Articles

Eggshell characteristics and yolk composition in the common cuckoo *Cuculus canorus*: are they adapted to brood parasitism?

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The developmental rate of cuckoo embryos and their hatching size is greater than that of host species, which may require more nutrient resources in the egg and more intensive gas exchange during development. In the present study, we compared various egg characteristics of a brood parasite, the common cuckoo *Cuculus canorus*, and its frequent host, the great reed warbler *Acrocephalus arundinaceus*. As maternally-derived testosterone is known to enhance growth rate of embryos and hatchlings, cuckoo eggs are expected to contain higher concentration of testosterone than host eggs. In addition, we expected higher concentration of antioxidants in cuckoo eggs to protect embryos from oxidative stress associated with accelerated growth. Our results showed that cuckoo eggs had thicker shells and higher pore density than great reed warbler eggs. Yolk was significantly heavier in cuckoo eggs and contained higher concentrations of carotenoids and vitamin E, however, yolk androgen and immunoglobulin concentrations were lower in cuckoo eggs as compared to great reed warbler eggs. We also examined whether eggshell colour was associated to egg quality, and detected a positive association between blue-green chroma and yolk antioxidant concentration in both species, suggesting that eggshell colour reflects the antioxidant investment of the female into the eggs. Our results suggest that cuckoo females increase the size, growth rate and competitive ability of their young by providing them with more nutrients and more dietary antioxidants for embryonic development, and not through elevated yolk testosterone or antibody levels. In addition, increased porosity of cuckoo eggshells may allow embryos to develop more rapidly because of a greater capacity of gas exchange.

The brood parasitic relationship between common cuckoos *Cuculus canorus* (hereafter 'cuckoo') and their host species represents a classic example of coevolutionary interactions. In this arms race, hosts have coevolved effective defence mechanisms to prevent brood parasitism, which at the same time have selected for more sophisticated responses by the cuckoo to overcome host defences (Davies and Brooke 1988, Davies 2000). The great reed warbler *Acrocephalus arundinaceus* is a well-known, high-quality cuckoo host (Kleven et al. 1999, Butchart et al. 2003). Egg rejection decisions in this species may depend on several factors including the mimicry of the parasite egg (Moskát and Honza 2002, Cherry et al. 2007), intraclutch variation of host eggs (Moskát et al. 2008), and whether cuckoos are observed near the nest (Bártol et al. 2002).

Cuckoo eggs typically need to be incubated for 12 days, while host eggs require 13 or 14 days (Wyllie 1981). The developmental rate of cuckoo embryos and their hatching mass is greater than that of host embryos (Honza et al. 2001, Hauber and Moskát 2008), which may require additional nutrient resources in the egg and more intensive

gas exchange during development. Moreover, cuckoo hatchlings evict all eggs and nestlings from the nest within 72 h after hatching (Wyllie 1981, Honza et al. 2007), and later use exaggerated begging calls to elicit food provisioning from the foster parents (Kilner et al. 1999, Butchart et al. 2003), which behaviour of the cuckoo may need hormonal stimulation. To understand the proximate basis of how cuckoos have adapted to brood parasitism, it is necessary to compare the characteristics of their eggs with those of host species.

Yolk mass, which reflects the amount of macronutrients transferred to the egg, is an important determinant of offspring fitness (Williams 1994, Christians 2002). Eggs with larger yolks may result in larger hatchlings or hatchlings with larger residual yolk reserves, assisting the survival and early growth of the hatchling (Parsons 1970, Peach and Thomas 1986). Therefore, we may expect cuckoo females to lay eggs with heavier yolks thus increasing the size, growth rate and viability of their young. Apart from macronutrients, there are several substances allocated to the yolk that can affect offspring development,

such as androgens, dietary antioxidants and immunoglobulins (Blount et al. 2000, Boulinier and Staszewski 2008, Gil 2008).

Elevated level of yolk androgens results in increased growth rate (Schwabl 1996, Eising et al. 2001, Navara et al. 2005; but see Sockman and Schwabl 2000), better competitive ability and begging vigour (Schwabl 1993, Eising and Groothuis 2003), and enhanced development of hatching muscle (Lipar and Ketterson 2000). Thus, obligate brood parasites may allocate more testosterone to their eggs than non-parasite females in order to enhance the competitiveness of their offspring (Winkler 1993, Gil 2003). Also, brood parasitic females may deposit more androstenedione into the eggs, which can function as a source of testosterone (Groothuis et al. 2005). Increased metabolic rate, which may be stimulated by high level of androgens, results in higher production of reactive oxygen species, which can damage several important molecules (von Schantz et al. 1999). An additional cost may arise as yolk steroids have been shown to suppress the immune system (Andersson et al. 2004, Navara et al. 2005). Thus, females of parasitic species may deposit more dietary antioxidants (e.g. carotenoids, vitamin E) and immunoglobulins into their eggs in order to protect the embryo from oxidative stress and infections (Surai 2002, Boulinier and Staszewski 2008), and to compensate for the potentially deleterious effects of steroids.

