



## Anti-parasitic egg rejection by great reed warblers (*Acrocephalus arundinaceus*) tracks differences along an eggshell color gradient

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### ARTICLE INFO

#### Keywords:

Brood parasitism  
Color  
Defense  
JND  
Maculation  
Metareplication  
Perception

### ABSTRACT

One of the most effective defenses against avian brood parasitism is the rejection of the foreign egg from the host's nest. Until recently, most studies have tested whether hosts discriminate between own and foreign eggs based on the absolute differences in avian-perceivable eggshell coloration and maculation. However, recent studies suggest that hosts may instead contrast egg appearances across a directional eggshell color gradient. We assessed which discrimination rule best explained egg rejection by great reed warblers *Acrocephalus arundinaceus*, a frequent host to an egg-mimetic race of common cuckoos *Cuculus canorus*. We deployed 3D-printed model eggs varying in blue-green to brown coloration and in the presence of maculation. Using visual modeling, we calculated the absolute chromatic and achromatic just-noticeable differences (JNDs), as well as directional JNDs across a blue-green to brown egg color gradient, between host and model eggs. While most model eggs were rejected by great reed warblers, browner eggs were rejected with higher probability than more blue-green eggs, and the rejection probability did not depend on maculation. Directional egg color discrimination shown here and in a suite of recent studies on other host species may shape the cognitive decision rules that hosts use to recognize foreign eggs and affect the course of evolution in parasitic egg mimicry.

### 1. Introduction

In obligate avian brood parasitism, host adults may forgo the costs of providing parental care for genetically unrelated parasitic young by rejecting foreign eggs from the nest (Rothstein, 1990). This system has been used as a model for animal cognition in the wild, with major research efforts focusing on the cues that hosts use to recognize and discriminate against foreign eggs (e.g., size, color, and/or maculation of eggs; Grim et al., 2011; Stoddard and Stevens, 2011; Stokke et al., 1999) and on the means by which hosts reject eggs (e.g., grasp or puncture egg ejection, nest desertion; Hauber et al., 2014; Hosoi and Rothstein, 2000). These experiments have provided critical insights into the sensory mechanisms and the cognitive decision rules used to recognize and reject parasitic eggs (reviewed by Manna et al., 2017).

Until recently, most cognitive models of foreign egg recognition have been built on the assumption that hosts use templates (memorized or innate) to compare their own eggs with potential foreign eggs to

assess host-parasite egg similarity (Hauber et al., 2015; Stoddard and Stevens, 2011). The eggs that fall beyond a specific acceptance threshold are rejected (Brooke et al., 1998; Reeve, 1989). Empirical data have been largely, but not unanimously, supportive of this model, providing experimental and statistical support for rejection decisions based on the absolute dissimilarity between own and foreign stimuli (Ruiz-Raya et al., 2015; Stevens et al., 2013).

Recently, however, an alternative model has been proposed, which suggests that hosts make egg rejection decisions using color categorization rather than evaluating the absolute differences between their own and potentially parasitic eggs (Hanley et al., 2017, 2019). This model predicts that a single acceptance/rejection threshold, falling along the color gradient of the natural diversity of avian eggs (Hanley et al., 2015), drives egg rejection decisions, in contrast to the models that assume discrimination in response to absolute foreign vs. own egg color differences, which predict multiple thresholds. Testing these two alternative models requires the use of egg colors that vary widely along

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the natural avian blue-green to brown eggshell color gradient in both directions, centered around the host egg's coloration (Canniff et al., 2018). The two alternative egg rejection models have now been tested in three different host-brood parasite systems (Hanley et al., 2017, 2019). These recent studies demonstrated that a single threshold based on directional egg color differences explains host egg rejection behavior statistically better than do the traditional multiple-threshold models, and, critically, showed that the location of the rejection threshold may depend on the maculation of the eggs (Hanley et al., 2019). Two of the systems in which these conceptual models have been tested include the hosts of obligate parasitic molothrine cowbirds. Testing these predictions across different parasite-host systems (i.e., metareplication, see Grim et al., 2011) is critical, because various host species may differ in the cognitive mechanisms that regulate their egg rejection behavior, which may depend on the host-parasite coevolutionary dynamics and/or species differences in the neural and other physiological substrates that regulate cognition and behavior (Abolins-Abols and Hauber, 2018).

