Ethology Ecology & Evolution
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/teee20

Does egg colouration signal female and egg quality in reed warbler (Acrocephalus scirpaceus)?

J. Krištofík [a], A. Darolová [a], M. Griggio [b], J. Majtán [a c], M. Okuliarová [d], M. Zeman [d], L. Zídková [e] & H. Hoi [b]

[a] Institute of Zoology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 06, Bratislava, Slovakia

[b] Konrad Lorenz Institute of Ethology, Department of Integrative Biology and Evolution, University of Veterinary Medicine, Savoyenstr. 1a, A-1160, Vienna, Austria

[c] Department of Microbiology, Faculty of Medicine, Slovak Medical University, 833 03, Bratislava, Slovakia

[d] Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University, Mlynská dolina, 842 15, Bratislava, Slovakia

[e] Department of Parasitology, Faculty of Science, Charles University, Viničná 2, 128 44, Prague, Czech Republic

Published online: 14 Apr 2013.

To cite this article: J. Krištofík , A. Darolová , M. Griggio , J. Majtán , M. Okuliarová , M. Zeman , L. Zídková & H. Hoi (2013): Does egg colouration signal female and egg quality in reed warbler (Acrocephalus scirpaceus)?, Ethology Ecology & Evolution, 25:2, 129-143

To link to this article: http://dx.doi.org/10.1080/03949370.2012.744357

Ethology Ecology & Evolution, 2013
Vol. 25, No. 2, 129–143, http://dx.doi.org/10.1080/03949370.2012.744357

Does egg colouration signal female and egg quality in reed warbler (*Acrocephalus scirpaceus*)?

J. KRIŠTOFÍK [1], A. DAROLOVÁ [1,6], M. GRIGGIO [2], J. MAJTÁN [1,3], M. OKULIAROVÁ [4], M. ZEMAN [4], L. ZÍDKOVÁ [5] and H. HOI [2]

[1] *Institute of Zoology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 06 Bratislava, Slovakia*
[2] *Konrad Lorenz Institute of Ethology, Department of Integrative Biology and Evolution, University of Veterinary Medicine, Savoyenstr. 1a, A-1160 Vienna, Austria*
[3] *Department of Microbiology, Faculty of Medicine, Slovak Medical University, 833 03 Bratislava, Slovakia*
[4] *Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University, Mlynská dolina, 842 15 Bratislava, Slovakia*
[5] *Department of Parasitology, Faculty of Science, Charles University, Viničná 2, 128 44 Prague, Czech Republic*

Received 15 February 2012, accepted 30 August 2012

To explain the evolution of egg colouration in open cup nesting species, a number of functions have been suggested. Recent studies focus on the role of eggshell colour as a postmating sexually selected trait of females which manipulates male parental investment. A basic prediction of this hypothesis is that egg pigmentation reflects female quality. In this study we examine whether there is a relationship between eggshell colouration and either female quality or egg quality in reed warblers. This open cup nesting species has eggs that are heavily spotted with brownish marks on a bluish-green background. We used several parameters describing female and egg quality, and measured eggshell colouration at the blunt pole and the egg centre, deriving four colour variables from colour spectrometry. To determine egg quality parameters, the third egg of each clutch was sampled and analysed. To determine female quality, females were trapped shortly after egg laying, and several morphological and a single conditional variable were determined. Additionally, a blood sample was taken to determine blood parasites (avian malaria and *Trypanosoma* spp.) and a faecal sample to determine intestinal parasites (*Isospora* spp). Our results revealed that eggshell pigmentation appears to be independent of female condition and parasites, but reflects concentrations of egg compounds such as testosterone and lysozyme. Egg colouration is also related to yolk weight and egg size. Our results further suggested that the information about colour varies depending

[6] Corresponding author: Alžbeta Darolová, Institute of Zoology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 06 Bratislava, Slovakia (Tel. 00421 2 59302619, Fax 00421 2 5930 26 46, Email: alzbeta.darolova@savba.sk).

on the position on the egg (blunt pole or egg centre). The only relationship with females was between female size (tarsus length) and egg colouration, which suggests a genetic component. We discuss reasons for the absence of a relationship between egg colouration and female quality.

KEY WORDS: reed warbler, eggshell colouration, egg yolk testosterone, lysozyme, egg quality, female quality, multiple signals.

INTRODUCTION

Originally, predation was thought to be the main selective force driving the evolution of eggshell colouration (WALLACE 1889; LACK 1958), which is partly supported by recent comparative analyses (UNDERWOOD & SEALY 2002; KILNER 2006). In the context of predation, as an alternative to using egg colour to mimic background colouration, an aposematic function (SWYNNERTON 1916) was suggested whereby conspicuous egg colours could, for example, serve as a warning signal for unpalatable eggs (LACK 1958). It has also been suggested that colourful egg ornamentation serves to discriminate own from conspecific eggs (BIRKHEAD 1978) or own from parasitic eggs (DAVIES & BROOKE 1989; GRIM et al. 2003). More recently, MORENO & OSORNO (2003) proposed that eggshell colouration signals female quality to their male mates. In species with biparental care, eggshell colour could be a post-mating sexually selected trait of females in order to induce male parental investment. The two main components of eggshell pigmentation, biliverdin and protoporphyrin, possess strong antioxidant activities (STOCKER et al. 1987; MCDONAGH 2001; KAUR et al. 2003), and may in fact signal a female's capacity to control free radicals despite the handicap (sensu ZAHAVI 1975) of removing antioxidant from her system. Consequently, deposition of these pigments to the eggshell is costly for laying females. However, ZHAO et al. (2006) pointed out that inclusion of biliverdin in the eggshell does not necessarily impair female antioxidant demands, because it is not originally synthesized in the blood and may therefore not constitute a handicap.

There are, in fact, contradictory results regarding the signalling function of egg colouration in relation to female quality. One set of studies found both correlative (MORENO et al. 2004; MORALES et al. 2006; SIEFERMANN et al. 2006; MARTÍNEZ-DE LA PUENTE et al. 2007; HARGITAI et al. 2008) and experimental (MORENO et al. 2006; SOLER et al. 2008; MORALES et al. 2012) support for this concept, whereas another set could not confirm these results (HARGITAI et al. 2008; LÓPEZ-RULL et al. 2008; HANLEY & DOUCET 2009; HONZA et al. 2011). There is therefore much ongoing discussion concerning the reliability of the signalling function of eggshell colouration (REYNOLDS et al. 2009).

