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Blunt egg pole holds cues for alien egg discrimination: experimental evidence

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Eggshell colour patterns play a crucial role in avian host–parasite coevolution. In contrast to many experiments investigating *general* host egg discrimination abilities, studies testing *where* specific recognition cues are located on the eggshells (on blunt, sharp or both egg poles) are lacking. Previous studies suggested that discrimination cues might be located at the blunt egg pole, where the shell patterning is typically concentrated. We tested this hypothesis experimentally in species subject to interspecific (great reed warblers *Acrocephalus arundinaceus*, reed warblers *A. scirpaceus*), and also intraspecific parasitism (song thrushes *Turdus philomelos*, blackbirds *T. merula*). We examined host responses towards two types of intraspecific eggs painted non-mimetic immaculate blue either at blunt or sharp poles. All four species rejected eggs manipulated at the blunt pole at significantly higher rates, indicating that they perceive the critical recognition cues in the same egg part. Thus, the presence of egg recognition cues at the blunt egg pole may be a general phenomenon in birds parasitized by both intraspecific and interspecific parasites.

Avian eggshell colour and patterning serve various structural, signalling and thermoregulatory functions (reviewed in Kilner 2006). In the context of avian brood parasite–host coevolution, eggshell patterns allow hosts to recognize and reject parasitic eggs in their own clutches (Davies and Brooke 1989, Stokke et al. 1999, Kilner 2006). In hosts of the best studied Old World parasite, the common cuckoo *Cuculus canorus*, almost 4000 individual egg experiments have been done so far (Grim 2007, own unpubl. data). In a striking contrast to the large number of experiments investigating *general* host egg discrimination abilities, studies testing *where* specific recognition cues are located on the eggshells (on blunt, sharp or both egg poles) are rare both in hosts of the common cuckoo and other brood parasites.

Three previous studies suggested that egg discrimination cues are located at the blunt egg pole (hereafter BP) – where spotting is concentrated – rather than at the sharp egg pole (hereafter SP). However, two of those studies were only correlational (Lahti and Lahti 2002, Polačiková et al. 2007) and the third study (Polačiková et al. 2010) was based on data from only one model host species, the song thrush *Turdus philomelos*. However, in ecology "no isolated experiment, however significant in itself, can suffice for the experimental demonstration of any natural phenomenon" (Fisher R. A., cited in Kelly 2006). The robustness and generality of conclusions in ecology can be established solely by replication (Kelly 2006). To test the robustness of the conclusion that egg rejection cues are located at the BP we collected additional data for song thrushes to perform true exact replication of the above mentioned study (Polačiková et al. 2010). To test the generality of the conclusion we studied three additional taxonomical replicates: blackbirds *T. merula*, great reed warblers *Acrocephalus arundinaceus* and reed warblers *A. scirpaceus*. All four species are ideal for this kind of study because the patterning of their eggs is concentrated at the BP and varies between individual clutches (see Results).

All four species possess good recognition abilities showing intermediate or high rejection rates of alien eggs (Table 1). However, the origin of their ability to discriminate foreign eggs might be different. Previous studies suggested that *Acrocephalus* warblers discriminate foreign eggs as a result of interspecific coevolution with the common cuckoo whereas *Turdus* thrushes evolved their egg discrimination abilities within the context of *intra*specific parasitism (Stokke et al. 1999, Grim and Honza 2001, Hale and Briskie 2007). Thus, the present study also addresses the question of whether hosts of both interspecific and intraspecific parasites base their egg discrimination on cues located in the same egg parts, namely the BP.

We tested the "blunt egg pole" hypothesis experimentally by examining host responses towards two types of intraspecific eggs painted non-mimetic immaculate blue either at the BP or the SP. We predicted that manipulation of the BP would elicit higher rejection rates than manipulation of the SP because: (1) the eggshell speckling of all our study host species was concentrated at the BP (Fig. 1 and Results), and (2) previous works suggested that it was

Table 1. Review of the rejection rates against model eggs painted immaculate blue on their whole surface in four host species tested in this study. Data from the various host populations are ranked in ascending order. Rejection rates pool ejections and desertions. "This study" used experimental eggs painted at either the sharp (SP), or the blunt pole (BP). See Methods for details.