To date, no published study has examined whether the directional discrimination mechanism explains egg rejection behavior in hosts of a mimetic race (gens) of the common cuckoo (*Cuculus canorus*). Common cuckoos are obligate brood parasites with diverse gentes that lay highly mimetic parasitic eggs in specific host species (Stoddard and Stevens, 2011). Cuckoo hosts, in turn have often evolved the ability to recognize and reject even highly mimetic eggs (Davies, 2000). Testing whether hosts of mimetic parasites use single or multiple-threshold discrimination rules, and whether these rejection thresholds are affected by egg maculation in such host-parasite systems, will allow us to better understand the generality of the single-threshold decision rule.

Here we test whether great reed warblers (*Acrocephalus arundinaceus*), a common cuckoo host with an intermediate rejection rate of naturally strongly-mimetic parasitic eggs (Moskát and Honza, 2002), use directional differences (single threshold) or absolute differences (multiple thresholds) when rejecting model eggs that vary in their ground color (the base color of the eggshell, excluding maculation) along the natural blue-green to brown gradient of avian eggshells as well as in the presence vs. absence of experimental maculation (spotting).

## 2. Materials and methods

### 2.1. Study area and field methods

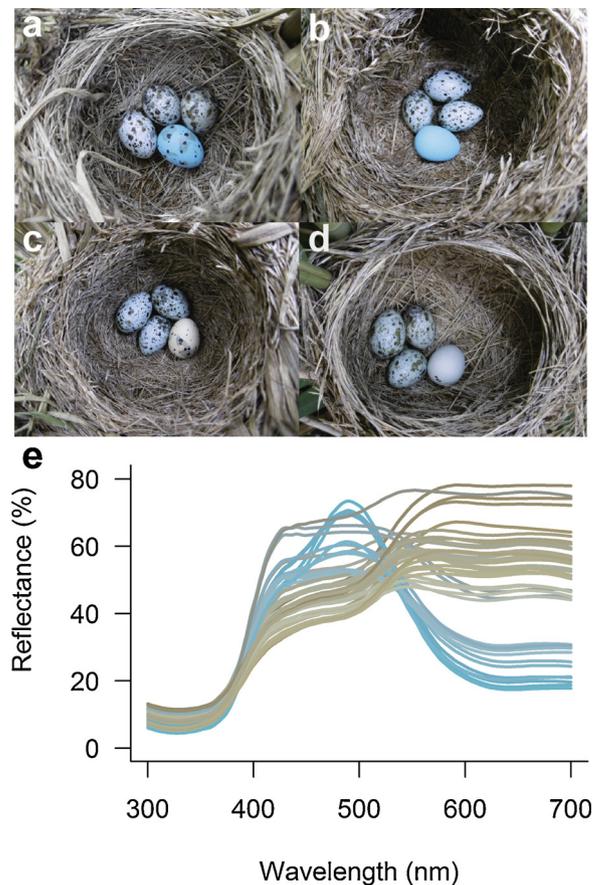
Our study was conducted with permission from The Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management, Budapest, Hungary (permit No. PE/KTF/17190-3/2015). The study took place in central Hungary around the village of Apaj (47°07' N, 19°06' E) in 2016 and 2017 between early May and early June, which is the peak of the great reed warblers' breeding season (Moskát et al., 2006). At Apaj, great reed warblers breed in reed beds along small irrigation channels and are parasitized by common cuckoos at a high rate (ca. 50%; Zölei et al., 2015). Cuckoo eggs show strong mimicry of host eggs when analyzed using the avian visual system (< 1 chromatic JND; Iqic et al., 2012) and are rejected naturally by this host species at a relatively low rate of ca. 30% (Moskát et al., 2009; Moskát and Honza, 2002; Zölei et al., 2015).