However, eggshell colouration may also reflect egg quality independently of female quality. The intensity of eggshell pigmentation may influence the amount of light crossing the shell (LAHTI 2008; SHAWKEY et al. 2008) and consequently egg hatchability (SHAWKEY et al. 2008). Eggshell pigmentation can indicate pesticide contamination (JAGANNATH et al. 2008). White colour intensity may indicate better resistance to overheating (MAGIGE et al. 2008). Egg colour may also reflect structural shell features; e.g. egg speckling may reflect inclusion of calcium (GOSLER et al. 2005; GARCÍA-NAVAS et al. 2011) or eggshell thickness (CHERRY & GOSLER 2010). Egg colour may also reflect egg components allocated by the mother into yolk or egg white. Important

egg ingredients could be nutritional compounds (e.g. lipids, proteins), antioxidants (e.g. vitamines, carotenoids), hormones (e.g. androgens, corticosterone) or immune compounds (e.g. immunoglobulins, lysozyme), which may consequently influence offspring quality (GROOTHUIS et al. 2005; ROSIVALL et al. 2005; MÜLLER et al. 2009; PITALA et al. 2009). All these egg quality features may be partly independent of female quality. For example, maternal components provided for embryo development in each egg may depend on female allocation strategies (SHELDON 2000; HARRIS & ULLER 2009; REMEŠ 2011). Ecological constraints (resource availability), socio-ecological conditions (e.g. breeding density or breeding synchronization), or mate quality may play an important role (GARCIA-FERNANDEZ et al. 2010; MCFARLANE et al. 2010) and mask the relationship between female and egg quality.

The relationship between egg colouration and female and egg quality is therefore complex, and both sets of studies, those which did or did not find a signalling function in relation to female quality, have probably explored partial links of a complex pattern.

In this study, we therefore investigated one prediction of the signalling function of eggshell colour in reed warblers (*Acrocephalus scirpaceus*) in relation to female as well as egg quality. Female reed warblers lay eggs that are heavily spotted on a blue-green background. Egg colouration in this species is probably also important in parasite defence, although cuckoos are still very successful in parasitizing reed warbler broods (SCHULTZE-HAGEN 1992; MOKSNES et al. 1993; ØIEN et al. 1998). The reed warbler is a socially monogamous species with male parental care (DUCKWORTH 1992; KLEINDORFER et al. 1995), whereby male investment significantly varies regarding offspring feeding (HOI et al. 1995) and predator defence (ILLE et al. 1996). Egg colouration in reed warblers seems also to be influenced by environmental components (spring climatic conditions such as rainfall and temperature) suggesting female condition is dependent on nutrition (insect) availability (AVILÉS et al. 2007). Intraclutch variation in egg colour and colour pattern is much smaller than variation between clutches (SOLER & MØLLER 1996). To examine eggshell colouration in relation to female quality we used body size, residual body mass, female parasite infestation in terms of haemosporidian blood parasites (*Plasmodium*, *Haemoproteus,* and *Leucocytozoon*), *Trypanosoma* spp., *Isospora* spp. prevalence, start of laying and clutch size (HOI & HOI-LEITNER 1997) as determinants of female quality.

For egg quality monitoring we used: (1) egg morphology in terms of size and egg weight, and (2) egg compounds in terms of yolk weight, yolk testosterone concentrations and egg white lysozyme antimicrobial activity. To evaluate the prediction related to the signalling function of egg colouration, we measured egg colouration in two positions on the egg, using spectrometry, and generated four standard variables frequently used to describe colour variation.

MATERIALS AND METHODS

Our reed warbler study population was located in the fishpond area of Veľké Blahovo, West Slovakia, (48°03´09´´N, 17°35´38´´E), and the data for this study were collected in the field seasons of 2009 and 2010.

In both years, the whole area was inspected daily between 6:00 and 18:00, from 6 April until 10 July, inclusive. Male arrival was determined by observations of singing males. Males were followed daily to determine female arrival and females were observed finding their nests. Through daily nest visits, we were able to determine start of egg laying. The third egg was removed from each nest and replaced by a dummy egg made of epoxid. No brood was deserted because of this manipulation.

The removed egg was immediately put into a cooling box (4 °C) for transportation and after arrival in the lab was frozen at – 80 °C. After chicks hatched, the females were trapped and individually colour-ringed. We determined female tarsus, length of wing, bill and tail, and body mass. A small blood sample (about 50 μL) was taken using brachial vein-puncture and stored in ethanol.

Female quality measurements

The study was done over 2 years but each female was only represented once in the data set. To determine female quality, we used: (1) female morphology; (2) female condition; (3) haemosporidian blood parasites; (4) *Trypansoma* spp.; (5) *Coccidia* spp. prevalence; (6) date of egg laying, and (7) clutch size. (1) Tarsus length is probably the best size indicator in reed warblers, given its importance for movement through reeds, whereas wing and tail length is probably a compromise to migration (LEISLER & WINKLER 1991). Therefore, we used tarsus length as a measure for female size. (2) Female condition was determined to be residual body weight not explained by size (tarsus). (3) Regarding avian blood parasites, we determined the presence or absence of haemosporidian blood parasites, including *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. However, only one female was infested by *Leucocytozoon*. DNA was extracted from the blood samples using a DNeasy Tissue Kit (Qiagen).

For amplification of partial segments of the cytochrome *b* gene of avian malaria of the genera *Plasmodium* and *Haemoproteus*, four primers (HaemNF, HaemNR2, HaemF, and HaemR2) and polymerase chain reaction (PCR) conditions according to WALDENSTRÖM et al. (2004) were used.

Amplification by first PCR was performed according to the following conditions: initial denaturation for 5 min at 95 °C, followed by 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 60 sec and 20 cycles, followed by final extension at 72 °C for 10 min. The 25 μL PCR reactions were performed with a final concentration of 50–70ng/μL template DNA, 10 mM of each primer (HaemF, HaemR2), 200 μM dNTPs (Fermentas), 2.5 mM magnesium chloride (MgCl) (Finnzymes), and one unit of Firepol DNA Polymerase (Solis Biodyne). Positive and negative controls were also used. PCR products were evaluated by running onto 2% agarose gels stained with ethidium bromide. For amplification of segments of *Leucocytozoon*, primer pairs of HaemNF1, HaemNR3, HaemFL and HaemR2L were used according to HELLGREN et al. (2004). PCR conditions were the same as above. (4) To determine the role of *Trypanosoma* spp., we used their presence/absence in the blood. Extracted DNA was used in nested PCR reactions to amplify small subunit (SSU) rRNA fragments, according to a protocol modified from SEHGAL et al. (2001). New primers for a second round of the nested PCR reaction were designed for this study to increase fragment size of the sequences from 326 to 770 bp. The first set of primers was Tryp763 (59-CATATGCTTGTTTCAAGGAC-39) and Tryp1016 (59-CCCCATAATCTCCAATGGAC-39), and the second set was Tryp99 (59-TCAATCAGACGTAATCTGCC-39) and Tryp957 (59-CTGCTCCTTTGT TATCCCAT-39). The cycling profile conditions were as follows: initial denaturation at 95 °C for 5 min, followed by five cycles at 95 °C for 1 min, 45 °C for 30 sec, and 65 °C for 1 min, followed by 35 cycles at 95 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min, and then a final extension at 65 °C for 10 min. We used 2 μL of the first PCR product as the template for the second PCR. The reaction conditions using the second primer set were as follows: initial denaturation at 96 °C for 3 min, followed by 25 cycles at 96 °C for 30 sec, 58 °C for 1 min, 72 °C for 30 sec, and then a final extension at 72 °C for 7 min (VALKIUNAS et al. 2011). (5) For each female, we also determined occurrence of *Coccidia* spp. in the faeces collected after 16:00. Since 100% of females were infested with *Coccidia* spp., we excluded this variable from the analyses. (6) Start of egg laying has been noted as an indicator of female quality (e.g. HOI & HOI-LEITNER 1997; GLADBACH et al. 2010); we therefore also used timing of egg laying in terms of the date when the collected (third) egg was laid and used the appropriate Julian date in the analyses. Since high quality females may start laying earlier, we expected a negative relationship between Julian date and egg colour measurements. (7) Additionally, we used clutch size as an indirect indicator of female quality (HOI & HOI-LEITNER 1997; MCCLEERY et al. 2008; GLADBACH et al. 2010). We predicted a positive relationship with egg colouration. As we had removed one egg, we used original clutch size (number of laid eggs) for the analyses.

