Anim Cogn (2013) 16:819–828
DOI 10.1007/s10071-013-0615-1

ORIGINAL PAPER

Egg arrangement in avian clutches covaries with the rejection of foreign eggs

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Received: 18 September 2012 / Revised: 24 January 2013 / Accepted: 5 February 2013 / Published online: 27 February 2013

Abstract In birds, the colour, maculation, shape, and size of their eggs play critical roles in discrimination of foreign eggs in the clutch. So far, however, no study has examined the role of egg arrangement within a clutch on host rejection responses. We predicted that individual females which maintain consistent egg arrangements within their clutch would be better able to detect and reject foreign eggs than females without a consistent egg arrangement (i.e. whose eggs change positions more often across incubation). We tested this "egg arrangement hypothesis" in blackbirds (*Turdus merula*) and song thrush (*T. philomelos*). Both species are suitable candidates for research on egg rejection, because they show high inter-individual variation and individual repeatability in egg rejection responses. As predicted, using our custom-defined metrics of egg arrangement, rejecter females' clutches showed significantly more consistent patterns in egg arrangement than acceptor females' clutches. Only parameters related to blunt pole showed consistent differences between rejecters and acceptors. This finding makes biological sense because it is already known that song thrush use blunt pole cues to reject foreign eggs. We propose that a disturbance of the original egg arrangement pattern by the laying parasite may alert host females that maintain a consistent egg arrangement to the risk of having been parasitized. Once alerted, these hosts may shift their discrimination thresholds to be more restrictive so as to reject a foreign egg with higher probability. Future studies will benefit from experimentally testing whether these two and other parasitized rejecter host species may rely on the use of consistent egg arrangements as a component of their anti-parasitic defence mechanisms.

Keywords Anti-parasite defences · Brood parasitism · Egg arrangement · Foreign egg recognition

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Introduction

Birds have evolved acute perceptual and memory systems and are able to make fine-tuned behavioural or physiological decisions through the use of visual signals (Shettleworth 2001). Specifically, many birds use diverse cues to recognize and remove foreign eggs from their nests and, thus, prevent the usually severe costs of providing care for unrelated brood parasites (Marchetti 2000; Hauber 2003; Moskát et al. 2008).

The recognition of foreign eggs by hosts of brood parasites may be based on differences in particular colours (Honza et al. 2007), maculation (Moskát et al. 2010), or size (Marchetti 2000) between host and parasite eggs within a clutch or a combination of those traits (de la Colina et al. 2012). The eggs' positions are critical in successful incubation (Boulton and Cassey 2012) and foreign, or otherwise less preferred eggs, may be pushed to the margins of the clutch (Lyon 2007). However, no study has considered whether, and how, the arrangement of eggs in the clutch affects a birds' ability to recognize its own and thus reject parasitic eggs.

Here, we propose an "egg arrangement hypothesis", stating that the consistent arrangement of eggs in a clutch may facilitate the detection of foreign eggs. This is because disruption of a temporally consistent pattern may alert hosts that a parasitic female has laid her egg into the host nest. The mechanism behind this could be that the replacement, or the addition, with a new egg inevitably changes the relative positions of eggs originally present in the nest cup. In contrast, a host that experiences variable, or changing, pattern of egg arrangement (e.g. due to her inability to keep eggs arranged in a consistent manner), even in the absence of parasitic egg laying, is less likely to notice that her nest was parasitized. Based on these assumptions, we predicted that hosts which reject foreign eggs would experience more consistent patterns of egg positions than hosts which accept foreign eggs.

We tested the egg arrangement hypothesis in blackbirds (*Turdus merula*) and song thrush (*T. philomelos*) which show high inter-individual variance in responses to foreign eggs (approx. 50 % egg rejection rate of non-mimetic immaculate blue eggs across many tested populations: Polačiková and Grim 2010; Grim et al. 2011; Samaš et al. 2011). Both species likely evolved foreign egg ejection in the context of conspecific brood parasitism (Hale and Briskie 2007; Polačiková and Grim 2010; Grim et al. 2009; Samaš et al. 2011). In addition, both species show very high individual repeatability of egg rejection responses against such egg types (Samaš et al. 2011). This is an important pre-requisite for tests of the egg arrangement hypothesis because it would be biologically unjustified to look for associations between *consistency* of egg arrangement and host egg rejection responses that were *not* highly repeatable (i.e. consistent). Interestingly, both species use blunt egg pole characteristics when recognizing foreign eggs (Polačiková and Grim 2010; Polačiková et al. 2010, 2011). These inter-specific patterns of behaviourally relevant similarities make both species reasonable taxonomic replicates for the present study (Johnson 2002).

Egg arrangement of a clutch is a metric of high dimensionality because it contains information on the positions and orientations of multiple eggs in the clutch, and there has previously been no standard metric to quantify it. We developed several novel measures of egg arrangement (i.e. blunt pole distance, blunt pole angle, blunt pole orientation, adjacent angle—see definitions of these custom-defined measures in our Methods) to test our hypothesis.