We searched for host nests twice a week, focusing on nests in the building stage. Once nests were discovered, we monitored their suitability for experimentation daily. We parasitized clutches using model eggs on the day when the 3<sup>rd</sup> host egg was laid. This corresponds to the middle of their laying stage (the modal clutch size in this population is 5 eggs, range 4–6), and cuckoos almost exclusively parasitize host nests during the great reed warbler's laying stage (Moskát and Honza, 2002).

We experimentally parasitized only those host nests that were not naturally parasitized by cuckoos during that breeding attempt, because host responses to foreign eggs might be weaker in multiply parasitized

nests (Moskát et al., 2009), and because hosts may show stronger responses to parasitism if a parasitic egg had previously been rejected from the nest (Hauber et al., 2006). We also did not remove a host egg when adding a model egg because our unpublished data from 1998 to 2000 showed that adding an experimental egg or replacing a host egg did not influence host responses to a non-mimetic model cuckoo egg (egg added: 13 out of 17 rejected; egg replaced: 18 out of 26 rejected; 2-tailed Fisher's exact test:  $p = 0.74$ ).

To experimentally parasitize warbler nests, we used 3D-printed, polished nylon plastic model eggs, which were sourced from Shapeways.com (the "Cow Bird" model egg; Iqic et al., 2015). These eggs closely resemble natural common cuckoo eggs in size (model egg dimensions, length: 23 mm; width: 18 mm; cuckoo egg dimensions (mean  $\pm$  standard error) length:  $22.26 \pm 0.09$  mm; width:  $16.57 \pm 0.06$  mm; Moskát and Honza, 2002) and weight (model egg weight after painting:  $3.35 \pm 0.02$  g; cuckoo eggs:  $3.40 \pm 0.06$  g; Grim et al., 2009). We used high-quality acrylic paints (see Canniff et al., 2018, and Hanley et al., 2019, for information on paint types) to paint model eggs along the blue-green to brown gradient of natural avian eggshell coloration (Hanley et al., 2015), which included the mean egg coloration of the great reed warbler eggs (from Hauber et al., 2015; see below). We generated two eggs per each color (a total of 29 different colors between across the blue-green to brown color gradient), to one of which we then added spotting, while the other remained immaculate (plain, Fig. 1). The immaculate and maculated model eggs with similar ground colors were used to parasitize different host nests on the same day or on consecutive days, thus limiting experimental date as a potential confounding variable. The ground color of model eggs



**Fig. 1.** Model eggs differing in coloration across the blue-green to brown gradient and spotting: a & b: host great reed warbler nest with either a) spotted or b) plain blue-green model cuckoo egg; c & d: great reed warbler nest with either c) spotted or d) plain brownish model cuckoo egg; e: reflectance spectra of model eggs. Photo credit: István Zsoldos.

added to nests was randomized within and between the two breeding seasons of the study.

We monitored the experimentally parasitized nests daily for up to 6 consecutive days to determine host responses (Grim et al., 2011). We categorized host responses either as *acceptance* if the parasitic egg was in the nest at the end of the monitoring period or *rejection* if the parasitic egg disappeared from the nest but all or some of the host eggs remained in the nest and were being incubated (*i.e.*, warm). No nests were deserted during the course of this study, but 14 out of 60 experimental nests were depredated. Out of the 14 depredated nests, 3 had more blue-green model eggs compared to hosts, while 11 had browner model eggs. Data from depredated nests (disappearance of the whole clutch, or nests with cold, broken eggs) were excluded from the analyses. Model eggs from some of the depredated nests were cleaned and reused in other active nests.