Egg quality measurements

The egg quality of the third egg of each of 80 clutches was determined. To do this, we measured: (1) egg size; (2) egg weight; (3) yolk weight; (4) estimated antimicrobial egg white lysozyme activity; and (5) estimated egg-yolk testosterone concentration for each egg. (1) Egg size (egg length and maximal width) was measured to the nearest 0.01 mm with an electronic caliper, immediately after egg removal. (2) Fresh egg weight was determined to the nearest 0.0001 g, using an electronic balance, about 3 hr after sampling. (3) Eggs were frozen for about 1 month. For the analyses, eggs were defrosted and egg yolks were carefully dissected from thawing albumen. Frozen yolks were weighed and both egg white and yolk were then stored at – 80 °C until lysozyme and testosterone analyses. Eggshells were rinsed under warm distilled water and dried at room temperature for later colour measurements. (4) For the antibacterial activity analyses we prepared the albumen samples. They were collected into pre-weighed 2.0-mL reagent tubes. Each tube with albumen sample was weighed and albumen was lyophilised. Lyophilised powder was subsequently dissolved in 200 μL of distilled water and the obtained albumen solution was used for determination of antibacterial activity. Radial diffusion assay was used in order to evaluate antibacterial activity of albumen samples. In brief, one bacterial colony from an overnight agar plate culture of *Micrococcus luteus* CCM 410 (Czech Collection of Microorganisms, Brno, Czech Republic) was suspended in phosphate buffered saline (PBS) and the suspension turbidity was adjusted to 10^8 CFU/mL. One-hundred μL aliquot of suspension was inoculated into 10 mL of melted Luria broth (LB) containing 0.9 % (w/v) agar pre-heated at 48 °C and poured into 90-mm Petri dishes. After solidification, 5 mm-diameter wells were punched into LB agar and 5 μL of sample was added to each well. The antibacterial activity of examined samples was compared on the basis of the radius of clear inhibition zone around the well against standard solutions of a chicken egg white lysozyme (Sigma-Aldrich, UK) after 24 hr of incubation at 37 °C. Antibacterial activity of albumen samples was expressed as concentration of egg-white lysozyme of equivalent activity. Results given represent mean values from duplicate measurements of each independent sample. The two repeated measurements are highly repeatable ($R = 0.93$, $P < 0.0001$, df = 1, 80). (5) Yolk T concentrations were determined by radioimmunoassay after yolk steroid extraction (Okuliarová et al. 2010). Sub-samples of 40–45 mg yolk were diluted in 500 μL of deionised water and vortexed with the addition of two glass beads for 3 min. Approximately 1500–2000 dpm of [^{3}H]-testosterone were added to each sample for individual recovery calculation (mean ± SE: 55.0 ± 0.8%). Samples were equilibrated overnight at 4 °C. Thereafter, they were applied on solid phase columns filled with Extrelut NT (Merck, Darmstadt, Germany) and extracted with 2 × 2 mL and 1 × 1 mL of a mixture of diethyl ether and petroleum ether (7:3). Following evaporation under a stream of nitrogen, dried extracts were reconstituted in 300 μL of phosphate buffer (pH = 7.5) and frozen until T assay. Yolk T concentrations were measured in 10 μL aliquots of the extract using [1,2,6,7-^{3}H]-testosterone (Amersham Biosciences, UK, specific activity 3.52 TBq/mmol) and a specific antibody generated in rabbits against testosterone-3-(carboxy-methyl) oxime bovine serum albumin conjugate. Cross reactivity of antiserum was 9.6% with 5α-dihydrotestosterone, and lower than 0.1% with other steroids (Zeman et al. 1986). All samples within each year were run in a single assay with intra-assay variation coefficients 7.6% and 2.2%, respectively.

Egg colour determination

To examine the role of eggshell colouration we determined four colour variables (see below). Since spottiness is not evenly distributed on the egg surface, we measured these variables at the blunt pole (B) and the centre of each egg (C) using spectrometry. The results revealed that brightness and hue are not correlated between the blunt pole and the egg centre (for both $P > 0.3$). However, measurements of Chroma and UV-Chroma are highly correlated between the two positions on the egg (for Chroma: $r = 0.91$, $P < 0.0001$, $n = 82$; for UV-Chroma: $r = 0.87$, $P < 0.0001$, $n = 82$).

Eggshell colouration of each egg was measured using a USB-2000 spectrometer and a DHS-2000-FHS deuterium halogen lamp, connected through a bifurcated fibre-optic probe (Ocean

Optics, Eerbek, The Netherlands). To exclude disturbance by external light sources and in order to maintain standardized distance and angle (90°), a black rubber cylinder was fitted on the top of the probe. Before each measurement, the spectrophotometer was recalibrated with a standard white (Avantes, Eerbek, The Netherlands); for calibration with black, the probe was removed from the light source and the cap of the plug closed. Standard descriptors (brightness, hue, Chroma and UV-Chroma) of reflectance spectra were used for quantification of colours. Since spottiness may not be evenly distributed over the whole egg surface, different parts on the egg surface may vary in importance from each other regarding information content, or may even provide different information (POLAČIKOVÁ & GRIM 2010; POLAČIKOVÁ et al. 2010). Measurements were taken from five spots (with a diameter of 1 mm) on the blunt pole and the central part of each egg. The five measurements showed a high repeatability (for all colour descriptors on the blunt and central part: $R > 0.85$, $P < = 0.001$). Calculations were carried out for reflectance in the 300–700 nm range. Mean brightness was calculated as the mean summed reflectance ($R_{300\ -\ 700\ nm}$). Thus brightness can be seen as the amount of light reflected by the sample in relation to the amount of light reflected by the white standard. Chroma was calculated as the difference between the highest and lowest reflectance divided by the average reflectance [$(R_{max} - R_{min})/R_{average}$)]. To quantify the UV-reflectance of each egg, we used the variable UV-Chroma, which is defined as the proportion of UV-reflectance over total reflectance ($R_{300} - R_{400}/R_{300} - R_{700}$) (DELHEY et al. 2006; ROBERTS et al. 2009; GRIGGIO et al. 2009, 2010). Hue was calculated as the wavelength at peak reflectance [λ (R_{max})] for reflectance in the 300–700 ηm range. Range for hue at the blunt pole varied between 300.3 and 698 and at the egg centre between 302.1 and 696.9.