We also tested the role of clutch "compactness" in egg rejection; this hypothesis assumes that more compact clutches allow for easier visual assessment of changes in egg arrangement. We measured compactness as an average blunt pole distance (see "Methods"). Blunt pole distance is a direct measure of compactness: The smaller the distance the more compact is the arrangement of eggs. We then predicted that rejecters would have higher compactness of their clutches than acceptors.

Methods

Study area and experimental procedures

We conducted the study in forests and parks in, and nearby, Auckland city (36°51′S, 174°46′E) in New Zealand from September to November 2009 following experimental protocols approved by the Animal Ethics Committee of the University of Auckland (AEC/09/2006/R512). Both blackbirds and song thrush were introduced to New Zealand in the nineteenth century from Europe (Hale and Briskie 2007). In their European source populations, both species are often found in sympatry with the brood parasitic cuckoo (*Cuculus canorus*). Although the introduced New Zealand populations are allopatric with the cuckoo and not impacted by other brood parasites, both species have maintained a fine-tuned ability to discriminate and reject alien eggs (Hale and Briskie 2007; Samaš et al. 2011).

We searched our study sites (for detailed descriptions, see Samaš et al. 2011) systematically for nests of both species. For nests not found during egg laying, we estimated an approximate laying date of all eggs by egg candling (Enemar and Arheimer 1980). We measured the eggs (length = L; breadth = B) to the nearest 0.1 mm using callipers and calculated egg volume ($V = 0.51 \times \text{length} \times \text{breadth}^2$;

Hoyt 1979). We marked all eggs by small numbers on the blunt pole of the eggshell using waterproof ink to allow individual egg identification and to determine egg-laying sequences. Both species always accepted their own marked eggs during previous studies (e.g. Honza et al. 2007; Polačiková and Grim 2010; Grim et al. 2011) including the New Zealand study populations studied here (Samaš et al. 2011). These findings exclude the possibility that eggshell numbering at the blunt pole had any effect on egg recognition by these hosts.

We included only clutches with three or four host eggs for our analyses because (1) the clutches with one or two host eggs could have been a consequence of a partial predation (both species usually lay three or four eggs in our study area; Cassey et al. 2006) and (2) clutches with more than four eggs were extremely rare, and we found them mostly during late incubation (unsuitable for testing our hypothesis, see below) or were depredated and excluded from our analyses.

We artificially parasitized nests of both species by adding an experimental model egg with size (mean ± SE: 22.9 ± 0.1 mm × 17.3 ± 0.2 mm, $N = 10$) and mass (3.5 ± 0.2 g, $N = 10$) within the range of natural cuckoo eggs from Europe (Perrins 1998). We used model eggs that were employed as a standard in previous studies of egg discrimination: They were made from polymer clay and painted with pale blue non-toxic colour to imitate eggs of the cuckoo gens parasitizing common redstarts (*Phoenicurus phoenicurus*) in Europe (reviewed in Polačiková and Grim 2010). For a representative spectral reflectance curve of the blue egg model and its photograph, see Figs. 1b and 2, respectively, in Samaš et al. (2011).

We did not remove any host egg(s), because (1) this was shown to have no effect on host responses (Davies 2000) and (2) our study *Turdus* species are most likely conspecific parasites (see above). Therefore, we methodologically "mimicked" the behaviour of conspecific parasites which do not typically remove any eggs from the clutches they parasitize (Davies 2000). All model eggs were placed in clutches of both future rejecters and acceptors by simply adding it next to the host eggs (i.e. not pushed between host eggs).

We added the egg to the clutch during the laying period (19 out of 20 experiments in blackbirds, 17 out of 20 experiments in song thrush) or immediately after the final egg was laid. Previous works showed no difference in *Turdus* responses to adding eggs between the laying and the early incubation stage (Davies 2000; Honza et al. 2007; Grim et al. 2011; in line with these studies, the rejection rate here did not differ between song thrush nests parasitized during laying (7 out of 17) and early incubation (1 out of 3; two-tailed Fisher's exact test: $P = 1.00$).