Brood parasites lay unusually strong eggs (Brooker and Brooker 1991), presumably to prevent them from puncture ejection by hosts (Spaw and Rohwer 1987, Picman 1989, Antonov et al. 2006; but see Antonov et al. 2008) or from damage during rushed laying (Lack 1968, Soler and Martínez 2000). The increased strength could be achieved by increased thickness of the shell and by the more rounded shape of the egg. As the shell is impervious to gases, it is perforated by microscopic pores, through which the exchange of respiratory gases takes place (Ar and Rahn 1985). The eggshells of brood parasites may have increased porosity than host eggs due to the more intense gas exchange needed for faster development, however, this comparison has not been performed before.

Blue-green egg colouration results from the pigment biliverdin accumulated in the eggshell (Kennedy and Vevers 1976, Mikšík et al. 1996). Since biliverdin shows an antioxidant activity (McDonagh 2001, Kaur et al. 2003), it has been proposed that only females of better condition and health state could afford the costs of depositing high amounts of this pigment into the eggshell during laying, a time of high oxidative stress (Moreno and Osorno 2003). This hypothesis was supported by a number of studies (Moreno et al. 2005, Siefferman et al. 2006, Krist and Grim 2007), although negative results were also presented (Hargitai et al. 2008, López-Rull et al. 2008, Hanley and Doucet 2009). Furthermore, egg colour may indicate the quality of offspring, as mediated through prenatal maternal investments, such as allocation of carotenoids, testosterone and antibodies to the yolk, which may be higher in females of better health state (Blount et al. 2002, Saino et al. 2002, Gil et al. 2006, Hargitai et al. 2006a), or laying eggs with stronger eggshells. This hypothesis has been tested in a few passerines in recent years, but the results are not consistent (Morales et al. 2006, Cassey et al. 2008, Hargitai et al. 2008, López-Rull et al. 2008).

In the present study, we compared various egg characteristics of a brood parasite, the cuckoo, and its frequent host, the great reed warbler to see if brood parasitic females enhance the competitiveness of their offspring through egg structure and composition. We expected that cuckoo eggs would contain heavier yolk and more pore channels than do host eggs so as to allow faster development, although egg size may not differ due to mimicry of cuckoo eggs. Moreover, as maternally-derived testosterone is known to enhance growth rate of embryos and hatchlings (Schwabl 1996, Eising et al. 2001, Navara et al. 2005), it was previously hypothesized that parasite eggs might contain higher concentration of testosterone than host eggs (Winkler 1993, Gil 2003). This hypothesis has been tested on the brood parasitic brown-headed cowbird *Molothrus ater*, however, the results showed an inconsistent pattern (Hauber and Pilz 2003, Hahn et al. 2005). The only test on the cuckoo revealed a nonsignificant trend for a lower yolk testosterone level in parasite eggs (Török et al. 2004), which is opposite to the prediction of the hypothesis. We aimed to repeat this comparison too, in order to reduce uncertainty, assuming that we would find a significant difference with a somewhat larger data set. In addition, we expected higher concentrations of antioxidants and antibodies in cuckoo eggs to protect the fast-growing embryos from oxidative stress and infections, which comparison has not been conducted before. Also, we predicted that the eggshell would be thicker in cuckoo eggs than in the eggs of its passerine host, which was reported earlier by Honza et al. (2001), but another study found no significant difference for the cuckoo (Brooker and Brooker 1991).

In addition, we expected an association between blue-green egg colour and egg quality in terms of yolk steroid, immunoglobulin, and antioxidant concentrations, yolk mass and eggshell thickness. Also, we tested if there was a correlation between concentrations of yolk carotenoids and testosterone. We expected a positive association, which could reflect a compensatory allocation of carotenoids to eggs with high testosterone level in order for carotenoids to offset the potentially harmful effects associated with high level of yolk testosterone (Navara et al. 2006, Török et al. 2007).

Methods

Field procedures

The study was conducted in the surroundings of the village Apaj (47° 07′N, 19° 06′E), ca. 40 km south of Budapest, Hungary, in 2006. In this great reed warbler population, approximately 64% of the nests are parasitized by cuckoos. Parasitized nests are found primarily within 50 m of trees, poles or electric wires, which serve as vantage points for the cuckoo (Moskát and Honza 2000).