Statistical analyses

Backward stepwise regression analysis was used to examine which of the egg quality and female quality parameters (P to remove > 0.05) were related to any of the four colour variables derived from our eggshell colour measurements. Regression models were performed separately for colour measurements taken from the blunt pole and the central region, using the four colour variables as dependent variables, respectively. Partial correlation coefficients were shown to determine the direction and strength of each relationship between pairs of variables separately. Whenever repeated measurements were taken their reliability was tested using the repeatability analyses (LESSELLS & BOAG 1987).

The importance of haemosporidian blood parasites and *Trypanosoma* spp. to eggshell pigmentation was examined by comparing the four eggshell measurements (dependent variable) for females infested or not infested with any of the parasites using an analysis of variance with haemosporidian blood parasites (yes/no) and *Trypanosoma* (yes/no) as independent factors. Since all females (100%) were infested with *Coccidia* spp. (Isospora) we excluded this variable from the analyses. For most variables we found no variation ($P > 0.1$) between years. For those variables which revealed a significant year effect ($P < 0.05$) we controlled for year differences using mean adjustments [mean for year 1 + (mean for year 1 – mean for year 2)]. Mean year adjustment was done for lysozyme and testosterone concentration, egg yolk weight and clutch size. Lysozyme was additionally corrected for clutch size. Therefore lysozyme concentration (dependent variable) was correlated to number of eggs/clutch (independent variable). The residual variance not explained by clutch size was further used in the analyses.

RESULTS

Egg colouration and female quality

When examining eggshell colouration at the blunt pole, no variable of female quality entered a stepwise regression model for any of the four colour variables, respectively (for hue, Chroma and UV Chroma, $P > 0.2$). Female tarsus length

approached significance only for brightness (stepwise regression model: $F = 2.64$, $R^2 = 0.33$, $P = 0.07$, df = 1, 80). The partial correlation coefficient indicated a positive relationship between brightness and female tarsus length ($r_{part} = 0.34$, $P = 0.08$).

Examining colouration of the central eggshell region, we found Chroma ($F = 4.12$, $R^2 = 0.37$, $P = 0.049$, df = 1, 80) and UV Chroma (stepwise regression model: $F = 3.86$, $R^2 = 0.23$, $P = 0.034$, df = 1, 80) to be related to female tarsus length. The partial correlation coefficient indicated a positive relationship between Chroma and female tarsus length ($r_{part} = 0.37$, $P = 0.04$) and a positive relationship between UV Chroma and female tarsus length ($r_{part} = 0.41$, $P = 0.03$). No female quality indicator entered the regression model using brightness and hue as dependent variables (for both, $P > 0.7$).

Females infested or not infested with haemosporidian blood parasites did not vary in egg colouration for the blunt pole as well as the central eggshell region (for all $P > 0.5$), using season as covariate, with the same being true for *Trypanosoma* spp. infestation for both egg regions ($P > 0.3$).

Egg colouration and egg quality

Examining egg colour variables at the blunt pole, egg quality variables entered the stepwise regression model only for brightness: $F = 4.05$, $R^2 = 0.24$, $P = 0.013$, df = 3, 80. In fact, three variables, yolk weight ($r_{part} = -0.30$, $P = 0.047$), testosterone concentration ($r_{part} = 0.33$, $P = 0.026$, Fig. 1) and lysozyme ($r_{part} = -0.28$, $P = 0.049$, Fig. 2), significantly entered the model, whereby yolk weight and lysozyme were negatively related to brightness, but testosterone concentration was positively related to brightness. However, no egg quality variable significantly entered a regression model examining hue ($P > 0.1$), Chroma ($P > 0.5$) and UV Chroma ($P > 0.6$) at the blunt pole.

When examining hue at the central region of the egg, two egg quality variables entered the stepwise regression model ($F = 2.98$, $R^2 = 0.15$, $P = 0.05$, df = 2, 80). We found a negative relationship between hue and yolk weight ($r_{part} = -0.28$, $P = 0.049$), and between hue and lysozyme ($r_{part} = -0.29$, $P = 0.045$). When using UV Chroma, one

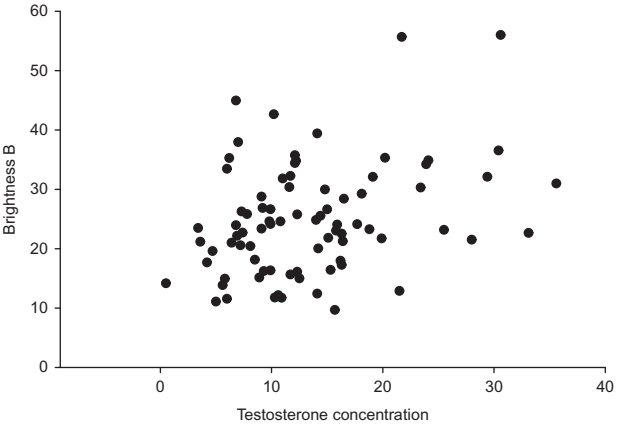

Fig. 1. — Relationship between testosterone egg yolk concentration (pk testosterone/mg yolk) and brightness measured on the blunt pole (B).

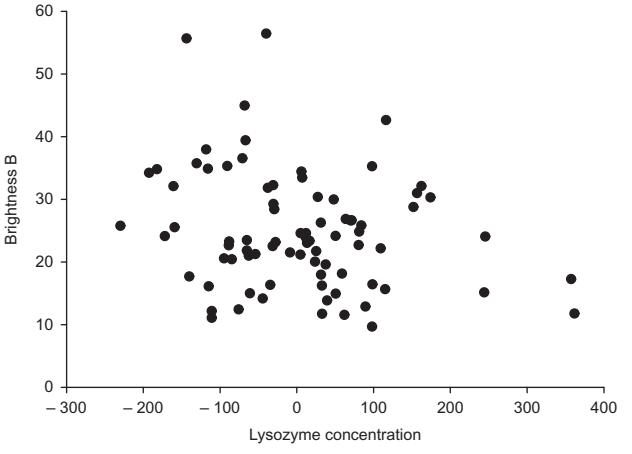

Fig. 2. — Relationship between eggshell brightness measured on the blunt pole (B) and lysozyme concentration. Lysozyme concentration is given as residuals not explained by clutch size and year.

variable entered the stepwise regression model ($F = 4.19$, $R^2 = 0.12$, $P = 0.045$, df = 1, 80). UV Chroma was positively related to egg size ($r_{part} = 0.34$, $P = 0.045$). However brightness ($P > 0.8$) and Chroma ($P > 0.7$) were not related to any egg quality variable.