Host species	Rejection rate (%)	n	References
Song thrush	25.0	16	This study – SP
	27.3	11	Davies and Brooke 1989
	35.3	14	Samaš 2007*
	35.7	17	Samaš 2007†
	58.3	12	Grim and Honza 2001
	76.4	17	Honza et al. 2007
	77.8	18	This study – BP
Blackbird	33.3	15	This study – SP
	53.3	15	Samaš 2007*
	58.1	31	Samaš 2007†
	59.1	22	Davies and Brooke 1989
	66.7	6	Grim and Honza 2001
	75.0	16	This study – BP
Great reed warbler	21.4	14	This study – SP
	66.6	15	This study – BP
	90.9	44	Požgayová et al. 2009
	92.3	39	Dyrcz and Halupka 2007
	100.0	5	Brown et al. 1990
Reed warbler	27.3	11	Stokke et al. 2008‡
	30.8	13	This study – SP
	33.3	24	Stokke et al. 2008‡
	44.8	29	Stokke et al. 2008‡
	47.4	38	Stokke et al. 2008‡
	48.6	105	Dyrcz and Halupka 2007
	56.3	48	Stokke et al. 1999
	59.0	39	Stokke et al. 2008‡
	59.6	52	Stokke et al. 2008‡
	60.7	28	Davies and Brooke 1989
	64.3	14	Davies and Brooke 1988
	68.9	45	Stokke et al. 2008‡
	75.0	12	This study – BP

*Population sympatric with the cuckoo.
†Population allopatric with the cuckoo.
‡Data from 7 populations regularly parasitized by the cuckoo.

the BP rather than the SP where recognition cues were located (Polačiková et al. 2007, 2010).

Previous work also showed that all four species reject model eggs whose whole surface was painted immaculate blue (Table 1). Thus we predicted that rejection rates of the SP would be lower, whereas rejection rates of the BP would be higher than rejection rates of the overall blue model eggs used in the previous studies.

Methods

Study area

We collected data in forests nearby Brno (49° 11′N, 16° 36′E) and Dolní Bojanovice (48° 51′N, 17° 02′E), the Czech Republic in 2007–2008. In both localities, all study species occur in sympatry with the cuckoo. Both warbler species are parasitized by cuckoos at relatively high rates within our study populations (Stokke et al. 1999, Požgayová et al. 2009). Both thrushes are currently not parasitized by cuckoos (Grim and Honza 2001). We also did not record any cases of intraspecific parasitism in any of our model species during our study.

Experimental eggs and field procedures

We searched for nests and marked all eggs using waterproof ink to allow individual egg identification. We measured the eggs (length, L; breadth, B) to the nearest 0.1 mm and calculated egg volume $V = 0.51 \times \text{length} \times \text{breadth}^2$ (Hoyt 1979).

We artificially parasitized host nests by adding an experimental egg to the clutch during laying or incubation periods. We used fresh intraspecific eggs collected from abandoned clutches with the natural range of colouration including UV wavelengths. The intraspecific eggs did not differ significantly in size from host eggs in any host species (paired comparison of parasite egg vs. average host egg size per clutch; Wilcoxon's matched pairs tests: all $Z < 1.57$, all $P > 0.12$).

We used two types of experimental eggs that were divided into two approximately equal-sized parts across their diagonal axis. *A posteriori* analyses confirmed that the BP and SP regions had the same areas (Wilcoxon's matched pairs tests: all $Z < 1.73$, all $P > 0.08$). Each egg part was painted with an immaculate blue non-toxic colour (regarded as "non-mimetic" in previous studies of all four study species, see references in Table 1, Fig. 2).

Each active nest of all study species was experimentally parasitized only once and each experimental egg was used in only one experimental trial. Nests were checked for six days after experimental parasitism (following Moksnes et al. 1991). If the parasitic egg disappeared (ejection) or the clutch was abandoned (desertion) within this period, we considered it "rejected". We included nest desertion as a method of rejection, because all four species desert regularly checked control nests less than experimentally parasitized nests (Davies and Brooke 1988, Grim and Honza 2001, Bártol et al. 2002). If the parasitic egg and the host eggs all remained unharmed in the nest, we considered it "accepted".

Measurements of egg appearance

We measured 108 eggs from 28 song thrush clutches, 84 eggs from 29 blackbird clutches, 94 eggs from 28 great reed warbler clutches and 73 eggs from 25 reed warbler clutches. We photographed clutches using a Canon Power Shot A520 digital camera. All photos were taken on a neutral grey background card. All host and parasitic eggs were photographed along the long egg axis at the BP and the SP.

We quantified the appearance of both experimental and host eggs (BP and SP) using LUCIA G image analysis software (version 5.0, Lab. Imaging, Prague, Czech Republic; www.lucia.cz). First, each photograph was calibrated in real units (mm^2). Second, each measured area at the egg pole was defined by a measurement frame and mask image. Third, we selected a spot with the palest shade to define what areas should be included as spots. All image analyses were done by one of the authors (LP).