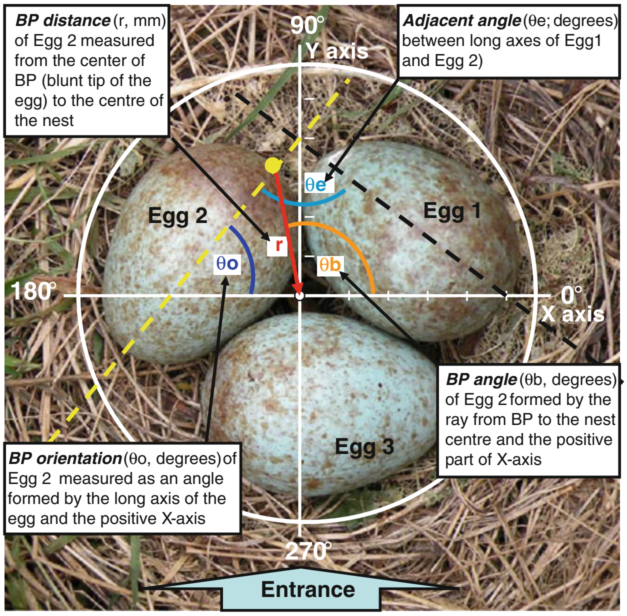

Fig. 1 An example of egg arrangement measurements. The focal egg to illustrate the metrics collected for each egg in a clutch is the second egg (*Egg 2*) within a blackbird's clutch. All eggs were numbered from the *X-axis*, which is perpendicular to the *Y-axis* that is defined by the entrance point and the centre of the nest. Eggs were then numbered in a counter-clockwise direction of a polar coordinate system. Long axis (*yellow dashed line*) of Egg 2 connects the blunt pole (hereafter BP; *yellow dot*) and sharp pole. We measured four different parameters of egg arrangement: (1) blunt pole distance (r, mm), (2) BP angle (θb, degrees), (3) BP orientation (θo, degrees), and (4) adjacent angle (θe, degrees). Each photograph was taken from the same side of the nest representing an "entrance" with the camera facing downwards. The distances were calibrated in real units (mm). See "Methods" for details and definition of terms (colour figure online)

We experimentally parasitized each active nest only once and used each model egg in one experimental trial only. We monitored nests for 6 days after experimental parasitism to assess rejection (Grim et al. 2011). If the parasitic egg disappeared within this period, we considered it "rejected" by "ejection". If all eggs remained intact in the nest, we considered the parasite egg "accepted". We excluded deserted nests from analyses because it was difficult to estimate precisely when and why these nests were deserted (some cold clutches were subsequently incubated again and later successfully hatched; own unpublished data). Imprecisely (late), estimates of desertion dates would artificially inflate our metric of egg arrangement repeatability (see below) because an apparently consistent pattern of egg arrangement would be a by-product of eggs not being attended and normally rotated by parents.

To test whether egg arrangement depended on nest design and whether nest architecture varied between rejecters and acceptors, we used several measures to characterize the nest cup as an extended phenotype. We measured inner and external diameter and depth of the nest

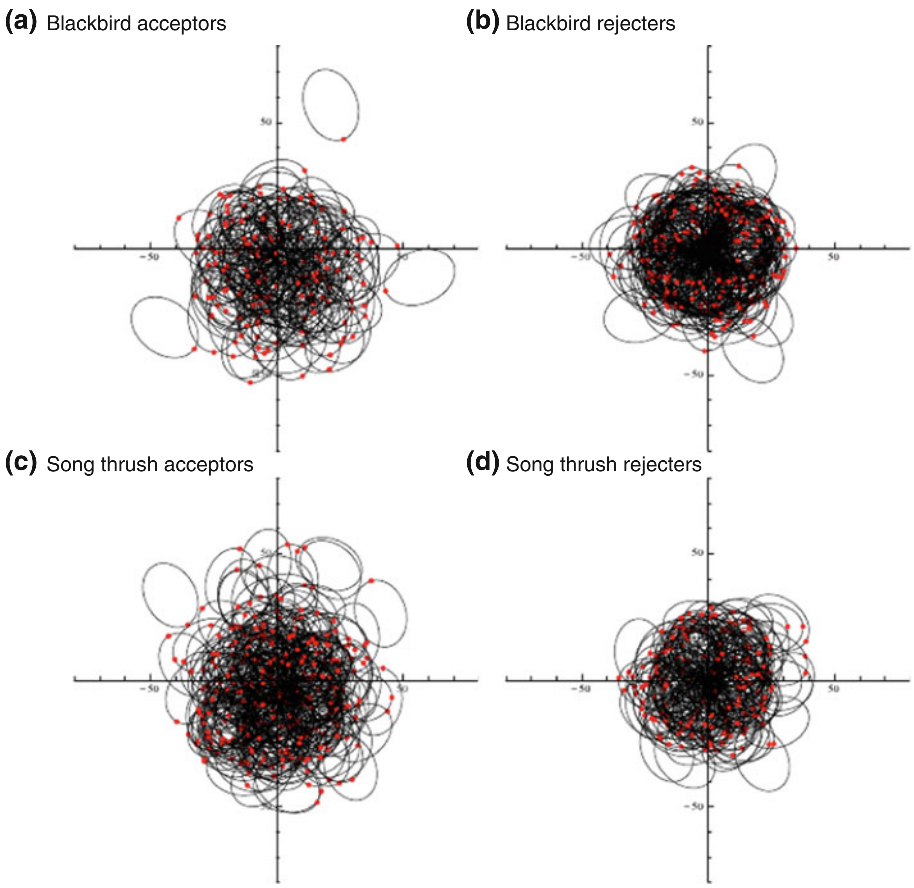
(a) Blackbird acceptors
(b) Blackbird rejecters
(c) Song thrush acceptors
(d) Song thrush rejecters

Fig. 2 Arrangements of all eggs in acceptor (**a**, **c**) versus rejecter (**b**, **d**) clutches superimposed above two axes (units: millimetres) that intersect in the centre of the nest cup. For sample sizes and generation of this figure, see "Methods". An egg is shown as an oval whose length and breadth is set equal to that of typical (average) host egg size (29 × 21 mm for the blackbird and 27 × 20 mm for the song thrush). The blunt pole is denoted as a small red dot for ease of visual assessment of egg compactness

cup to the nearest 0.5 cm. We calculated nest cup volume as a fraction of an ellipsoid ($V = 4/3 \times \pi \times$ smallest radius^2 $\times$ largest radius of the ellipsoid $\times$ 0.5; Soler et al. 1998). As an additional parameter, describing nest design, we calculated an index of nest cup steepness (nest depth/nest cup diameter ratio; Grim et al. 2009).