We collected one great reed warbler egg from 13 nests and one cuckoo egg from 12 nests before incubation began. Cuckoo eggs were not collected from the same nests as great reed warbler eggs, except for 3 cuckoo eggs. In a previous study it was shown that egg laying order had no effect on

yolk mass and yolk testosterone concentration (Török et al. 2004), so we analysed one randomly selected great reed warbler egg per clutch. One great reed warbler and two cuckoo eggs turned out to contain embryos, so we did not analyse the yolks of those eggs. Egg length and breadth were measured with a caliper (to the nearest 0.1 mm), egg size was estimated from Hoyt's formula (Hoyt 1979; size = length $\times$ breadth$^2 \times 0.51$). An eggshape index was calculated by dividing breadth by length.

Immunoglobulin assay

Yolks were separated from albumen, their mass was weighed, and thoroughly homogenized in an equal (1 ml per gram of yolk) amount of distilled water, and stored at -20° C until analysis. Antibody (IgG) concentrations were determined using an enzyme-linked immunosorbent assay (ELISA). ELISA plates (F96 Maxisorp, Nunc-Immunoplate) were coated with yolk samples diluted 1:10 000 (cuckoo yolk) or 1:20 000 (great reed warbler yolk) in carbonate-bicarbonate buffer (pH 9.6). One hundred µl of this dilution was added to plates in duplicates of each sample and incubated overnight at 4° C. To assess total antibody concentration, a serial dilution of a standard of pooled cuckoo and great reed warbler yolks (n = 5 and 5, respectively) was added in duplicate to each plate, and a negative control was included as well. The linear range of the sigmoid curve of the dilutions was between 1:2 000 and 1:12 8000, and 1:2 000 was assigned a concentration of 10 000 IU. All values were subsequently expressed relative to this standard. Eggs from the same species were estimated in one plate. The plates were washed three times with 200 µl PBS-Tween20 (0.05%) buffer, and blocked with 100 µl of 5% milk powder for 1 h at 37° C. After washing the wells with 200 µl of PBS-Tween 20 (0.05%) buffer (three times), 100 µl peroxide-conjugated goat-antichicken IgG (Sigma A-9046) diluted 1:125 in PBS-Tween20 were added to each well and incubated overnight at 4° C. Subsequently, plates were washed three times with 200 µl PBS-Tween20, and a substrate (ABTS, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid), Sigma A-1888) and hydrogen peroxide in citrate buffer were added (100 µl). Plates were covered with an aluminium foil (to keep them protected from light) and incubated at room temperature for 2.5 h. Absorbances were measured with a Tecan ELISA reader using a 405 nm wavelength filter and Magellan software.

Steroid assay

One hundred mg of the yolk samples were homogenized in 1 ml of distilled water by vigorous mixing, and was allowed to equilibrate overnight at 4° C. Free steroids were then extracted by adding 2.8 ml petroleum ether/diethyl ether (30:70, v/v) to the samples, then tubes were vortexed for 5 min in a Multivortex (Multi-tube vortexer VWR, VX-2500), and centrifuged for 5 min at 3 000 rcf. The ether fraction was decanted after snap-freezing the tubes in a dry ice bath (-70° C) and evaporated with a Techne sampler concentrator (D8-3, Dri-Block) at 40° C. The dried residue was redissolved in 1 ml of 90% ethanol and kept overnight at -20° C. Precipitated proteins and lipids were separated

from the ethanol phase by decanting after centrifugation at 1 300 rcf for 5 min. The ethanol phase was evaporated with a Techne sampler concentrator at 40° C, and the dried extract was resuspended with 1 ml of steroid-free serum (DRG-EIA-1559 kit) and vortexed for 10 min. Yolk concentrations of testosterone and androstenedione were determined by two different ELISA kits highly specific for each hormone (DSL Labs, USA for testosterone and DRG Labs, USA for androstenedione). Manufacturer's inserts report cross-reactivities of the androstenedione antibody of $<1\%$ for all hormones tested, and cross-reactivities of the testosterone antibody of $<1\%$ for all hormones tested except for 5-α-dihydrotestosterone which was 6.6%. Assays were performed following manufacturer's instructions. All samples were analysed in duplicate in the same plate, and concentrations calculated with respect to standard curves. The intra-assay coefficient of variation was 9.6% for testosterone and 3.4% for androstenedione.