## 2.2. Color analyses

We collected and summarized the avian visible (300–700 nm) reflectance spectra of the ground colors from each experimental egg, as well as from eight natural host eggs collected from abandoned clutches (Hauber et al., 2015), using locally weighted Gaussian second-degree polynomial regression. We modeled great reed warbler visual perception by calculating photoreceptor quantum catch using the UV-sensitive blue tit's (*Cyanistes caeruleus*) photoreceptor sensitivity (Hart, 2001) and density estimates (Hart et al., 2000), based on a D65 standard illuminant. We then calculated the perceivable chromatic (differences based on color hue) and achromatic (differences based on color darkness) differences between host and model eggs using receptor noise-limited visual models accounting for neural noise (Siddiqi et al., 2004; Vorobyev et al., 1998; Vorobyev and Osorio, 1998). This generated estimates of discriminability in just-noticeable-difference (JND) units, such that values less than one would not be noticeable, a value of one would be noticeable under ideal conditions, and the likelihood of discrimination would increase proportionally with JNDs above one.

To test which cognitive model great reed warblers use to differentiate between own and parasitic eggs, we calculated three different JND scores. First, because the natural color of most bird eggs has been shown to fall on a gradient from blue-green to brown (Hanley et al., 2015), we classified directional chromatic contrast based on its relative position to the hosts' own egg color (*i.e.*, more blue-green or browner). Specifically, if the color of the model egg fell on the blue-green side of the average host color, its chromatic contrast was multiplied by  $-1$ , but if it fell on the brown side of the average host color it was multiplied by  $1$  (hereafter directional JND). Second, we also calculated chromatic and achromatic JNDs based on the absolute difference between host species' own and the model eggs. Because chromatic and achromatic JNDs represent the absolute perceivable difference in color (chromatic JND) or darkness (achromatic JND) without assigning directionality to that difference (*e.g.*, a parasitic egg that is bluer than the host egg can have the same chromatic JND as a browner parasitic egg), they are necessarily all non-negative values.

Despite our best efforts, we were unable to generate model eggs that matched the chroma of the ground color of natural host eggs within 2 chromatic JNDs using the acrylic paints (Fig. 2). However, because in this host species the variation in conspecific egg coloration does not exceed 2 JNDs (Hauber et al., 2015), this ensured that model eggs that were more blue-green than the average host egg were always more blue-green than the focal individual's clutch, and that model eggs that were browner than the average host egg were always browner than the focal individual's clutch.

## 2.3. Data analyses

We used a binomial generalized linear model (GLM) approach in R (R Core Team, 2017) to quantify which aspects of egg color best

explained variation in the egg rejection behavior by hosts. The three measures of contrasts between host and model eggs were positively correlated with each other (directional JNDs across the blue-green to brown gradient and achromatic JNDs: Spearman's  $r = 0.69$ ;  $p < 0.01$ ; directional JNDs and chromatic JNDs:  $r = 0.55$ ;  $p < 0.01$ ; achromatic JNDs and chromatic JNDs:  $r = 0.24$ ,  $p = 0.07$ ). Therefore, to avoid issues related to the collinearity of the predictor variables, we ran separate models to investigate which of the contrasts best statistically explained host egg rejection patterns.

Specifically, we constructed three separate GLMs using base R function *glm* that predicted host behavior (acceptance vs rejection) using either directional JNDs (continuous), chromatic JNDs (continuous), or achromatic JNDs (continuous). Each model also included egg maculation (categorical) as a fixed effect. We did not include either date or year in our models, because the experiment was fully randomized within and between breeding seasons, and neither date (Moskát et al., 2014) nor year (Zölei et al., 2015) predicted egg rejection behavior in this species. Competing models were evaluated based on their AIC<sub>c</sub> weights, calculated using the *MuMIn* package (Bartoń, 2018), and compared to the null model with likelihood ratio tests using the package *lmerTest* (Zeileis and Hothorn, 2002) to determine the model that best explained variation in host rejection behavior.