Egg arrangement within the clutch

During the 6 days after experimental parasitism, we photographed the arrangement of all eggs, including the model egg, daily using a Nikon Coolpix P80 digital camera. All photographs were taken in the same orientation (from the same spot vertically above the nest cup) by the same observer (LP). We photographed each nest from the same side representing the most open access to the nest and likely used as a landing area or access point to the nest by incubating females (only females incubate in our two study species; Perrins 1998; J. Weiszensteinová and T. Grim, unpublished data). We took photographs of each clutch in the morning (three photographs: 07:00–12:00 hours) and the afternoon (three photographs: 12:00–17:00 hours).

Based on the photographs, we analysed the arrangement of all eggs within each clutch using image software Nikon NIS-Elements (Nikon Inc.). Photographs were analysed in temporal sequence, and all measurements of egg arrangement were conducted blind with respect to host response to foreign eggs (until the day when the experimental egg went missing).

First, we calibrated each photograph according to the known (i.e. measured) egg dimensions in real linear distance units (mm). Second, we overlaid each photograph with a two-dimensional circle grid with the horizontal *X-axis* and the vertical *Y-axis* having the origin where the two axes crossed (Fig. 1). This origin was placed in the centre of the nest and was identical among all six photographs per each clutch (Fig. 1). Third, we defined each egg in the nest by: (1) a long axis of the egg (crossing both egg poles); and (2) a point where the long axis crossed the blunt pole of the egg (Fig. 1). An alternative approach would have been to use the egg centroid or egg sharp pole, but it is known that our study species use blunt egg pole features for their egg discrimination decisions (Polačiková and Grim 2010 and references therein). Further, centroid-based measures are inevitably highly correlated with blunt pole-based measures because the position of the egg centroid is highly associated with the position of blunt egg pole if additional information on egg size is taken into account (this inevitably follows from geometry of solid objects placed in a 2D plane).

We employed the polar coordinate system to produce four different measures of egg arrangement (Fig. 1):

1. *blunt pole distance* (r, mm) is defined as the distance between the nest centre and the egg's blunt pole (i.e. blunt tip point of the egg).
2. *blunt pole angle* (θb, °) is defined as the angle between the *X-axis* and the ray connecting the blunt pole with the nest centre (measured in the counter-clockwise direction in the polar coordinate system).
3. *blunt pole orientation* (θo, °) is defined as the angle between the egg's long axis and the *X-axis* (measured in the counter-clockwise direction).
4. *adjacent angle* (θe, °) is defined as the angle between the long axes of the adjacent eggs within the clutch (between Egg 1 and Egg 2, Egg 2 and Egg 3, etc.; Fig. 1).

The first three custom-defined measures record the *absolute* position and orientation of each egg separately. This is because a host female may arrange and remember positions and orientations of all eggs *or* only one particular egg (e.g. an egg that is located on the other side of the nest cup from her arrival point of view). Therefore, we analysed r, θb, and θo for each egg separately in order to detect a non-random consistency in position and orientation even if such consistency was limited to a single egg. Alternatively, a host female may not care about such absolute positions but may instead focus on *relative* patterns, that is, position of two adjacent eggs. This alternative hypothesis is tested by analysing variation in the fourth measure (adjacent angle) which describes the relative positional relationships among the eggs in the clutch.

An arrangement of eggs is uniquely determined once positions, and orientations of all eggs in the clutch are recorded (here we assume that all eggs are identical in size and shape which is a reasonable assumption, see very small variation in egg sizes in our study species, Table 1). The *position* of an egg is uniquely determined by the position of the blunt pole using the distance from the nest centre to the blunt pole (r) and the angle from the *X-axis* (θb) in polar coordinate system. The *orientation* of an egg is uniquely determined by the angle of the long egg axis and the *X-axis* (θo). With information of these three measures for all eggs, the egg arrangement within a clutch is fixed. Using the first three measures, we generated plots showing actual egg positions and orientations (Fig. 2), using the program Mathematica (ver. 8.0, Wolfram Research, Champaign, IL, USA). We stress that many alternative measures of any biological phenomenon, including egg arrangement, can be devised. In the present study, we developed one of them and future studies will benefit from assessing and refining our approaches, where appropriate.

During incubation, birds rotate all eggs within the clutch frequently to aid embryonic development (Boulton and Cassey 2012). Therefore, the arrangement of eggs within the clutch varies when it is incubated. We quantified this variation across each of the egg arrangement parameter as SD (standard deviation). For each photograph, separately, we numbered all eggs including the model egg in the nest from the *X-axis* in the counter-clockwise direction (Fig. 1). For each egg, separately, we calculated SD in (1) blunt pole distance, (2) blunt pole angle, (3) blunt pole orientation, and (4) adjacent angles within the 6-day monitoring period after experimental parasitism. We evaluated adjacent angles in two ways: (1) only host eggs were included in calculations and (2) both host eggs and the model egg were included in the calculations. We use these SDs as the metric of egg arrangement; the smaller these are, the more ordered the arrangement of eggs in the clutch. We also quantified "compactness" as the average blunt pole distance between adjacent eggs for each of the whole clutches.

