fact that we cross-fostered eggs between pairs of nests with the same laying date (which is unavoidable in this sort of experimental study) and, due to time constraints, usually between nests that were located in the same of the four study plots. Correlations among predictors in multiple regression (collinearity) might reduce the power of statistical tests (Quinn and Keough 2002). To assess the effect of collinearity on our results, we looked at variance inflation factors (VIF) for our predictors in individual models with continuous response variable. Predictors with VIFs <10 are generally accepted as giving unbiased results (Quinn and

Keough 2002). Recently, it has been suggested that VIF as small as two might substantially bias results (Graham 2003). All VIFs were smaller than 1.2 in our analyses, therefore, collinearity did not seriously bias our results. It is important to realize that despite the positive correlation between the colour of original and cross-fostered eggs, more than 92% of variation in cross-fostered BGC was uniquely generated by our cross-fostering experiment (i.e. this part of variation was independent on colour of original eggs). The respective figure for cross-fostered hue is 97%.

Results

The reflectance spectra of eggs of the collared flycatcher have a bimodal shape. The major peak of reflectance lays in the blue-green part of the spectrum which agrees with the human-perceived colour of these eggs, whereas the minor one lays in the UV part of the spectrum which is invisible to humans (Fig. 1a). Both hue and BGC changed significantly throughout the laying order. Relative hue increased linearly ($F_{1,445}=100.2$, $p<0.001$, $R^2=0.184$; Fig. 1c), whereas relative BGC decreased non-linearly in the laying order (quadratic regression; $F_{2,444}=241.1$, $p<0.001$, $R^2=0.520$; Fig. 1b). These relationships hold even when the relative laying order is used for these tests (Hue: $F_{1,445}=95.4$, $p<0.001$, $R^2=0.177$; BGC-quadratic regression: $F_{2,444}=365.1$, $p<0.001$, $R^2=0.622$).

Despite the significant intra-clutch trends, both spectral measures were also significantly repeatable within clutches (BGC: $r=0.509$, $F_{69,377}=6.71$, $p<0.001$; Hue: $r=0.315$, $F_{69,377}=4.05$, $p<0.001$), which is a premise of signalling function at a clutch-level. Therefore, we used clutch means of spectral measures in subsequent analyses. The summed reflectance in the UV part of the spectrum was most variable between clutches (SD=326.8). We use SD instead of coefficient of variation as an estimate of variability in particular segments of spectrum, as the use of the latter parameter would be statistically flawed (see Dale 2006). Summed reflectance in the red (286.2), blue (248.0) and green (235.1) part of the spectrum was less variable. The above differences in standard deviations should not be greatly affected by precision differences of measuring apparatus in particular parts of spectra, as they are based on repeated measurements of eggs in a clutch (8–16 measurements depending on clutch size).