Antioxidant assay

After thawing, astaxanthin (internal standard) was added to a representative portion of the sample. The sample was placed in a mortar containing 4 times more C_{18} phase than sample, and they were manually blended for about 30 s. The blended material was then transferred into a column suitable for conducting sequential elution and pressed to a compact column bed between two frits. The column was then washed with 6 ml water to elute the polar impurities. Before eluting the analytes a second column packed with sodium sulphate was placed under the first column in order to remove the water residue from the effluent. The carotenoid fraction was eluted with 6 ml dichloromethane. After evaporating the solvent to dryness under nitrogen stream at 40° C, the residue was redissolved in 200 µl dichloromethane/methanol (1:4) solution. The solution was analysed with two different high pressure liquid chromatographic methods. In the first method a reversed phase Develosil C_{30} (150 mm $\times$ id 4.6 mm, 3 µm) column was used with a Zorbax C_{18} (12.5 mm $\times$ id 4.6 mm, 5 µm) precolumn. Gradient elution was performed with flow and solvent gradient to separate the compounds. The mobile phase was prepared from water and acetonitrile/ethyl acetate (2:1) with 0.01% (v/v) triethylamine. The diode array detector was operated at 295 nm (α-tocopherol) and 450 nm (carotenoids). In the second method a normal phase Zorbax Rx-SIL (150 mm $\times$ id 4.6 mm, 5 µm) column was employed for the separation of lutein and zeaxanthin. In the gradient elution 2-propanol and hexane were used with a flow rate of 1 ml min^{-1}. The target compounds were detected at 450 nm. Peaks were identified and quantified by comparisons with retention times and calibration curves of carotenoid and tocopherol standards.

Eggshell thickness and pore density

Pieces of eggshell were taken from the equatorial region of each egg. Prior to analysis, organic shell membranes were removed by immersing shells in household bleach (5.4% aquous sodium hypochlorite) for 1–3 min. Eggshells were then rinsed in tap water, drained and allowed to dry.

Table 1. Comparison of egg characteristics between cuckoos and great reed warblers shown as means ± SD. The two species were compared by Student's t-test. Bold indicates that the effect was significant.

Egg characteristics	Great reed warbler	Cuckoo	t	n (great reed warbler)	n (cuckoo)	P-value
Egg volume (cm^3)	2.94 ± 0.31	3.07 ± 0.20	−1.21	13	12	0.239
Breadth/length (mm/mm)	0.71 ± 0.03	0.73 ± 0.03	−2.08	13	12	**0.049**
Shell thickness (μm)	80.99 ± 3.90	104.02 ± 3.64	−14.60	12	11	**<0.001**
Pore density (pores/25 mm^2)	9.79 ± 4.60	18.74 ± 3.78	−5.07	12	11	**<0.001**
Blue-green chroma (%)	0.52 ± 0.01	0.50 ± 0.01	2.65	12	11	**0.015**

Shell thickness was measured with a Mitutoyo Digimatic Micrometer (395–271) with a ball-point end at a precision of 0.001 mm at six places of the shell. Variation in eggshell thickness was greater among eggs than within eggs (great reed warbler: $F_{11,60} = 24.71$, P < 0.001, r = 0.80; cuckoo: $F_{10,55} = 35.67$, P < 0.001, r = 0.85), indicating that these measurements were repeatable (Lessells and Boag 1987). We used the mean of the six measurements as an average eggshell thickness of an egg.

The inner surface of shell fragments was painted with 1% aqueous Evans blue. Capillary forces filled the pore channels rendering them visible as fine blue dots on the external surface of the shell. A paper mat with an exposed area of 25 mm^2 was placed over the shell, and pores were counted in 2–3 fields using a stereomicroscope with a magnification of 40×. Variation in pore density was higher among eggs than within eggs (great reed warbler: $F_{11,18} = 6.06$, P < 0.001, r = 0.67; cuckoo: $F_{10,18} = 7.54$, P < 0.001, r = 0.71), showing that these measurements were repeatable.

Spectrophotometry

Reflectance spectra were measured using an Ocean Optics USB2000 spectrophotometer (range: 300–700 nm) with a DT-Mini-2GS deuterium-halogen light source and a quartz optic fibre (QR400-7-UV-BX). The measurement illuminated a region of 6 mm in diameter and were taken at a 90° angle to the eggshell surface. Reflectance was measured of pieces of eggshells placed on a matte black cardboard in a dark room. Integration time was 250 ms. We took 3–4 readings of the background colour, moving the probe before taking each new reading. A white reference (WS-1) was measured after every four spectra for new calibration. Data from the spectrophotometer were passed into a PDA, where a OOIPS2000 software calculated reflectance spectra at 0.37 nm intervals. Reflectance ratios as estimates of blue-green chroma ($R_{400–580}/R_{320–700}$) were used in later analyses. Variation in blue green chroma was greater among

eggs than within eggs (great reed warbler: $F_{11,26} = 2.94$, P = 0.012, r = 0.38; cuckoo: $F_{10,30} = 10.74$, P < 0.001, r = 0.72), indicating that these measurements were repeatable.