DISCUSSION

An earlier study on reed warblers revealed high variation in eggshell pigmentation between years, depending on environmental factors such as temperature and rain (AVILÉS et al. 2007). Assuming that these environmental variables directly affect female condition (e.g. nutritional availability), egg colouration would consequently be an indicator for female condition. However our results do not support this assumption and suggest instead that female quality, in terms of condition, seems not to be reflected in eggshell pigmentation. In fact, we found evidence contradicting the assumption that eggshell pigmentation signals female condition. There is no evidence regarding female blood parasite infection. To our knowledge, no study has yet investigated the relationship between egg colour and blood parasites. However, some studies have found detrimental effects of haemosporidian blood parasites on female condition and reproductive success (KNOWLESS et al. 2010). TOMÁS et al. (2006) found an effect of *Trypanosoma* on behaviour and immune capacity in blue tits *Cyanistes caeruleus*, and cases of a marked negative impact on wild birds, including morbidity and mortality, have also been documented (MOLYNEUX & GORDON 1975; MOLYNEUX et al. 1983). In our reed warblers, more than half (52%) of females were infested with one or more of the three haemosporidian blood parasites and 82% with *Trypanosoma*, but both avian malaria and *Trypanosoma* infection did not seem to be reflected in egg colour variation, and presence or absence of parasites did not affect female condition [analysis of variance (ANOVA): haemosporidian blood parasites: $F = 0.04$, $P > 0.8$, or *Trypanosoma:* $F = 1.08$, $P > 0.3$ and there is also no interaction effect between the two $F = 0.04$, $P > 0.8$, df = 1, 15].

Assuming that female clutch size is an indicator of female quality, one would expect a positive relationship between clutch size and eggshell pigmentation, meaning that better females should produce larger clutches with more colourful eggs. Such a positive relationship between clutch size and the proportion of bluish eggs and spot patterns was found in house sparrows *Passer domesticus* (López de Hierro & de Neve 2010). However, similar to Cassey et al. (2008), we did not find a relationship between egg pigmentation and clutch size for our reed warbler females. If egg pigmentation is costly, one would additionally expect that intraclutch variation should decrease with clutch size. Unfortunately, we were unable to derive any evidence in that respect, since we collected only one egg (third egg) from each clutch.

Several studies report a positive relationship between female condition and egg colouration (Morales et al. 2006; Siefferman et al. 2006; Martínez-de la Puente 2007).

We found no relationship between residual body weight (a measure for female condition) and eggshell pigmentation in reed warbler. Thus, in conclusion there is no evidence for eggshell pigmentation signalling female quality in terms of condition.

There is evidence that eggshell colouration is under genetic control (Gosler et al. 2000; Morales et al. 2010) and, according to Moreno & Osorno (2003), eggshell colouration could also signal female genetic quality. In line with these findings we found a positive relationship between tarsus length and several eggshell colour measurements. Thus, bigger females tend to lay brighter eggs with eggshell colouration at the blunt pole, with Chroma and UV Chroma at the egg centre being positively related to body size. These suggest genetic quality differences rather than condition-dependent distribution of colour substances.

Thus, the question that arises is: what drives between-year variation in egg colouration in reed warblers in relation to climate variables such as temperature or rain (Avilés et al. 2007)? Colouration itself might be important to tolerance to overheating from solar radiation (Gosler at al. 2005; Magige et al. 2008) or similar, or the risk of bacterial infection of eggs may increase with humidity: eggshell pigments show a photodynamic antibacterial activity against gram-positive bacteria (Ishikawa et al. 2010). To increase antimicrobial activity in humid years, females may also increase lysozyme concentration in the egg white, which may also be reflected in egg colour. Females may therefore vary their investment in egg colour as a direct or indirect response to these environmental needs, but without this investment being a handicap (Zhao et al. 2006). If egg colour substances are synthesized in the shell gland, it may not necessarily constitute a handicap. Furthermore, lysozyme production may not be that costly (Shawkey et al. 2008).

A compatible assumption for female quality to be involved in the "sexually selected egg colour" hypothesis is that egg colouration signals egg instead of female quality (Moreno et al. 2004; Morales et al. 2006) or maybe both. A lack of evidence for a missing link between egg colour and female attributes does not of course imply that egg quality is independent of female quality. Eggs are entirely produced by the females and egg quality therefore is certainly somehow an extension of female quality.

Evidence that eggshell colouration is reflected in egg compounds is also found in other studies. For example, eggshell colour intensity was found to be positively related to the concentration and amount of carotenoids, vitamin E and other antioxidants in the yolk (Navara et al. 2006a; Hargitai et al. 2010), and a positive association was found between egg colour and yolk lutein concentration (Hargitai et al. 2008). In reed warblers, we found that brightness of colour on the egg blunt pole was positively correlated with testosterone levels in the egg yolk. This suggests a negative correlation

between biliverdin and testosterone. On the contrary, a positive relationship between yolk testosterone concentrations and the amount of biliverdin in the eggshell was found in the spotless starling (López-Rull et al. 2008). Testosterone is an important egg component. It is known that females may deposit more testosterone into the eggs when mated with more attractive males (Gil et al. 1999, 2006; Loyau et al. 2007). However, there is divergent evidence on the benefits to offspring of yolk androgens. Yolk androgens, for example, may increase nestling begging behaviour in some species (Schwabl 1996; Eising & Groothuis 2003), and begging may increase the male contribution to the feeding rate (Ottosson et al. 1997). In contrast, yolk androgens may have detrimental effects on nestling growth and survival, especially reducing immune capacity (Sockmann & Schwabl 2000; Gil 2003; Groothuis et al. 2005), and some studies have shown a negative effect of yolk androgens, but only on male chicks (Müller et al. 2005; Saino et al. 2006).

We also found a relationship between egg colouration and lysozyme concentration in the egg white. Lysozyme is an important substance, showing an antibacterial function. It enhances phagocytic activity and stimulates monocytes (Wellman-Labadie et al. 2007), and might have positive consequences, in particular for early post-hatching survival (Prusinowska et al. 2000).

Interestingly, in contrast to yolk testosterone, lysozyme concentrations decreased with increasing brightness on the egg blunt pole. This could be a consequence of female allocation strategies. It is known that female allocation of testosterone and lysozyme in the egg varies with laying order. For example, Maddox et al. (2008) and López-Rull & Gil (2009) showed that yolk testosterone increases with laying order, whereas lysozyme declines with laying order (Saino et al. 2002; Bonisoli-Alquati et al. 2010).

Besides the fact that the same colour may provide information about the concentration of different egg components, we also found that colouration can signal different information depending on the different parts of an egg. For example, the yolk weight, which may indicate the nutritional status of an egg, is reflected by hue on the central part of an egg.

A morphological egg characteristic was also reflected by a colour variable at the egg centre, namely egg size, which correlated with the UV Chroma at the centre of the egg. In this case, colour may work as an amplifier of egg size and content as egg size and yolk weight are positively correlated ($R = 0.44$, $P = 0.001$, $\mathrm{df} = 48$).

Thus, in principle, egg colouration may have a multiple signalling function. However, the question remains as to whether the relationships we found (1) can be recognized and (2) have any meaning for the birds. Further investigations would therefore be essential to clarify whether or not birds are able to perceive the information provided by the eggs.

In conclusion, egg colour in the study species seems to reflect some aspects of female and/or egg quality but not others. Thus it partly supports the prediction of the signalling function. It may depend on the reproductive strategy a female follows in relation to environmental factors (Avilés et al. 2007) or mate quality (Burley 1988). It is known that females may allocate resources depending on male attractiveness (Safran et al. 2008; D'Alba et al. 2010; Martínez-Padilla et al. 2010) or alternatively to compensate for low male quality (Navara et al. 2006a, 2006b; Bolund et al. 2009). Allocation strategy may also depend on the need for paternal care; for example, high-quality females may be more independent from male parental investment and hence may invest less in egg pigmentation. Low-quality females, in contrast, may overproportionally invest in egg pigmentation (Hargitai et al. 2008) in order to recruit male

parental investment. Therefore, to what extent egg quality may also reflect male quality remains a subject for further investigation.