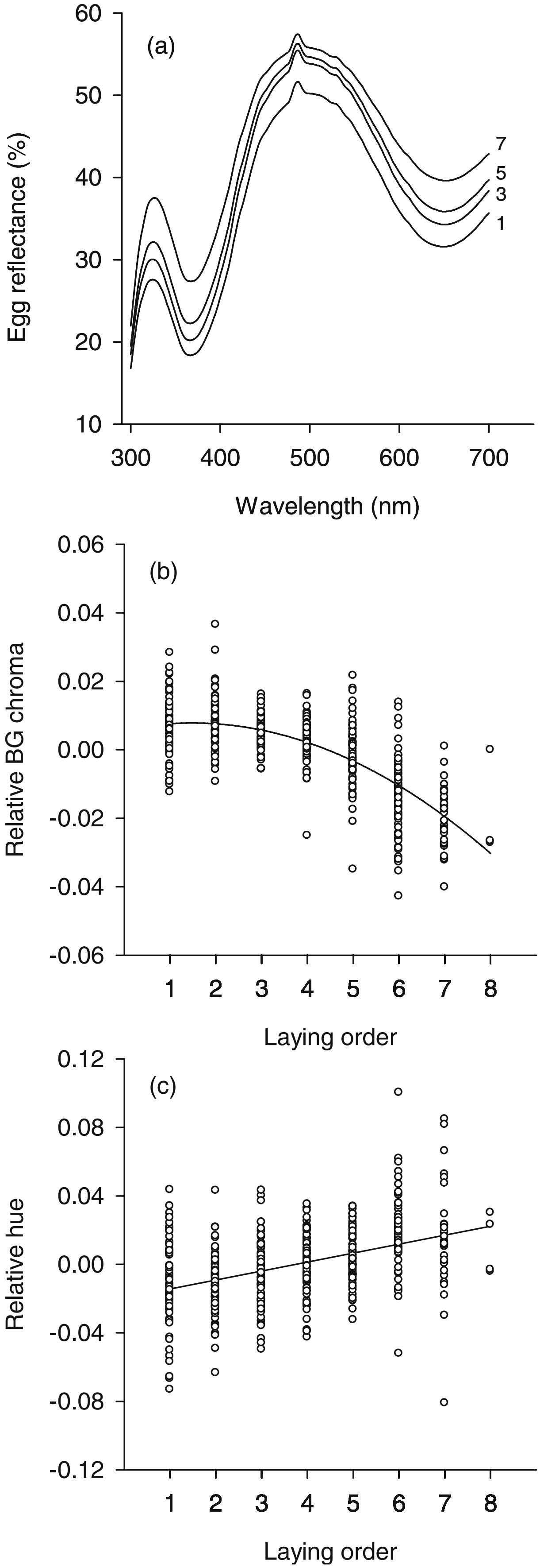


Fig. 1 Relationship between laying order and **a** shape of mean ▶ reflectance spectra (laying order is indicated by numbers alongside the reflectance curves) and **b**–**c** individual spectral qualities (centered within clutches); lines of best fit are displayed. See text for statistical tests

Egg colour and paternal effort

Three nests were not attended by the male at age 6 days and five at age 12 days (including the three nests that were already not attended at age 6 days). One nest was not attended by the female at age 12 days. We did not include these nests into the analyses of feeding frequencies presented below, as they might be caused either by polygyny or by predation on adults, which are factors that might have little relevance to the SSEC hypothesis. Moreover, inclusion of these cases would also be problematic from the statistical point of view because they would represent outliers in analyses of feeding frequencies. Nevertheless, nests attended by males at age 12 days did not differ from those where males were absent (BGC_{CF}: $t=1.97$, $p=0.055$; BGC_{OR}: $t=0.70$, $p=0.488$; Hue_{CF}: $t=-0.76$, $p=0.451$; Hue_{OR}: $t=0.68$, $p=0.500$; $df=49$ in all cases; subscripts CF and OR refer to cross-fostered and original eggs, respectively). The only result approaching statistical significance was in the opposite direction than predicted by the SSEC hypothesis, as BGC_{CF} was lower in nests attended by the male (mean±SE, 0.603±0.0019) than in nests without males (0.615±0.0057).

In nests attended by males, paternal feeding frequency at nestling age 6 days was not related to any spectral quality of original or cross-fostered eggs, whereas it was negatively affected by hour of observation (Table 1; Fig. 2a). Similarly, no spectral quality predicted paternal feeding frequency at age 12 days, whereas this was positively affected by brood size (Table 1). The only result approaching significance was in the opposite direction than predicted by SSEC hypothesis. Males fed young less frequently in nests where original eggs were highly saturated (Fig. 2b). The overall intensity of nest defence by males was not affected by any spectral quality of original or cross-fostered eggs (Table 1; Fig. 2c). In the subgroup of males that attacked the dummy, BGC_{OR} and brood size negatively affected the intensity of attacks (Table 1; Fig. 2d), whereas the hue of both cross-fostered and original eggs did not predict the intensity of attacks (Table 1).

Table 1 Effects of BGC and hue of cross-fostered (subscript CF) and original (subscript OR) eggs on four measures of paternal effort. Presented models include statistically significant covariates

	Model number	DF	F*	P
Feeding frequency 6				
BGC_{CF}	1	1, 49	2.76	0.103
BGC_{OR}	1	1, 49	0.02	0.899
Time of day	1	1, 49	5.50	0.023
Hue_{CF}	2	1, 49	0.77	0.383
Hue_{OR}	2	1, 49	<0.01	0.993
Time of day	2	1, 49	5.15	0.028
Feeding frequency 12				
BGC_{CF}	3	1, 41	0.70	0.409
BGC_{OR}	3	1, 41	3.57	0.066
Brood size	3	1, 41	7.08	0.011
Hue_{CF}	4	1, 41	0.23	0.633
Hue_{OR}	4	1, 41	1.89	0.177
Brood size	4	1, 41	7.82	0.008
Intensity of nest defence				
BGC_{CF}	5	1, 47	2.00	0.157
BGC_{OR}	5	1, 47	0.92	0.336
Hue_{CF}	6	1, 47	0.02	0.898
Hue_{OR}	6	1, 47	0.04	0.850
PC1 attacks				
BGC_{CF}	7	1, 19	0.64	0.435
BGC_{OR}	7	1, 19	10.48	0.004
Brood size	7	1, 19	5.34	0.032
Hue_{CF}	8	1, 20	0.55	0.469
Hue_{OR}	8	1, 20	0.06	0.813