We used two variables representing eggshell spotting: "spottiness" (percentage of a cumulative area of all measured spots divided by the total measured area) and "spot density" (average number of spots per mm^2).

We quantified background appearance using image analysis software (see Soler et al. 2000). We evaluated three image parameters representing eggshell background using an IHS (Intensity, Hue, Saturation) system where values range from 0 (black object) to 255 (white object; for details see Pilin et al. 2007). Brightness was defined as the average value of brightness per measured area. Saturation was defined as purity of light per measured area. Hue was defined as the characteristic tint within the measured area. All these parameters were measured from 15 random locations for each egg pole in the area with no spots each covering 1 mm^2. We found significant repeatability among 15 measurements per each host egg for the BP and SP separately (Lessells and Boag 1987; spottiness: all $r > 0.20$, all $P < 0.05$; spot density: all $r > 0.43$, all $P < 0.001$; brightness: all $r > 0.70$, all $P < 0.001$; hue: all $r > 0.87$, all $P < 0.001$; saturation: all $r > 0.94$, all $P < 0.001$). Therefore, for each host clutch we calculated the average of each shell characteristic (separately for the two egg poles).

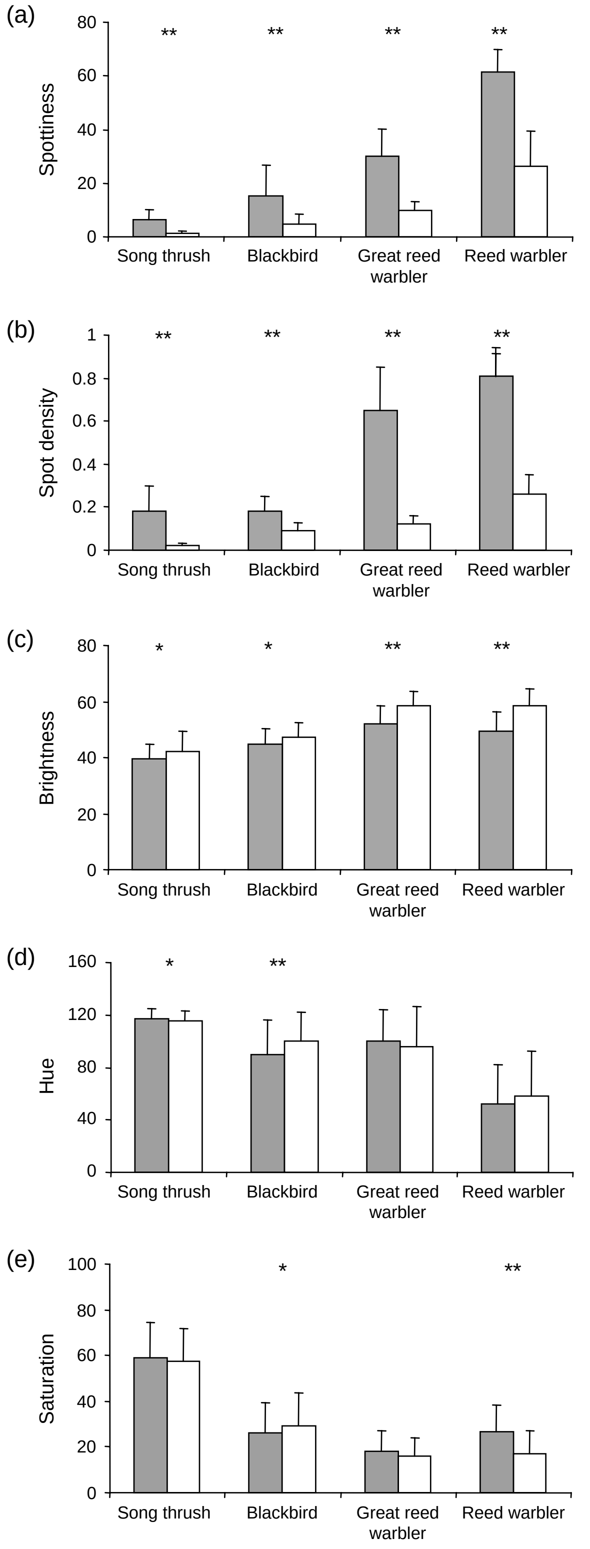

Figure 1. Within-egg between-pole variance (means+SD) in appearance of host own eggs. The differences between the blunt (full bars) and sharp poles (open bars) of host eggs in spottiness (a), spot density (b), brightness (c), hue (d), and saturation (e). For definitions and measurement details see Methods. Differences tested with Wilcoxon's matched pairs test. $^{**}P < 0.001$, $^{*}P < 0.05$. Sample sizes (clutch averages): song thrush = 29, blackbird = 27, great reed warbler = 28, reed warbler = 25.