ACKNOWLEDGEMENTS

This study was supported by VEGA Project No. 02/0071/09. We thank Milena Svobodová for analysing faeces samples regarding *Coccidia* infestation. We also thank Jana Bohová for technical support in the laboratory.

REFERENCES

AVILÉS J.M., STOKKE B.G., MOKSNES A., RØSKAFT E. & MØLLER A.P. 2007. Environmental conditions influence egg color of reed warblers *Acrocephalus scirpaceus* and their parasite, the common cuckoo *Cuculus canorus*. *Behavioral Ecology and Sociobiology* 61: 475–485.

BIRKHEAD T.R. 1978. Behavioural adaptations to high density nesting in the common guillemot *Uria aalge*. *Animal Behaviour* 26: 321–331.

BOLUND E., SCHIELZETH H. & FORSTMEIER W. 2009. Compensatory investment in zebra finches: Females lay larger eggs when paired to sexually unattractive males. *Proceedings of the Royal Society of London (B)* 276: 707–715.

BONISOLI-ALQUATI A., RUBOLINI D., ROMANO M., CUCCO M., FASOLA M., CAPRIOLI M. & SAINO N. 2010. Egg antimicrobials, embryo sex and chick phenotype in the yellow-legged gull. *Behavioral Ecology and Sociobiology* 64: 845–855.

BURLEY N. 1988. The differential allocation hypothesis: an experimental test. *The American Naturalist* 132: 611–628.

CASSEY P., EWEN J.G., BLACKBURN T.M., HAUBER M.E., VOROBYEV M. & MARSHALL N.J. 2008. Eggshell colour does not predict measures of maternal investment in egg of *Turdus* thurshes. *Naturwissenschaften* 95: 713–721.

CHERRY M.I. & GOSLER A.G. 2010. Avian eggshell coloration: new perspectives on adaptive explanations. *Biological Journal of the Linnean Society* 100: 753–762.

D'ALBA L., SHAWKEY M.D., KORSTEN P., VEDDER O., KINGMA S.A., KOMDEUR J. & BEISSINGER S.R. 2010. Differential deposition of antimicrobial proteins in blue tit (*Cyanistes caeruleus*) clutches by laying order and male attractiveness. *Behavioral Ecology and Sociobiology* 64: 1037–1045.

DAVIES N.B. & BROOKE M.DE L. 1989. An experimental study of co-evolution between the Cuckoo, *Cuculus canorus*, and its hosts. I. Host egg discrimination. *Journal of Animal Ecology* 58: 207–224.

DELHEY K., PETERS A., JOHNSEN A. & KEMPENAERS B. 2006. Seasonal changes in blue tit crown color: do they signal individual quality? *Behavioral Ecology* 17: 790–798.

DUCKWORTH J.W. 1992. Effects of mate removal on the behaviour and reproductive success of reed warblers *Acrocephalus scirpaceus*. *Ibis* 134: 164–170.

EISING C.M. & GROOTHUIS T.G.G. 2003. Yolk androgens and begging behaviour in black-headed gull chicks: an experimental field study. *Animal Behaviour* 66: 1027–1034.

GARCIA-FERNANDEZ V., GUASCO B., TANVEZ A., LACROIX A., CUCCO M., LEBOUCHER G. & MALACARNE G. 2010. Influence of mating preferences on yolk testosterone in the grey partridge. *Animal Behaviour* 80: 45–49.

GARCÍA-NAVAS V., SANZ J.J., MERINO S., MARTÍNEZ-DE LA PUENTE J., LOBATO E., DEL CERRO S., RIVERO J., RUIZ-DE-CASTAÑEDA R. & MORENO J. 2011. Experimental evidence for the role of calcium in eggshell pigmentation pattern and breeding performance in blue tits *Cyanistes caeruleus*. *Journal of Ornithology* 152: 71–82.

GIL D. 2003. Golden eggs: maternal manipulation of offspring phenotype by egg androgens in birds. *Ardeola* 50: 281–294.

GIL D., GRAVES J.A., HAZON N. & WELLS A. 1999. Male attractiveness and differential testosterone investment in zebra finch eggs. *Science* 286: 126–128.

GIL D., NINNI P., LACROIX A., DE LOPE F., TIRARD C., MARZAL A. & MØLLER A.P. 2006. Yolk androgens in the barn swallow (*Hirundo rustic*a): a test of some adaptive hypotheses. *Journal of Evolutionary Biology* 19: 123–131.

GLADBACH A., GLADBACH D.J. & QUILLFELDT P. 2010. Seasonal clutch size decline and individual variation in the timing of breeding are related to female body condition in a non-migratory species, the Upland Goose *Chloephaga picta leucoptera*. *Journal of Ornithology* 151: 817–825.

GOSLER A.G., BARNETT P. & REYNOLDS S.J. 2000. Inheritance and variation in eggshell patterning in the great tit *Parus major*. *Proceedings of the Royal Society of London (B)* 267: 2469–2473.

GOSLER A.G., HIGHAM J.P. & REYNOLDS S.J. 2005. Why are birds' eggs speckled? *Ecology Letters* 8: 1105–1113.

GRIGGIO M., DEVIGILI A., HOI H. & PILASTRO A. 2009. Female ornamentation and directional male mate preference in the rock sparrow. *Behavioral Ecology* 20: 1072–1078.

GRIGGIO M., ZANOLLO V. & HOI H. 2010. UV plumage color is an honest signal of quality in male budgerigars. *Ecological Research* 25: 77–82.

GRIM T., KLEVEN O. & MIKULICA O. 2003. Nestling discrimination without recognition: a possible defence mechanism for hosts towards cuckoo parasitism? *Proceedings of the Royal Society of London (B)* 270 (*Suppl.*) 1: 573–575.

GROOTHUIS T.G.G., EISING C.M., DIJKSTRA C. & MÜLLER W. 2005. Balancing between costs and benefits of maternal hormone deposition in avian eggs. *Biology Letters* 1: 78–81.

HANLEY D. & DOUCET S.M. 2009. Egg coloration in ring-billed gulls (*Larus delawarensis*): a test of the sexual signaling hypothesis. *Behavioral Ecology and Sociobiology* 63: 719–729.

HARGITAI R., HERÉNYI M. & TÖRÖK J. 2008. Eggshell colouration in relation to female condition, male attractiveness and egg quality in the collared flycatcher (*Ficedula albicollis*). *Journal of Avian Biology* 39: 413–422.

HARGITAI R., MOSKÁT C., BÁN M., GIL D., LÓPEZ-RULL I. & SOLYMOS E. 2010. Eggshell characteristics and yolk composition in the common cuckoo *Cuculus canorus*: are they adapted to brood parasitism? *Journal of Avian Biology* 41: 177–185.

HARRIS W.E. & ULLER T. 2009. Reproductive investment when mate quality varies: differential allocation versus reproductive compensation. *Philosophical Transactions of the Royal Society (B)* 364: 1039–1048.