* In models 5 and 6 χ^2 is presented

Egg colour and offspring performance

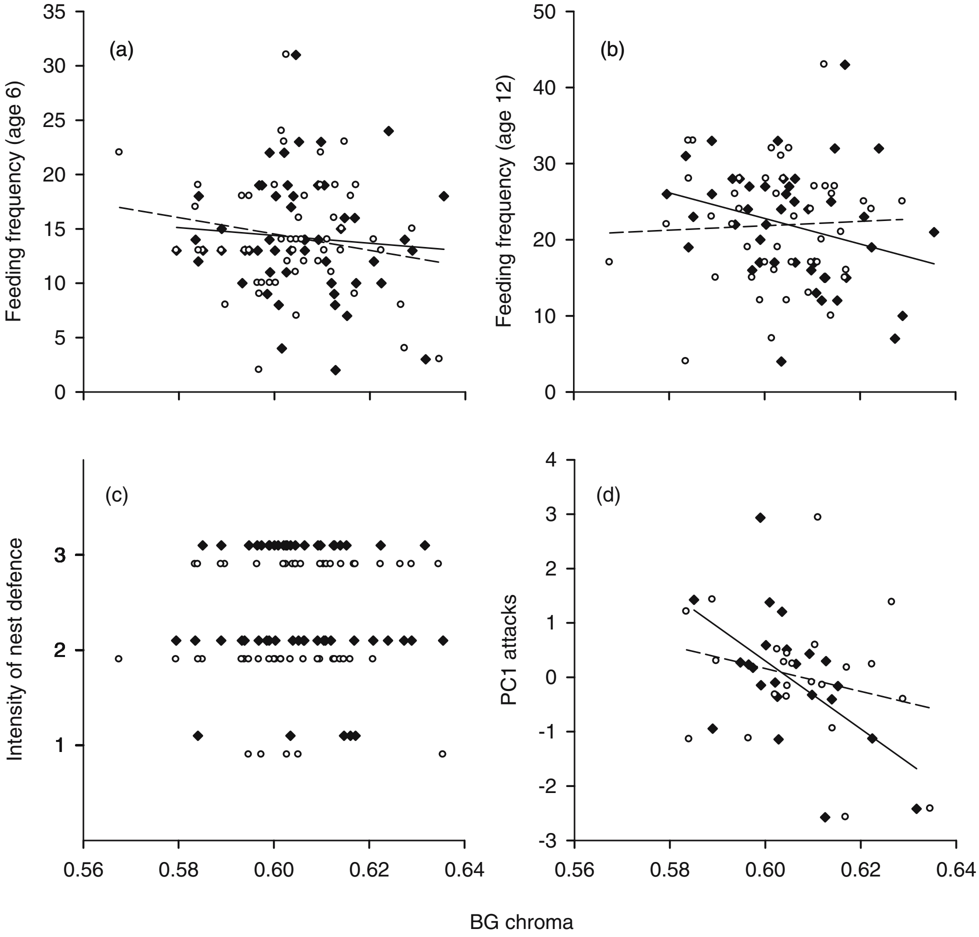
Out of 70 experimental clutches, 56 hatched. Complete hatching failure was caused by marten (*Martes martes*) predation of incubating female (12 cases) and by clutch infertility (two cases) and, therefore, has no relevance to the SSEC hypothesis. In 56 hatched clutches, 339 eggs hatched and 20 failed to hatch. No variable was predictive of hatching success in these nests (Table 2). Out of 56 hatched broods, 50 fledged and six suffered total failure. Egg colour did not differ in the two categories of nests (BGC_{CF}: $t=-1.20$, $p=0.236$; BGC_{OR}: $t=0.41$, $p=0.681$; Hue_{CF}: $t=0.27$, $p=0.786$; Hue_{OR}: $t=-1.79$, $p=0.079$; $df=54$ in all cases). Within 50 fledged nests, 249 young fledged and 52 died. Fledging success was lower in nests that were attended by only one parent, infested by mites and initiated late in the season. In contrast to these significant effects of covariates, no spectral quality predicted fledging success (Table 2).

Nestling tarsus length was positively affected by mid-tarsus length of genetic parents and negatively affected by laying date in both models. From the spectral qualities, only BGC_{OR} had a significant and positive effect on nestling tarsus length (Table 2; Fig. 3b). Nestling weight (Fig. 3a) and T cell mediated immunity (Fig. 3c) were not affected by either covariates or spectral qualities (Table 2).