## 3. Results

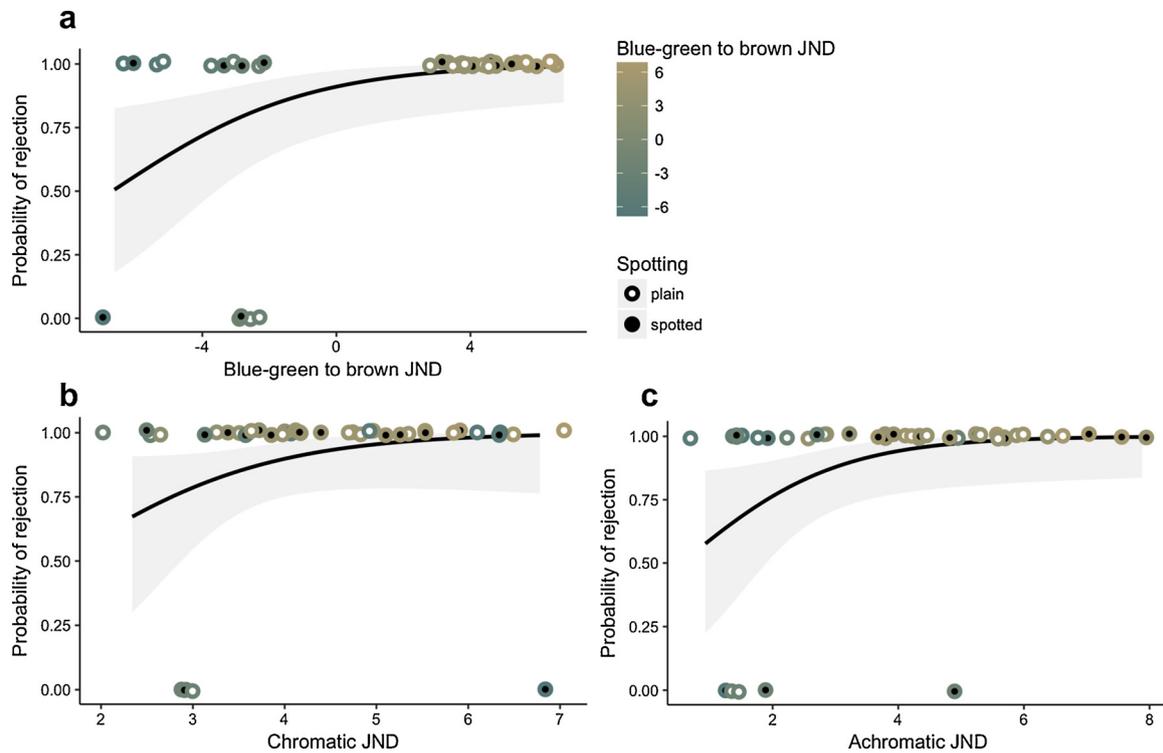
Great reed warblers rejected most (89%) model eggs (41 out of 46). Out of 41 rejected eggs, 85% (35 eggs) were rejected within a day, while 12% (5) were rejected within 2 days, and 2% (1) were rejected within 3 days. Out of the three statistical models tested, the probability of egg rejection was best explained by model that included directional JNDs between host and model eggs across the blue-green to brown gradient (Table 1): more blue-green eggs were significantly more likely to be accepted than browner eggs (Fig. 2a). Achromatic absolute JNDs were also significantly related to the probability of egg rejection (Table 1, Fig. 2c), but the model including the achromatic JNDs was less informative than the model investigating directional blue-green to brown JNDs ( $\Delta AIC_c > 2.0$  between these two models, Table 2). The chromatic JNDs did not explain significant variation in egg rejection, and this model also showed  $\Delta AIC_c > 2.0$  compared to the top model (Table 1, Fig. 2b). Likelihood ratio tests indicated that the directional JND model as well as achromatic JND model were significantly better than the null model (Table 2).

The presence (spotted) or absence (plain) of maculation did not explain the probability of egg rejection in any of these models (Table 1).

## 4. Discussion

Great reed warblers have been classified as intermediate rejecters of mimetic parasite eggs: ca. 30% of naturally laid common cuckoo eggs are rejected in this population (Moskát and Honza, 2002). In our study population, the species also rejects the vast majority (ca. 70–100%) of artificial model eggs, even when painted to resemble the host eggs (*e.g.*, Bártol et al., 2002; Honza and Moskát, 2008).