HELLGREN O., WALDENSTRÖM J. & BENSCH S. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *Journal of Parasitology* 90: 797–802.

HOI H. & HOI-LEITNER M. 1997. An alternative route to coloniality in the bearded tit: females pursue extra-pair fertilizations. *Behavioral Ecology* 8: 113–119.

HOI H., KLEINDORFER S., ILLE R. & DITTAMI J. 1995. Prey abundance and male parental behaviour in *Acrocephalus* warblers. *Ibis* 137: 490–496.

HONZA M., POŽGAYOVÁ M., PROCHÁZKA P. & CHERRY M.I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. *Naturwissenschaften* 98: 493–499.

ILLE R., HOI H. & KLEINDORFER S. 1996. Brood predation, habitat characteristics and nesting decisions in *Acrocephalus scirpaceus* and *A. palustris*. *Biologia* 51: 219–225.

ISHIKAWA S., SUZUKI K., FUKUDA E., ARIHARA K., YAMAMOTO Y., MUKAI T. & ITOH M. 2010. Photodynamic antimicrobial activity of avian eggshell pigments. *FEBS Letters* 584: 770–774.

JAGANNATH A., SHORE R.F., WALKER L.A., FERNS P.N. & GOSLER A.G. 2008. Eggshell pigmentation indicates pesticide contamination. *Journal of Applied Ecology* 45: 133–140.

KAUR H., HUGHES M.N., GREEN C.J., NUGHTON P., FORESTI R. & MOTTERLINI R. 2003. Interaction of bilirubin with reactive nitrogen species. *FEBS Letters* 543: 113–119.

KILNER R.M. 2006. The evolution of egg colour and patterning in birds. *Biological Reviews* 81: 383–406.

KLEINDORFER S., FESSL B. & HOI H. 1995. More is not always better: male incubation in two *Acrocephalus* warblers. *Behaviour* 132: 607–625.

KNOWLES S.C.L., PALINAUSKAS V. & SHELDON B.C. 2010. Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. *Journal of Evolutionary Biology* 23: 557–569.

LACK D. 1958. The significance of the colour of urdine eggs. *Ibis* 100: 145–166.

LATHI D. 2008. Population differentiation and rapid evolution of egg colour in accordance with solar radiation. *The Auk* 125: 796–802.

LEISLER B. & WINKLER H. 1991. Results and concepts in the ecomorphology of birds. *Journal of Ornithology* 132: 373–425.

LESSELLS C.M. & BOAG P. 1987. Unrepeatable repeatabilities: a common mistake. *The Auk* 104: 116–121.

LÓPEZ DE HIERRO M.D.G. & DE NEVE L. 2010. Pigment limitation and female reproductive characteristics influence egg shell spottiness and ground colour variation in the house sparrow (*Passer domesticus*). *Journal of Ornithology* 151: 833–840.

LÓPEZ-RULL I. & GIL D. 2009. Elevated testosterone levels affect female breeding success and yolk androgen deposition in a passerine bird. *Behavioural Processes* 82: 321–318.

LÓPEZ-RULL I., MIKSIK I. & GIL D. 2008. Egg pigmentation reflects female and egg quality in the spotless starling *Sturnus unicolor*. *Behavioral Ecology and Sociobiology* 62: 1877–1884.

LOYAU A., JALME M.S., MAUGET R. & SORCI G. 2007. Male sexual attractiveness affect the investment of maternal resources into eggs in peafowl. *Behavioral Ecology and Sociobiology* 64: 1043–1052.

MADDOX J.D., BOWDEN R.M. & WEATHERHEAD P.J. 2008. Yolk testosterone and estradiol variation relative to clutch size, laying order and hatching asynchrony in Common Grackles. *Journal of Ornithology* 149: 643–649.

MAGIGE F.J., MOE B. & RØSKAFT E. 2008. The white colour of the Ostrich (*Struthio camelus*) egg is a trade-off between predation and overheating. *Journal of Ornithology* 149: 323–328.

MARTÍNEZ-DE LA PUENTE J., MERINON S., MORENO J., TOMÁS G., MORALES J., LOBATO E., GARCÍA-FRAILE S. & MARTÍNEZ J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? *Journal of Avian Biology* 38: 377–384.

MARTÍNEZ-PADILLA J., DIXON H., VERGARA P., PÉREZ-RODRÍGUEZ L. & FARGALLO J.A. 2010. Does egg colouration reflect male condition in birds? *The Auk* 127: 453–459.

MCCLEERY R.H., PERRINS C.M., SHELDON B.C. & CHARMANTIER A. 2008. Age-specific reproduction in a long-lived species: the combined effects of senescence and individual quality. *Proceedings of the Royal Society of London (B)* 275: 963–970.

MCDONAGH A.F. 2001. Turning green to gold. *Nature Structural & Molecular Biology* 8: 198–200.

MCFARLANE M.L., CHERRY M.I. & EVANS M.R. 2010. Female Cape sugarbirds (*Promerops cafer*) modify egg investment both for extra-pair mates and for male tail length. *Journal of Evolutionary Biology* 23: 1998–2003.

MOKSNES A., RØSKRAF E., BIČÍK V., HONZA M. & ØIEN I.J. 1993. Cuckoo *Cuculus canorus* parasitism on *Acrocephalus* warblers in Southern Moravia in the Czech Republic. *Journal of Ornithology* 134: 425–434.

MOLYNEUX D.H., COOPER J.E. & SMITH W.J. 1983. Studies on the pathology on avian trypanosome (*T. bouffardi*) infection in experimentally infected canaries. *Parasitology* 87: 49–54.

MOLYNEUX D.H. & GORDON E. 1975. Studies on immunity with three species of avian trypanosomes. *Parasitology* 70: 181–187.

MORALES J., KIM S.-Y., LOBATO E., MERINO S., TAMÁS G. & MARTÍNEZ-DE LA PUENTE J.J. 2010. On the heritability of blue-green eggshell coloration. *Journal of Evolutionary Biology* 23: 1783–1791.

MORALES J., SANZ J.J. & MORENO J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. *Biology Letters* 2: 334–336.

MORALES J., TORRES R. & VELANDO A. 2012. Safe betting: males help dull females only when they raise high-quality offspring. *Behavioral Ecology and Sociobiology* 66: 135–143.

MORENO J., LOBATO E., MORALES J., MERINO S., TOMÁS G., MARTÍNEZ-DELLA PUENTE J., SANZ J.J., MATEO R. & SOLER J.J. 2006. Experimental evidence that egg color indicates female condition at laying in a songbird. *Behavioral Ecology* 17: 651–655.

Moreno J. & Osorno J.L. 2003. Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? *Ecology Letters* 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*. *Journal of Avian Biology* 35: 300–304.

Müller W., Groothuis T.G.G., Eising C.M., Daan S. & Dijkstra C. 2005. Within clutch covariation of egg mass and sex in the black-headed gull. *Journal of Evolutionary Biology*18: 661–668.

Müller W., Vergauwen J. & Eens M. 2009. Long-lasting consequences of elevated yolk testosterone levels on female reproduction. *Behavioral Ecology and Sociobiology* 63: 809–816.