Original eggs and female traits

Female age was positively associated with BGC of original eggs ($r_s=0.399$, $n=37$, $p=0.014$; Fig. 4a), whereas hue of original eggs was unrelated to female age ($r_s=0.214$, $n=37$, $p=0.204$). The relationship between female morphology and the colour of eggs that she laid was marginally non-significant (condition: BGC_{OR}, $r=0.248$, $p=0.070$, Fig. 4b;

Fig. 2 Relationship between BG chroma of original (*solid diamonds*, *solid lines*) and cross-fostered (*open circles*, *dashed lines*) eggs and four measures of paternal effort. Regression lines are displayed. The displayed data and fitted regression lines are uncontrolled for effects of covariates. See Table 1 for statistical tests



Hue$_{OR}$, r=−0.136, p=0.328; tarsus length: BGC$_{OR}$, r=0.251, p=0.068; Hue$_{OR}$, r=0.169, p=0.223; n=54 in all cases).

Female feeding frequencies were positively affected by brood size and hour of feeding at nestling age 6 days but unrelated to colour of original eggs (BGC$_{OR}$: $F_{1,42}$=1.17, p=0.286, brood size at age six: $F_{1,42}$=21.99, p<0.001, Fig. 4c; Hue$_{OR}$: $F_{1,41}$=0.24, p=0.629, brood size at age six: $F_{1,41}$=17.43, p<0.001, hour of feeding at age six: $F_{1,41}$= 4.47, p=0.041). Neither spectral quality of original eggs predicted the overall intensity of nest defence by females: $\left(BGC_{OR} : \chi^2_{1,52} = 0.06, p = 0.809;\ Hue_{OR} : \chi^2_{1,52} = 0.24, p = 0.62\right)$.

Mean egg volume of the clutch was positively correlated with BGC of that clutch (r=0.261, n=70, p=0.029; Fig. 4d), but was unrelated to the hue of that clutch (r=0.114, n=70, p=0.348). In contrast to mean egg volume, neither clutch size (BGC$_{OR}$: r=−0.035, n=70, p=0.772; Hue$_{OR}$: r=0.113, n=70, p=0.350) nor laying date (BGC$_{OR}$: r=0.156, n=70, p=0.199; Hue$_{OR}$: r=0.023, n=70, p=0.853) was related to egg colour.

The relationships that we found between egg colour and female morphology and egg volume were probably not mediated through female age, as the age was not related to any of the former variables (condition: r_s=0.053, n=37, p=0.758; tarsus length: r_s=−0.002, n=37, p=0.989; egg volume: r_s=0.069, n=37, p=0.686).

Discussion

In our experiments, we found that egg colour changed in the laying order; hue increased linearly, whereas BGC decreased non-linearly in the course of laying. We also found correlations between egg colour and female traits. Eggs with higher BGC were laid by older females and, independently of this, by females tended to be in better condition and to have longer tarsi. Saturation of egg colour was also positively associated with the mean egg volume that females laid. These findings are consistent with the assumptions of the SSEC hypothesis (Moreno and Osorno 2003). Firstly, the blue biliverdin pigment colouring eggs may be a limited source for laying females. Secondly, female quality was positively associated with egg colour. However, in contrast to the tentative support for the assumptions of the SSEC hypothesis, we have found no evidence in support of its prediction. None of our three

Table 2 Effects of BGC and hue of cross-fostered (subscript CF) and original (subscript OR) eggs on five measures of offspring performance. Presented models include significant covariates