For logistic and technical reasons, and due to high predation rates, we were able to reliably photograph the complete 6-day period after experimental parasitism in only 20 clutches per species (12 rejecters and 8 acceptors in blackbirds and 8 rejecters and 12 acceptors in song thrush).

Data analyses

We analysed data for blackbirds and song thrush separately. Most of the variables departed from the normal distribution (Shapiro–Wilk tests, $P < 0.05$). Therefore, we used unequal variance *t* tests to compare breeding and nest design characteristics of blackbird and song thrush rejecters versus acceptors. To compare SD of each metric of the clutch between rejecters and acceptors, we used Mann–Whitney *U* tests.

We tested the "compactness" prediction by building general linear mixed models with egg id nested within clutch id as random effects, nest owner response status (rejecter versus acceptor) as a predictor and blunt pole distance as a response variable. We checked the models for normality of residuals and found them satisfactory.

Egg arrangement parameters were mostly independent (i.e. not statistically significantly correlated), except that in both species, the adjacent angle SD between Egg 1 and Egg 2 was positively correlated with blunt pole distance and negatively correlated with blunt pole orientation of these eggs (blackbird: for distance—Kendall Tau = 0.34, $P = 0.04$; for orientation—Kendall Tau = −0.41, $P = 0.01$; song thrush: for distance—Kendall Tau = 0.49, $P = 0.002$; for orientation—Kendall Tau = −0.33, $P = 0.04$).

Statistical analyses were conducted in JMP 8.0.1. Results are presented as mean ± SE if not stated otherwise. All tests were two-tailed ($\alpha = 0.05$).

Table 1 Breeding and nest design characteristics of blackbird and song thrush acceptors versus rejecters of experimentally introduced model eggs (mean ± SD)

Host species	Acceptors		Rejecters		t	P
	Mean ± SD	n	Mean ± SD	n		
Blackbird						
Clutch size (eggs)	3.5 ± 0.5	8	3.3 ± 0.5	12	−0.68	0.49
Average egg volume (cm^3)	7.1 ± 0.5	8	6.8 ± 0.9	12	−0.75	0.46
Nest cup volume (cm^3)	542.1 ± 138.3	7	538.9 ± 138.5	12	−0.05	0.96
Nest cup steepness	0.82 ± 0.17	7	0.78 ± 0.13	11	−0.57	0.58
Laying date	18.9 ± 13.2	8	29.8 ± 13.4	12	1.81	0.09
Song thrush						
Clutch size (eggs)	3.9 ± 0.3	12	3.8 ± 0.5	8	−0.91	0.38
Average egg volume (cm^3)	5.3 ± 0.4	11	5.8 ± 0.4	8	2.55	0.02
Nest cup volume (cm^3)	452.7 ± 110.3	10	462.3 ± 54.4	5	0.23	0.82
Nest cup steepness	0.88 ± 0.1	9	0.91 ± 0.1	5	0.57	0.58
Laying date	24.3 ± 16.1	12	18.3 ± 11.8	8	−0.96	0.35

Nest cup volume was calculated according to Soler et al. (1998) and nest cup steepness calculated according to Grim et al. (2009), see "Methods" for details. Laying date was counted starting from 1 September = day 1. Sample sizes are unequal and differences were tested using the unequal variance *t* test

Results

Blackbirds ejected 60.0 % of model eggs ($N = 20$ nests; latency to ejection = 1.67 ± 0.40 days). Song thrush ejected 40.0 % of model eggs ($N = 20$ nests; latency to ejection = 1.63 ± 0.34 days). In both species, rejecters and acceptors did not statistically significantly differ in the majority of their breeding and nest design characteristics (Table 1). In the song thrush, rejecters showed significantly larger average egg volumes than acceptors, but the effect size was small (average difference of 0.5 cm^3 egg; Table 1).

Importantly, blunt pole distance, angle, orientation, and adjacent angles did not significantly differ between the photographs taken in the morning and afternoon (for both species: Wilcoxon's matched pairs test, for all $Z < 1.40$, $P > 0.16$). Therefore, we pooled the photographs to calculate SD in further analyses.

Blunt pole distance, angle and orientation

A visual inspection of representative summary plots of position of all eggs superimposed for both rejecters and acceptors suggested that positions of rejecter's eggs are more compactly arranged than those of acceptors in both study species (Fig. 2). Accordingly, the average blunt pole distances (a direct measure of compactness, see "Methods") were significantly lower in clutches of rejecters than acceptors (Table 2). In both species, rejecters had lower variation in all blunt pole distances within the clutch compared to the acceptors, although not all these differences reached statistical significance (Fig. 3a). Variation in blunt pole angles (Fig. 3b) and orientations (Fig. 3c) were generally not consistently related to rejecter versus acceptor status of either blackbirds or song thrush.