Results

Comparison of egg characteristics between cuckoos and great reed warblers

Egg volume did not differ significantly between cuckoo and great reed warbler eggs, however, cuckoo eggs were more rounded than host eggs (Table 1). A comparison of eggshell thickness showed that cuckoo eggs had much thicker shells than great reed warbler eggs (Table 1). As predicted, we found that the pore density of cuckoo eggshells was higher than that of host eggshells (Table 1).

We also analysed whether yolk mass and composition differed between the host and brood parasite species. Our results showed that the yolk was significantly heavier in cuckoo eggs (Table 2). Yolk androstenedione and testosterone concentrations were lower in cuckoo eggs as compared to great reed warbler eggs (Table 2). Similarly, yolk immunoglobulin concentration was significantly lower in cuckoo eggs (Table 2). However, we found that total concentration of carotenoids was higher in cuckoo eggs (114.7 ± 36.7 μg/g) than in great reed warbler eggs (51.6 ± 13.4 μg/g; t = 5.11, df = 18, P < 0.001). Our results revealed that cuckoo eggs contained significantly higher concentration of lutein, zeaxanthin, and α-tocopherol, while great reed warbler eggs showed higher concentration of β-cryptoxanthin (Table 2). Our observation showed that the colour of great reed warbler yolks was orange, while it was yellow in cuckoo eggs.

Relationships between eggshell characteristics and egg composition

We also examined whether the blue-green background colour of the eggshell was associated with egg quality, and found no

Table 2. Comparison of egg yolk mass (g), yolk antioxidant concentration (μg/g) and androgen concentration (ng/g) between cuckoos and great reed warblers shown as means ± SD. The two species were compared by Student's t-test. Bold indicates that the effect was significant.

| Yolk composition	Great reed warbler	Cuckoo	t-value	n (great reed warbler)	n (cuckoo)	P-value
Yolk mass	0.61 ± 0.07	0.78 ± 0.09	−5.04	12	10	**<0.001**
Androstenedione	10.26 ± 3.19	5.38 ± 1.43	4.46	12	10	**<0.001**
Testosterone	20.65 ± 12.18	4.51 ± 2.54	4.10	12	10	**0.001**
Immunoglobulin	1329.96 ± 364.01	489.75 ± 73.46	6.39	9	8	**<0.001**
Lutein	15.52 ± 9.95	64.89 ± 48.34	−3.16	10	10	**0.010**
Zeaxanthin	2.48 ± 0.87	7.21 ± 5.75	−2.57	9	10	**0.029**
Beta-cryptoxanthin	22.33 ± 8.65	3.71 ± 1.90	6.96	11	10	**<0.001**
Beta-carotene	11.51 ± 8.13	38.87 ± 44.51	−1.91	11	10	0.086
Alpha-tocopherol	193.94 ± 86.44	415.05 ± 137.17	−4.40	11	9	**<0.001**
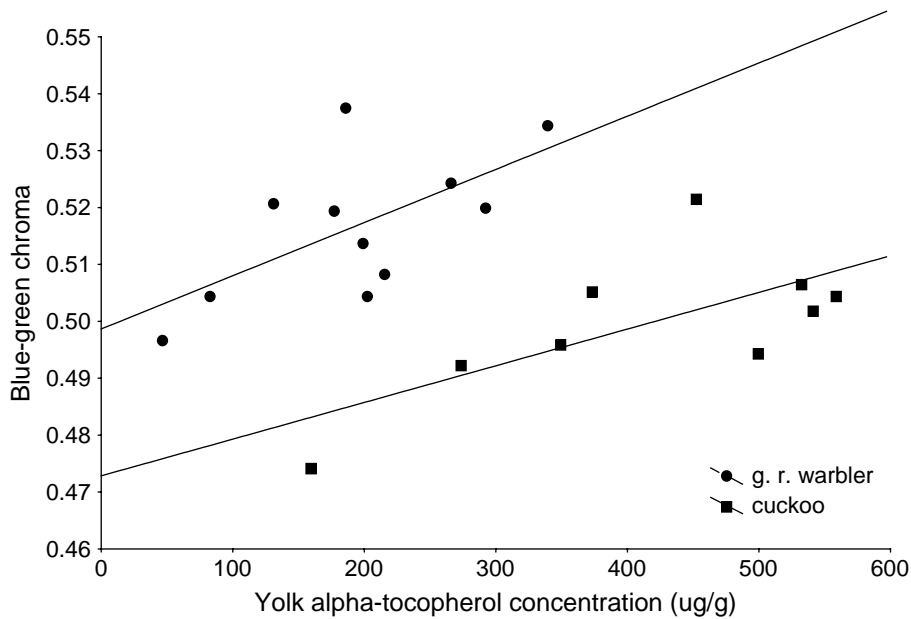
Figure 1. Relationship between yolk alpha-tocopherol concentration (µg/g) and eggshell blue-green chroma ($R_{400–580}/R_{320–700}$) obtained by Ocean Optics spectrophotometer of great reed warbler and cuckoo eggs. Sample sizes are 11 and 9 eggs, respectively.