	Model number	DF	F	P
Hatching success				
$\mathrm{BGC}_{\mathrm{CF}}$	1	1, 53	0.66	0.419
$\mathrm{BGC}_{\mathrm{OR}}$	1	1, 53	1.74	0.192
$\mathrm{Hue}_{\mathrm{CF}}$	2	1, 53	0.90	0.347
$\mathrm{Hue}_{\mathrm{OR}}$	2	1, 53	0.25	0.619
Fledging success				
$\mathrm{BGC}_{\mathrm{CF}}$	3	1, 45	0.04	0.838
$\mathrm{BGC}_{\mathrm{OR}}$	3	1, 45	0.64	0.426
Laying date	3	1, 45	7.51	0.006
Number of parents	3	1, 45	18.41	<0.001
$\mathrm{Hue}_{\mathrm{CF}}$	4	1, 36	2.51	0.122
$\mathrm{Hue}_{\mathrm{OR}}$	4	1, 36	0.22	0.641
Number of parents	4	1, 36	12.01	0.001
Presence of mites	4	1, 36	4.27	0.046
Nestling tarsus length				
$\mathrm{BGC}_{\mathrm{CF}}$	5	1, 32	0.59	0.449
$\mathrm{BGC}_{\mathrm{OR}}$	5	1, 32	6.67	0.015
Laying date	5	1, 32	15.47	<0.001
Parental midtarsus	5	1, 32	9.60	0.004
$\mathrm{Hue}_{\mathrm{CF}}$	6	1, 32	1.73	0.198
$\mathrm{Hue}_{\mathrm{OR}}$	6	1, 32	1.30	0.264
Laying date	6	1, 32	12.33	0.001
Parental midtarsus	6	1, 32	9.67	0.004
Nestling weight				
$\mathrm{BGC}_{\mathrm{CF}}$	7	1, 47	1.77	0.190
$\mathrm{BGC}_{\mathrm{OR}}$	7	1, 47	0.98	0.326
$\mathrm{Hue}_{\mathrm{CF}}$	8	1, 47	1.02	0.319
$\mathrm{Hue}_{\mathrm{OR}}$	8	1, 47	2.52	0.119
Nestling immunity				
$\mathrm{BGC}_{\mathrm{CF}}$	9	1, 47	<0.01	0.952
$\mathrm{BGC}_{\mathrm{OR}}$	9	1, 47	0.33	0.566
$\mathrm{Hue}_{\mathrm{CF}}$	10	1, 47	2.78	0.102
$\mathrm{Hue}_{\mathrm{OR}}$	10	1, 47	2.81	0.100

measures of paternal care was associated with the colour of cross-fostered eggs. Furthermore, we did not find any positive association between paternal care and colour of original eggs. The only significant relationship was in the unexpected direction, as males defended nests more intensely in which original eggs were less saturated.

Similar to our study, Moreno et al. (2005) previously found that eggs decrease in colouration with laying order in the closely related pied flycatcher. Both adaptive and non-adaptive explanations were previously proposed to explain the pale colour of last eggs. Yom-Tov (1980) suggested that