Table 2 Rejecters of both study species have shorter distances (cm) between blunt poles of their eggs and the nest centre (BP distance), than acceptors

Species	BP distance (cm)		*ddf*	*F*	*P*
	Acceptor	Rejecter			
Blackbird	2.6 ± 0.1	2.2 ± 0.1	16.45	7.23	0.016
Song thrush	2.5 ± 0.1	2.2 ± 0.1	17.83	10.93	0.004

Estimates from a general linear mixed model (see "Methods"). *ddf* denominator degrees of freedom

The rejecters who delayed rejection of model eggs beyond the first 24 h did not significantly differ in egg arrangement variation before (host eggs including model egg) and after ejection of model egg (only host eggs; blackbird: all variables $Z_4 > 3$, $P > 0.19$; song thrush: all variables $Z_3 > 3$, $P > 0.66$).

Adjacent angles

In both species, rejecters had significantly lower variation in adjacent angle between Egg 1 and Egg 2 than acceptors when model eggs were either excluded (blackbird: $U_{12,8} = 2$, $P = 0.004$; song thrush: $U_{8,12} = 6$, $P = 0.001$) or included in the calculations (blackbird: $U_{12,8} = 4$, $P = 0.001$; song thrush: $U_{8,12} = 6$, $P = 0.001$). There was no significant variation in other adjacent angles between

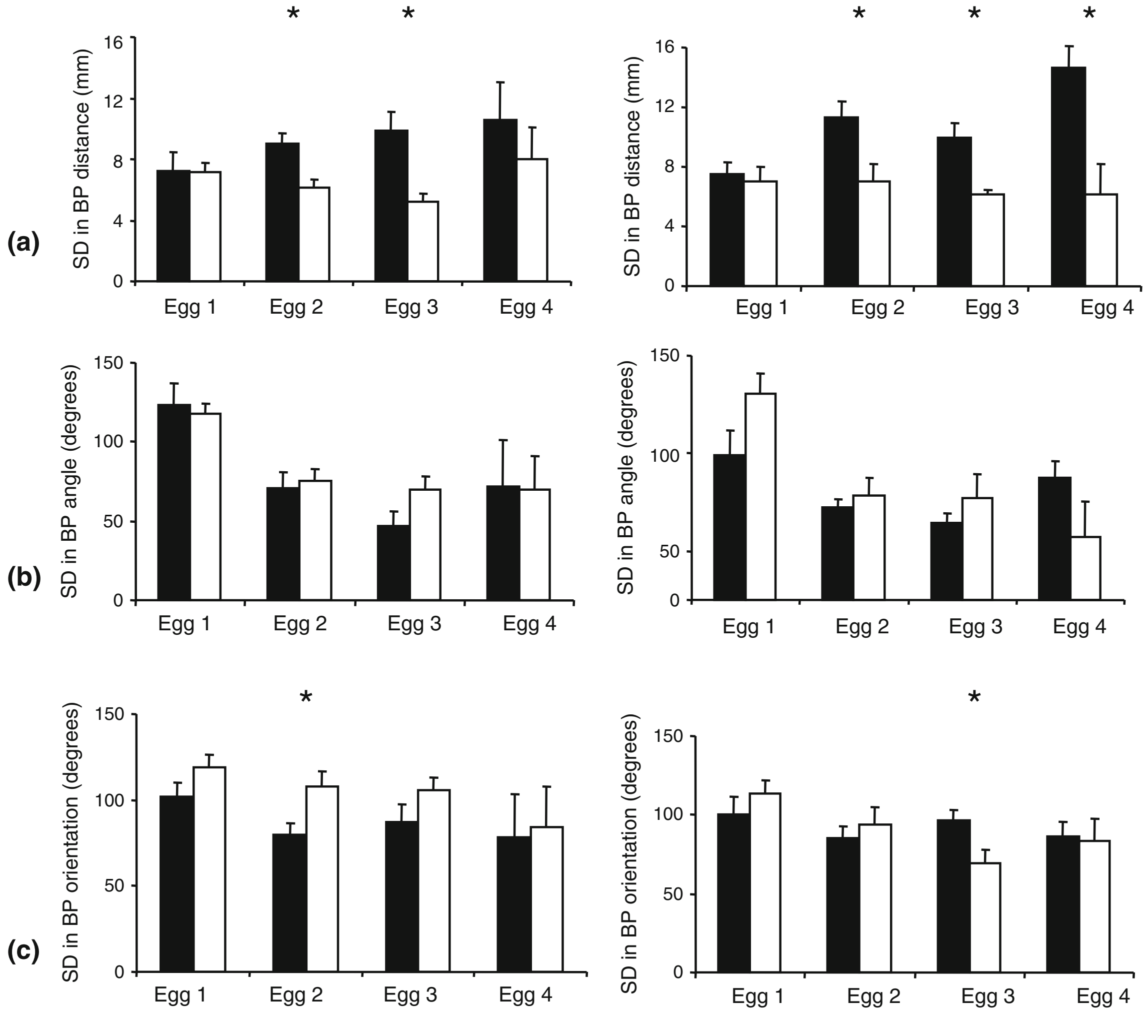

Fig. 3 Comparison of variation (SD) in egg arrangement between acceptors (*full bars*) and rejecters (*open bars*) of model eggs by individual blackbirds (*left*) and song thrush (*right*). For sample sizes see Fig. 2. Differences were tested using Mann–Whitney *U* tests, $*P < 0.05$. Bars are means + SE

the rejecters and acceptors (for all variables: blackbird—all $U > 27$, all $P > 0.11$; song thrush—all $U > 31$, all $P > 0.67$).