Here we used spotted and plain model eggs along a natural avian blue-green to brown eggshell color gradient to assess which of the three color contrasts best explain egg rejection behavior in a cuckoo host. As we noted above, however, we were unable to generate model eggs that matched the ground color of natural host eggs within 2 JNDs. Perhaps as a result, only five model eggs were accepted for at least six days in our study. Nevertheless, egg rejection behavior by great reed warblers was best predicted by directional blue-green to brown difference between warbler and model egg color as opposed to the absolute difference in egg color – warblers accepted some dissimilar eggs that were more blue-green, but always rejected equally dissimilar eggs (*i.e.*, an absolute distance in the avian visual space) that were browner than their own eggs. This result corroborates findings of Hanley et al. (2017,



**Fig. 2.** The probability of model egg rejection in relation to color contrasts and spotting. a: egg-rejection in relation to directional JNDs across the blue-green to brown color gradient; b: egg-rejection in relation to chromatic JNDs; c: egg-rejection in relation to achromatic JNDs. The line indicates the probability of rejection across different egg colors, calculated using a model that included only each specific JND type as a predictor. Shaded area indicates 95% confidence interval for the best fit line. The outline of each datapoint reflects the model egg color relative to the average warbler egg: blue-green circles are more blue-green model eggs, while brown circles represent browner eggs. Circle fill indicates if the model eggs were plain (no fill) or spotted (dark fill).

**Table 1**

The effect of color contrasts and spotting on model egg rejection. Subheadings indicate the type of JND tested and the overall GLM model statistics compared to null model. We report the parameter estimates along with their standard errors and z-scores. Factors that explain significant ( $p < 0.05$ ) amount of variation in egg rejection are in bold.

Model	Factor	Estimate	SE	z	p
Directional JND ( $\chi^2 = 8.79$ , $R^2 = 0.35$ , $AIC_c = 29.41$ , $n = 46$ , $p = 0.01$ )					
	Intercept	3.51	1.16	3.02	< 0.01
	<b>Blue-green to brown JND</b>	1.49	0.62	2.38	<b>0.02</b>
	Spotting	- 0.76	1.10	- 0.69	0.49
Chromatic JND ( $\chi^2 = 4.02$ , $R^2 = 0.17$ , $AIC_c = 34.18$ , $n = 46$ , $p = 0.13$ )					
	Intercept	2.93	0.97	3.01	< 0.01
	Chromatic JND	1.19	0.69	1.72	0.09
	Spotting	- 0.61	1.03	- 0.60	0.55
Achromatic JND ( $\chi^2 = 6.73$ , $R^2 = 0.27$ , $AIC_c = 31.47$ , $n = 46$ , $p = 0.03$ )					
	Intercept	3.38	1.14	2.96	< 0.01
	<b>Achromatic JND</b>	1.54	0.74	2.09	<b>0.04</b>
	Spotting	- 0.77	1.07	- 0.71	0.48

**Table 2**

Comparison of  $AIC_c$  scores and model weights between competing models testing the effect of color contrasts and spotting on egg rejection; “+ 1” denotes the intercept.

Model	$AIC_c$	$\Delta AIC_c$	weight
Egg rejection = directional JND + spotting + 1	29.41	-	0.64
Egg rejection = achromatic JND + spotting + 1	31.47	2.06	0.23
Egg rejection = 1	33.72	4.31	0.07
Egg rejection = chromatic JND + spotting + 1	34.18	4.77	0.06

2019), who demonstrated that three other host species reject increasingly dissimilar brown eggs but accept equally dissimilar blue-green eggs.