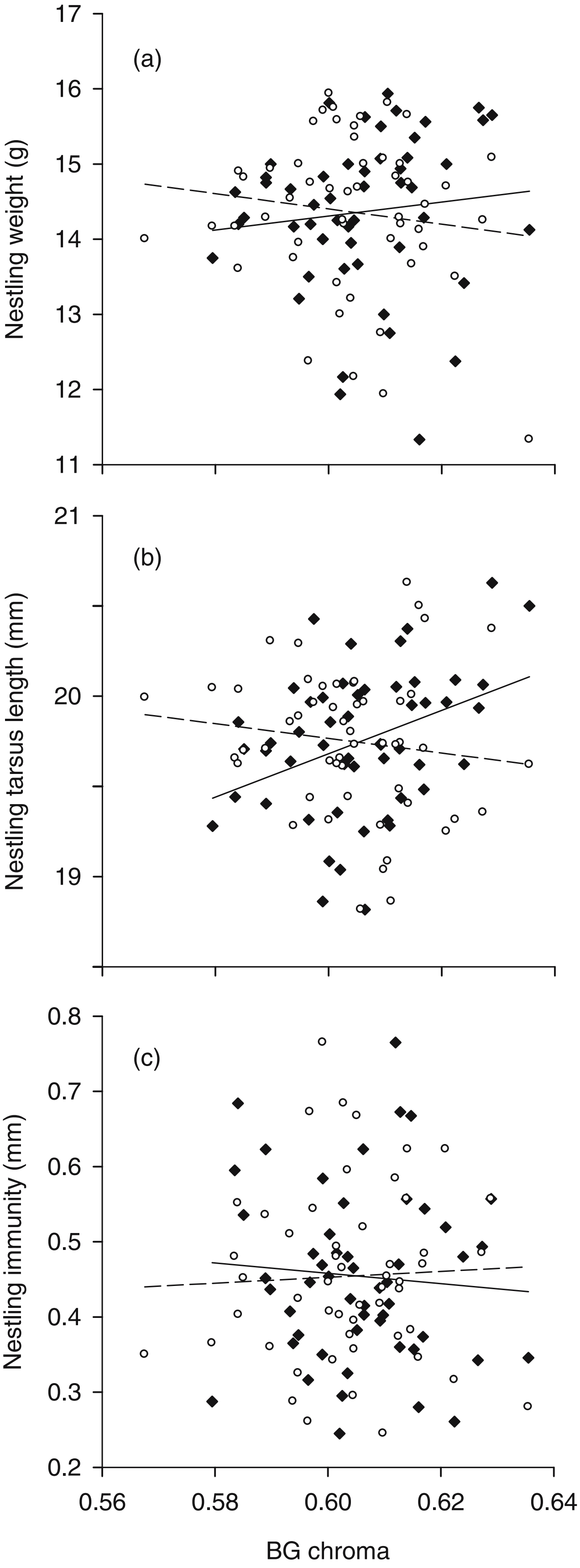


Fig. 3 Relationship between BG chroma of original (*solid diamonds and lines*) and cross-fostered (*open circles*, *dashed lines*) eggs and three measures of nestling performance. The displayed data and fitted regression lines are uncontrolled for effects of covariates. See Table 2 for statistical tests ▶

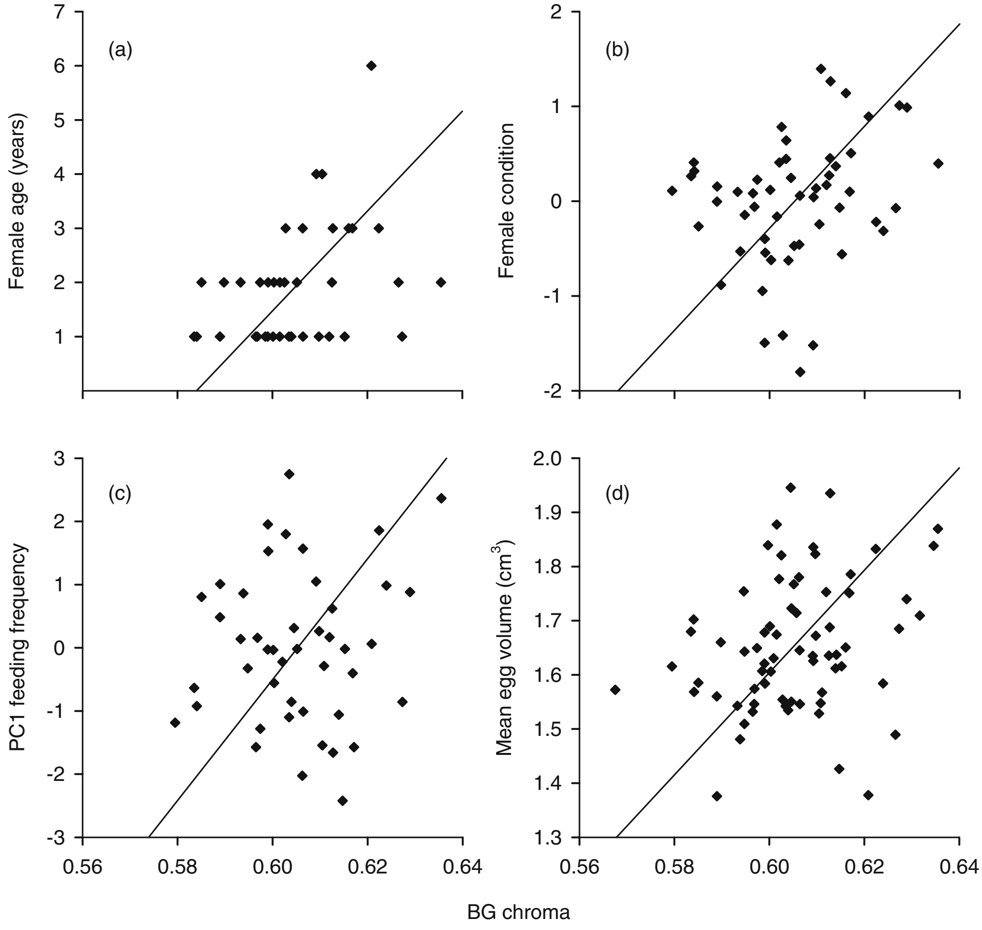


Fig. 4 Relationship between BG chroma of original eggs and female age (**a**), condition (**b**), feeding frequency (**c**) and mean egg volume (**d**). The displayed data and *fitted lines* are uncontrolled for effects of covariates. The standard (reduced) major axes from model II regressions (Sokal and Rohlf 1995) are fitted instead of ordinary regression lines, as there is no clear causality between the pairs of variables. See text for statistical tests