In acceptors of both species, there was no significant variation among the adjacent angles when the model egg was excluded or included in the calculations (data not shown; for all variables: blackbird—all $U > 22$, all $P > 0.32$; song thrush—all $U > 56$, all $P > 0.37$) indicating that the presence or absence of the model egg had no effect on variation in adjacent angles.

Discussion

As predicted, rejecters of experimentally introduced model eggs in two species of *Turdus* thrushes had a relatively more consistent egg arrangement within the clutch than acceptors. Interestingly, only parameters related to blunt pole distance, including its average ("compactness"; Table 2) and variation (Fig. 3a), showed consistent differences between rejecters and acceptors. These results make biological sense because both species use blunt pole cues to reject foreign eggs (Polačiková and Grim 2010 and references therein). The consistency of egg arrangement within a host clutch may parallel the cognitive rules assessing intraclutch variability of eggshell colouration (maculation, size, etc.; Soler et al. 2000; Stokke et al. 2007) in detecting parasitic eggs or being alerted to parasitism events. Accordingly, similarity of individual hosts' eggs within clutches may facilitate the discrimination and recognition of a parasite egg from own eggs more easily (Moskát et al. 2008). We suggest that future studies should investigate experimentally whether the relatively greater uniformity in clutch arrangement (i.e. a more consistent egg arrangement) detected in our study for rejecters is an actual cue to facilitate the recognition of foreign eggs or is only a correlated behavioural trait of egg rejecters.

Importantly, both thrushes maintained a consistent egg arrangement across the day (see no difference between morning and afternoon egg arrangements). If individuals use a consistent egg arrangement when recognizing a foreign egg, adding or replacing one of their eggs with a parasitic one inevitably disturbs the original egg arrangement. Such changes in the egg arrangement might present a first signal informing host females about the presence of a foreign egg within their clutch. This first signal could function as an alert and induce the hosts to use another recognition cue(s) and/or more restrictive acceptance thresholds to discriminate foreign eggs (Hauber et al. 2006; Reeve 1989). Therefore, we propose that egg arrangement could present a visual pattern serving as an important initial signal informing nest owners about presence of foreign eggs in the clutch.

Rejecters of both study species appeared to maintain a fixed angle between two of their own eggs (adjacent angle) within the clutch. Each clutch was photographed from the same side representing the putative parental access to the nest, which may also have been used as a landing area and then as "an entrance" to the nest by the nest owners (Fig. 1). If bird parents use egg arrangement as a cue to focus on and compare own egg characteristics, it may be advantageous for them to monitor this pattern from a preferred and consistent landing direction (Lessells et al. 2006). Therefore, we propose that the hosts may use the fixed angle between two of their eggs as a basis of this pattern.

In acceptor nests, the model egg was present throughout the whole experimental period, and thus, the overall egg number was artificially increased within their clutches. Increased number of eggs within a limited space of the nest could lead to difficulties in keeping a consistent egg arrangement. However, we believe that our results are not a by-product of the increased clutch size in acceptors for the following reasons: (1) within acceptors, including or excluding the model egg did not affect estimates of clutch arrangement variation, (2) acceptors with three host eggs and one model egg (four eggs together) had significantly higher variation in the egg arrangement than the rejecters with four own host eggs, (3) the rejecters who delayed rejection of model eggs beyond the first 24 h did not significantly differ in egg arrangement variation before (host eggs including model egg) or after ejection of model egg (only host eggs). In addition, contrary to the role of any spatial constraints on consistency of egg orientation, rejecters and acceptors did not significantly differ either in the nest cup volume or the nest cup steepness in both species, and finally, both species lay fewer of eggs in New Zealand than in Europe (Cassey et al. 2006; Samaš et al. 2013) but do not differ in the nest volume (own unpublished data). Therefore, the nest cup space should be sufficient for maintaining a consistent clutch pattern also after parasitism when the overall number of the eggs in the nest is increased.

Methodological issues

Any study of a new hypothesis inevitably faces methodological innovations and constraints which hopefully will be replicated or ameliorated, respectively, by future studies. To facilitate such scientific progress, we identify and discuss various possible sources of limitations in the present study.

Egg position was idealized for analyses (all eggs simply lay flatly in nest, on the same plane), but in nature eggs are often tilted in the nest cup (angles and distances must be different in tilted eggs in 3D than measured from 2D photographs). However, the inaccuracy in our egg position estimates could only add noise to the measurements. Such noise, by definition, cannot generate consistent patterns, including those that we detected. This means that our metrics and results should be conservative and robust.