Data analyses

First, we evaluated responses of study species towards two types of experimental eggs by a likelihood ratio chi-square test (G-statistic; Table 2). We then used logistic regressions to examine the effect of the egg type (nominal: painted BP vs. SP) on host response (nominal: acceptance vs. rejection) and also included potential confounders: year, locality, breeding stage (nominal: laying vs. incubation), laying date of the first egg, final clutch size, and mean volume of host eggs (continual variables). We used backward elimination of non-significant terms.

Some clutches were not photographed due to technical reasons, thus, sample sizes differ among analyses. Results are shown as mean ± SD, and statistical analyses were made in JMP and SPSS 15.0 (SPSS Inc.).

Results

Appearance of eggs

In all four study species, both spotting and background characteristics significantly differed between the BP and the SP of naturally laid eggs (Fig. 1). Specifically, spottiness and spot density were consistently and significantly higher at the BP (Fig. 1). Backgrounds of the BP were also significantly duller than backgrounds at the SP in all four species (Fig. 1). However, directions of differences in hue and saturation were less consistent across species and were not significant in all cases (Fig. 1).

Host responses towards egg models

All four species rejected experimental eggs painted blue at the BP at significantly higher rates than those painted at the SP (Table 2). In all four species, there were no significant differences in host responses between the experimental

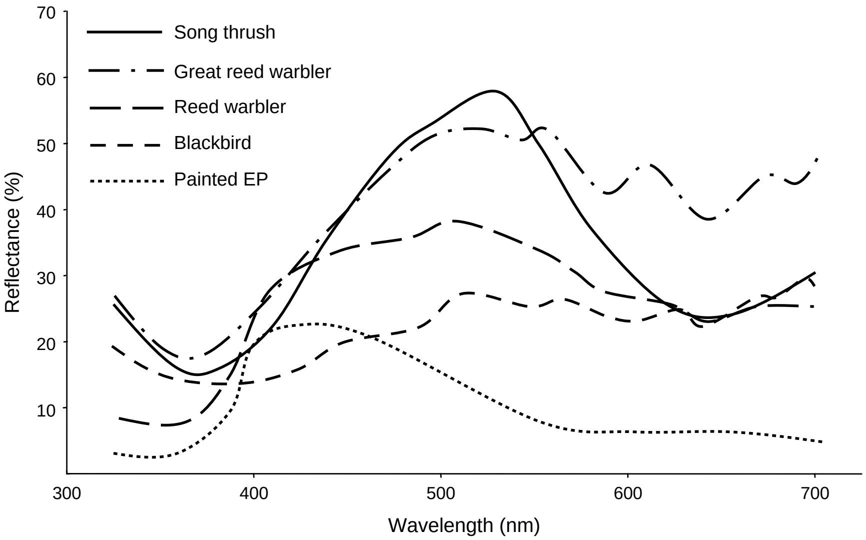

Figure 2. Representative reflectance spectra of all four study species and eggshell surface (ES) painted by non-mimetic blue colour (for each species and model eggs n = 5).

groups in which the parasitic egg was added during laying or incubation periods (Fisher's exact test: song thrush: $n_1 = 16$, $n_2 = 18$, $P = 0.44$; blackbird: $n_1 = 20$, $n_2 = 11$, $P = 0.71$; great reed warbler: $n_1 = 12$, $n_2 = 17$, $P = 0.72$; reed warbler: $n_1 = 13$, $n_2 = 12$, $P = 0.43$). Latencies to rejection of the BP and the SP were similar in all host species (Table 3). Logistic regressions showed that the effects of egg type on egg rejection were not confounded by any of the co-variables (see Methods). The egg type (egg painted at BP vs. SP) remained the only significant variable in all final models (Table 2).

Discussion

As predicted, experimental eggs with the blunt egg pole (BP) manipulated were rejected at higher rates than eggs manipulated at the sharp egg pole (SP) in all four model host species. Thus, BP characteristics might provide cues for egg discrimination in birds in general. There was a striking pattern when we put results of this study into the context of previous studies (Table 1). In almost all cases the rejection rates of SP eggs were lower than any previously reported rejection rates of whole blue eggs. In contrast, in almost all cases the rejection rates of BP eggs were higher than any of the many previously published rejection rates of plain blue eggs (Table 1). These comparative results further support the view that egg discrimination cues are located at the BP rather than the SP. The variability in frequency of the blue egg rejection between populations (Table 1) could be influenced by: (1) the fact that eggshell pigmentation also varies between different individuals within the same population, and (2) subtle differences in the composition of blue paints used in different studies, although all of them were regarded as "non-mimetic".