significant associations between eggshell colour and yolk immunoglobulin and testosterone levels and yolk mass in either species (great reed warbler: immunoglobulin: $r = 0.20$, $n = 9$, $P = 0.61$; testosterone: $r = -0.31$, $n = 12$, $P = 0.32$; yolk mass: $r = 0.49$, $n = 12$, $P = 0.11$; cuckoo: immunoglobulin: $r = -0.26$, $n = 8$, $P = 0.54$; testosterone: $r = 0.12$, $n = 10$, $P = 0.74$; yolk mass: $r = 0.11$, $n = 10$, $P = 0.76$). However, blue-green chroma was marginally significantly positively related to β-cryptoxanthin ($r = 0.59$, $n = 11$, $P = 0.058$), and significantly to α-tocopherol concentrations ($r = 0.63$, $n = 11$, $P = 0.036$, Fig. 1) in great reed warbler eggs, and to β-cryptoxanthin ($r = 0.71$, $n = 10$, $P = 0.021$), β-carotene ($r = 0.74$, $n = 10$, $P = 0.015$), and α-tocopherol

($r = 0.69$, $n = 9$, $P = 0.041$, Fig. 1) concentrations in cuckoo eggs. Intensities of the blue-green background colour were not related to eggshell thickness in either species (great reed warbler: $r = 0.05$, $n = 12$, $P = 0.88$; cuckoo: $r = -0.04$, $n = 11$, $P = 0.91$).

We also analysed if there were significant relationships between yolk carotenoid and testosterone concentrations. We found that yolk β-carotene concentration was positively related to yolk testosterone concentration in great reed warbler eggs ($r = 0.66$, $n = 11$, $P = 0.027$, Fig. 2), but there was no significant correlation between concentrations of testosterone and any carotenoid types in cuckoo eggs ($| r | < 0.28$, $n = 10$, $P > 0.44$).
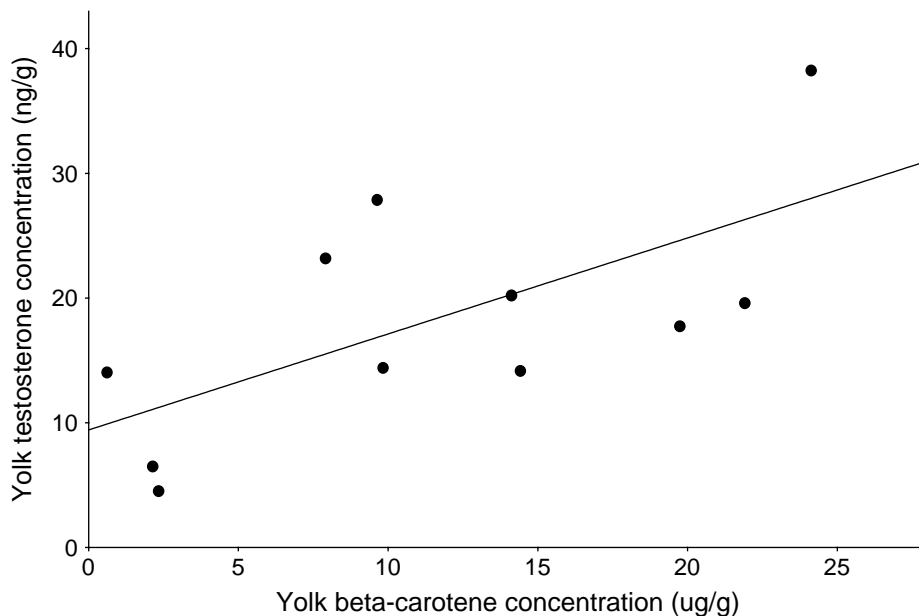

Figure 2. Relationship between yolk beta-carotene concentration (µg/g) and yolk testosterone concentration (ng/g) of great reed warbler eggs. Sample size is 11 eggs.