Achromatic contrasts also showed a significant positive relationship with the probability of model egg rejection by great reed warblers. However, according to the  $AIC_c$  comparison, this model was less informative than the model including blue-green to brown JNDs. Importantly, a previous study revealed high achromatic contrast between cuckoos and great reed warblers in Hungary (Cherry et al., 2007), much higher than in a similar cuckoo-host system in Japan (Moskát et al., 2012). This suggests that hosts may indeed use difference in the egg darkness as a cue when making decisions about egg rejection. We suggest that future studies should better distinguish (and independently experimentally manipulate) between directional, chromatic, and achromatic differences in this and other cuckoo-host systems.

Whereas eggs that were more blue-green were more likely to be accepted, blue-green eggs were nevertheless also often rejected. The predicted acceptance of eggs did not reach 50% at any point along the blue-green to brown color range (Fig. 2), which prevents us from pinpointing the precise location of the color acceptance threshold in this host species. Both spotted and immaculate eggs were accepted, suggesting that in this host species, spotting does not override egg rejection decisions based on ground coloration. This is in contrast with studies in a different brood parasitic study system, where hosts lay immaculate eggs and are more likely to reject spotted eggs (Dainson et al., 2017; Dinets et al., 2015). Our results are also in contrast with a recent study in yet another brood host-parasite system, where hosts lay spotted eggs but the parasites can lay both immaculate or spotted eggs; in this system, hosts have been shown to accept more dissimilar browner model eggs when they are spotted (Hanley et al., 2019). However, the effect of maculation on host behavior is likely driven by more than simply its presence or absence, and it most likely depends on several

parameters, such as spot size, density, distribution, and color (Cherry et al., 2007; Dainson et al., 2017; Li et al., 2016; Stoddard and Stevens, 2010). For example, a previous study in our study population revealed that the efficiency of foreign egg rejection by great reed warblers was reduced when small artificial spots on host eggs were used to reduce clutch uniformity (Moskát et al., 2008).

The perceptual decision rules that hosts use to reject parasitic eggs have important potential consequences for parasite-host arms races. Specifically, host decision rules shape the fitness landscape for parasitic egg colors. In this system, cuckoos that lay more blue-green eggs than hosts may experience lower egg rejection rates than cuckoos that lay browner eggs. This predicts that cuckoo eggs in this population should be, on average, biased towards being more blue-green. This is a prediction that is yet to be tested in great reed warblers, but a comparative analysis of 25 European cuckoo hosts, including 3 congeneric reed warblers (*Acrocephalus* spp.), reported that hosts of the blue-green egg-laying cuckoo host race have lower egg rejection rates of model cuckoo eggs (Soler et al., 2012). If parasites indeed evolve to exploit a single-threshold directional discrimination, this may compromise the ability of hosts to reject parasitic eggs. However, it remains unclear if these decision rules have arisen by natural selection or are a result of a perceptual constraint.

In summary, our findings suggest that great reed warblers parasitized by common cuckoos use differences across a natural blue-green to brown color gradient to differentiate between own and parasitic eggs. However, we could not exclude the possibility that warblers may also be differentiating between eggs based on differences in egg color darkness. Overall, these results support previous conclusions in this species that show that great reed warblers can use specific aspects of egg appearance to make adaptive decisions about rejecting parasitic cuckoo eggs from their nests. The specific decision rules used by hosts to reject parasitic eggs have important consequences for host-parasite co-evolutionary dynamics, and we suggest that further studies in this and other systems should focus on what rules hosts use to reject parasitic eggs, and how (or if) these rules evolve. Future research should also address how the neural integration of color perception may drive the evolution of cognitive decision rules demonstrated in this and other host-parasite systems.

## Acknowledgements

We thank István Zsoldos for the assistance with fieldwork. We also thank anonymous referees for their comments.

Funding for this project was provided by a Human Frontier Science Program award (to TG and MEH), the European Social Fund and the state budget of the Czech Republic, project no. CZ.1.07/2.3.00/30.0041 (to TG and DH), and by the National Research, Development and Innovation Office, Hungary (OTKA No. NN118194 to CM). Additional support was provided by the Harley Jones Van Cleave Professorship of the University of Illinois and funds from Palacky University.

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