All four species rejected the experimental eggs painted at the BP at similar rates (Table 2) and with similar latencies (Table 3). Previous studies suggested that *Acrocephalus* warblers discriminate foreign eggs as a result of *inter*specific coevolution with the common cuckoo whereas *Turdus* thrushes evolved their egg discrimination abilities within the context of *intra*specific parasitism (Stokke et al. 1999, Grim and Honza 2001, Moskát et al. 2003). Therefore, in the case that rejection behaviour in the *Turdus* species evolved due to intraspecific parasitism, the hosts of both interspecific and intraspecific parasites might base their egg discrimination on cues located in the same egg parts, namely the BP.

Methodological issues

Acrylic paints used in both the present and previous studies have different spectral reflectances than natural eggshells (Fig. 2). This inability of artificial colours to closely mimic

Table 2. Avian responses (E – ejection; D – desertion) towards experimental conspecific eggs painted blue at the blunt (BP) or the sharp (SP) poles. Differences in overall rejection (ejection and desertion pooled) were tested with likelihood ratio chi-square test (G-statistic).

Species	BP			SP				
	E (%)	D (%)	n	E (%)	D (%)	n	G	P
Song thrush	61.1	16.7	18	25.0	0.0	16	9.95	0.002
Blackbird	50.0	25.0	16	20.0	13.3	15	5.59	0.018
Great reed warbler	53.3	13.3	15	14.3	7.1	14	6.25	0.012
Reed warbler	75.0	0.0	12	30.8	0.0	13	5.07	0.024

Table 3. Latency (in days) to egg rejection responses (ejection and desertion pooled) towards experimental conspecific eggs painted blue at the blunt (BP) or the sharp (SP) poles. Differences (mean ± SD) were tested with Mann-Whitney U-test.

Species	BP		SP		U	P
	Latency	n	Latency	n		
Song thrush	1.8 ± 1.2	14	2.1 ± 0.5	4	0.38	0.70
Blackbird	1.8 ± 0.9	12	2.5 ± 1.1	5	1.34	0.18
Great reed warbler	1.9 ± 1.4	10	2.0 ± 1.0	3	0.45	0.66
Reed warbler	1.9 ± 1.1	9	1.9 ± 1.1	4	0.00	1.00

natural egg phenotypes might pose problems for interpretation of results of interspecific comparisons (see interspecific variation in visual acuity in birds: Renoult et al. 2010). However, this issue is unlikely to affect the conclusions of the present study. This is because we compared differences in responses to: (1) eggs painted "in an unnatural way" at the BP, and (2) eggs painted "in an unnatural way" at the SP *within* particular host species. Regarding our hypothesis ("is it the blunt or sharp pole that holds the discrimination cues?") it does not matter what the particular cues for recognition are – we asked *where* the cues are, not *what* the cues are (or how do the birds perceive the colours of artificial paint).

Theoretically, the results of the present study could be a by-product of egg orientation in the nest cup: if the BP is the more visible part of the eggshell then manipulation of this would be more visible to nest owners. However, our study subjects had a chance to see both the BP and the SP because we defined BP vs. SP as *halves* of the eggs (see Methods). Thus, even if the long egg axis would be oriented under some steep angle (say 30 degrees), it would still be possible for birds to see at least part of both egg poles (as defined in the present work).

Further, egg anatomy might make it easier to detect any egg feature at the BP over the SP because the BP would provide hosts with a higher surface area with which to evaluate egg features. However, the surface areas of the BP and the SP did not differ statistically (see Methods).

Future directions

In all study host species, the eggshell spotting is focused at the BP and the SP is usually spotted sparsely. Comparative studies revealed that eggshell markings at the BP vary considerably among clutches in some bird species (Brown and Sherman 1989, Bischoff and Murphy 1993). Birds use BP cues for egg recognition as suggested by both correlative (Lahti and Lahti 2002, Polačiková et al. 2007) and experimental studies (present study, Polačiková et al. 2010). Therefore, the colour pattern located at the BP and not at the SP might be characteristic for a female bird and serve as a "fingerprint" of individual females, facilitating foreign egg recognition. This hypothesis provides an impetus for future research in this so far neglected area.

To conclude, the presence of egg recognition cues at blunt egg poles may be a general phenomenon in birds. In addition, it seems that hosts of both intraspecific and interspecific parasites might use recognition cues located in the same egg parts. In the future, studies experimentally manipulating background and spotting in the blunt egg part are necessary to reveal what are the specific cues for egg discrimination in birds.

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