Discussion

Differences between cuckoo and great reed warbler eggs

Our results showed that yolk mass of cuckoo eggs was significantly higher than that of great reed warbler eggs, although egg volume did not differ (see also Török et al. 2004). Nestlings from eggs with larger yolks may grow more quickly as they have more nutrients available during embryonic growth and development (e.g. Parsons 1970, Blomqvist et al. 1997). Honza et al. (2001) reported that cuckoo hatchlings were heavier, had longer forearms and somewhat longer tarsi than great reed warbler hatchlings. Cuckoo females could thus increase the size, growth rate and viability of their young by providing them with more resources for embryonic development and improving their competitive abilities for ejection of host eggs and young. In addition, shell porosity was higher in cuckoo eggs, which may allow cuckoo embryos to develop more rapidly than hosts because of a greater capacity of oxygen consumption (Ar and Rahn 1985). Indeed, a negative association between eggshell porosity and incubation period has been reported in previous studies (Massaro and Davis 2004, Zimmermann and Hipfner 2007). Alternatively, it is also possible that the larger yolk and the higher number of shell pores of cuckoo eggs are related to phylogenetic differences between cuckoo and host species, and is not a consequence of an adaptation process to brood parasitism.

In agreement with our expectations, cuckoo eggs contained higher concentrations of carotenoids and vitamin E (α-tocopherol), which may provide protection against the oxidative stress associated with accelerated growth. Carotenoids and tocopherols are efficient antioxidants (Surai and Speake 1998, Surai et al. 1999), protecting the vulnerable, lipid-rich tissues of bird embryos from the attack of free radicals and other reactive oxygen metabolites (von Schantz et al. 1999). The concentration and profile of carotenoids depend to a large extent on the maternal dietary intake of these components (Bortolotti et al. 2003, Karadas et al. 2005), consequently it may vary according to foraging mode and habitat use (Cassey et al. 2005). Cuckoos mostly feed on caterpillars, while great reed warblers feed on insects, spiders, and snails captured in reeds (Avilés et al. 2007). It is possible that the diet of cuckoos is richer in carotenoids, or cuckoo females actively search for carotenoid-rich food items during egg formation. Great reed warbler eggs contained more β-cryptoxanthin, which could be accounted for some food component lacking from the cuckoo's diet. Relatively high level of β-cryptoxanthin was also encountered in the eggs of the American coot *Fulica americana* and the common moorhen *Gallinula chloropus*, which also prey on invertebrates near bodies of water (Surai et al. 2001). In contrast, the carotenoid profile of cuckoo eggs was similar to that of insectivorous passerines feeding mainly on caterpillars (Partali et al. 1987, Hargitai et al. 2006b). Probably this difference caused the redder yolk colour of great reed warber eggs, as the absorbance maximum of β-cryptoxanthin is higher (452 nm) than that of lutein (445 nm; Goodwin 1976).

In contrast to the prediction of Winkler (1993) and Gil (2003), we did not find higher concentration of yolk testosterone or androstenedione in brood parasite eggs as compared to host eggs. Similarly, in the brown-headed cowbird, no consistent differences were found in yolk testosterone level between the brood parasitic and host species (Hauber and Pilz 2003, Hahn et al. 2005). Our results strengthen the findings of an earlier study, which showed a tendency for cuckoo eggs to contain lower concentration of testosterone than host eggs (Török et al. 2004). These results therefore do not support that interspecific differences in competitive ability and growth rate of nestlings are related to variation in yolk testosterone concentration across species.

However, it is possible that target tissue sensitivity to hormones is more important in determining the phenotype (growth rate, begging behaviour) of hatchlings than the absolute amount of yolk androgens (Hauber and Pilz 2003). Comparative studies of target tissue sensitivity should be conducted so that we understand what are the effective species-specific levels of yolk androgens. In addition, it is possible that the costs of large amounts of yolk testosterone are higher for the brood parasitic than for the host species. Such adverse effects could be the immunosuppressive effects of yolk androgens on nestlings (Andersson et al. 2004, Müller et al. 2005, Navara et al. 2005). We may assume that the costs of immunosuppression are higher for cuckoo nestlings as cuckoo females cannot provide their offspring via the egg with maternal antibodies against the various pathogens and ectoparasites they may face in the host's nest. Indeed, we found lower level of yolk immunoglobulins in cuckoo as compared to host eggs.