The presence of the artificial egg differed, by definition, between rejecters (shorter presence) and acceptors (longer presence), which might have affected the resulting egg arrangement consistency in time. However, the presence of an extra egg *per se* is irrelevant because we do not measure *absolute* positions of eggs but their *relative changes* in time (as SD of various egg arrangement measures). What is important is that there was only one major disturbance at acceptor nests; the addition of the model egg. In contrast, at rejecter nest, there were two disturbances: (1) the addition of a model egg by the experimenter and (2) model egg removal by the host. Although rejecters experienced a larger number of disturbances in clutch composition, they were still able to maintain more consistent egg arrangements than acceptors. This excludes a possibility that our results are a by-product of the presence of model egg *per se*.

Measurements of egg positions may be biased if not all of the photographs are taken from the same spot vertically to the nest cup. Of course, it is impossible for people to be absolutely precise. Although we tried to take photographs from exactly the same spot above the nest cup (see "Methods"), inevitably there must have been some minor variation in the position of the camera. However, this again introduces noise in the resulting data. Importantly, it is unlikely that the chosen camera positions were consistently biased between rejecter and acceptor nests. As stressed above, noise cannot in principle generate statistically significant differences. This again underlies our belief that the patterns we detected are robust, despite the various sources of measurement error (imprecision) and despite host behaviour affecting egg arrangement in the direction that is opposite to our predictions and findings.

Another source of noise in our data might be the accuracy with which we were able to determine the exact location of the blunt pole. If an egg is tilted, then the blunt pole (point) may not be visible, making it difficult to locate, for either researchers or the hosts themselves. However, this was not a great problem in our study because the thrush species relatively small clutch sizes (Samaš et al. 2013) combined with their relatively large open cup nests make tilting not very pronounced. Indeed, only 2 eggs were tilted so much that they were standing with their sharp poles up (out of 870 egg/day measurements). In those 2 cases, we had to estimate the location of the blunt pole. Similar to other factors discussed in this section, this slightly increased the noise in the data but the amount of noise (2 out of 870) is clearly negligible. Moreover, a large body of evidence consistently shows that various hosts, including species with larger clutch sizes and smaller nests than *Turdus* thrushes in New Zealand, use blunt egg poles to recognize foreign eggs (Lahti and Lahti 2002; Polačiková et al. 2007, 2010, 2011; Polačiková and Grim 2010). Thus, *empirically*, tilting seems not to prevent hosts to *estimate* where the blunt pole is located.

Before taking the photographs, most of the female thrushes were flushed from their nest. However, it would be very difficult to adequately measure how flushing the female changes egg arrangement simply because eggs are hidden from view below the incubating female before she leaves the nest. Also, females turn the eggs during incubation, but during this process, she just slightly raises above the nest cup, rotates the eggs, and resumes brooding, thus, again blocking the view (J. Weiszensteinová and T. Grim, unpublished data). Importantly, it would be logistically difficult to control for the flushing because most nests were well hidden in vegetation and could not be checked from a distance to, for example, visit all nests when the female was absent. However, again we stress that any variation caused by our experimental approaches could only add noise to the data making the observed non-random patterns robust.

We assumed that thrushes arrived to their nests from a single “entrance” direction. Indeed, bird parents have been documented to be individually consistent in their use of feeding positions (analogous to our “nest entrance”; Lessells et al. 2006). Regardless, birds are capable of very precise recognition of objects and patterns even though those that change their position from the bird’s point of view or are partially hidden (e.g. Tvardíková and Fuchs 2010). Therefore, it is quite possible that our assumption does not need to be met in the real world. Nonetheless, future research should test whether different individuals, populations, or species vary in their arrival direction consistency and whether this affects egg arrangement consistency and egg rejection probability.

Finally, there have been thus far no standard metrics to measure egg arrangement of a clutch. We custom-defined several metrics of egg arrangement, namely blunt pole distances, angles, orientations, and adjacent angles. The smaller the variations in these metrics, the more consistent the egg arrangement (this directly follows from simple geometry of non-overlapping physical objects placed in 2D plane, see above). Some of our metrics were weakly intercorrelated (but the majority did not correlate with each other). We do not see this as a problem because the aim of the present study is inevitably explorative, that is, to for the first time seek if any—ideally simple and intuitive—measurements of egg arrangement are able to reveal differences in egg arrangement consistency between rejecters and acceptors of foreign eggs. The development of more sophisticated measures and statistical methods that quantify “arrangement” provides a challenge for future studies.

Conclusions

Our study revealed that egg rejecter individuals in two species of *Turdus* thrushes experienced a relatively more consistent and compact arrangement of their blunt egg poles in the clutch than did acceptors. This is the first study proposing and supporting the hypothesis that hosts of avian brood parasites might use a consistent clutch pattern as one of the first lines of defence against brood parasitism. In the future, studies experimentally manipulating original egg arrangement will be necessary to assess the relative importance of clutch pattern consistency in individual bird’s egg discrimination decisions.

Acknowledgments Comments by two anonymous reviewers greatly improved the manuscript. We thank to P. Samaš for help with the fieldwork. The study was supported by the Human Frontier Science Program (RGY69/07 to TG, PC and MEH; RGY83/12 to MEH and TG), and the Research Council of Norway (grant no. 218144) to BGS. PC is an ARC Future Fellow.

Conflict of interest The authors declare that they have no conflict of interest.

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