Navara K.J., Badyaev A.V., Mendonça M.T. & Hill G.E. 2006a. Yolk antioxidants vary with male attractiveness and female condition in the house finch (*Carpodacus mexicanus*). *Physiological and Biochemical Zoology* 79: 1098–1105.

Navara K.J., Hill G.E. & Mendonça M.T. 2006b. Yolk androgen deposition as a compensatory strategy. *Behavioral Ecology and Sociobiology* 60: 392–398.

Øien I.J., Moksnes A., Røskraf E. & Honza M. 1998. Costs of Cuckoo *Cuculus canorus* parasitism to reed warbler *Acrocephalus scirpaceus*. *Journal of Avian Biology* 29: 209–215.

Okuliarová M., Šárniková B., Rettenbacher S., Škrobánek P. & Zeman M. 2010. Yolk testosterone and corticosterone in hierarchical follicles and laid eggs of Japanese quail exposed to long-term restraint stress. *General and Comparative Endocrinology* 165: 91–96.

Ottosson U., Bäckman J. & Smith H.G. 1997. Begging affects parental effort in the pied flycatcher, *Ficedula hypoleuca*. *Behavioral Ecology and Sociobiology* 41: 381–384.

Pitala N., Ruuskanen S., Laaksonen T., Doligez B., Tschirren B. & Gustafsson L. 2009. The effects of experimentally manipulated yolk androgens on growth and immune function of male and female nestling collared flycatchers *Ficedula albicollis*. *Journal of Avian Biology* 40: 225–230.

Polačiková L. & Grim T. 2010. Blunt egg pole holds cues for alien egg discrimination: experimental evidence. *Journal of Avian Biology* 41: 111–116.

Polačiková L., Stokke B.G., Procházka P., Honza M., Mosknes A. & Røskraf E. 2010. The role of blunt pole characteristics for recognition of eggs in the song trush (*Turdus philomelos*). *Behaviour* 147: 465–478.

Prusinowska I., Jankowski J., Sowinski G. & Wawro K. 2000. An evaluation of lysozyme usability in turkey improvement. *Czech Journal of Animal Science* 45: 225–228.

Remeš V. 2011.Yolk androgens in great tit eggs are related to male attractiveness, breeding density and territory quality. *Behavioral Ecology and Sociobiology* 65: 1257–1266.

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

Roberts M.L., Ras E. & Peters A. 2009. Testosterone increases UV reflectance of sexually selected crown plumage in male blue tits. *Behavioral Ecology* 20: 535–541.

Rosivall B., Szöllösi E. & Török J. 2005. Maternal compensation for hatching asynchrony in the collared flycatcher *Ficedula albicollis*. *Journal of Avian Biology* 36: 531–537.

Safran R.J., Pilz K.M., McGraw K.J., Correa S.M. & Schwabl H. 2008. Are yolk androgens and carotenoids in barn swallow eggs related to parental quality? *Behavioral Ecology and Sociobiology* 62: 427–438.

Saino N, Dall'ara P., Martinelli R. & Møller A.P. 2002. Early maternal effects and antibacterial immune factor in the eggs, nestlings and adults of the barn swallow. *Journal of Evolutionary Biology* 15: 735–743.

Saino N., Ferrari R.P., Romano M., Martinelli R., Lacroix A., Gil D. & Møller A.P. 2006. Maternal allocation of androgens and antagonistic effects of yolk androgens on sons and daughters. *Behavioral Ecology* 17: 172–181.

Schulze-Hagen K. 1992. Parasitism and egg losses due to the Cuckoo (*Cuculus canorus*) in Reed and Marsh Warblers (*Acrocephalus scirpaceus*, *A. palustris*) in central and western Europe. *Journal of Ornithology* 133: 237–249.

SCHWABL H. 1996. Maternal testosterone in the avian eggs enhances postnatal growth. *Comparative Biochemistry and Physiology (A)* 114: 271–276.
SEHGAL R.N.M., JONES H.I. & SMITH T.B. 2001. Host specificity and incidence of *Trypanosoma* in some African rainforest birds: a molecular approach. *Molecular Ecology* 10: 2319–2327.
SHAWKEY M.D., KOSCIUCHB K.L., LIUC M., ROHWERD F.C., LOOSE E.R., WANGA J.M. & BEISSINGERA S.R. 2008. Do birds differentially distribute antimicrobial proteins within clutches of eggs? *Behavioral Ecology* 19: 920–927.
SHELDON B.C. 2000. Differential allocation: tests, mechanisms and implications. *Trends in Ecology & Evolution* 15: 397–402.
SIEFFERMAN L., NAVARA K.J. & HILL G.E. 2006. Egg coloration is correlated with female condition in eastern bluebirds (*Sialia sialis*). *Behavioral Ecology and Sociobiology* 59: 651–656.
SOCKMAN K.W. & SCHWABL H. 2000. Yolk androgens reduce offspring survival. *Proceedings of the Royal Society (B)* 267: 1451–1456.
SOLER J.J. & MØLLER A.P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. *Behavioral Ecology* 7: 89–94.
SOLER J.J., NAVARRO C., CONTRERAS T.O.P., AVILÉS J.M. & CUERVOK J.J. 2008. Sexually selected egg coloration in spotless starlings. *The American Naturalist* 171: 183–194.
STOCKER R., YAMAMAOTO Y., MCDONAGH A.F., GLAZER A.N. & AMES B.N. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043–1046.
SWYNNERTON C.F.M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. *Ibis* 4: 529–606.
TOMÁS G., MERINO S., MORENO J., SANZ J.J., MORALES J. & GARCÍA-FRAILE S. 2006. Nest weight and female health in Blue Tit (*Cyanistes caeruleus*). *The Auk* 123: 1013–1021.
UNDERWOOD T.J. & SEALY S.G. 2002. Adaptive significance of egg coloration, pp. 280–298. In: Deeming, D.C., Ed. Avian incubation, behaviour, environment and evolution. *Oxford*: *Oxford University Press*.
VALKIUNAS G., IEZHOVA T.A., CARLSON J.S. & SEHGAL R.N.M. 2011. Two new *Trypanosoma* species from African birds, with notes on the taxonomy of avian trypanosomes. *Journal of Parasitology* 97: 924–930.
WALDENSTRÖM J., BENSCH S., HASSELQUIST D. & ÖSTMAN, Ö. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology* 90: 191–194.
WALLACE A.R. 1889. Darwinism: An exposition of the theory of natural selection with some its applications. *London*: *Macmillan*.
WELLMAN-LABADIE O., PICMAN J. & HINCKE M.T. 2007. Avian antimicrobial proteins: structure, distribution and activity. *World's Poultry Science Journal* 63: 421–438.
ZAHAVI A. 1975. Mate selection – a selection for a handicap. *Journal of Theoretical Biology* 53: 205–214.
ZEMAN M., KOŠUTZKÝ J. & BOBÁKOVÁ E. 1986. Testosterone concentration in the seminal plasma of cocks. *British Poultry Science* 27: 261–266.
ZHAO R., XU G.Y., LIU Z.Z., LI J.Y. & YANG N. 2006. A study on eggshell pigmentation: biliverdin in blue-shelled chickens. *Poultry Science* 85: 546–549.