laying a pale last egg may be adaptive for females to prevent conspecific brood parasitism. By laying a pale last egg, females might indicate to conspecific females that they are already incubating the clutch. Conspecifics should then avoid laying a parasitic egg in the nest which contains a pale egg, as the probability of successful hatching would be diminished. Ruxton et al. (2001) formalized this idea and showed that such signalling may indeed theoretically work under some conditions. An alternative, non-adaptive explanation for the occurrence of pale eggs at the end of the laying sequence is that pigments deposited in eggshells are depleted in the course of laying (Nice 1937). As a gradual change in eggshell hue was detected, our data, therefore, do not provide support for the adaptive scenario of Yom-Tov (1980) which predicts only the last egg to be of a different appearance (Ruxton et al. 2001). Blue-green chroma decreased at an accelerating rate in the laying order; however, the decrease was evident as early as in the pre-penultimate egg (results not shown). Moreover, in our population, there is no evidence for conspecific brood parasitism (see Krist et al. 2005) which suggests little opportunity for the evolution of such an anti-parasitic signalling system. However, the decrease in colour of eggs with laying order may still be adaptive for females for other reasons. For example, if pigments strengthen eggshells (Gosler et al. 2005), females may allocate more pigments to early laid eggs because the risk of breakage may be greater for them. As our study was not designed to test for such a possibility, we can conclude that our data are only consistent with the assumption of the SSEC hypothesis that eggshell pigments are a limited source for females (see Moreno and Osorno 2003).

To be an honest signal for males, egg colour should be correlated with female and/or offspring quality (Moreno and Osorno 2003). We have found that females laying presumably more costly (saturated) eggs tended to be in better condition and to have longer tarsi. They also laid larger eggs and were older than females laying less saturated eggs. These results are generally in the direction expected under the SSEC hypothesis and as such provide some support for its assumption. For example, egg size has been suggested to be an indicator of female quality in another hole-nester with altricial young, the great tit (Hõrak et al. 1997). Older individuals may be regarded as superior to young ones either due to increased breeding experiences (Cichoń 2003) or because they have already proved their survival abilities (Mauck et al. 2004). Furthermore, we have found that young that hatched in nests where originally laid eggs were more saturated had longer tarsi, which is a trait positively associated with fitness in this species (Kruuk et al. 2001). As this finding was independent of total feeding frequency of the young, it suggests that some unmeasured type of parental or territory quality is associated with egg colour. However, as the colour of cross-fostered eggs did not affect offspring performance, we can exclude the possibility that egg colour was associated with offspring genetic quality or superior egg composition which would be a more direct support for the SSEC hypothesis.

Four previous studies looked for an association between colour of eggs and other female traits (Moreno et al. 2005, 2006; Morales et al. 2006; Siefferman et al. 2006). All of them found some evidence that egg colour depends on female quality. Similar to our study of the collared flycatcher, female condition was also positively associated with blue-green chroma of eggs in the pied flycatcher (Morales et al. 2006) and the bluebird (Siefferman et al. 2006). Siefferman et al. (2006) found that older females laid more saturated eggs, which is in agreement with our results but in contrast to those of Moreno et al. (2005) who found that females who were 5 years or older laid less pigmented eggs than younger individuals. In the pied flycatcher, immunocompetence of females (Moreno et al. 2005) and eggs (Morales et al. 2006) was positively associated with saturation of egg colour. In the same species, food-supplemented females laid more saturated eggs than control ones, which suggests a direct link between female nutritional state and egg colour (Moreno et al. 2006). Taken together, the results of these studies support the view that egg colour contains some degree of information about female and/or offspring quality.

However, in this experimental study, we did not find any support for the main prediction of the SSEC hypothesis, as no type of paternal care was related to colour of cross-fostered eggs. Perhaps the most parsimonious explanation for this negative result would be the flycatchers' inability to perceive subtle differences in egg colour in poor light conditions inside cavities. However, neither was paternal care positively related to saturation of original eggs. Taken together, these two negative results are in contrast to those of a previous correlative study on the pied flycatcher (Moreno et al. 2004) in which males fed young hatching from more saturated eggs with a higher frequency. Therefore, we need to look for such explanations of our negative results that would be consistent with positive results of Moreno et al. (2004). There is a number of such potential explanations.

First, the two studies were carried out on different species, and it is possible that differential allocation of paternal effort evolved in one but not in the other species. However, we consider such an explanation unlikely. The collared flycatcher is a sister species of the pied flycatcher from which it divided in relatively recent past, and the reproductive isolation between the two species is currently incomplete (Saetre et al. 1997).