Our results revealed that cuckoo eggs had a more rounded shape than great reed warbler eggs (see also Moskát and Honza 2002), but egg size was not different between the parasitic and host species (see also Honza et al. 2001, Török et al. 2004). These results suggest that cuckoo eggs adapted in size to host eggs to avoid that the host detects and rejects them (Marchetti 2000), and to improve incubation efficiency (Davies and Brooke 1988). This hypothesis is also supported by the correlation between the size of cuckoo and host eggs as demonstrated in comparative studies (Moksnes and Røskaft 1995, Krüger and Davies 2004). In addition, we found that cuckoos laid eggs that were more thickly shelled than those of their host, similarly to an earlier study of cuckoos (Honza et al. 2001), but in contrast to the results of Brooker and Brooker (1991). Likewise, Rahn et al. (1988) showed that shell thickness and shell mass were significantly larger in parasitic than in nonparasitic Icteridae species of equivalent egg mass. Cuckoos are known to lay eggs in two-day intervals (Davies 2000), so the slower passage of an egg allows the deposition of higher amount of eggshell constituents and the compression of the eggshell material in a more compact way, thereby increasing eggshell thickness and density (Picman and Pribil 1997). The increased strength of cuckoo eggs may be an adaptation to parasitism, precluding rejection of cuckoo eggs by small hosts through puncture ejection (Spaw and Rohwer 1987, Picman 1989, Antonov et al. 2006; but see Antonov et al. 2008), or making eggs more resistant to damage during rushed laying (Lack 1968, Soler and Martínez 2000). Alternatively, thicker eggshell of cuckoo eggs could be a residual characteristic of the originally larger cuckoo eggs before it evolved to its smaller

size, as nonparasitic cuckoos also have somewhat higher eggshell density than that of other non-passerine species (Picman and Pribil 1997).

Eggshell characteristics and yolk composition

In our study, biliverdin-based eggshell colour was unrelated to shell thickness similarly to the findings of a study of the sparrowhawk *Accipiter nisus* (Jagannath et al. 2008) and the spotless starling *Sturnus unicolor* (López-Rull et al. 2008). Moreover, in contrast to the results of Morales et al. (2006), blue-green eggshell colour was not related to yolk immunoglobulin concentration. However, we found that blue-green chroma was positively related to yolk α-tocopherol concentration in great reed warbler eggs, and to β-cryptoxanthin, β-carotene, and α-tocopherol concentrations in cuckoo eggs. Thus, eggshell colour may reflect the antioxidant investment by the female into her eggs and thus the quality of offspring. It is possible that the availability or the regulation and stimulation of the deposition of dietary antioxidants are affected by similar mechanisms as the deposition of eggshell biliverdin pigment. In a previous study of collared flycatchers *Ficedula albicollis* there was also a significant positive correlation between the intensity of blue-green colour and yolk lutein concentration (Hargitai et al. 2008), although in two *Turdus* thrushes only weak associations were reported (Cassey et al. 2008).

We found that concentration of yolk β-carotene was positively related to concentration of yolk testosterone in great reed warbler eggs. We suggest that females laying eggs with elevated testosterone concentration allocated more β-carotene to their eggs to counteract the potentially detrimental effects of steroids. Recently, it was demostrated that high level of plasma testosterone upregulated lipoprotein status and increased bioavailability of carotenoids (Blas et al. 2006, McGraw et al. 2006). Positive correlation between yolk carotenoid and testosterone concentrations was also shown in a study of collared flycatchers (Török et al. 2007) and house finches *Carpodacus mexicanus* (Navara et al. 2006). In cuckoo eggs, we could not find evidence for this relationship, which may be related to its relatively low testosterone and high carotenoid levels, with no need for compensatory adjustment. It is possible that the transfer of these components to the yolk is regulated by different processes across species, and cuckoos cannot interactively allocate these yolk compounds.

Conclusions

In summary, our data showed that cuckoo yolks were larger, contained greater concentration of dietary antioxidants, and the eggshell had higher pore density, which could be beneficial for the fast-growing brood parasitic embryo. However, steroid hormone and antibody levels were lower in cuckoo eggs, which may reflect that the costs of allocating these important biomolecules to eggs is higher for the cuckoo. Our results revealed a positive association between eggshell blue-green chroma and yolk antioxidant level, suggesting that eggshell colour reflects the antioxidant investment of the female. Although the found associations between eggshell characteristics and yolk composition are suggestive, similar work with higher sample size is necessary to definitely establish the link between these components. We encourage future research to compare yolk antioxidant and antibody levels and shell porosity of brood parasitic and host species to see if the differences observed in our study could be generalized.

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