Second, the discordance might be caused simply by sampling effect, either by a type II error in our study or a type I error in Moreno et al. (2004) study. However, as we have used a somewhat larger sample size than Moreno et al. (2004), statistical power to detect effect of the magnitude detected by these authors (r=0.38 and r=0.41) was relatively high in our study (feeding frequency at age 6 days: power=0.82 and 0.88 for the two effect sizes, respectively; feeding frequency at age 12 days: power=0.75 and 0.82, respectively). Type I error rate in the study of Moreno et al. (2004) has been controlled at the conventional level (α<0.05). Therefore, this explanation for the discordance results is also unlikely.

Third, in the study of Moreno et al. (2004), males cared for their own nestlings, whereas in our study, parents cared for unrelated cross-fostered offspring. This could, in theory, cause the absence of predicted effects of egg colour on paternal care in our study. However, such an explanation is highly unlikely because birds were so far never shown to discriminate against unrelated conspecific nestlings hatched in their nests (Kempenaers and Sheldon 1996).

Fourth, whereas one of our three indicators of paternal effort was exactly the same as the one of Moreno et al. (2004), feeding rates to 12-day-old nestlings recorded for an hour, the description of egg colour differed between the two studies. Moreno et al. (2004) recorded egg reflectance in the human-visible spectrum only, whereas we also measured egg reflectance in the UV spectrum (301–400 nm). This difference might be of importance, as most passerines see also in UV part of spectrum (Cuthill et al. 2000). This seems to be also the case in the pied flycatcher, as females in this species mate preferentially with males with high UV reflectance of plumage (Siitari et al. 2002). To test directly for the possibility that different spectral range caused discordance results of the two studies, we computed a second estimate of blue-green chroma ($BGC_{401-700}$) that was restricted to the spectral range used by Moreno et al. (2004). $BGC_{401-700}$ was calculated as the reflectance between 401–600 nm divided by the total reflectance between 401–700 nm. This estimate was very strongly correlated with our original estimate of BGC, which was based on the spectral range 301–700 nm (r=0.976, n=70, p<0.001). Such high correlation means, at least in the context of biliverdin signalling, that the information contained in the UV part of the spectrum is redundant to that of human-visible part of spectrum. Consequently, different results of the two studies cannot be explained by the use of different spectral range.

Fifth, it may be possible that males increase paternal care only when superior egg colour is accompanied by superior female quality. In a natural situation, these traits may covary, whereas our cross-fostering approach separated them. However, this would mean that in such a scenario, egg colour per se is not predictive of paternal effort.

Finally, it may be that the two flycatchers' populations diverged in the type of paternal care that is differentially allocated. In the Spanish population, feeding frequency seems to be the plastic trait with an effect on offspring performance (Moreno et al. 2004), whereas in our population, some dimension of paternal care that we did not measure (for example quality of diet) might evolve this plasticity. Remarkably, feeding frequency was important for offspring performance in Spanish population (Moreno et al. 2004), whereas we did not detect effect of total feeding frequency on any offspring trait. This hypothesis requires further testing.

In conclusion, results of the studies performed so far have given evidence supporting the view that the egg colour is informative about female and offspring quality. Such a correlation is a critical assumption of the SSEC hypothesis (Moreno and Osorno 2003). However, in this experimental study, we have found no evidence for increased paternal effort with more saturated egg colour. This suggests that the correlation between female quality and egg colour is not strong enough to select for the probably costly discriminatory abilities in males to allocate paternal care differently according to varying egg colour. Males can perhaps assess their offsprings' reproductive value more precisely and allocate their care more appropriately according to some more direct and informative cue, such as offspring size. This explanation implies that either egg colouration is not very costly or serves other functions (e.g. eggshell strengthening; Gosler et al. 2005). However, as our results are in contrast with those of previous studies on related species and the egg colouration could, in theory, be even more informative about female quality than colouration of its plumage (see "Introduction"), tests of the SSEC hypothesis might be a fruitful area for further research.

Acknowledgement We thank Vladimír Remeš, Manuel Soler, Karel Weidinger, and several anonymous referees for valuable comments and/or discussion on the manuscript. We owe J. Stříteský and forest enterprise Prostějov for providing the nest-boxes. T. Koutný helped with the analyses of video records. We are grateful to Dana Cambell for correcting our English. M. K. thanks Kačka for support. This study was supported by grants from the Czech Ministry of Education (MSM 6198959212) and from Grant Agency of the Czech Republic (No. 206/03/D234 and 206/03/0215). The experiments were done under the license from The Central Commission for Animal Welfare of the Czech Republic (No. 065/2002-V2) and in accordance with the laws and ethical guidelines of the Czech Republic.

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